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Immune and endocrine alterations at the early stage of inflammatory assemblage in toads after stimulation with heat-killed bacteria (*Aeromonas hydrophila*)

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ABSTRACT

The red-leg syndrome in amphibians is a condition commonly associated with the bacteria *Aeromonas hydrophila* and has led to population declines. However, there is little information concerning the inflammatory assemblage in infected anurans. We evaluated immune and endocrine alterations induced by stimulation with heat-killed *A. hydrophila* injected in *Rhinella diptycha* toads. Control animals were not manipulated, while the others were separated into groups that received intraperitoneal injection of 300 μl of saline or heat-killed bacteria: groups A1 (3×10^7 cells), A2 (3×10^8 cells), and A3 (3×10^9 cells). Animals were bled and euthanized six hours post-injection. We evaluated neutrophil: lymphocyte ratio (NLR), plasma bacterial killing ability (BKA), testosterone (T), melatonin (MEL), and corticosterone (CORT) plasma levels. Heat-killed *A. hydrophila* increased CORT and NLR, and decreased MEL, especially at higher concentrations. There was no effect of treatment on T and BKA. We then selected the saline and A3 groups to conduct mRNA expression of several genes including glucocorticoid receptor (GR), toll-like receptor-4 (TLR-4), interferon-γ (IFN-γ), interleukin (IL)-1β, IL-6, and IL-10. We found higher expression of IL-6, IL-1β, IL-10, and IFN-γ in group A3 compared to the saline group. These results indicate the beginning of an inflammatory assemblage, notably at the two highest concentrations of bacteria, and give a better understanding of how anurans respond to an infection within an integrated perspective, evaluating different physiological aspects. Future studies should investigate later phases of the immune response to elucidate more about the inflammation in amphibians challenged with *A. hydrophila*.

1. Introduction

Declines in amphibian populations have been reported across the globe in the last decades and are associated with multiple factors that act synergistically (Carey et al., 1999; Daszak et al., 1999; Rollins-Smith, 2001, 2017; Scheele et al., 2019). This includes factors of anthropic origin (*e.g.*, habitat fragmentation and exposition to contaminants), as well as infectious diseases caused by different types of pathogens, such as the fungi *Batrachochytrium salamandrivorans* (*Bsal*) and *Batrachochytrium dendrobatidis* (*Bd*), the virus *Ranavirus,* and the bacteria *Aeromonas hydrophila* (Carey et al., 1999). Easily found in aquatic environments, the bacteria *A. hydrophila* can be responsible for causing the red-leg syndrome in amphibians, which is characterized by the formation of edemas, cutaneous ulcers, and erythema on the abdominal region and ventral part of the posterior members (Hill et al., 2010; Rivas, 2016). This pathogen is an opportunistic agent, proliferating mainly when the host is immunosuppressed (Miller et al., 2008; Hill et al., 2010). Considering how it is associated with the mortality of individuals

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in wild and captive populations (Nyman, 1986; Miller et al., 2008), more studies are needed to evaluate how amphibians' immune system responds to infections by *A. hydrophila*.

Similar to other vertebrates, the amphibians' immune system can be divided into adaptive and innate immunity (Robert and Ohta, 2009; Zimmerman et al., 2014; Ruiz and Robert, 2023). One of the mechanisms for innate immune activation is the recognition of pathogen-associated molecular patterns (PAMPs) by toll-like receptors (TLR) present in the membrane of tissue-resident immune cells (*e.g.*, macrophages and dendritic cells). This interaction will consequently initiate cytokine production to recruit other leukocytes (*e.g.*, neutrophils) to boost cytokine production at the infection site (Bornstein et al., 2006; Robert and Ohta, 2009). Cytokines are immunomodulatory molecules, including the pro-inflammatory interleukin (IL)-1β, IL-6, and interferon-γ (IFN-γ), that will stimulate phagocytosis, migration of leukocytes to the infection site, and induce the production of other cytokines with similar effects (Scapigliati et al., 2006). Additionally, anti-inflammatory cytokines, as IL-10, inhibit the pro-inflammatory effects, avoiding an exacerbated immune response that would damage the organism (Zimmerman et al., 2014).

The inflammatory response can also be modulated by hormones, including glucocorticoids, melatonin (MEL), and testosterone (T) (Cain and Cidlowski, 2017; Foo et al., 2017; Markus et al., 2018). Corticosterone (CORT) is the main glucocorticoid in anurans (Kloas and Hanke, 1990). CORT immunomodulatory effects vary according to the inflammatory context, and its production is stimulated by pro-inflammatory cytokines during the first phase of the inflammatory response (Givalois et al., 1994; Chrousos, 1995; Floreste et al., 2022), while enhancing the release of anti-inflammatory cytokines by leukocytes during the resolution phase (Cain and Cidlowski, 2017; Gardner et al., 2018). Furthermore, CORT secretion also modulates the migration of leukocytes from the bloodstream to other tissues during inflammation, increasing the proportion of neutrophils to lymphocytes in the circulation (Dhabhar, 2006; Davis et al., 2008; Titon Junior et al., 2021). Glucocorticoid immunomodulation can be mediated by glucocorticoid receptors (GRs) present in many types of cells, including macrophages, lymphocytes, and dendritic cells (Cain and Cidlowski, 2017). In addition, in the hypothalamus, hippocampus, and pituitary gland, GRs regulate the production of CORT by the negative feedback loop (Keller-Wood, 2015; Wingfield and Romero, 2015).

