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Full Length Article

Short-term stressors and corticosterone effects on immunity in male toads (*Rhinella icterica*): A neuroimmune-endocrine approach

Stefanny Christie Monteiro Titon *, Braz Titon Junior, Fernando Ribeiro Gomes, Vania Regina Assis

Departamento de Fisiologia, Instituto de Biociências, Universidade de São Paulo, São Paulo, São Paulo, Brazil

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ABSTRACT

In the last decades, it is growing the idea that stress-induced immunomodulation is bimodal: with acute stress associated with enhancing effects while chronic stress with suppressive effects. However, the immune-endocrine interactions and its implications are often overlooked in ectotherms. We investigated the impact of corticosterone (CORT) treatment and short-term stressors on CORT plasma levels and the immunity of male toads (*Rhinella icterica*), using three distinct protocols: restraint, immune challenge (with lipopolysaccharide, LPS), and CORT transdermal application (TA). Our results showed increased CORT and neutrophil: lymphocyte ratio (NLR) regardless of the stress input (restraint, LPS challenge) or CORT TA. In the meantime, the bacterial killing ability (BKA) was not affected by any treatment, suggesting this immune parameter might be a more constitutive and robust response. Interestingly, the cellular immune response showed distinct patterns. Increased phagocytosis of blood leukocytes and phytohemagglutinin edema followed LPS and CORT TA (15 μg), respectively. In contrast, the phagocytosis of peritoneal leukocytes decreased after CORT TA (1 and 10 μg), indicating that short-term increases in CORT levels might impair local immune function. Such differences in cellular immunity might also be associated with CORT doses or the interaction between CORT and other immune mediators, such as melatonin, testosterone, and cytokines. Overall, our results highlight the immune-enhancing effects of the acute stress response and CORT TA, and the complexity of the immune-endocrine interaction in anurans. It also highlights the relevance of investigating distinct contexts for CORT increase arising from different situations, as well as diverse immune components for a better understanding of the stress-induced immunomodulation.

1. Introduction

Stress stimuli can be from distinct nature, in which a situation can be perceived as a stressor (psychological or neurogenic stimulus) or a perturbation in the internal state (physiological stimulus) can be the stressor (Dhabhar, 2014). While the input can come from different situations, the stressors activate biological stress responses involving the release of local and systemic mediators, leading to an integrated physiological response (Goldstein and Mcewen, 2002; Pakos-Zebrucka et al., 2016; Sapolsky et al., 2000). One of the most known stress-induced and glucocorticoid-mediated effects is the immunomodulation, which plays a central role in individual survival (Dhabhar, 2014; Sapolsky et al., 2000). In fact, all immune cells have glucocorticoid receptors and are modulated by these hormones during a stress response (Wiegers and Reul, 1998). Therefore, an extensive literature has been dedicated to describe the immune-enhancing and protective effects of the acute/short-term stress and the suppressive and dysregulatory effects of chronic/long-term stress exposure, particularly in mammals and birds (reviewed in: Dhabhar, 2014; Martin, 2009).

The stress response duration and intensity have been described as a key determinant for the stress and glucocorticoid-mediated immunological enhancing *vs.* suppressive effects (Dhabhar, 2014, 2018). Acute/short-term stress-induced immune-enhancing effects have been reported in studies investigating the impact of different stressors, such as restraint, exercise, and exposure to predators (reviewed in: Dhabhar, 2018, 2014). In amphibians, handling and restraint can increase the immune function, e.g., phagocytosis activity and plasma bacterial killing ability (BKA) (Graham et al., 2012b; Hopkins and DuRant, 2011). Besides, toads submitted to an immune challenge with lipopolysaccharide (LPS) had increased plasma corticosterone (CORT) levels and BKA,

* Corresponding author. , Departamento de Fisiologia – Instituto de Biociências, Universidade de São Paulo, Rua do Matão, trav. 14, 101, 05508-090, São Paulo, Brazil.

E-mail address: stefannychristie@gmail.com (S.C.M. Titon).

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highlighting the immunoprotective effect of the acute stress response (Gardner et al., 2020). Additionally, high intensity stressors and exposure to supraphysiological concentrations of CORT (values above the highest ones under natural and intense stressful situations already reported for the species) or chronic/long-term stress have been associated with immunosuppressive/dysregulatory effects in vertebrates (Dhabhar, 2014; Dhabhar and McEwen, 1997; Martin et al., 2005). In this context, stressors eliciting extremely high CORT levels often induce immunosuppression (Dhabhar, 2014, 2018). Namely, *Rhinella icterica* toads subjected to restraint with movement restriction showed the highest CORT levels and neutrophil: lymphocyte ratio (NLR), a measure commonly related to the stress response, associated with the lowest BKA, when comparing with toads under restraint without movement restriction (Assis et al., 2019).

Complementary evidence has been provided by studies manipulating CORT to determine its effects on immunity. Adrenalectomized mammals, for example, show increased skin cell-mediated immunity when treated with physiological doses of CORT (Dhabhar and McEwen, 1996; Dhabhar and Viswanathan, 2005). In anurans, studies using CORT transdermal application (TA) and waterborne solution showed stimulatory effects on the phagocytosis activity of blood leukocytes (Assis et al., 2017; Falso et al., 2015). Additionally, *R. marina* toads treated with CORT TA showed increased gene expression of inflammatory mediators following LPS exposure (Gardner et al., 2018), highlighting the immunoprotective effects of an acute increase in CORT. In contrast, daily CORT TA for 20 days decreased the skin cell-mediated immunity in *R. ornata* toads (Titon et al., 2019). In salamanders, chronically increased CORT, induced by silastic implant, impaired wound healing (Thomas and Woodley, 2015), demonstrating that keeping high CORT levels for an extended period can compromise amphibian's immune response.

Despite some studies describing acute and chronic stress-induced increases in CORT levels associated with immunomodulation in amphibians, the immune-enhancing effects of the acute stress response and its implications are often overlooked in anurans. Herein, we investigated the impact of CORT treatment and short-term stressors on CORT levels and immunity of male toads (*Rhinella icterica*), using three distinct protocols: restraint, immune challenge, and CORT TA. The restraint protocol was used to generate a neuroimmune-endocrine response. The LPS was used to induce an immune response, followed by activation of the hypothalamic-pituitary-interrenal axis, culminating in elevation of CORT. Additionally, the CORT TA was used to directly promote CORT elevation in the circulation (physiological and supraphysiological levels). We expect increased values of stress biomarkers (CORT and NLR) in response to all treatments with different outcomes on immunity (phagocytosis activity and phytohemagglutinin edema). Immune enhancing effects for the neuro-endocrine stressor (restraint), immune challenge, and a low dose of CORT TA, contrasting with suppression effects from high doses of CORT TA.

