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SYMPOSIUM

The Impacts of Transdermal Application of Corticosterone on Toad (*Rhinella icterica*) Immunity

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Synopsis Recent studies have shown that acute physiological increases in endogenous glucocorticoid levels have immunostimulatory effects. Although post-acute stress immunosuppressive effects have also been described, the difference between enhancing and suppressing the immune response seems mediated by the stressor's duration, intensity, and the immune component under analysis. To elicit physiologically relevant corticosterone levels that can be found in *Rhinella icterica* toads after stressful events (e.g., restraint or captivity) and understand how acute increased glucocorticoid levels of different intensities affect corticosterone and testosterone plasma levels and immune parameters (*in vitro* plasma bacterial killing ability, neutrophil-to-lymphocyte ratio, and *in vivo* phagocytosis of peritoneal leukocytes), we submitted toads to the transdermal application of two corticosterone doses (1 and 10 μg). Corticosterone transdermal application increased corticosterone plasma levels with different intensities: 3 times for 1 μg and fourteen times for 10 μg, compared to the vehicle, and the neutrophil-to-lymphocyte ratio increased regardless of the corticosterone dose. However, there was no effect on testosterone levels and bacterial killing ability. Interestingly, both corticosterone doses promoted immunosuppression, decreasing peritoneal leukocytes' phagocytosis activity by 60% for toads receiving the dose of 1µg and 40% for those receiving 10 μg. Our results show the complexity of the relationship between increased corticosterone levels and immunomodulation. The different corticosterone doses promoted increases of distinct magnitudes in corticosterone plasma levels, with the less intense increase in corticosterone levels generating greater cell-mediated immunosuppression. Future studies using different corticosterone doses to achieve and compare physiological vs. pharmacological hormone levels are imperative to understanding these interrelationships between corticosterone and immune response.

Introduction

The immune response is modulated by many physiological factors, including hormones like glucocorticoids (Webster et al. 2002). Glucocorticoids modulate several processes, including metabolism, development, and immunity (Crespi et al. 2013; Koutsos and Klasing 2014; Cain and Cidlowski 2017). Lately, particular attention has been devoted to understanding the immunomodulatory effects of glucocorticoids in ectotherms, using an exogenous application of hormones to mimic glucocorticoid-related changes that occur during stress events (Meylan et al. 2010; Wack et al. 2010; Bliley and Woodley 2012; Assis et al., 2015, 2017; Kaiser et al. 2015; Thomas and Woodley 2015; Barsotti et al. 2017; Fonner et al. 2017; Gardner et al. 2018; Madelaire et al. 2019; Titon et al. 2019; Billig et al. 2020), stress protocols such as restraint (Hopkins and DuRant 2011; Narayan et al. 2012; Neuman-Lee et al. 2015; Assis et al. 2019; Hudson et al. 2020; Titan et al. 2022b), captivity (Assis et al. 2015; Titon et al. 2018; Fischer and Romero 2019; Gastón et al. 2019; Claunch et al. 2022), and even under natural conditions (Assis et al. 2020; Spence et al. 2020; Cassettari et al. 2022; Garcia Neto et al. 2022).

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Based on these studies exploring the immunomodulatory effects of glucocorticoids in ectotherms, we know that adrenocorticotropic hormone injection increases corticosterone, the main glucocorticoid in amphibians, and reduces testosterone, suggesting that corticosterone may inhibit reproductive activity in tree frogs (*Boana faber*) (Barsotti et al. 2017). Additionally, corticosterone and adrenocorticotropic hormone injection resulted in high circulating corticosterone levels and inhibition of antipredator behavior in rough-skinned newts (*Taricha granulosa*), indicating that corticosterone may mediate the antipredator behavior (Neuman-Lee et al. 2015). In snakes (*Crotalus helleri*), corticosterone implants were a reliable method for increasing corticosterone levels, although no effects on testosterone levels or defensive behavior were detected (Claunch et al. 2017).

Concerning immunomodulation, toads (*Rhinella icterica*) treated with transdermal application (hormone solution placed on top of the skin, where it is absorbed into the bloodstream) of corticosterone (15 μg) had increased corticosterone and neutrophil-to-lymphocyte ratio 1 h after corticosterone transdermal application, but no effect was found on plasma bacterial killing ability, an *in vitro* protein-based immune response (Assis et al. 2017). Interestingly, eastern fence lizards (*Sceloporus undulatus*) treated with a low (6 μg) corticosterone dose showed enhanced hemagglutination, another *in vitro* protein-based immune response (McCormick et al. 2015), while a higher (18 μg) corticosterone dose suppressed hemagglutination, suggesting that the immune outcomes are corticosterone dose-dependent (McCormick et al. 2015). Finally, gray tree frog tadpoles (*Dryophytes versicolor*) raised in corticosterone-treated water developed higher trematodes parasitic loads than controls, providing evidence for corticosterone-mediated immunosuppression in tadpoles (Belden and Kiesecker 2005).

Like corticosterone, testosterone has immunomodulatory effects (Roberts et al. 2004; Foo et al. 2017). The immunocompetence handicap hypothesis posits that testosterone has a dualistic role in stimulating sexual signal expression while causing immunosuppression (Folstad and Karter 1992). To investigate the effects of both steroids on amphibian immunity, Madelaire et al. (2019) subjected toads (*Rhinella jimi*) to a transdermal application of different doses of corticosterone (7 and 14 μg) and testosterone (12 and 20 μg). Plasma levels of both hormones increased 1 h post-application, with no dose-dependent effect. Although no effect of testosterone application was found on immune responses, toads that received corticosterone transdermal application showed maximal phytohemagglutinin-induced edema earlier than those that received testosterone or vehicle, indicating that an acute experimental increase in corticosterone can anticipate the resolution of inflammatory processes (Madelaire et al. 2019). In contrast, toads (*Rhinella ornata*) treated with corticosterone transdermal application (15 μg) once a day for 20 consecutive days showed increased corticosterone and reduced phytohemagglutinin-induced edema (Titon et al. 2019), which means that long-term increases in corticosterone can promote immunosuppression. Moreover, there was a decrease in phytohemagglutinin-induced edema response in marine iguanas (*Amblyrhynchus cristatus*) submitted to restraint or corticosterone injections, but testosterone levels were not related to immunosuppression (Berger et al. 2005). In wood frogs (*Lithobates sylvaticus*), the likelihood of having a ranavirus infection increased with decreased body condition, and if animals had an infection, viral titers were positively correlated to testosterone levels (Crespi et al. 2015). Similarly, in European tree frogs (*Hyla arborea*), males that received testosterone transdermal application with a high body mass presented an immune enhancement, represented by the strength of the edema response to phytohemagglutinin injection (Desprat et al. 2015).