MEL is the hormone produced by the pineal gland when the retina detects the absence of light (Reiter et al., 2010). In healthy individuals, increased nocturnal MEL reduces the expression of adhesion molecules by endothelial cells, impairing the migration of leukocytes to peripheral tissues (Lotufo et al., 2001; Markus et al., 2007). However, during the first phase of the inflammatory assemblage, the inhibition of pineal melatonin secretion allows the migration of leukocytes to the site of infection, where macrophages start synthesizing MEL locally, stimulating phagocytic activity (Fernandes et al., 2017; Yi and Kim, 2017). The immune regulatory properties of MEL are mostly explored in mammals, but recent studies suggest that it also has an immune role in amphibians, with plasma MEL levels being decreased in individuals after an inflammatory stimulus (Ferreira et al., 2021; de Figueiredo et al., 2021; Bastos et al., 2022; Floreste et al., 2022). On the other hand, the sexual steroid hormone T has controversial immunomodulatory properties, mainly being known for suppressing cell-mediated and humoral immune responses (Grossman, 1984, 1985; Foo et al., 2017), though other studies also reported immune-enhancing effects, increasing antibody production and response (Evans et al., 2000; Peters, 2000). In the context of an immune challenge, the production of testosterone can be inhibited in vertebrates, as a sign of reallocating energy for survival and avoiding its potential immunosuppressive effects (Boonekamp et al., 2008; Titon Junior et al., 2021; Sonnweber et al., 2022). In mammals, it is known that this may occur as a result of different factors, including glucocorticoids acting on Leydig cells (Dong et al., 2004), and/or pro-inflammatory cytokines inhibiting the production of the gonadotropin-releasing hormone in the hypothalamus (Spratt, 2001). However, in amphibians we still need more information on the effects of an immune challenge on testosterone production. Evaluating how corticosterone, melatonin and testosterone interact with the immune function in the context of *A. hydrophila* infection could elucidate more about how amphibians cope with this pathogen.

The inflammatory response in amphibians has been usually assessed using inoculation with lipopolysaccharide (LPS), a component of the outer membrane of gram-negative bacteria that is recognized by TLR-4 and initiates part of the innate response (Lu et al., 2008). In different anuran species, LPS injection increases plasma CORT levels, bacterial killing ability (BKA), neutrophil: lymphocyte ratio (NLR), gene expression of pro and anti-inflammatory cytokines, and decreases plasma MEL and T levels (Gardner et al., 2020; Ferreira et al., 2021; de Figueiredo et al., 2021; Titon Junior et al., 2021; Bastos et al., 2022; Floreste et al., 2022; Garcia Neto et al., 2022). However, exploring other methods for assessing inflammatory assemblage, as injection of heat-killed *A. hydrophila*, could bring new information regarding amphibian immune regulation, since it may include more signaling pathways beyond those activated by TLRs (Jang et al., 2004; Qi et al., 2016). This study evaluates the early immune and endocrine alterations induced by injection with heat-killed *A. hydrophila* in the Brazilian Cururu toad (*Rhinella diptycha*) at three different concentrations. We hypothesize that heat-killed *A. hydrophila* induces concentration-dependent endocrine and immune alterations. Therefore, we predict that the highest plasma CORT levels, BKA, NLR, and lowest plasma MEL and T levels will be detected in the group injected with the highest concentration of bacteria and progressively less in the other groups. Additionally, we hypothesize that injection with heat-killed *A. hydrophila* will alter mRNA levels of cytokines and receptors in toads. We predict an upregulation of pro-inflammatory cytokines (IL-1β, IL-6, IFN-γ), the anti-inflammatory cytokine IL-10, and receptors (GR, TLR-4), showing an immune profile associated with the first phase of the inflammatory response in toads stimulated with the bacteria.

2. Material and methods

2.1. Study site and animals

Adult males (body size ≥70 mm, [Krakauer, 1968]) of *R. diptycha* were collected in the municipality of Botucatu (22°46′55"S, 48 28′38"W) in the State of São Paulo, Brazil, in September 2021 ($N = 48$; mean SVL = 126,06 ± 12,11 mm; mean weight = 177,84 ± 55,71 g). Toads were manually captured while foraging and placed in plastic containers (43.0 cm × 28.5 cm × 26.5 cm). After capture, toads were transported to the laboratory, where they were maintained in individual plastic containers (43.0 cm × 28.5 cm × 26.5 cm) with perforated lids to allow air circulation, fasting, and free water access. Animals were exposed to a 13/11 LD cycle and 21 ± 2 °C for 10 days for acclimation before treatments started. This photoperiod and temperature were chosen based on the natural condition that the toads were found.

Toads were collected under authorization from Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio, 8132–1). All the procedures were approved by the Animal Ethics Commission of the Institute of Bioscience, University of São Paulo (CEUA/IB-USP - n° 371/2020).

2.2. Bacterial stimulation

Aeromonas hydrophila (IOC/FDA 110–36), donated by the Oswaldo Cruz Foundation (FIOCRUZ; RJ), were cultured overnight at 37 °C (10 μl of bacteria in 50 ml of tryptic soy broth [TSB]) with a negative control containing only TSB in sterile Erlenmeyer flasks of 125 ml. The following day, aliquots of 25 ml of the bacteria mixture were prepared in sterile conical tubes of 50 ml. The tubes were agitated, and 300 μl of bacteria samples were transferred to a 96-well microplate, including the

negative control (TSB). To determine the bacteria concentrations, samples were read in a plate spectrophotometer (595 nm; Spectramax 250), and the negative control value was subtracted from the bacteria sample to determine bacteria concentration. Then, the tubes containing the bacteria mixture were boiled for 1 h and centrifuged (5000 rpm, at 23 °C for 10 min) for pellet formation. After centrifugation, the supernatant was discarded, and 5 ml of phosphate-buffer saline (PBS) was added to all tubes which were agitated. Tubes were centrifuged again (5000 rpm, at 23 °C for 10 min), and the supernatant was discarded. Next, the pellets were resuspended in different volumes of PBS to achieve the following bacteria concentrations: 1×10^8/ml, 1×10^9/ml, and 1×10^{10}/ml. These concentrations were based on previous studies investigating immune effects induced by *A. hydrophila* in other amphibian species (Marr et al., 2005; De Jesús Andino et al., 2012; Robert et al., 2014). Considering this is the first study with injection of heat-killed *A. hydrophila* in *R. diptycha*, three concentrations were tested to evaluate their immune and endocrine alterations and thus determine which of them are more appropriate for future studies involving the same immune challenge.

The animals were weighed (0.01 g), and the snout-vent length (SVL) was measured (0.01 mm) 3 days before the experiment. Then, toads were randomly divided, considering these morphometric parameters. A body index (BI) was calculated as unstandardized residuals of a linear regression of body mass as a function of SVL. Animals were grouped as follows: control (no injection and no manipulation), saline (toads received an intraperitoneal injection [i.p.] of 300 μl of amphibian PBS [APBS]), *Aeromonas* 1 (A1, i.p. with 300 μl of solution with heat-killed bacteria in the concentration of 1×10^8/ml, corresponding to 3×10^7 bacteria), *Aeromonas* 2 (A2, i.p. in the concentration of 1×10^9/ml, corresponding to 3×10^8 bacteria) and *Aeromonas* 3 (A3, i.p. in the concentration of 1×10^{10}/ml, corresponding to 3×10^9 bacteria). The control group was included in the study to identify potential differences between the control and saline-injected groups, which may arise due to the injection's effects.