2. Materials and methods

2.1. Study sites and animal collection

Adult male toads (*Rhinella icterica*) were collected at the City of São Luis do Paraitinga (23°13′23″ S; 45°18′38″ W), São Paulo/Brazil, in February 2012 (N = 14); January (N = 20) and November (N = 30) 2013, March 2017 (N = 24); September (N = 14) and December (N = 19) 2018, and in Botucatu (22°53′21.1″S 48°28′20.1″W), São Paulo/Brazil, in July 2019 (N = 24).

Toads were collected under license from the Instituto Chico Mendes de Conservação da Biodoversidade (ICMBio n° 29896). The Ethics committee of the Bioscience Institute of the University of Sao Paulo approved all procedures for this study (CEUA: n° 142/2011; 242/2016; 254/2016 and 323/2018).

2.2. Field procedures

Toads were visually located, and hand captured. Blood samples were collected in the field only for the toads submitted to the restraint challenge (Exp. 1 and 2) and after the LPS challenge in the field (Exp. 3). Animals submitted to the restraint challenge were weighed (0.01 g) and measured (0.01 mm) after the restraint, and then returned to the field. Individuals collected for the LPS challenge in the field (Exp. 3) were processed in the field. The individuals collected for the Exp. 4–7 were transported to the laboratory for the respective experimental procedures. Detailed information on data collection and experimental methods are in Table 1.

2.3. Restraint challenge (experiments 1 and 2)

Following blood collection (200 μl) for baseline (field) measures, the same toads were subjected to a restraint challenge (using wet cloth bags) for 1 h (Exp. 1: December 2018, N = 19) or 24 h (Exp. 2: January 2013, N = 10), according to (Assis et al., 2019; Titon et al., 2019). After 1 or 24 h, toads were bled again (200 μl) to reassess the variables measured at baseline (CORT, BKA, and NLR).

2.4. Lipopolysaccharide (LPS) challenge (experiments 3 and 4)

We first performed an LPS challenge in the field (Exp. 3), with toads

Table 1
Detailed information on data collection, experimental procedures, statistical comparisons, and studied parameters of *Rhinella icterica* toads.

Year	Experiment	Comparison	Parameters	Analysis
	Experiment 1	Field *vs.* 1 h	CORT	Wilcoxon
2018	Restraint	post-restraint	NLR	Paired *t*-test
	1 h	(repeated measure)	BKA	Wilcoxon
	Experiment 2	Field *vs.* 24 h	CORT	Wilcoxon
2013	Restraint	post-restraint	NLR	Paired *t*-test
	24 h	(repeated measure)	BKA	Wilcoxon
	Experiment 3	LPS *vs.* SAL	CORT	Independent *t*-test
2018	LPS	LPS: 2 mg/kg	NLR	Independent *t*-test
	2 h post-injection	(independent sample)	BKA	Independent *t*-test
	Experiment 4	LPS *vs.* SAL	CORT	ANOVA
2019	LPS	10 a.m. *vs.* 10 p.m.	NLR	ANOVA
	2 h post-injection	LPS: 2 mg/kg	BKA	ANOVA
		(independent sample)	PP	ANOVA
	Experiment 5	Placebo *vs.* CORT-treated	CORT	Independent *t*-test
2012	CORT TA	P *vs.* C15	NLR	Mann-Whitney
	1 h post-application	(independent sample)	BKA	Independent *t*-test
			PHA	Independent *t*-test
	Experiment 6	Placebo *vs.* CORT-treated	CORT	Mann-Whitney
2013	CORT TA	P *vs.* C15	NLR	Mann-Whitney
	1 h post-application	(independent sample)	BKA	Mann-Whitney
	Experiment 7	Placebo *vs.* CORT-treated	CORT	ANOVA
2017	CORT TA	P *vs.* C1 and C10	NLR	ANOVA
	1 h post-application	(independent sample)	BKA	ANOVA
			PP	ANOVA

Abbreviation as follow: **CORT TA:** corticosterone transdermal application; **CORT:** corticosterone plasma levels; **NLR:** neutrophil: lymphocyte ratio; **BKA:** bacterial killing ability; **SAL:** saline; **LPS:** lipopolysaccharide; **PHA:** phytohemagglutinin edema; **PP:** phagocytosis percentage; **P:** placebo; **C1:** corticosterone 1 μg; **C10:** corticosterone 10 μg; **C15:** corticosterone 15 μg.

collected in September 2018. Immediately after being caught in the field, toads were weighed to adjust the volumes of LPS (2 mg/kg) or saline injections, according to Gardner et al. (2018). Individuals were randomly selected to receive an LPS or saline treatment throughout the night between 10:00 and 11:20 p.m. (N = 7 for LPS, and N = 6 for saline). After intraperitoneal injections, toads were placed in a plastic bin (20 L; 43 × 28.5 × 26.5 cm) with free water access. The lids of the bins had holes to allow air circulation. Two hours post-injection, individuals were bled (600 μl), measured, and then killed by decapitation.

Toads from experiment 4 were collected in July 2019, and kept in the laboratory for eight days prior to the experiment, in a bin (20 L), inside a controlled room: 21 ± 1 °C and photoperiod of 11:13LD (lights on 6:00 a.m.). Toads were weighed and measured two days before the experiment to distribute the groups evenly. On the 9th day, individuals were intraperitoneally injected with LPS (2 mg/kg), or saline at 10:00 a.m. or 10:00 p.m. (N = 6 in each group) and then returned to their bins. Two hours post-injection, individuals were bled (600 μl), and then killed by decapitation.

2.5. Corticosterone transdermal application (experiments 5, 6 and 7)

To assess the effects of distinct CORT concentrations on immunity, we performed three experiments using the CORT TA protocol. The first two with the same CORT concentration (15 μg, CORT Sigma – 27840), but investigating BKA and NLR in one (Exp. 6) and another, including BKA, NLR, and a PHA challenge (Exp. 5). The third CORT TA experiment with two CORT doses (1 and 10 μg) included investigating their effects on BKA, NLR, and an *in vivo* phagocytosis test (Exp. 7).

Independent of the experiment, all toads were kept under constant temperature 22 ± 2 °C and photoperiod 13:11LD (lights turn on 06:30 a.m.), in individual plastic bins (20 L) with free water access, and being weekly fed and cleaned. All animals were weighed and had their snout-vent length (SVL) measured two days before the TA. Inside the placebo group, toads always received one drop of 5 μl sesame oil on their backs using a micropipette. Inside the CORT-treated group, toads received one drop of 5 μl working solution adjusted to deliver the desired concentration (a mixture of sesame oil + CORT), as described in (Assis et al, 2015, 2017).