Most studies assessing immune responses in amphibians use *in vitro* tests (e.g., bacterial killing ability, antibody titers, lymphocyte proliferation, and phagocytosis) (Morales et al. 2010; Assis et al. 2020; Lima et al. 2020). However, *in vivo* tests like the phytohemagglutinin-induced edema challenge (Assis et al. 2015; Titon et al. 2016), antiviral T-cell responses (Morales and Robert 2008), organismal responses to fungal pathogens (Friday et al. 2020), and wound healing (Thomas and Woodley 2015; Prado et al. 2021) have also been used. In a recent publication, Titon et al. (2021a) highlighted that *in vivo* and *in vitro* immune responses (e.g., bacterial killing ability, phagocytosis, and phytohemagglutinin-induced edema) might be enhanced or suppressed, with differences associated with the intensity in corticosterone elevation, time, and the interaction between corticosterone and other immune mediators (e.g., melatonin, testosterone, and cytokines) (Titon et al. (2021a).

Despite recent efforts, immune–endocrine interactions in anurans remain underexplored. Therefore, to understand the immunomodulatory effects of an acute experimental elevation of different corticosterone levels, we used transdermal application to expose toads to two doses of corticosterone: 1 and 10 μg. These doses were selected to elicit physiologically relevant corticosterone levels similar to those found in *R. icterica* toads after stressful events (e.g., restraint (Assis et al. 2019; Titon et al. (2021a)) or captivity (Titon et al. 2018, 2021a). We had the following predictions: (1) a low dose (1 μg) of corticosterone transdermal application should

increase the corticosterone plasma levels, neutrophil-to-lymphocyte ratio, and the immune response (*in vitro* bacterial killing ability and *in vivo* phagocytosis of peritoneal cells) while decreasing testosterone plasma levels; and (2) corticosterone transdermal application of a high dose (10 μg) should cause a higher increase in corticosterone plasma levels and neutrophil-to-lymphocyte ratio, followed by a decrease in the immune response (bacterial killing ability and phagocytosis), with a greater reduction in testosterone levels.

Materials and methods

Animals and study site

Adult male toads *R. icterica* were collected in March 2017 in São Luiz do Paraitinga, SP, Brazil (23°13′23″S, 45°18′38″W). These are large toads (Maciel et al. 2010), associated with forested habitats in the Atlantic rainforest, and often found in anthropomorphized areas (Maciel et al. 2010). Toads ($N = 24$) were visually located, hand captured, and transported to the laboratory in plastic bins covered with lids with holes for air circulation.

Animal collections were performed under authorization from the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio: 17895–1) and the laboratory procedures were performed under the approval of the Comissão de Ética no Uso de Animais (CEUA) do Instituto de Biociências da Universidade de São Paulo, Brazil (CEUA—#242/2016).

Experimental design

Toads were in captivity for 60 days under constant conditions of light (13:11, LD) and temperature (25 ± 2°C) before experiments. All individuals were weighed weekly (0.01 g), and there was no body mass loss throughout time ($F_{4,88} = 1.861$, $P = 0.128$). Animals were randomly assigned to the groups ($N = 8$ per group): vehicle, low dose (1 μg), and high dose (10 μg) of corticosterone (Merck-Millipore, Darmstadt, Germany–27840). Toads from the vehicle group received 5 μL sesame oil, while those treated with corticosterone received 5 μL of the mixture of sesame oil + hormone in the desired concentration (1 or 10 μg), similar to that found in Assis et al. (2015). Transdermal application occurred between 9 and 10 am. After one hour, toads were bled (250 μL) via cardiac puncture with heparinized 1 mL syringes and 26G needles to assess the effects of treatment on neutrophil-to-lymphocyte ratio, bacterial killing ability, corticosterone, and testosterone. Only blood samples collected within 3 min were used to ensure that corticosterone levels were not elevated due to handling (Romero and Reed 2005). We did the transdermal application during the day because it is when the lowest corticosterone values are found in nocturnal amphibians (Jessop et al. 2014; Falso et al. 2015; Titon et al. 2021b), allowing a greater amplitude of increase in corticosterone levels after the treatment. Following blood collection, the animals received an intraperitoneal injection of carboxyfluorescein succinimidyl ester-labeled zymosan (5×10^7 particles) for the *in vivo* phagocytosis assay, as described below.