2.3. Blood and spleen collection

Injections started at 6:00 pm, and blood and tissue collection began 6 h later (12:00 am), representing when toads are more active in the wild and captivity (Jessop et al., 2014; Bastos et al., 2022) and, therefore, more likely to interact with pathogens during foraging activities. Moreover, we chose to collect samples 6 h post-injection because that is when LPS-injected *R. diptycha* toads exhibit a more diverse spectrum of endocrine and immune alterations in the plasma and immune organs compared to earlier and later time-points (Floreste et al., 2022, 2023). Toads were injected sequentially, picking one animal from each group for each injection round, in the following order: saline, A1, A2, A3. Blood sampling was performed by cardiac puncture using heparinized 1 ml syringes and 26 G × 1/2″ needles within 3 min of handling. Individuals were bled in the same order of injections. After blood sampling, animals were euthanized by decapitation, and then the spleen was extracted. All procedures were performed in darkness using lamps with red filters to avoid light-induced alterations in MEL levels (Trinder et al., 1996). One drop of blood was used to perform a smear and measure the NLR. Then, blood samples were centrifuged (3000 rpm, 4 min) to isolate the plasma. Plasma samples were transferred to a − 80 °C freezer for posterior analyses of CORT, MEL, T, and BKA. For cytokine and receptor gene expression, spleens were collected, immediately frozen in liquid nitrogen, and then transferred to a − 80 °C freezer.

2.4. Blood leukocyte profile

The increased proportion of neutrophils in relation to lymphocytes in the blood is often associated with higher CORT plasma levels after the application of an immune challenge (Titon Junior et al., 2021; Garcia Neto et al., 2022), making it a relevant parameter to evaluate the cellular component of the inflammatory response. A blood smear was prepared on a microscope slide using a drop of blood to evaluate the proportion of leukocytes in circulation. The smears were stained using a panoptic dye (LaborClin - #620529). Slides were observed through an optical microscope at ×1000 magnification (Nikon E200, 104c) using immersion oil. To determine the proportion of each cell type, one hundred leukocytes were counted and identified in each slide based on the cellular morphology in amphibians as described by Stacy et al. (2022): lymphocyte, neutrophil, monocyte, eosinophil, and basophil. NLR was calculated by dividing the number of neutrophils by the number of lymphocytes counted on each slide.

2.5. Plasma bacterial killing ability (BKA)

The plasma BKA assesses innate immune response efficacy by evaluating plasma complement proteins and natural antibodies' bactericidal capacity (Demas et al., 2011; Assis et al., 2013; Titon Junior et al., 2021). The protocol followed the procedures outlined in Assis et al. (2013) and Moretti et al. (2019). Plasma samples (10 μl) diluted in 190 μl of ringer for amphibians (NaCl: 6.5 g, KCl: 1 g, NaH2PO4: 0.1 g, CaCl2: 1.125260 g, NaHCO3: 0.2 g, C6H12O6: 2 g, diluted in 1 l of distilled water) were incubated with *A. hydrophila* (10 μl – 2.5×10^5 bacteria) for 1 h at 22 °C. The positive control consisted of 10 μl of bacteria in 200 μl of ringer, while the negative control contained only the ringer solution (210 μl). After the incubation period, 500 μl of TSB was added to each sample and transferred in duplicates to a 96-well sterile microplate. After 1 h at 37 °C, the optical density of the samples was measured hourly in a plate spectrophotometer (595 nm; Spectramax 250), totaling 4 readings. BKA was calculated as: 1 – (optical density of sample / optical density of positive control), representing the proportion of killed bacteria in the samples compared to the positive control. The plasma BKA was assessed during the initial stage of bacterial exponential growth. Their maximum growth becomes apparent at this moment, and the highest BKA index for each sample can be scored.

2.6. Hormone assays

Plasma CORT, T, and MEL levels were determined using ELISA kits (CORT, Cayman Chem. – #501320; T, Cayman Chem. – #582701; MEL, IBL – RE54021), according to the manufacturer's instructions and previous studies conducted with the same or closely related species (Titon et al., 2017, 2022; Assis et al., 2019; Bastos et al., 2022).

Steroids were initially extracted from 10 μl of plasma by adding 3 ml of ethyl ether, according to Assis et al. (2015, 2017). For CORT and T quantification, samples were resuspended in ELISA buffer and assayed in the plate following the fabricant instructions. Inter and intra-assay coefficients of variation were 10.35% and 11.66% for CORT, 9.92% and 11.91% for T, respectively. Assay sensitivity was 33.03 pg/ml and 9.85 pg/ml for CORT and T, respectively. For MEL determination, samples were extracted through silica columns (Waters Sep- Pak® Vac), using 150 μl of plasma, as previously described by de Figueiredo et al. (2021), and assayed in the plate according to fabricant instructions. Intra-assay coefficient of variation for MEL was 4.54%, and assay sensitivity was 3.00 pg/ml.

2.7. Gene selection

To further evaluate the inflammatory assemblage, including molecular data, we chose animals from A3 (injected with the highest bacteria concentration) and the saline group since A3 showed the most evident physiological response, and there was no difference between control and saline-treated toads. Due to their role in modulating the inflammatory response, we selected the genes of the pro-inflammatory cytokines IL-1β, IL-6, IFN-γ, and anti-inflammatory cytokine IL-10 (Zimmerman et al., 2014). Additionally, we designed primers to evaluate GR and TLR-4 mRNA levels based on nucleotide data from other animal species

using the pick primer tool available at the National Center for Biotechnology Information (NCBI) as in (Ferreira et al., 2021). We used the β-actin gene as the reference gene. All primers were purchased from Thermo Fisher Scientific (Table 1).

2.8. RNA extraction and reverse transcription

RNA extraction and conversion to complementary DNA (cDNA) were done according to Ferreira et al. (2021). Briefly, around 50 mg of spleen were homogenized in TRIzol reagent (Thermo Scientific, # 15596018), and the total RNA was isolated according to the manufacturer's instructions. The RNA concentration and quality were measured using a spectrophotometer at A260 / A280 (Nanodrop ND1000, Thermo Scientific, USA). Next, to eliminate the remaining genomic DNA, samples were treated with DNase I (Thermo Scientific, # EN0521). Then, the reverse transcription was performed using 2 μg of total RNA, random primers (100 μM, Thermo Scientific, # SO142), dNTPs (10 mM, Thermo Scientific, # R0192), and reverse transcriptase (Revertaid H minus Reverse Transcriptase kit, Thermo-Scientific, # EP0451), according to the manufacturer's instructions.