The first CORT TA protocol was performed with toads collected in 2012 (Exp. 5), under captivity for three months. Animals were then divided into two groups: placebo (P; N = 15) and CORT-treated with 15 μg (C15; N = 15). The TA was performed at 5 p.m. for 13 consecutive days. At the end of the 13th day, 1 h after the TA, a blood sample was taken (200 μl) to assess immune parameters and CORT. After that, individuals were submitted to a phytohemagglutinin (PHA) challenge (see section 2.12). The second CORT TA was performed with individuals collected in 2013 (Exp. 6), under captivity for one week. On the 8th day in captivity, toads were divided into two groups: placebo (P; N = 7) and CORT-treated with 15 μg (C15; N = 7) at 10:30 a.m. The third CORT TA was performed with individuals collected in 2017 (Exp. 7), under captivity for two months. Toads were then divided into three groups: placebo (P; N = 8) and CORT-treated with 1 μg (C1; N = 8) or 10 μg (C10; N = 8). The TA happened at 10:30 a.m. in a single day. In Exp. 6 and 7, toads were bled (200 μl) 1 h after the TA to assess immune parameters and CORT. For Exp. 7, after that, toads were submitted to an *in vivo* phagocytosis assay (see section 2.10).

2.6. Blood collection and isolation

Blood samples were collected by cardiac puncture using a heparinized 1 ml syringe and 26Gx1/2" needle within 3min of animal capture and handling. Blood samples were identified and kept on ice (<2 h), blood smears were performed, and then the blood was centrifuged (604 g × 4 min, 23 °C) to isolate the plasma. Plasma samples were stored at −80 °C freezer for CORT quantification and BKA assays.

2.7. Neutrophil: lymphocyte ratio (NLR)

A drop of blood was used to perform a blood smear for each individual. The slide was dried for 30 min, then fixed with methanol, stained with Giemsa solution (Merck KGaA, Darmstadt, Hessen, Germany), and observed under an optical microscope (100X objective, using oil immersion – Nikon E200, 104c). One hundred leukocytes were counted on each slide and classified based on morphology as neutrophils, lymphocytes, monocytes, eosinophils, and basophils. The NLR was calculated as the number of neutrophils divided by the number of lymphocytes on each slide (Davis et al., 2008).

2.8. Bacterial killing ability (BKA)

This assay was performed against two pathogens: *Escherichia coli* (Assis et al., 2013) or *Aeromonas hydrophila* (Moretti et al., 2019). For Exp. 1, 3, 4, and 7, the BKA was conducted with *A. hydrophila,* and for Exp. 2, 5, and 6, the BKA assay was performed with *E. coli*. Briefly, plasma (10 μl) diluted in amphibian Ringer's (NaCl 6.5 g; KCl: 1 g; NaH_2PO_4: 0.1 g; $CaCl_2$: 1.125 g; $NaHCO_3$: 0.2 g; $C_6H_{12}O_6$: 2 g; diluted in 1 L of distilled water) solution (190 μl) were mixed with 10 μl of *A. hydrophila* (2.5×10^5 microorganisms) or *E. coli* (5×10^4 microorganisms). Positive controls consisted of 10 μl of *A. hydrophila* or *E. coli* in 200 μl of Ringer's solution and negative control 210 μl of Ringer's solution. Samples, positive and negative controls were incubated at 37 °C for 1 h. After the incubation, 500 μl of tryptone soy broth was added to each sample. The suspensions were thoroughly mixed, and 300 μl of each was transferred (in duplicate) to a 96 wells microplate. The microplate was incubated at 37 °C for 1 h (*A. hydrophila*) or 2 h (*E. coli*), and after that, the optical density (OD) of the samples was measured hourly in a plate spectrophotometer (*A. hydrophila*: 595 nm; *E. coli*: 600 nm), totaling four readings. The plasma BKA was evaluated at the beginning of the bacterial exponential growth phase and calculated according to the formula: 1 - (sample OD/positive control OD), representing the proportion of killed microorganisms in the samples compared to the positive control.

2.9. In vitro phagocytosis assay from blood leukocytes

This assay was conducted according to (Titon et al, 2017, 2019). Blood samples (200 μl) from individuals of LPS challenge in the lab (Exp. 4) were decanted (30min, 23 °C), the leukocytes were collected and transferred to a 15 ml conical tube with 10 ml of 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). Leukocyte concentration was determined by the Trypan Blue exclusion method (viable cells exceeded 95%). The leukocyte suspension was centrifuged (259 g × 9 min, 4 °C), the supernatant discarded, and the cells were resuspended in APBS to 1×10^6 cells/1 ml APBS.

Thereafter, 200 μl of the resuspended cells (2×10^5 cells) were diluted in 800 μl of APBS. Then 100 μl of zymosan-CFSE work solution (1×10^6 particles) (60 mg zymosan A from *Saccharomyces cerevisiae*, Sigma-Aldrich Z4250, labeled with 25 μg/100 μl green fluorescence carboxyfluorescein succinimidyl ester - CFSE, Sigma 21888F (Dagher et al., 2018)) was added to the samples. Samples were protected from light, incubated in an orbital shaker for 1 h, 22 °C (mean thermal preference for the toads, Moretti et al., 2018). After incubation, the reactions were stopped by adding 2 ml of cold (4 °C) EDTA (6 mM) followed by subsequent centrifugation (259 g × 7 min, 4 °C). The supernatant was then discarded, and 200 μl of cold (4 °C) paraformaldehyde (PFA 1%) was added. Samples were then kept for 1 h at 4 °C for cell fixation. After this period, 500 μl of APBS solution was added, and samples were centrifuged (259 g × 7 min, 4 °C). The supernatant was discarded, and 100 μl of APBS solution was added for flow cytometry.

2.10. In vivo phagocytosis assay from peritoneal leukocytes

This assay was performed for toads from the CORT TA with two doses

(Exp. 7), according to (Titon et al., 2017; Young et al., 2004), with modifications. After blood collection, toads were intraperitoneally injected with APBS + zymosan- CFSE (5×10^7 particles in 500 μl/150 g). The zymosan concentration was determined by previous studies conducted with this same species, in which we recovered the mean of 1×10^7 cell/10 ml APBS from the lavage fluid *per* individual (Titon et al., 2018). One hour after zymosan injection, toads were euthanized by immersion in a lethal solution of benzocaine (0.2%) followed by spinalization. After euthanasia, the abdomen was cleansed with ethanol 70%, and a small incision was made into the peritoneal cavity with a sterile surgical instrument. Using a sterile Pasteur pipette, 10 mL of sterile APBS was injected into the peritoneum, and the abdominal region of the toad was carefully massaged. Lavage fluid (resulting solution of APBS + cells) was then removed with a sterile Pasteur pipette and placed in a 50 ml conical tube. Thereafter, reactions were stopped adding 20 ml of EDTA (6 mM, 4 °C) followed by subsequent centrifugation (259 g × 7 min, 4 °C). The supernatant was discarded, then the cells were resuspended in 2 ml of cold (4 °C) APBS and transferred to a polypropylene centrifuge tube (8 ml), followed by centrifugation (259 g × 7 min, 4 °C) (this process was repeated once again). The supernatant was discarded, and 400 μl of cold (4 °C) PFA (1%) was added. Samples were then kept for 1 h at 4 °C for cell fixation. After this period, 1 ml of APBS solution was added, and samples were centrifuged (259 g × 7 min, 4 °C). The supernatant was discarded, and 100 μl of APBS solution was added for flow cytometry.