In vivo phagocytosis assay and imaging flow cytometry

The *in vivo* phagocytosis assay of peritoneal cells was performed according to Titon et al. (2021b). One hour after transdermal application, all toads were intraperitoneally injected with amphibian phosphate buffered saline + zymosan (zymosan A-carboxyfluorescein succinimidyl ester, Merck-Millipore, Darmstadt, Germany–Z4250, Dagher et al. 2018) at a concentration of 5×10^7 zymosan particles diluted in 500 μL/150 g of the animal. The zymosan concentration was determined by previous studies conducted with *R. icterica* toads, in which we recovered on average 1×10^7 cell/10 mL amphibian phosphate buffered saline from the peritoneal lavage fluid per individual (Titon et al. 2018). One hour after zymosan injection, toads were killed by immersion in a lethal benzocaine solution (0.2%). The abdomen was cleaned with 70% ethanol, and a small incision was made into the peritoneal cavity with sterile surgical material (e.g., tweezers, scissors, and scalpel). Next, amphibian phosphate buffered saline (10 mL) was injected with a sterile Pasteur pipette into the peritoneum. The abdominal region was carefully massaged, and lavage fluid (resulting solution of amphibian phosphate buffered saline + cells) was removed with a sterile Pasteur pipette and placed in a 50 mL sterile conical tube. Cold ethylenediaminetetraacetic acid (20 mL, 6 mM, 4°C) was added to stop the reactions followed by centrifugation (1200 rpm, 4°C, 7 min). The supernatant was discarded and 2 mL of cold amphibian phosphate buffered saline was added, followed by centrifugation (1200 rpm, 4°C, 7 min), and this process was repeated one more time. Pellets were resuspended in 400 μL of cold paraformaldehyde (1%) for cell fixation. After 1 h, 1 mL of amphibian phosphate buffered saline solution was added and samples were centrifuged (1200 rpm, 4°C, 7 min). The supernatant was discarded and 100 μL of amphibian phosphate buffered saline was added for flow cytometry. Direct measurements of mean fluorescence in the green channel were recorded as phagocytosis.

Cells were analyzed on an image flow cytometer (AMNIS Flowsight imaging flow cytometer, Merck-Millipore, Darmstadt, Germany) interfaced with a DELL computer with 20,000 events collected using the 488 nm laser and 20x magnification through INSPIRE software. Phagocytes (macrophages and neutrophils) and lymphocytes were identified through gate images in focused-single cells plotted on bright detail intensity—brightfield vs. SSC plot (Fig. 1A) following the methods of Titon et al. (2021b). The number of internalized particles was calculated using the *"Spot Count"* wizard of the IDEAS analysis software (Merck-Millipore, Darmstadt, Germany) version 6.1 for Windows as in Park et al. (2020) (Fig. 1B). The algorithm determines whether a pixel is connected to a particular spot (in this study, the green-zymosan) or the background. The algorithm first obtains bright regions from an image, regardless of the intensity differences from one spot to another. Next, using the spot-to-cell background ratio, the program identifies the number of intensity areas with local maxima (brightness). Finally, the spot to cell background ratio is calculated by dividing the spot pixel value by the background intensity in the bright detail image. Phagocytosis of peritoneal cells was measured as phagocytosis percentage, representing the percentage of cells that engulfed at least one zymosan particle (Fig. 1B); and phagocytosis efficiency was calculated as the percentage of cells that ingested three or more zymosan particles (Fig. 1B).

Blood processing

All blood samples were kept on ice until they were divided into two aliquots on the same night (<6 h). One was used to obtain blood smears (for analysis of leukocyte profile), and the other was centrifuged to isolate plasma (4 min, 3000 rpm). Plasma samples (∼200 μL) were stored in the −80°C freezer in the lab. The assay for bacterial killing ability was done within 15 days, and the hormone assays (corticosterone and testosterone) within 30 days. To avoid potential deleterious effects of freezing/thawing, samples were kept in different microtubes and thawed only on the assay day.

Neutrophil-to-lymphocyte ratio

A drop of blood was used to perform a blood smear for each individual. The slide was dried for 30 min, fixed with methanol for 2 min, stained for 15 min with Giemsa solution (Merck-Millipore, Darmstadt, Germany), and observed using optical microscopy (100x magnification, using oil immersion—Nikon E200,104c, Nikon Instruments Inc., Tokyo, Japan). One hundred leukocytes were counted on each slide and classified based on morphology as neutrophils, lymphocytes, monocytes, eosinophils, and basophils (Davis et al. 2008; Davis and Maney 2018; Stacy et al. 2022). The neutrophil-to-lymphocyte ratio was calculated as the number of neutrophils divided by the number of lymphocytes on each slide (Davis et al., 2008).

Plasma bacterial killing ability

Plasma bacterial killing ability measurements followed established protocols (Assis et al. 2013). Plasma samples diluted (1:20) in Ringer's solution (10 μL plasma:190 μL Ringer) were mixed with 10 μL of *Escherichia coli* (Merck-Millipore, Darmstadt, Germany-ATCC 8739) working solution (∼10^4 microorganisms). Positive controls consisted of 10 μL of *E. coli* working solution in 200 μL of Ringer's solution, and negative control contained 210 μl of Ringer's solution only. All samples and controls were incubated for 60 min at 37°C. After incubation, 500 μL of tryptic soy broth was added to each sample. The bacterial suspensions were thoroughly mixed, and 300 μL of each was transferred (in duplicates) to a 96-well microplate. The microplate was incubated at 37°C for 2 h, and the optical density of the samples was measured hourly in a plate spectrophotometer (wavelength 600 nm) for a total of four readings. The bacterial killing ability was calculated according to the formula: (1 – optical density of sample/optical density of positive control), representing the proportion of killed microorganisms in the samples compared to the positive control. The plasma bacterial killing ability was evaluated at the beginning of the bacterial exponential growth phase.