2.9. Primers' test

To verify the efficiency of the newly designed primers (GR and TLR-4), the cDNA was used to generate the amplicons of the target genes in the conventional polymerase chain reaction (PCR). The PCR mix was prepared for each primer using a total volume of 100 μl with 50 μl of 2× DreamTaq Master Mix (Thermo Scientific, # K1081), 2 μl of specific target-genes primer (10 μM stock; 1 μl forward primer +1 μl reverse primer), 5 μl sample (cDNA 50 ng) and 43 μl of water. The reaction was performed in a thermocycler (Mastercycler Gradient, Eppendorf) according to the following steps: 94 °C for 5 min, followed by 40 cycles at 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, and then 1 cycle of 72 °C for 5 min, and withholding at 10 °C. All PCR products were analyzed by electrophoresis (3% agarose gel). Once the amplicon was observed, they were cut from the gel using sterile sharp blades and transferred to 2 ml sterile microtubes. The gel pieces containing the amplicon were purified using the Pure Link Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen, # K220001) following the manufacturer's instructions. After purifying the amplicon, cDNA was quantified by a spectrophotometer (Nanodrop N8000, Thermo Scientific, USA). Calculations to verify the copy numbers of each PCR product were based on fragment length and molecular mass. A standard curve was prepared in a ten-fold dilution, and the efficiency was analyzed using the quantitative PCR (qPCR) in a thermocycler (QuantStudio3 Real-Time PCR System, Applied Biosystems), following this program: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min, and then dissociation-curve cycles 95 °C for 15 s, 60 °C for 60 s and 95 °C for 15 s. Results were obtained at the end of the test using QuantStudio Design & Analysis Software version 1.5.2. Both primers showed specificity ($R^2 \geq 0.99$ and efficiency ≥93%; Fig. A1).

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

For the RT-qPCR, a reaction mix was prepared to contain 5 μl sample (cDNA 50 ng); 10 μl SYBR Green 2× (Thermo Fisher Scientific, # K0223); 0.1 μl target primer (10 μM; forward + reverse mix) and water to obtain a final volume of 20 μl. The reaction was performed using the StepOne™ real-time PCR System (Thermo Scientific, Finland) following the steps: 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, 60 °C for 60 s, and then dissociation-curve cycles at 95 °C for 15 s, 60 °C for 60 s and 95 °C for 15 s. Results were obtained at the end of the test using StepOne Software Version 2.3.

Gene expression rate was calculated by relative quantification using the ΔΔCt method (Livak and Schmittgen, 2001), and it is shown as a fold change of each cytokine and receptor normalized by the reference gene β-actin.

2.11. Statistical analysis

Shapiro-Wilk tests for normality and Levene's tests for homogeneity of variance were performed for all variables. Firstly, to evaluate the possible effects of body condition, independent sets of ANCOVAs were performed considering all physiological parameters (NLR, BKA, MEL, T and CORT) as dependent variables, treatment (Control, Saline, A1, A2, and A3) as factors, and BI as a covariate. There was no effect of BI as covariate. Then, BI was removed from models, and one-way ANOVA was used to analyze CORT, T, BKA, and NLR, with these physiological parameters as dependent variables, and treatment (Control, Saline, A1, A2, and A3) was an independent factor. Kruskal-Wallis test was used to evaluate MEL as dependent variable, and treatment (Control, Saline, A1, A2, and A3) as independent factor. When appropriate, ANOVAs tests were followed by Tukey, while Kruskal-Wallis tests were followed Dunn's multiple comparisons tests. Molecular data were evaluated using the Mann-Whitney test, considering treatment (saline and A3) as a factor and gene expression (IFN-γ, IL-6, IL-1β, IL-10, GR, and TLR-4) as a dependent variable. Additionally, Pearson or Spearman correlation tests were used according to the assumptions of each test to investigate the possible relations among variables within different treatments. Statistical significance was considered at $p \leq 0.05$. IBM SPSS Statistics 26 was used for analyses, and GraphPad Prism 7.04 for figures.

3. Results

The BI did not affect any of the investigated variables ($p \geq 0.211$). Among the physiological parameters, the bacterial challenge induced changes in CORT, MEL, and NLR (Fig. 1). Toads from groups stimulated with the higher concentrations of heat-killed bacteria (A2 and A3) showed increased CORT ($F_{4, 42} = 2.735$, $p = 0.041$; Fig. 1A) and NLR ($F_{4, 42} = 5.457$, $p = 0.001$; Fig. 1B), contrasting with decreased MEL ($H_4 = 24.812$, $p < 0.0001$; Fig. 1C). However, there was no treatment effect for T ($F_{4, 42} = 0.423$, $p = 0.791$; Fig. 1D) and BKA ($F_{4, 42} = 1.677$, $p = 0.173$; Fig. 1E).

As for the molecular parameters, toads from the group challenged with the highest dose of heat-killed bacteria (A3) showed upregulation of IL-1β ($U < 0.0001$, $p < 0.0001$; Fig. 2A), IL-6 ($U = 1$, $p < 0.0001$;

Table 1
Primers' sequence used to evaluate gene expression.

Gene	Forward (5′) primer	Reverse (3′) primer	Length (bp)	Design
IL-1β	GAGAACATTGCGCAAGAAGC	AAATAGAGTTGACGGCCTGC	110	Gardner et al., 2018
IL-6	CAGTGATCTCCTGACGTTCC	AGCATTTGCCAAGGAGATGG	112	Gardner et al., 2018
IL-10	TGTGAGCAGCCACAAGACAT	GCATGCGGCCTTGGATCTTA	84	Ferreira et al., 2021
IFN-γ	AGGACAAGCTCCTAGACCTGA	TCCAACTGCCTTGTACATCCC	140	Ferreira et al., 2021
TLR-4	GTCCGCAACTGTGTTCTCCT	GCAACTCCCAGCACTACGAA	131	Made in this study
GR	ACACTCAGCAAGCTCCACTC	TGGCACTCTCTCTGTTGCAG	110	Made in this study
Actin[a]	ATGACACAGATAATGTTTGAGAC	ATCACCAGAGTCCATCACAAT	117	Halliday et al., 2008

Note. IL-1β: interleukin-1β; IL-6: interleukin-6; IFN-γ: interferon-γ; IL-10: interleukin-10; TLR-4: toll-like receptor 4; GR: glucocorticoid receptor; [a] Reference gene.