2.11. Phagocytosis quantification

Phagocytosis from peritoneal and blood leukocytes was acquired on an AMNIS Flowsight flow cytometer (Merck-Millipore, Darmstadt, Germany) interfaced with a DELL computer. Data from 20,000 events were obtained, utilizing the 488 nm laser, at a 20x magnification, using the INSPIRE software. Direct measurements of mean fluorescence in the green channel were recorded as phagocytosis. Phagocytosis percentage (PP) was recorded as the number of cells engulfing at least one zymosan particle (with green fluorescence divided by the total number of cells [multiplied by 100]). Acquired data were analyzed using IDEAS analysis software (EMD Millipore) version 6.1 for windows.

2.12. Phytohemagglutinin (PHA) swelling assay

This assay was performed to assess the cell-mediated innate immune response at the end of the CORT TA during the morning (Exp. 5), as described in (Assis et al., 2015). The fleshy hind base of the right foot was injected with 10 μl of a PHA (20 mg PHA diluted in 1 ml of sterile saline solution; PHA Sigma L8754) using a 10 μl syringe and 30Gx1/2" needles. As a control, the fleshy hind base of the left foot was injected with 10 μl of sterile saline. The thickness of each injected hind fleshy base of each animal was measured (three times) with a thickness gauge (Digimess, accuracy 0.01 mm) before and 12 h after PHA and saline injections. The edema in response to PHA and saline was calculated from the proportional change in foot thickness before and 12 h after injection. The PHA edema was calculated as the PHA relative swelling minus the saline relative swelling.

2.13. Corticosterone assays

Plasma samples were extracted with ether, and CORT levels were determined using ELISA kits (CORT number 501320; Cayman Chemical), according to the manufacturer's instructions and previous studies conducted with *R. icterica* species (Assis et al. 2017). The mean values for intra and inter-assay variations were 6.86% and 8.52%, respectively. The sensitivity of the assays was 32.94 pg/ml.

2.14. Statistical analyses

Shapiro-Wilk normality test and Levene's test for homogeneity of variance were performed to determine parametric or non-parametric analyses within each experiment. For restraint (exp. 1 and 2), we used paired *t*-test for variables that fit the parametric assumptions or Wilcoxon for the non-parametric ones. Independent samples *t*-test were used for parametric variables, and Mann-Whitney for non-parametric ones for LPS in the field (Exp. 3) and CORT TA C15 (Exp. 5 and 6). Sets of ANOVAs were used to investigate the treatment effect for LPS at the laboratory (Exp. 4) and CORT TA with two doses (Exp. 7). For the LPS at the laboratory (Exp. 4), CORT, NLR, BKA, and PP were used as dependent variables, treatment (LPS and Saline), and period (10 a.m. and 10 p.m.) were used as factors. For the CORT TA with two doses (Exp. 7), CORT, NLR, BKA, and PP were used as dependent variables, and treatment (placebo, C1, and C10) was used as a factor. All statistical analyses were performed in IBM SPSS Statistics 26.

It is worth to mention that our main goal here was to show different short-term stressors impacts on corticosterone plasma levels and different immune responses. Each experiment has its control, which means we are not making comparisons among experiments but highlighting the patterns that arose from each one.

3. Results

All treatments increased plasma CORT levels (Fig. 1, Tables 2–4), although the statistical difference was unclear for the LPS in the field (Exp. 3, Fig. 1C). For the NLR, increased values were also described after almost all treatments, except for Restraint 1 h (Exp. 1) and CORT TA C15 (Exp. 5) (Fig. 2, Tables 2–4). The BKA was not affected by any treatment (Fig. 3, Tables 2–4). Regarding cellular immune response, the blood PP was affected by the interaction treatment*period in toads submitted to the LPS challenge in the laboratory (Exp. 4, Fig. 4A, Table 4). The PHA edema increased after CORT TA in the C15 group, compared with the placebo group in CORT TA C15 (Exp. 5, Fig. 4B, Table 2). The peritoneal PP was affected by treatment in CORT TA with two doses (Exp. 7), with both C1 and C10 displaying lower peritoneal PP than the placebo group (Fig. 4C, Table 4).

4. Discussion

Increased stress biomarkers (CORT and NLR) were observed in *R. icterica* following different challenges, indicating that distinct stress inputs promoted similar CORT and NLR outcomes. When restrained, individuals perceived the stressor and generated a stress response that included increased CORT and NLR (Assis et al., 2019; Davis and Maney, 2018). LPS-induced increases in CORT and NLR were also expected, since LPS activates the hypothalamic-pituitary-interrenal axis, culminating in glucocorticoid release, promoting the immune cell redistribution (Bornstein et al., 2006; Cain and Cidlowski, 2017). Additionally, CORT TA was a handy tool enabling direct CORT increases and allowing a more direct study of CORT-mediated effects (Assis et al., 2017; Dhabhar and McEwen, 1999). Similar increases in NLR (e.g., twice as high) were found following the three CORT doses (C1, C10, and C15), showing that CORT evoked immunoredistribution independently of the CORT levels achieved by TA (physiological or supraphysiological). The use of these combined protocols allowed an integrative and broad comprehension of the stress and CORT-mediated impact on the immune cell redistribution, demonstrating that augmented CORT and NLR are conservative responses following acute stress in *R. icterica*.

Increased glucocorticoid levels associated with immune-enhancing effects following acute stressors are postulated as adaptive, increasing protection under threat or challenging conditions, such as fight or flight responses (Dhabhar, 2018). Accordingly, immune-enhancing effects of acute stressors and CORT treatment have been widely demonstrated in mammals (Dhabhar and McEwen, 1997; Dhabhar and Viswanathan, 2005; Hernández-Arciga et al., 2018), reptiles (Neuman-Lee and French, 2017), fishes (reviewed in: Tort et al., 2019), and amphibians (Assis et al., 2017; Hopkins and DuRant, 2011). This is corroborated by