Hormonal assays

Plasma samples were extracted with ether following Assis et al. (2017). Briefly, 3 mL of ether was added to 10 μL of each sample, vortexed for 30 s, and centrifuged (1800 rpm, 4°C, 9 min). The samples were transferred to −80°C for 7 min, and the liquid phase was pipetted off in another tube. These tubes were kept in a laminar flow hood at room temperature (23 ± 2°C) until ether evaporation (∼24 h). According to the manufacturer's instructions, samples were resuspended in enzyme-linked immunosorbent assay buffer, and corticosterone and testosterone were assayed using enzyme-linked immunosorbent assay kits (corticosterone #501320; testosterone #582701, Cayman Chemical, Ann Arbor, Michigan, USA). We estimated intra-assay variation for corticosterone to be 6.64% and for testosterone to be 2.71%. Inter-assay variation was calculated using the average of four intermediate values from the standard curve (recommended

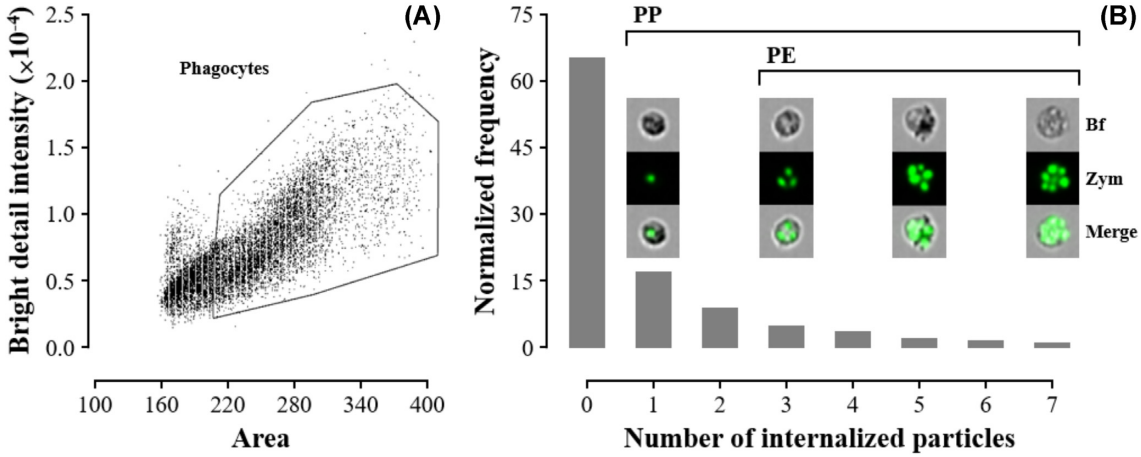

Fig. 1 Representative histograms and flowsight cytometry analysis of toads (*R. icterica*) peritoneal cells. **(A)** Flowsight plot: bright detailed intensity—brightfield vs. area—SSC dot plot, with phagocytes gated. **(B)** Flowsight green fluorescence (488 nm) spot count histogram shows the number of zymosan particles internalized by phagocytes. Abbreviations: Bf: brightfield; Zym: zymosan; Merge: brightfield + zymosan; PP: phagocytosis percentage; PE: phagocytosis efficiency.

by the kit instructions). It was 2.70% for corticosterone and 4.81% for testosterone. Sensitivity of the assays was 17.47 pg/mL for corticosterone and 15.31 pg/mL for testosterone.

Statistical analyses

The sample is the same ($N = 8$) for all variables in each group, except for testosterone levels (vehicle: $N = 7$; 1 µg: $N = 8$; 10 µg: $N = 6$), due to methodological limitations. Descriptive statistics were performed for all variables. Shapiro–Wilk normality test and Levene's test for homogeneity of variance were used to test assumptions of ANCOVAs and ANOVAs. Corticosterone, phagocytosis percentage, and phagocytosis efficiency were $\log_{10}$ transformed to satisfy assumptions. Unstandardized residuals of a linear regression of body mass as a function of snout–vent length were used as body index (Schulte-Hostedde et al. 2005). Body index (an index that indicates animal nutritional condition) was used as a covariate on a set of ANCOVAs using treatment (vehicle, low [1 µg], and high [10 µg] dose) as factors, and corticosterone, testosterone, bacterial killing ability, neutrophil-to-lymphocyte ratio, phagocytosis percentage, and phagocytosis efficiency as dependent variables. When the covariate did not affect the variables, it was removed from the model, and an ANOVA was performed instead. When there was a covariate effect, a parametric correlation test (Pearson) between the covariate and the dependent variable was used to determine the direction of the correlation. Following ANCOVAs and ANOVAs, simple independent contrasts were performed to compare corticosterone doses (1 and 10 µg) with the vehicle group. Statistical analyses were run using SPSS 26 (IBM statistics, Armonk, New York, USA).

Results

The effects of corticosterone transdermal application on morphological, endocrine, and immune parameters in *R. icterica* toads can be found in Tables 1 and 2. Body index affected corticosterone levels ($F_{1,20} = 0.5735$, $P = 0.027$), phagocytosis percentage ($F_{1,20} = 7.782$, $P = 0.011$), and phagocytosis efficiency ($F_{1,20} = 9.544$, $P = 0.006$) with a trend of an inverse relation with those variables ($r_{(24)} = -0.098$, $P = 0.650$; $r_{(24)} = -0.576$, $P = 0.003$; and $r_{(24)} = -0.622$, $P = 0.001$, respectively). The two corticosterone transdermal application doses affected all variables (Table 2) except testosterone levels ($F_{2,18} = 2.311$, $P = 0.128$) and bacterial killing ability ($F_{2,21} = 0.008$, $P = 0.992$). Regarding endocrine response, the corticosterone low dose increased corticosterone levels 3 times ($P = 0.001$), while the high dose increased it 14 times ($P < 0.001$) compared with vehicle-treated toads (Fig. 2A); in contrast, there was no effect on testosterone levels (Fig. 2B; $P \geq 0.136$). Concerning immune response, bacterial killing ability was not affected by transdermal application (Fig. 3A; $P > 0.999$), while the neutrophil-to-lymphocyte ratio increased ($F_{2,21} = 4.604$, $P = 0.022$) following the application of both low and high doses of corticosterone (Fig. 3B; $P \leq 0.011$). Also, the phagocytosis percentage was reduced by 60 and 40% in response to low- and high-dose treatment, respectively (Fig. 3C), compared with vehicle-treated toads. Phagocytosis efficiency was reduced by 70% ($P = 0.001$) in the low-dose treatment compared with vehicle-treated toads (Fig. 3D).

Discussion

As we predicted, the higher corticosterone dose resulted in a higher plasma level of corticosterone, indicating

Table 1 Descriptive statistic of morphological, endocrine, and immune parameters for *R. icterica* following transdermal application.