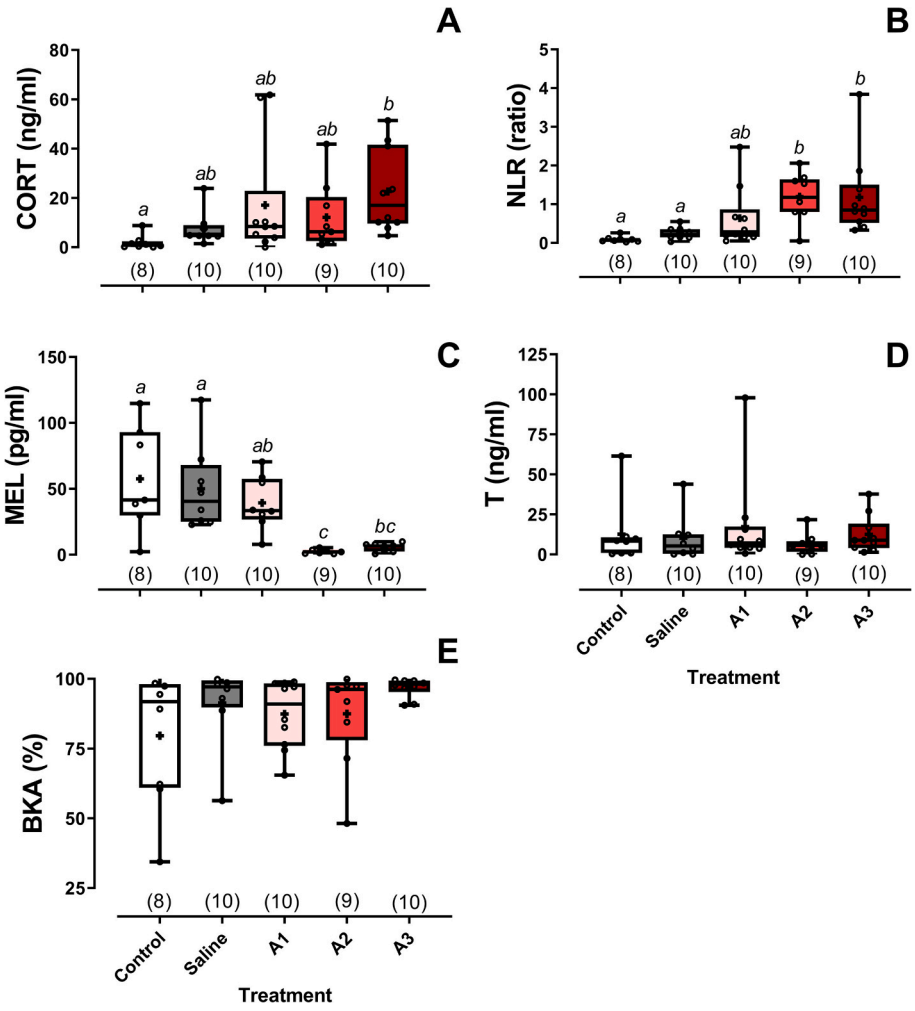

Fig. 1. Immune and endocrine modulation in *Rhinella diptycha* toads post-stimulation with different heat-killed bacteria (*Aeromonas hydrophila*) concentrations. Plasma corticosterone levels (A), neutrophil: lymphocyte ratio (B), plasma melatonin levels (C), plasma testosterone levels (D), and bacterial killing ability (E). Boxplots display the median and interquartile range (IQR), with whiskers indicating min and max values, and plus signals show the mean. Letters above boxplots represent statistical differences for Tukey (CORT and NLR) or Dunn (MEL) tests, with different letters representing statistical differences within groups with $p \leq 0.05$. The small circles represent each individual data point. The N is indicated in parentheses below the boxplots. Control = not manipulated; Saline = 300 μl of intraperitoneal injection of saline; A1 = intraperitoneal injection of 3×10^7 heat-killed bacteria; A2 = intraperitoneal injection of 3×10^8 heat-killed bacteria; A3 = intraperitoneal injection of 3×10^9 heat-killed bacteria.

Fig. 2B), IL-10 ($U < 0.0001$, $p < 0.0001$; Fig. 2C), and IFN-γ ($U = 11$, $p = 0.031$; Fig. 2D) mRNA transcripts when compared to those from the saline group, but there was no treatment effect for GR ($U = 38$, $p = 0.897$; Fig. 2E) and TLR-4 ($U = 37$, $p = 0.829$; Fig. 2F) transcripts. Heat-killed bacteria increased cytokine IL-1β by 40-fold, IL-6 by 500-fold, IL-10 by 1000-fold, and IFN-γ by six-fold.

Additionally, we observed that toads from the A3 group showed a positive correlation between IL-6 and IL-1β ($r_{(9)} = 0.792$, $p = 0.011$; Fig. 3A), IFN-γ and IL-6 ($r_{(9)} = 0.764$, $p = 0.017$; Fig. 3B) and IL-6 and CORT ($r_{(9)} = 0.700$, $p = 0.036$; Fig. 3C). Moreover, there was also a positive correlation between GR and TLR-4 in both saline ($r_{(8)} = 0.943$, $p < 0.0001$) and bacteria-injected toads ($rs_{(10)} = 0.903$, $p < 0.0001$; Fig. 3D).

4. Discussion

Our results demonstrate the induction of the inflammatory response in toads 6 h after immune stimulation with heat-killed *A. hydrophila*. Specifically, the highest bacteria concentration showed the more evident response, as shown by the increase in plasma CORT levels, NLR, and decrease of plasma MEL levels. Moreover, gene expression analysis showed upregulation of the pro-inflammatory cytokines IL-1β, IL-6, IFN-γ, and the anti-inflammatory cytokine IL-10 in animals stimulated with the highest concentration of bacteria (A3) compared to the saline group. Similar patterns were observed after LPS injection in anurans (Ferreira et al., 2021; de Figueiredo et al., 2021; Titon Junior et al., 2021; Floreste et al., 2022; Garcia Neto et al., 2022), birds (Owen-Ashley et al., 2006), fishes (Haukenes and Barton, 2004), mammals (Terrazzino et al., 1997; Tamura et al., 2010), and after *A. hydrophila* stimuli in reptiles (Glassman et al., 1981) and fishes (Marinho de Mello et al., 2019).