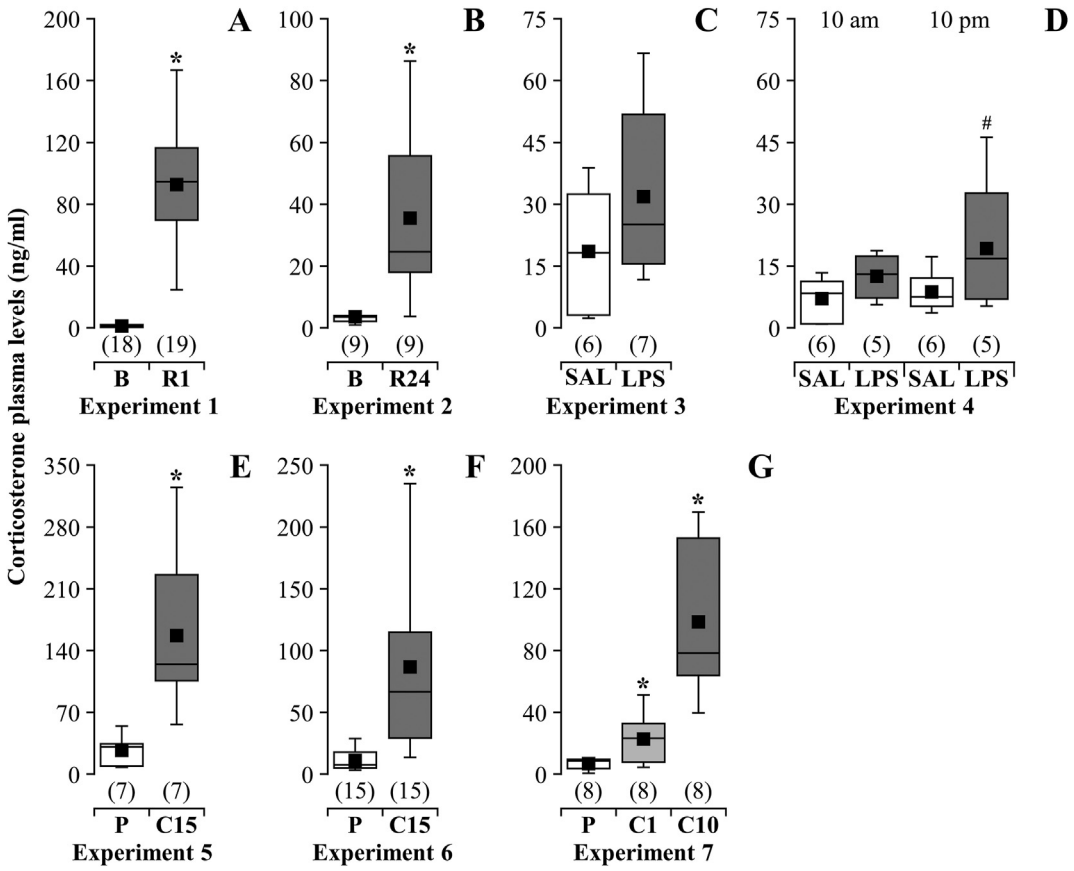

Fig. 1. Corticosterone plasma levels following restraint, LPS, and corticosterone transdermal application in *Rhinella icterica* toads. (A) Exp. 1: Restraint – Field *vs*. 1 h post-restraint, (B) Exp. 2: Restraint – Field *vs*. 24 h post-restraint, (C) Exp. 3: LPS *vs*. Saline – Field, (D) Exp. 4: LPS *vs*. Saline, 10 a.m. and 10 p.m., (E) Exp. 5: corticosterone transdermal application C15, placebo *vs*. corticosterone 15 μg, (F) Exp. 6: corticosterone transdermal application C15, placebo *vs*. corticosterone 15 μg, and (G) Exp. 7: corticosterone transdermal application at two doses, placebo *vs*. corticosterone 1 μg *vs*. corticosterone 10 μg. The boxplot inside lines indicates medians, lower and upper borders represent 1st and 3rd quartiles, respectively. Black squares indicate means, and whiskers represent upper and lower limits of 1.5 times inter-quartile range. The N is indicated below bars in parentheses. "*" denotes differences $P \leq 0.05$. "#" represents a trend $P = 0.063$.

Table 2
Detailed information regarding statistical analysis of experiments 1, 2, 3, 5, and 6 of *Rhinella icterica* toads.

Experiment	Variable	Test	Z/t	DF/U	P	MD
Restraint (Experiment 1)	CORT	Paired *t*-test	−11.128	17	**< 0.001**	−91.406
	NLR	Wilcoxon	−1.374		0.170	−0.010
	BKA	Wilcoxon	−1.351		0.177	0.041
Restraint (Experiment 2)	CORT	Wilcoxon	−2.666		**0.008**	−31.970
	NLR	Paired *t*-test	−3.074	9	**0.013**	−0.504
	BKA	Wilcoxon	−1.897		0.058	0.122
LPS - Field (Experiment 3)	CORT	Independent *t*-test	−1.341	11	0.207	−13.339
	NLR	Independent *t*-test	−3.675	11	**0.004**	−0.072
	BKA	Independent *t*-test	−0.182	11	0.859	−0.004
CORT TA C15 (Experiment 5)	CORT	Independent *t*-test	−3.754	6.39	**0.008**	−130.036
	NLR	Mann-Whitney	−0.802	13.00	0.423	−0.048
	BKA	Independent *t*-test	−1.909	6.66	0.100	−0.301
	PHA	Independent *t*-test	−2.316	10	**0.043**	−0.353
CORT TA C15 (Experiment 6)	CORT	Mann-Whitney	−4.376	7	**0.000**	−150.996
	NLR	Mann-Whitney	−2.773	41.50	**0.006**	−0.345
	BKA	Mann-Whitney	−1.467	71.50	0.142	−0.198

Abbreviation as follow: **CORT TA:** corticosterone transdermal application; **CORT:** corticosterone plasma levels; **LPS:** lipopolysaccharide; **NLR:** neutrophil: lymphocyte ratio; **BKA:** bacterial killing ability; **PHA:** phytohemagglutinin edema; **PP:** phagocytosis percentage.

LPS-induced immune augmentation in the present study. NLR and phagocytosis activity of blood cells were both increased following LPS exposure in *R. icterica* toads. LPS exposure promotes the enhancement of several aspects of immune function, including phagocytosis and BKA activity, recruitment of cells to the inflammatory site, and proinflammatory gene expression (e.g., IL1β, TNF), in several vertebrates (Chiaramonte et al., 2019; Connor et al., 2005; Millet et al., 2007). In anurans, Gardner et al. (2018) described LPS-induced increase gene expression of proinflammatory cytokines in *R. marina*. Another study demonstrated *R. jimi* toads subjected to CORT TA had a more pronounced PHA response, associated with a decrease in the time for inflammatory resolution (Madelaire et al., 2019). Besides, Dhabhar (2002) describes

Table 3
Detailed information regarding statistical analysis of LPS in the laboratory (exp. 4) of *Rhinella icterica* toads.