Variable	Treatment	Mean ± *SD*	Minimum	Maximum	*N*
BM (g)	Vehicle	143.17 ± 51.60	72.41	244.22	8
	Low dose	144.25 ± 21.24	114.22	178.33	8
	High dose	149.76 ± 44.43	61.59	198.26	8
SVL (mm)	Vehicle	118.64 ± 11.49	104.00	140.54	8
	Low dose	116.68 ± 6.33	104.92	124.16	8
	High dose	117.72 ± 9.92	99.92	132.43	8
CORT (ng/mL)	Vehicle	6.86 ± 3.56	0.68	10.69	8
	Low dose	22.80 ± 15.77	4.40	51.25	8
	High dose	98.51 ± 48.93	39.62	169.73	8
T (ng/mL)	Vehicle	16.81 ± 9.09	2.18	26.87	7
	Low dose	6.50 ± 6.21	1.07	16.19	8
	High dose	11.28 ± 12.48	0.14	28.10	6
NLR	Vehicle	0.12 ± 0.05	0.04	0.20	8
	Low dose	0.20 ± 0.04	0.13	0.25	8
	High dose	0.19 ± 0.08	0.09	0.30	8
BKA (%)	Vehicle	74.00 ± 15.23	50.00	100.00	8
	Low dose	75.00 ± 15.18	54.00	93.00	8
	High dose	74.87 ± 19.49	46.00	100.00	8
PP (%)	Vehicle	24.75 ± 8.61	17.04	39.80	8
	Low dose	9.91 ± 2.86	6.10	15.59	8
	High dose	15.50 ± 7.39	7.85	29.20	8
PE (%)	Vehicle	5.62 ± 3.96	2.15	11.85	8
	Low dose	1.52 ± 0.84	0.57	2.91	8
	High dose	2.89 ± 1.82	1.20	5.72	8

Abbreviations are as follows: *SD*: standard deviation; BM: body mass; SVL: snout-vent length; CORT: corticosterone plasma levels; T: testosterone plasma levels; NLR: neutrophil-to-lymphocyte ratio; BKA: plasma bacterial killing ability; PP: phagocytosis percentage; PE: phagocytosis efficiency. The vehicle group received sesame oil, low (1 μg) and high (10 μg) doses received corticosterone.

that the amount of hormone absorbed through the skin was proportional to the dose used. Also, plasma levels of corticosterone were physiologically relevant, similar to plasma corticosterone levels after restraint challenge with and without movement restriction in this species (for details, see Assis et al. 2019; Titon et al. 2021a).

Contrary to our predictions, there was no effect of corticosterone application on plasma testosterone levels. Increases in glucocorticoid levels, through activation of the hypothalamus–pituitary–adrenal/interrenal axis, usually interfere with the hypothalamus–pituitary–gonadal axis through genomic mechanisms, reducing the activity of the hypothalamus–pituitary–gonadal axis, decreasing the luteinizing hormone, and consequently, the testosterone levels (Sapolsky et al. 2000). However, a reduction in testosterone may occur without decreasing luteinizing hormone, which is attributed to corticosterone's rapid non-genomic mechanisms and cytosolic effects, probably reducing cyclic adenosine monophosphate, the second messenger involved in the acute regulation of steroidogenesis or accelerating testosterone clearance (Dong et al. 2004; Deviche et al. 2012). We believe the lack of effect of the corticosterone treatment in testosterone levels is time-related. In birds (*Peucaea carpalis*) subjected to restraint stress, a reduction in testosterone can be observed within 10 min (Deviche et al. 2012) and in mice (C57BL/6) within 30 min (Dong et al. 2004), without a decrease in luteinizing hormone. Considering toads are ectotherms, this process may take longer. Indeed, five-lined skinks (*Plestiodon inexpectatus*) submitted to 2 h of confinement showed increased corticosterone levels without reduced testosterone levels, and the authors thought that a longer confinement duration might suppress testosterone levels in these reptiles (Seddon and Klukowski 2012). Moreover, testosterone levels decreased in tree lizards (*Urosaurus ornatus*) in response to restraint stress, but this response was not as rapid as the corticosterone response, nor was the relative magnitude of change as great. Although testosterone levels had dropped after 1 h, this decrease was significantly lower only 4 h after restraint (Moore

Table 2 Results of ANCOVAs and ANOVAs evaluating the effects of corticosterone application on endocrine and immune variables of *R. icterica*.

Variable	Source	Type III SS	df	MS	*F*	*P*
CORT (ng/mL)	Intercept	40.864	1	40.864	429.013	**<0.001**
	Body index	0.546	1	0.546	5.735	**0.027**
	Treatment	6.471	2	3.235	33.967	**<0.001**
	Error	1.905	20	0.095		
	Total	49.276	24			
T (ng/mL)	Intercept	2752.862	1	2752.862	32.066	**<0.001**
	Treatment	396.719	2	198.359	2.311	0.128
	Error	1545.302	18	85.850		
	Total	4623.963	21			
NLR	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			
BKA (%)	Intercept	13.365	1	13.365	475.968	**<0.001**
	Treatment	<0.001	2	< 0.001	0.008	0.992
	Error	0.590	21	0.028		
	Total	13.955	24			
PP (%)	Intercept	32.728	1	32.728	1746.037	**<0.001**
	Body index	0.146	1	0.146	7.782	**0.011**
	Treatment	0.485	2	0.242	12.930	**<0.001**
	Error	0.375	20	0.019		
	Total	33.868	24			
PE (%)	Intercept	3.640	1	3.640	69.991	**<0.001**
	Body index	0.496	1	0.496	9.544	**0.006**
	Treatment	0.858	2	0.429	8.244	**0.002**
	Error	1.040	20	0.052		
	Total	6.327	24			

Sets of ANCOVAs/ANOVAs with endocrine and immune parameters used as dependent variables, body index as a covariate, and treatment (vehicle, low [1 μg], and high [10 μg] corticosterone dose) as factors. Abbreviations as follows: Type III SS: Type III sum of squares; df: degrees of freedom; MS: mean square; CORT: corticosterone plasma levels; T: testosterone plasma levels; NLR: neutrophil-to-lymphocyte ratio; BKA: plasma bacterial killing ability; PP: phagocytosis percentage; PE: phagocytosis efficiency. Variables with $P \leq 0.05$ are highlighted in bold.

et al. 1991). Another possibility is that testosterone levels were already low in these toads due to captivity time. We know from previous work in our lab, including with this same species (Titon et al. 2018), that the testosterone levels decrease with time in captivity. Then, the effects of the applied corticosterone would be less pronounced and more difficult to detect.