Corroborating our initial prediction, the immune challenge with heat-killed bacteria increased the plasma CORT levels, indicating activation of the hypothalamus-pituitary-adrenal/interrenal axis (HPA/I) in the highest bacteria concentrations (Rivier et al., 1989). Similar results have been observed in fish inoculated with *A. hydrophila* (Marinho de Mello et al., 2019) and in several vertebrates treated with LPS (Terrazzino et al., 1997; Haukenes and Barton, 2004; Owen-Ashley et al., 2006; Acerete et al., 2007), including anurans of the same or closely related species (Ferreira et al., 2021; Titon Junior et al., 2021; Bastos et al., 2022; Floreste et al., 2022; Garcia Neto et al., 2022). This could be explained by the activation of TLR-4 and TLR-2, responsible for interacting with PAMPs, in the hypothalamus and the adrenocortical cells,

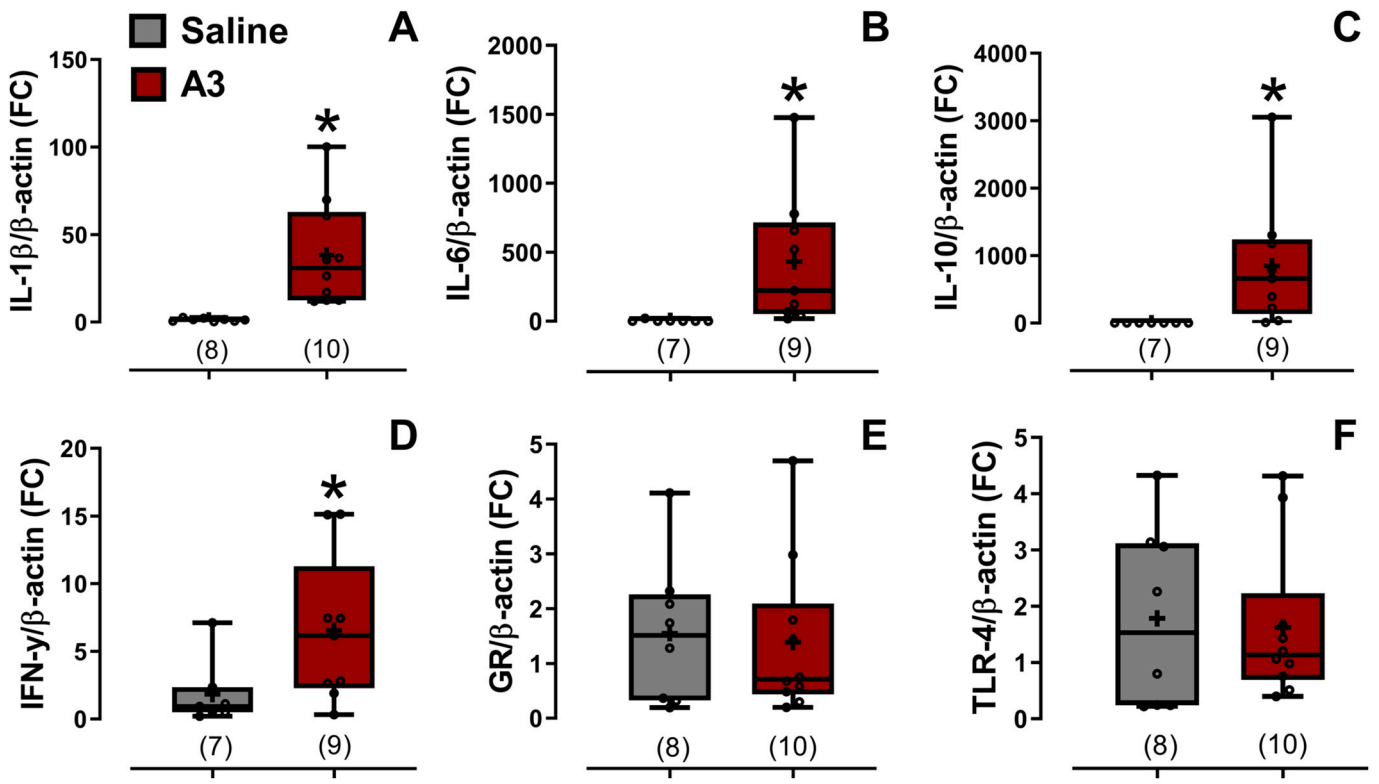

Fig. 2. Modulation of mRNA expression in *Rhinella diptycha* toads post-injection with the highest (3×10^9 cells) heat-killed bacteria (*Aeromonas hydrophila*) concentration. Interleukin-1β (A), interleukin-6 (B), interleukin-10 (C), interferon-γ (D), glucocorticoid receptor (E), and toll-like receptor-4 (F). A3: the highest dose of bacteria injection (3×10^9 bacteria). FC: fold change. Boxplots display the median and interquartile range (IQR), with whiskers indicating min and max values, and plus signals show the mean. The small circles represent each individual data point. Asterisks (*) denote significant differences ($p \leq 0.05$) between groups.

leading to CORT release (Bornstein et al., 2004, 2006; Ishii et al., 2007; Muduli et al., 2021). Moreover, there was a positive correlation between IL-6 expression and CORT levels in toads injected with the highest concentration of bacteria. Increased production of glucocorticoids could also be a result of stimulation of the HPA/I axis by pro-inflammatory cytokines, such as IL-1β and IL-6, which bind to receptors in the hypothalamus and increase the secretion of corticotropin-releasing hormone (Bumiller et al., 1999; Holland et al., 2002; Dunn, 2006). We did not detect alterations in the expression of TLR-4 and GR in the spleen, although both genes are essential in the modulation of CORT and immune variables (Bornstein et al., 2006; Cain and Cidlowski, 2017). This could be due to the sampling timing since all individuals in this study were euthanized 6 h after injection. Changes in the expression of both GR and TLR-4 were reported 24 h after an immune challenge with LPS in mammals (Ibeagha-Awemu et al., 2008; Kamiyama et al., 2008). Nonetheless, we found a positive correlation between both receptors independently of treatment, indicating a possible interaction for CORT regulation in and out of the inflammatory context. Future studies aiming to evaluate the gene expression of these two receptors should explore later hours after applying an inflammatory stimulus and include other receptors associated with the innate immune response, such as TLR-2 (Muduli et al., 2021), to investigate possible differences in the expression dynamics between amphibians and mammals.