Variable	Source	Type III SS	df	MS	F	P
CORT	Corrected Model	469.526	3	156.509	2.012	0.148
	Intercept	3075.755	1	3075.755	39.546	< **0.001**
	Period	95.167	1	95.167	1.224	0.283
	Treatment	347.319	1	347.319	4.466	**0.049**
	Period * Treatment	36.788	1	36.788	0.473	0.500
	Error	1399.990	18	77.777		
	Total	4784.308	22			
	Corrected Total	1869.515	21			
NLR	Corrected Model	0.073	3	0.024	17.383	< **0.001**
	Intercept	0.423	1	0.423	303.285	< **0.001**
	Period	0.001	1	0.001	0.616	0.442
	Treatment	0.069	1	0.069	49.671	< **0.001**
	Period * Treatment	0.001	1	0.001	0.668	0.424
	Error	0.027	19	0.001		
	Total	0.514	23			
	Corrected Total	0.099	22			
BKA	Corrected Model	0.028	3	0.009	2.550	0.086
	Intercept	13.286	1	13.286	3639.405	< **0.001**
	Period	0.019	1	0.019	5.296	**0.033**
	Treatment	0.000	1	0.000	0.073	0.790
	Period * Treatment	0.009	1	0.009	2.484	0.132
	Error	0.069	19	0.004		
	Total	13.397	23			
	Corrected Total	0.097	22			
PP	Corrected Model	10.838	3	3.613	3.143	**0.048**
	Intercept	117.882	1	117.882	102.566	< **0.001**
	Period	0.134	1	0.134	0.116	0.737
	Treatment	3.278	1	3.278	2.852	0.107
	Period * Treatment	7.426	1	7.426	6.461	**0.019**
	Error	22.987	20	1.149		
	Total	151.707	24			
	Corrected Total	33.824	23			

Abbreviation as follow: **CORT TA:** corticosterone transdermal application; **CORT:** corticosterone plasma levels; **NLR:** neutrophil: lymphocyte ratio; **BKA:** bacterial killing ability; **PHA:** phytohemagglutinin edema; **PP:** phagocytosis percentage.

Table 4
Detailed information regarding the statistical analysis of CORT TA two doses (exp. 7) of *Rhinella icterica* toads.

Variable	Source	Type III SS	df	MS	F	P
CORT	Corrected Model	5.960	2	2.980	25.531	< **0.001**
	Intercept	40.864	1	40.864	350.082	< **0.001**
	Treatment	5.960	2	2.980	25.531	< **0.001**
	Error	2.451	21	0.117		
	Total	49.276	24			
	Corrected Total	8.412	23			
NLR	Corrected Model	0.029	2	0.015	4.604	**0.022**
	Intercept	0.704	1	0.704	221.168	< **0.001**
	Treatment	0.029	2	0.015	4.604	**0.022**
	Error	0.067	21	0.003		
	Total	0.800	24			
	Corrected Total	0.096	23			
BKA	Corrected Model	0.000	2	0.000	0.008	0.992
	Intercept	13.365	1	13.365	475.968	< **0.001**
	Treatment	0.000	2	0.000	0.008	0.992
	Error	0.590	21	0.028		
	Total	13.955	24			
	Corrected Total	0.590	23			
PP	Corrected Model	0.620	2	0.310	12.493	< **0.001**
	Intercept	32.728	1	32.728	1319.812	< **0.001**
	Treatment	0.620	2	0.310	12.493	< **0.001**
	Error	0.521	21	0.025		
	Total	33.868	24			
	Corrected Total	1.140	23			

Abbreviation as follow: **CORT TA:** corticosterone transdermal application; **CORT:** corticosterone plasma levels; **NLR:** neutrophil: lymphocyte ratio; **BKA:** bacterial killing ability; **PHA:** phytohemagglutinin edema; **PP:** phagocytosis percentage.

mammals treated with CORT prior to an immune challenge exposure exhibited a more pronounced inflammatory response; thus, demonstrating subjecting individuals to increased CORT can improve immune reactivity (Dhabhar, 2002). Therefore, during the acute increased CORT, immune-enhancement resulting from the stress response could favor the individual's survival by preparing the host to cope with a possible infection.

The increases we found in *R. icterica* CORT levels due to the LPS challenge and restraint were physiological and generated from the perception of the stressor and activation of the hypothalamic-pituitary-interrenal axis (maximum values of 66 ng/ml for LPS challenge, and 166 ng/ml for Restraint 1 h). While those generated by CORT TA ranged from physiological (maximum values of 51 ng/ml for C1, and 170 ng/ml for C10) to supraphysiological (maximum values of 325 ng/ml for C15), and are derived from the direct absorption of the hormone administered to the toads' skin. Direct effects of increased CORT on immune function and cell distribution are depicted as immune-enhancing when physiological CORT levels are achieved (Dhabhar et al., 1995; Dhabhar and McEwen, 1999; Falso et al., 2015; McCormick and Langkilde, 2014). Otherwise, immunosuppressive effects are often associated with supraphysiological CORT treatments in several vertebrate species (Berger et al., 2005; Dhabhar and McEwen, 1999; McCormick et al., 2015; Thomas and Woodley, 2015; Titon et al., 2019). However, decreased phagocytosis of the peritoneal cells was observed in our toads, even following the lowest CORT TA dose (C1, 1 μg). This decrease in PP was similar to that observed after a higher CORT dose (C10, 10 μg), which elicited physiological and restraint-comparable increases in CORT levels. Decreased immune function following acute stress and treatment with low CORT doses have been previously described for birds (Gao and Deviche, 2019; Shini et al., 2010) and fishes (Maule et al., 1989); demonstrating impaired immune function and reactivity can also be observed following acute physiological increases in CORT. It is important to consider that the *in vivo* phagocytosis in this study was performed by injecting zymosan in the peritoneal cavity, and cells were collected 1hr after stimulation. In this way, the observed immunomodulation might result from multiple interacting neuro-endocrine mediators (Barriga et al., 2002; Cain and Cidlowski, 2017; Markus et al., 2018). As an example, it is possible that increased CORT due to TA induced decreased local melatonin synthesis in the peritoneum, therefore, reducing phagocytosis activity by peritoneal phagocytes (Silva, 2017) at both low (C1) and high (C10) CORT doses.