In our previous study (Assis et al. 2017), we found no effect of corticosterone application (15 μg) effect on testosterone levels. Still, we observed an interaction with time such that animals treated for 30 consecutive days exhibited the lowest testosterone levels 12 h post-application (Assis et al. 2017). Supporting our assumptions, restraint stress increased corticosterone levels from 30 min to 48 h, while decreasing testosterone values were only significantly different following 12 h restraint (Titon et al. 2022a). Based on these studies, we believe that waiting longer after the corticosterone transdermal application might increase the likelihood of seeing a decrease in testosterone levels. However, the mechanism by which exogenous corticosterone decreases testosterone levels remains to be determined in amphibians.

Regarding the immune parameters, corticosterone transdermal application did not affect bacterial killing ability. Bacterial killing ability tests the ability of plasma proteins (e.g., natural antibodies and complemented proteins) to kill a pathogen. Natural antibodies mediate the direct neutralization of bacteria or viruses in the bloodstream (Ochsenbein et al. 1999) and activate the complement cascade (Boes et al. 1998). The complement cascade is a complex series of molecular events that result in the permeabilization of microbial membranes (Godbey 2022), contribute to protection by en-

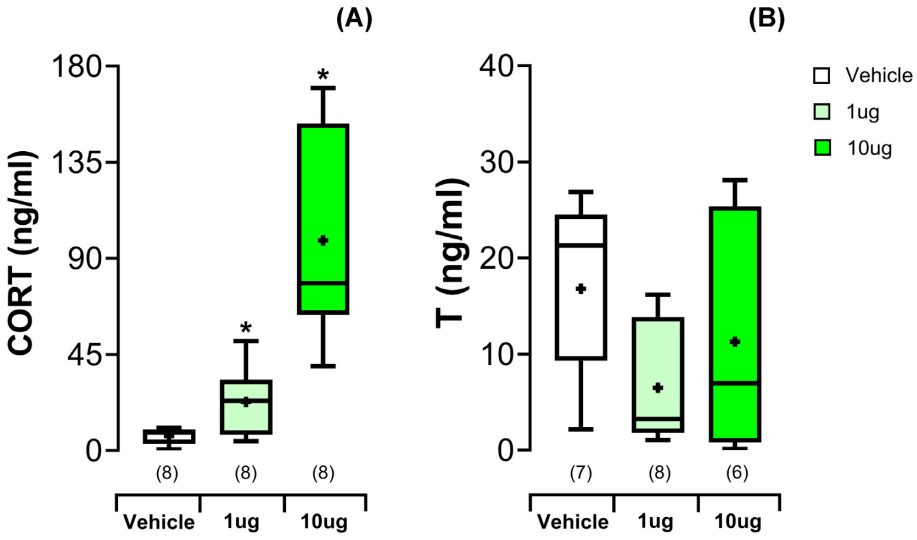

Fig. 2 Endocrine effects of corticosterone transdermal application in *R. icterica*. Toads received a transdermal application of sesame oil (vehicle) or two different corticosterone doses: 1 μg (low dose) or 10 μg (high dose). **(A)** Corticosterone plasma levels (CORT); and **(B)** testosterone plasma levels (**T**). Boxplot inside lines indicate medians, lower and upper borders represent first and third quartiles, respectively, black crosses (+) indicate means, and whiskers represent upper and lower limits of 1.5 times inter-quartile range. Asterisk (∗) denotes independent contrast significant differences ($P \leq 0.05$) compared with the vehicle group.

hancing phagocytosis of parasites and bacterial clearance via the formation of the lytic complex (Baumgarth et al. 2000), and provide an essential link between the innate and the adaptive response (Carroll 2004). How stress or corticosterone levels affect bacterial killing ability in ectotherms has been quite variable in the literature, with studies showing an increase (Hopkins and DuRant 2011; Billig et al. 2020; Hudson et al. 2020; Cassettari et al. 2022), decrease (Graham et al. 2012; Assis et al. 2015), or no change (McCormick and Langkilde 2014; Titon et al. 2021a; 2022a).

Many factors could contribute to variation in bacterial killing ability, including assay temperature and nutritional state. The plasma bacterial killing ability may be strongest when assays are conducted at the animals' thermal preferendum (Moretti et al. 2019; Lima et al. 2020). Since we ran the bacterial killing ability at 37°C, the optimal temperature for bacterial growth, and not 22°C, the toad's thermal preferendum during the winter (Moretti et al. 2018), it is possible that the assay temperature was limiting the effects of corticosterone application. The animals' nutritional state or energetic conditions could also affect the plasma bacterial killing ability (Bonneaud et al. 2003; Hanssen et al. 2004; Viney et al. 2005; Neuman-Lee et al. 2015). However, considering that there were no signs of malnourishment in the toads in our study (there was no body mass loss throughout time), nor an influence of body index on bacterial killing ability, we suggest that bacterial killing ability might be constitutively activated as part of normal immune surveillance (Ricklin et al. 2010).