We observed an increase in NLR in toads after bacterial stimulation, especially in the groups treated with the two highest concentrations of bacteria. This has been documented after LPS treatment in other anurans (Gardner et al., 2020; de Figueiredo et al., 2021; Titon Junior et al., 2021; Garcia Neto et al., 2022), and alligators infected with *A. hydrophila* (Glassman et al., 1981). The pattern of higher NLR is often associated with increased CORT levels (Davis et al., 2008; Titon et al., 2021) due to the role of glucocorticoids as modulators of leukocyte redistribution (Dhabhar, 2006). Though there was no correlation between CORT and NLR in this study, it is known that elevated glucocorticoid levels induce the migration of lymphocytes from the bloodstream to tissues while concomitantly increasing the influx of neutrophils to the circulation (Dhabhar et al., 1995; Dhabhar, 2002, 2006). This immune redistribution is also associated with higher pro-inflammatory gene expression (Garcia Neto et al., 2022) and with the suppression of MEL (Lotufo et al., 2001; Markus et al., 2007), enhancing the inflammatory assemblage.

As predicted, bacterial stimulation markedly decreased plasma MEL levels in toads injected with the two highest concentrations of bacteria. Other studies reported decreased MEL after different types of inflammatory stimuli in mammals (Tamura et al., 2010; Fernandes et al., 2017), birds (Piesiewicz et al., 2012), and anurans (Ferreira et al., 2021; de Figueiredo et al., 2021; Titon Junior et al., 2021; Bastos et al., 2022). In mammals, during the first phase of the inflammatory response, this suppression occurs due to the activation of TLR-4 by PAMPs and the concomitant interaction of high levels of cytokines, glucocorticoids, and catecholamines in the bloodstream that reach the pineal gland, thus inhibiting the MEL synthesis pathway (Fernandes et al., 2006, 2017; Markus et al., 2018). This decrease in MEL levels allows the migration of leukocytes through the endothelial layer to the site of infection (Markus et al., 2007). This communication between the pineal gland and the immune system is known as the Immune-Pineal Axis (Markus et al., 2018). While the Immune-Pineal axis has been better evaluated in mammals, recent studies with amphibians reported results that suggest the existence of this axis also in this group (Barsotti et al., 2017; Ferreira et al., 2021; de Figueiredo et al., 2021; Floreste et al., 2022). Furthermore, we did not observe differences between the control and saline groups as initially predicted. A decrease in plasma MEL levels was reported in rats injected with a vehicle (treatment with saline solution) compared to a naïve group, showing the inhibitory effect of the injection *per se* (Tamura et al., 2010). However, considering that the main site of

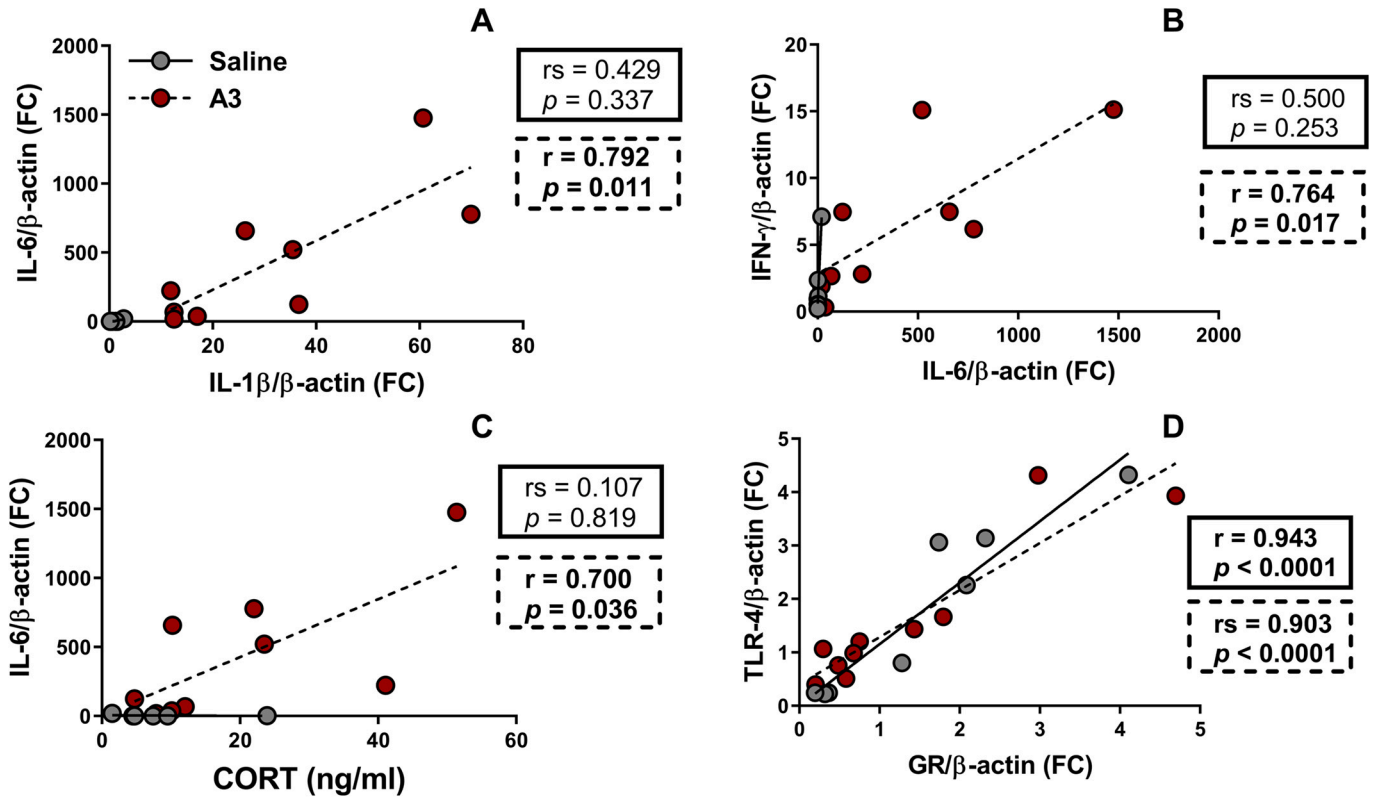

Fig. 3. Correlations between parameters assessed in *Rhinella diptycha* toads considering both treatments (saline and bacterial stimulation). IL-6 *vs.* IL-1β gene expression (A), IFN-γ *vs.* IL-6 gene expression (B), IL-6 gene expression *vs.* corticosterone plasma levels (C), TLR-4 *vs.* GR gene expression (D). A3: the highest dose of bacteria injection (3×10^9 bacteria). Within the squares, "rs" represents the coefficient of Spearman, while "r" represents the coefficient of Pearson. FC: fold change. Gray circles with a solid line represent animals from the saline group, and dark red circles with a dashed line represent animals from the A3 group. Trend lines illustrate the correlation between variables. Values in bold have a significant correlation ($p \leq 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