Meanwhile, when a higher dose of CORT was used (15 μg), and

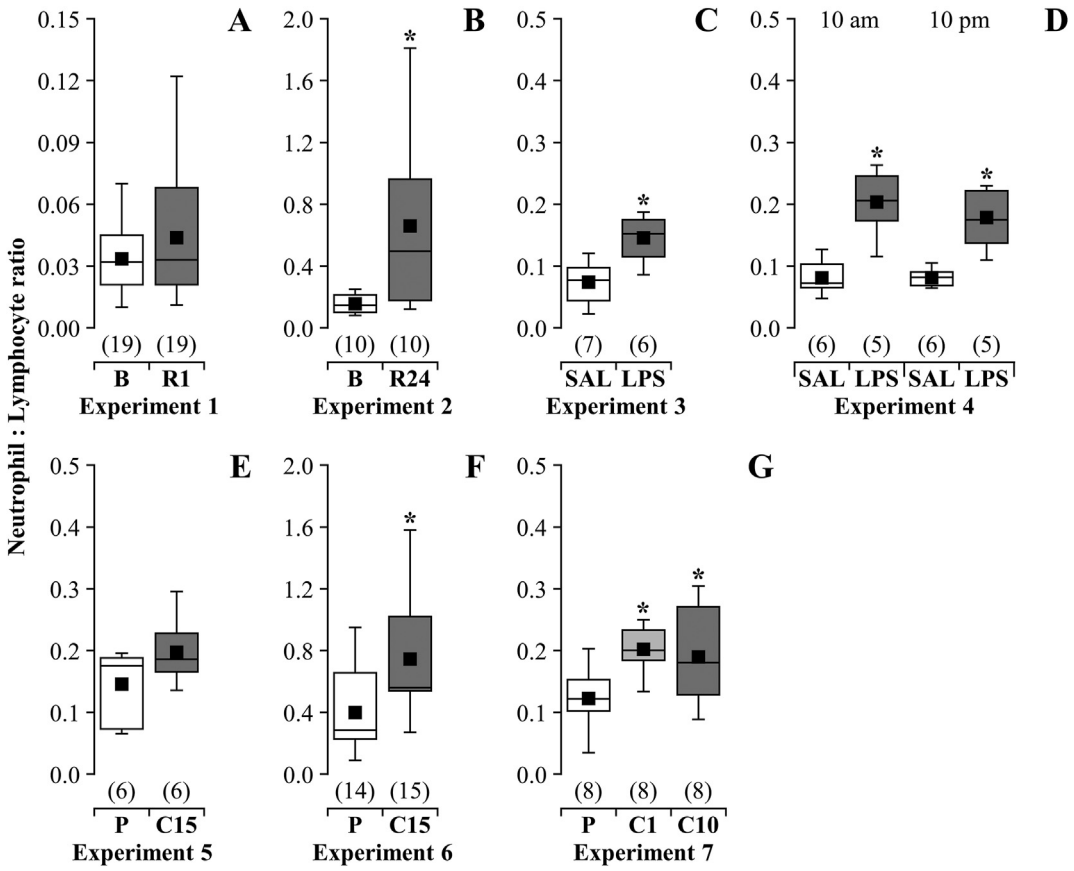

Fig. 2. Neutrophil: lymphocyte ratio following restraint, LPS, and corticosterone transdermal application in *Rhinella icterica* toads. (A) Exp. 1: Restraint – Field *vs*. 1 h post-restraint, (B) Exp. 2: Restraint – Field *vs*. 24 h post-restraint, (C) Exp. 3: LPS *vs*. Saline – Field, (D) Exp. 4: LPS *vs*. Saline, at 10 a.m. and 10 p.m., (E) Exp. 5: corticosterone transdermal application C15, placebo *vs*. corticosterone 15 μg, (F) Exp. 6: corticosterone transdermal application C15, placebo *vs*. corticosterone 15 μg, and (G) Exp. 7: corticosterone transdermal application at two doses, placebo *vs*. corticosterone 1 μg *vs*. corticosterone 10 μg. The boxplot inside lines indicates medians, lower and upper borders represent 1st and 3rd quartiles, respectively. Black squares indicate means, and whiskers represent upper and lower limits of 1.5 times inter-quartile range. The N is indicated below bars in parentheses. "*" denotes differences $P \leq 0.05$.

supraphysiological CORT levels were achieved, a different type of local cellular immune response related to inflammation (PHA edema) increased. Although general immunosuppressive effects are observed (Dhabhar and McEwen, 1997; French et al., 2007; Roberts et al., 2007), increased immunity have been described in supraphysiological CORT-treated anurans (Assis et al., 2017; Falso et al., 2015). Increased CORT can decrease central melatonin production reducing plasma melatonin levels (Fernandes et al., 2017). The decrease in melatonin allows the leukocyte adhesion and migration to an inflammatory site during situations of high CORT and infections (Markus et al., 2018). Therefore, the increased CORT in C15 might have inhibited central melatonin secretion (Fernandes et al., 2017), consequently favoring the leukocyte migration to the inflammatory site. Besides, another important hormone playing crucial immunomodulatory effects is testosterone (Foo et al., 2017). Testosterone plasma levels are usually decreased after increased CORT levels, including those elicited by the TA, and the reduction in testosterone might be directly related to impaired immunity in toads (Titon et al., 2018). Interestingly, by describing the decreased phagocytosis of peritoneal cells and increased PHA edema following CORT TA, we observed the CORT increase might interact with other immune mediators to promote the resulting immune outcome. Moreover, it highlights the use of diversified protocols is the best way to obtain more integrative results when investigating CORT-immune interactions.

Regarding the plasma BKA, no changes were observed following restraint, LPS challenge, and CORT TA in *R. icterica* toads. Several studies with lizards (Graham et al., 2012a; McCormick and Langkilde, 2014) and anurans (Assis et al, 2017, 2019, 2020; Madelaire et al., 2019; Titon et al., 2019) reported that the BKA is often sustained following acute stressors and short-term CORT TA, indicating this could be a more constitutive immune response in ectotherms. The proteins present in the plasma associated with BKA (e.g., lysozymes, C3-complex) circulate as inactive proteins. After a pathogen detection, it promotes its cleavage by activating the complement cascade (French and Neuman-Lee, 2012; Lubbers et al., 2017). Complement proteins target the cell membranes of bacteria and promote cell lysis (Ricklin et al., 2010); thus, sustained BKA could be an essential immune response associated with the first line of defense. It is possible that long-term stressors could have a more pronounced effect on plasma proteins by decreasing BKA response, a result previously found in toads (Titon et al, 2017, 2019), including *R. icterica* (Assis et al., 2015; Titon et al., 2018). Interestingly, differences in the BKA for the saline group were observed in experiment 4, in which increased BKA values were described at night. Daily variations in immunity are described for mammals (Scheiermann et al., 2013) and frogs (Titon et al., 2021), with increased immune functions observed at night for nocturnal species. Thus, increased BKA in the saline group at 10 p.m. could be associated with circadian variation in *R. icterica* toads.

5. Conclusions

We demonstrated restraint, LPS challenge, and CORT TA increased CORT, NLR, local (PHA edema), and systemic (phagocytosis of blood cells) immunity in *R. icterica* toads. These results show that acute/short-term stressors and CORT TA can enhance immune function in anurans. However, physiological increases in CORT levels promoted by two doses