We also found that corticosterone transdermal application resulted in an increase in the neutrophil-to-lymphocyte ratio. Corticosterone levels and neutrophil-to-lymphocyte ratio usually increase in parallel (Müller et al. 2011; Assis et al. 2015, 2017, 2019). This is due to the role of glucocorticoids in leukocyte trafficking: lymphocytes are sent to peripheral tissues and inflammatory sites, while neutrophils remain in the bloodstream (Davis et al. 2008; Davis and Maerz 2010; Dhabhar 2014). In this study, plasma corticosterone levels were not directly correlated with the neutrophil-to-lymphocyte ratio. It is possible that this lack of dose-dependent effect was related to the temporal nature of changes in corticosterone levels and neutrophil-to-lymphocyte ratio, which usually differs in the context of stress. Although both indexes change after short- and long-term stress, the response in corticosterone levels tends to be faster and short-lived. Meanwhile, changes in the neutrophil-to-lymphocyte ratio appear to take a little longer to detect and remain elevated for longer (reviewed in Goessling et al. 2015; Davis and Maney 2018). Indeed, in our previous study with this same species (Assis et al. 2017), the corticosterone levels peaked 1 h post-transdermal application, and even though the neutrophil-to-lymphocyte ratio increased after 1 h, it peaked 6 h post-application (Assis et al. 2017).

Our most exciting result was the decrease in phagocytosis activity of the peritoneal cells after application of corticosterone. We predicted an enhanced immune response with the low dose (1 μg) and suppres-

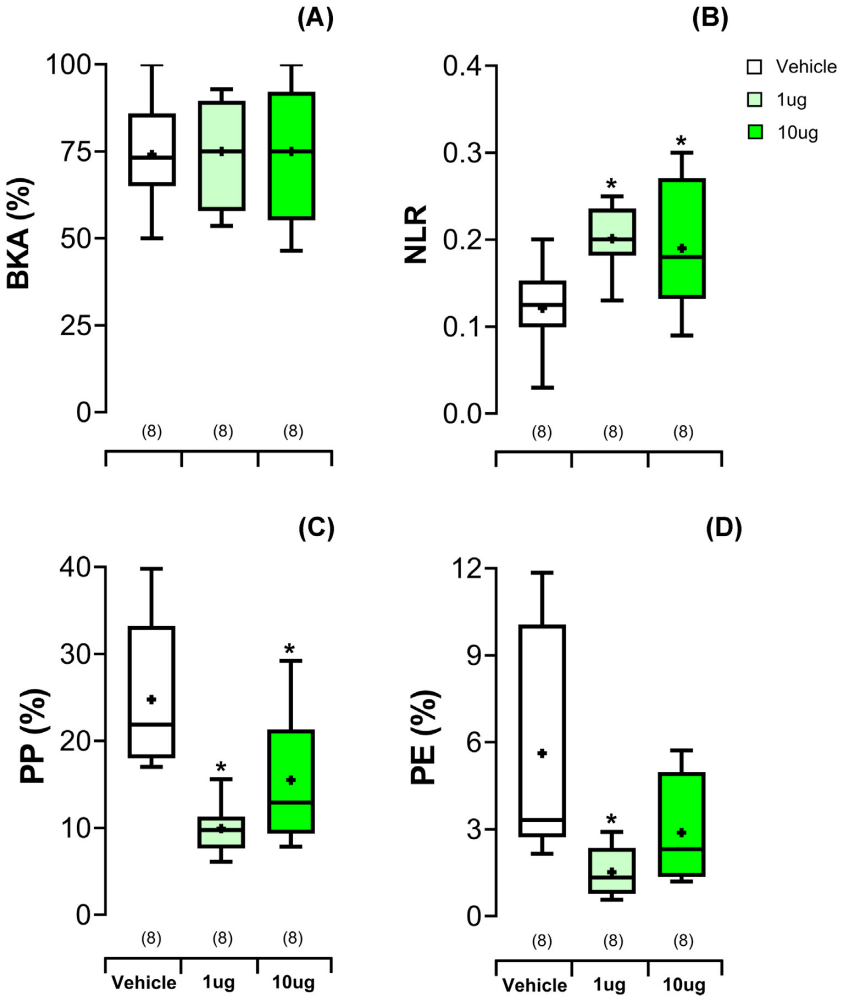

Fig. 3 Immune effects of corticosterone transdermal application in *R. icterica*. Toads received a transdermal application of sesame oil (vehicle) or two corticosterone doses: 1 μg (low dose) or 10 μg (high dose). **(A)** Plasma bacterial killing ability (BKA); **(B)** neutrophil-to-lymphocyte ratio (NLR); **(C)** phagocytosis percentage (PP); and **(D)** phagocytosis efficiency (PE). Boxplot inside lines indicate medians, lower and upper borders represent first and third quartiles, respectively, black crosses (+) indicate means, and whiskers represent upper and lower limits of 1.5 times inter-quartile range. Asterisk (∗) denotes independent contrast significant differences ($P \leq 0.05$) compared with the vehicle group.

sion with the high dose (10 μg) of corticosterone. Instead, we had a lower phagocytosis percentage, and an additional decrease in phagocytosis efficiency in toads that received the low-dose-corticosterone application compared to vehicle-treated toads. In tiger salamanders (*Ambystoma tigrinum*), treatment with dexamethasone reduced the phagocytosis percentage of peritoneal neutrophils (Froese et al. 2005). Natural and exogenous glucocorticoids exert their activities through the mineralocorticoid and glucocorticoid receptors. The effects of baseline glucocorticoid levels, which have daily and seasonal fluctuations, are mediated by mineralocorticoid receptors, whereas glucocorticoid receptors predominantly mediate physiological and behavioral changes to restore homeostasis after stressors (Conway-Campbell et al. 2007; Liebl and Martin 2013; Desmet and De Bosscher 2017). Thus, increased glucocorticoid levels due to corticosterone transdermal application might have acted mostly on glucocorticoid receptors, which mainly present immunosuppressive actions (Conway-Campbell et al. 2007). Additionally, glucocorticoid exposure before inflammation might induce anti-inflammatory actions (Desmet and De Bosscher 2017), and in this experiment, toads were treated with corticosterone or vehicle 1 h before the immune challenge.