MEL production in amphibians is the retina (Serino et al., 1993), it is possible that the injection *per se* could be detected in the eyes (*i.e.*, ocular melatonin), even though not detected in the plasma. Additionally, ocular MEL in amphibians is modulated upon stimulation of the HPI axis and in an inflammatory context (Barsotti et al., 2017; Bastos et al., 2022). Future studies should explore this possibility, also evaluating the gene expression of key enzymes in the MEL synthesis pathway in the eyes. Moreover, considering that there was no difference between the control, saline, and A1 (toads injected with the lowest concentration of bacteria) groups regarding plasma MEL levels and NLR, we suggest that the threshold of bacteria cell for triggering an inflammatory response probably lies between 3×10^7 (group A1) and 3×10^8 bacteria (group A2).

As expected, the pattern of cytokines gene expression we observed is associated with the first phase of the inflammatory response, characterized by the upregulation of the pro-inflammatory cytokines IL-1β, IL-6, and IFN-γ. These cytokines are responsible for initiating the innate immune response, enhancing phagocytic activity, recruitment of leukocytes to the site of infection, induction of sickness behavior, and production of acute phase proteins (Commins et al., 2010; Zimmerman et al., 2014). Similar results were observed in fish post-inoculation with *A. hydrophila* (Reyes-Becerril et al., 2011; Hajirezaee and Hossein Khanjani, 2021) and after immune challenge with LPS in anurans (Ferreira et al., 2021; Floreste et al., 2022, 2023; Garcia Neto et al., 2022; Titon et al., 2022). In addition, we also observed positive correlations of IL-1β with IL-6, and IFN-γ with IL-6 in toads injected with bacteria. Accordingly, IL-1β and IL-6 are known for stimulating their own production in leukocytes, and IL-1β is also responsible for enhancing the synthesis of IL-6 (Dinarello, 1998; Staeheli et al., 2001; Zimmerman et al., 2014). As for IFN-γ, considered the hallmark of the Th1 immune response, it enhances the production of IL-6 mRNA post-LPS treatment in monocytes of mammals (Biondillo et al., 1994). In bacteria-stimulated toads, there was also higher gene expression of IL-10, an anti-inflammatory cytokine with a central role in regulating the immune response by inhibiting the production of pro-inflammatory cytokines, preventing it from causing damage to host tissues (Commins et al., 2010; Zimmerman et al., 2014). Other studies with anurans reported increased expression of IL-10 in individuals treated with LPS after 6, 18, and 24 h (Qi et al., 2015; Ferreira et al., 2021; Floreste et al., 2022). In later hours, when the inflammatory response reaches its resolution phase, the expression of IL-10 may increase even more while that of pro-inflammatory cytokines is severely reduced, but this still needs to be better characterized in amphibians.

There was no treatment effect for BKA and plasma T levels. We expected increased BKA in animals injected with heat-killed bacteria compared to the saline and control groups, resulting from increased activity of complement system proteins (Assis et al., 2013; Gardner et al., 2020). Interestingly, though not a statistically significant difference, we observed that toads in the A3 group (injected with the highest concentration of bacteria) presented less variation and higher BKA than the control group. Possibly, for toads in A3, there was a higher activity in the plasma of innate immune components, such as humoral proteins and natural antibodies, acting upon the recognition of PAMPs (Gardner et al., 2020; Titon Junior et al., 2021). Regarding T levels, we predicted a decrease in bacteria-stimulated animals since an inflammatory assemblage would induce suppression of the hypothalamus-pituitary-gonadal axis activity, as observed in other studies with anurans after immune challenge with LPS (Gregorio et al., 2018; Titon Junior et al., 2021). The lack of modulation of BKA and T by heat-killed bacteria in this study might be associated with the short time between administering the

injection and collecting the blood sample. Indeed, studies with other anuran species only reported changes in these parameters in 20 and 24 h after applying inflammatory stimulus (Gardner et al., 2020; de Figueiredo et al., 2021). Studies aiming to evaluate the effects of similar inflammatory stimuli for these parameters should explore longer intervals of time between initial stimulation and sampling.

5. Conclusions

This study evaluated the immune and endocrine effects induced by stimulation with heat-killed *A. hydrophila* in adult anurans. Toads injected with heat-killed bacteria showed decreased plasma MEL levels, increased NLR, plasma CORT levels, and mRNA levels of the cytokines IL-1β, IL-6, IL-10, and IFN-γ. Similar patterns were observed in other studies involving different groups of vertebrates and inflammatory stimuli, especially LPS, a component found in the outer membrane of *A. hydrophila*. Immune-challenged toads showed an integrated response between physiological and molecular parameters typical of the onset of inflammatory response when there is the initial assemblage to deal with the infection. Future research should concentrate on the later stages of the immune response when it is expected to occur a shift in the production and expression of some of these parameters to better understand the complete progression of the inflammatory assemblage in amphibians.

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CRediT authorship contribution statement

Patrício G. Garcia Neto: Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Stefanny C.M. Titon:** Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Conceptualization. **Sandra M. Muxel:** Investigation. **Braz Titon:** Writing – original draft, Visualization, Investigation, Formal analysis. **Aymam C. de Figueiredo:** Writing – original draft, Investigation. **Felipe R. Floreste:** Writing – original draft, Investigation. **Alan S. Lima:** Investigation. **Vania R. Assis:** Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Conceptualization. **Fernando R. Gomes:** Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Raw data - Bacteria 2023 (Original data) (Mendeley Data)

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpa.2024.111606.

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