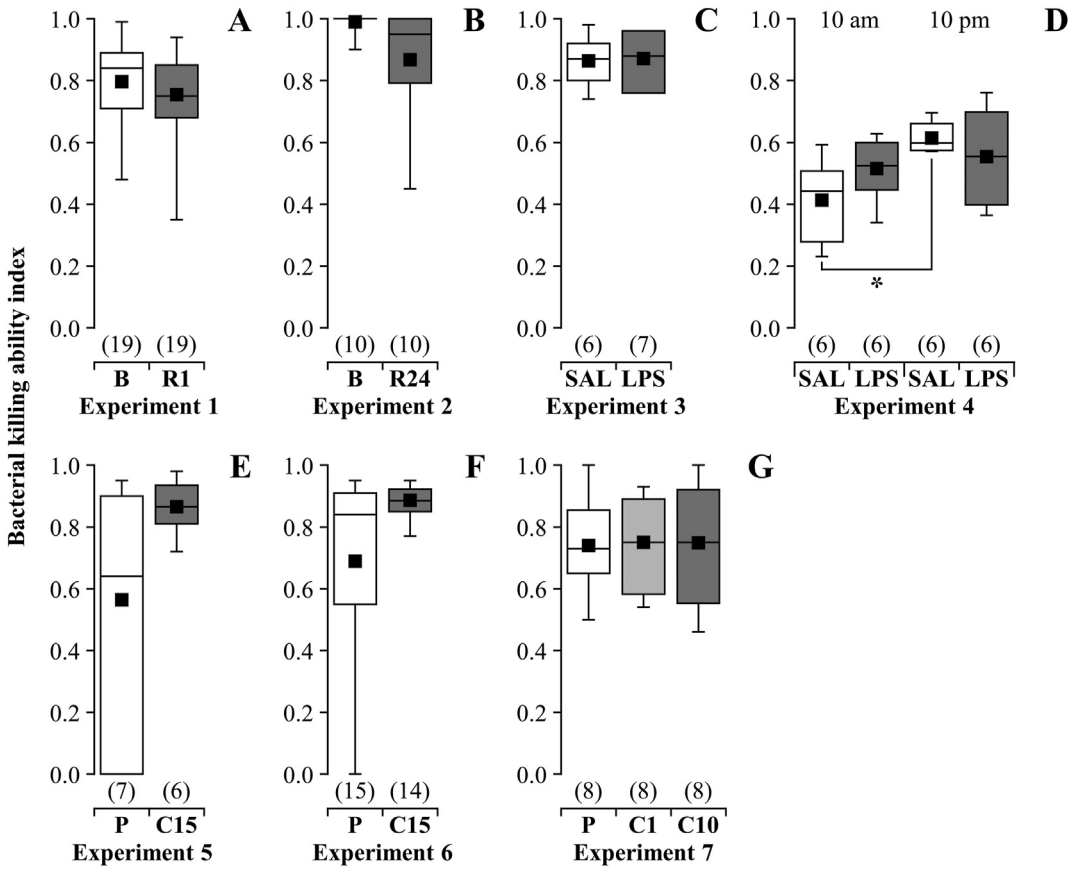

Fig. 3. Plasma bacterial killing ability following restraint, LPS, and corticosterone transdermal application in *Rhinella icterica* toads. (A) Exp. 1: Restraint – Field *vs*. 1 h post-restraint, (B) Exp. 2: Restraint – Field *vs*. 24 h post-restraint, (C) Exp. 3: LPS *vs*. Saline – Field, (D) Exp. 4: LPS *vs*. Saline, at 10 a.m. and 10 p.m., (E) Exp. 5: corticosterone transdermal application C15, placebo *vs*. corticosterone 15 μg, (F) Exp. 6: corticosterone transdermal application C15, placebo *vs*. corticosterone 15 μg, and (G) Exp. 7: corticosterone transdermal application at two doses, placebo *vs*. corticosterone 1 μg *vs*. corticosterone 10 μg. The boxplot inside lines indicates medians, lower and upper borders represent 1st and 3rd quartiles, respectively. Black squares indicate means, and whiskers represent upper and lower limits of 1.5 times inter-quartile range. The N is indicated below bars in parentheses. "*" denotes differences $P \leq 0.05$.

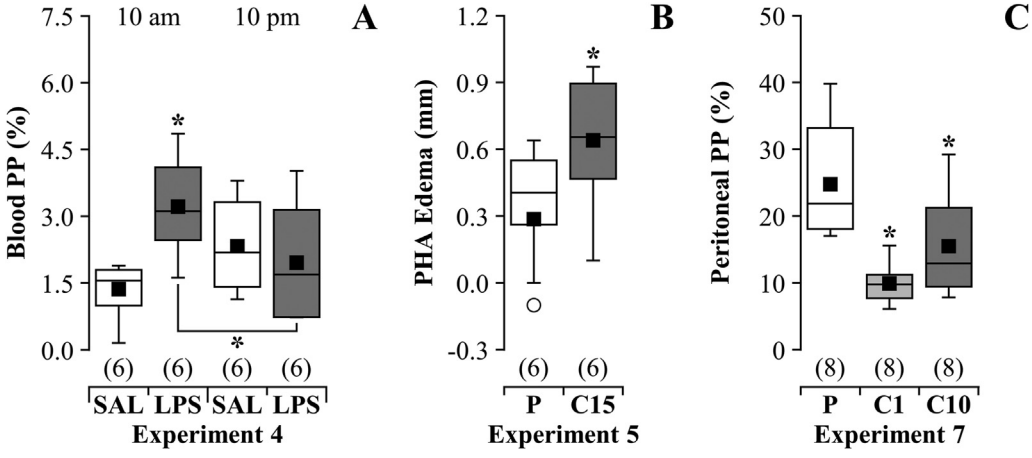

Fig. 4. Phagocytosis activity and inflammatory response following LPS and corticosterone transdermal application in *Rhinella icterica* toads. (A) Exp. 4: LPS *vs*. Saline, at 10 a.m. and 10 p.m., (B) Exp. 5: corticosterone transdermal application C15, placebo *vs*. corticosterone 15 μg, and (C) Exp. 7: corticosterone transdermal application at two doses, placebo *vs*. corticosterone 1 μg *vs*. corticosterone 10 μg. The boxplot inside lines indicates medians, lower and upper borders represent 1st and 3rd quartiles, respectively. Black squares indicate means, and whiskers represent upper and lower limits of 1.5 times inter-quartile range. The N is indicated below bars in parentheses. "*" denotes differences $P \leq 0.05$.

of CORT TA had local immunosuppressive effects (phagocytosis of peritoneal cells). In this way, these results show the immune-enhancing effects of acute stressors. However, the immune response following CORT TA varied according to the investigated immune parameter, with decreased phagocytosis associated with the lowest CORT doses. Otherwise, increased PHA edema was observed following the highest CORT dose. These results suggest that the immune function might vary according to other immune mediators that would be key participants in the CORT-induced immunomodulation interaction.

Therefore, our results highlight that it is crucial studying several aspects of the immune system at once since distinct immune response can show opposite effects induced by different doses of CORT TA. For future studies evaluating the immunomodulatory effects of acute stressors and the acute increase in CORT, we advise the more immune parameters and endocrine mediators, the better. We recommend using the protocols presented here and the inclusion of other innate measures, such as

natural antibodies (hemagglutination assay). Additionally, it is necessary to develop a toolbox to evaluate amphibians' acquired immune response (proliferation of lymphocytes, production of antibodies, and cytokines, among others). Moreover, it would also be important to consider other possible mediators of the immune response, such as the hormones melatonin and testosterone. As we have seen, the effects of CORT do not seem to happen isolated.

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Declaration of competing interest

The authors have no competing interests to declare.

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