We believe the less pronounced immunosuppressive effect we found with the higher dose (10 μg) is probably due to a down-regulation of the glucocorticoid receptors. The higher amount of plasma corticosterone

added to the bloodstream in such a short time might have initiated the process of down-regulation. Upon ligand binding, the glucocorticoid receptor undergoes a rapid decline of receptor levels, which has been well demonstrated in many glucocorticoid receptor target tissues, and diminishes cellular responsiveness to the ligand (Wallace et al. 2010). By down-regulating receptor numbers, organisms can be protected from the harmful effects that occur with prolonged hormonal stimulation (Burnstein et al. 1991). Consequently, although higher corticosterone levels can be found with the higher transdermal application dose, that does not result in higher immunosuppression (lower phagocytosis percentage and/or efficiency). Future studies exploring mineralocorticoid and glucocorticoid receptors and their role in modulating the biological response to corticosterone during inflammation are critical and deserve more attention in amphibians, where they are still largely unexplored.

Lastly, body index was negatively associated with corticosterone levels, phagocytosis percentage, and phagocytosis efficiency. Usually, glucocorticoids have catabolic effects, important in mobilizing energy resources (DuRant et al. 2008; Wack et al. 2012; Madelaire and Gomes 2016), and it is common to observe a negative relationship between body index and corticosterone. Additionally, there are possible trade-offs between the immune system and metabolism (Martin et al. 2003; Demas et al. 2011). The increase in corticosterone in animals with low body index may be causally related to immunosuppression. Indeed, increased corticosterone levels were associated with decreased body index, bacterial killing ability, and phagocytosis activity of the peritoneal cells in *R. diptycha* toads (Titon et al. 2017, 2018). In this context, toads with lower body index would have lower energy reserves to dedicate to the assemblage of a cellular immune response, highlighting the possible predictive role of body index as a cofactor mediating corticosterone effects on the immune response in toads. Evidence of interaction between food restriction and chronic stress treatments was previously found in snakes (Neuman-Lee et al. 2015). The suppression of innate immune function was associated with changes reflecting the use (increased glycerol) and storage (decreased triglycerides) of energy metabolites (Neuman-Lee et al. 2015). Accordingly, poor body condition and reduced energy availability increase the likelihood of immunosuppression (Demas 2004; Carlton et al. 2012; Ashley and Demas 2017). Therefore, body index and glucocorticoids can play an essential and integrative role in regulating immune responses (Titon et al. 2018). Hence, an experimental approach with controlled fasting could be important to test this hypothesis in amphibians.

Conclusions

Our results show the complexity of the relationship between increased corticosterone levels and immunomodulation. The different corticosterone doses promoted increases of distinct magnitudes, with the less intense increase in corticosterone generating greater cell-mediated immunity suppression. It is worth noting that there was no effect on humoral immunity represented here by complement, lysozyme, and natural antibodies. Additionally, the immunomodulatory effects seem to be associated with body condition in addition to corticosterone. Future studies using different corticosterone doses and manipulating the body index of animals are fundamental to understanding these interrelationships between body index, corticosterone, and immune response. Furthermore, exploring mineralocorticoid and glucocorticoid receptors might help better understand the dose-dependent effects of corticosterone on immunity.

Author contribution

V.R.A., S.C.M.T., and F.R.G. conceived the ideas. V.R.A. and S.C.M.T. designed the methodology. V.R.A., S.C.M.T., and B.T.Jr collected the data. V.R.A. and B.T.Jr analyzed the data. V.R.A. led the writing of the manuscript. All authors gave final approval for publication.

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Competing interests

The authors declare no competing interests.

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Data availability statement

The original data used in this manuscript are available at the Mendeley Data, through the DOI: 10.17632/4zrvwrrxx7.1

References

Ashley NT, Demas GE. 2017. Neuroendocrine-immune circuits, phenotypes, and interactions. Horm Behav 87:25–34.

de Assis VR, Titon SCM, Barsotti AMG, Spira B, Gomes FR. 2013. Antimicrobial capacity of plasma from anurans of the Atlantic forest. South Am J Herpetol 8:155–60.

de Assis VR, Gardner ST, Smith KM, Gomes FR, Mendonça MT. 2020. Stress and immunity: field comparisons among populations of invasive cane toads in Florida. J Exp Zool A Ecol Integr Physiol 333:779–91.

de Assis VR, Titon SCM, Barsotti AMG, Titon B, Jr, Gomes FR. 2015. Effects of acute restraint stress, prolonged captivity stress and transdermal corticosterone application on immunocompetence and plasma levels of corticosterone on the cururu toad (*Rhinella icterica*). PLoS One 10:e0121005.

de Assis VR, Titon SCM, Gomes FR. 2019. Acute stress, steroid plasma levels, and innate immunity in Brazilian toads. Gen Comp Endocrinol 273:86–97.

de Assis VR, Titon SCM, Queiroz-Hazarbassanov NGT, Massoco C de O, Gomes FR. 2017. Corticosterone transdermal application in toads (*Rhinella icterica*): effects on cellular and humoral immunity and steroid plasma levels. J Exp Zool A Ecol Integr Physiol 327:200–213.

Barsotti AMG, de Assis VR, Titon SCM, Titon B, da Silva Ferreira ZF, Gomes FR. 2017. ACTH modulation on corticosterone, melatonin, testosterone and innate immune response in the tree frog *Hypsiboas faber*. Comp Biochem Physiol A: Mol Integr Physiol 204:177–84.

Baumgarth N, Herman OC, Jager GC, Brown LE, Herzenberg LA, Chen J. 2000. B-1 and B-2 cell – derived immunoglobulin M antibodies are nonredundant components of the protective response to influenza virus infection. J Exp Med 192: 271–80.

Belden LK, Kiesecker JM. 2005. Glucocorticosteroid hormone treatment of larval treefrogs increases infection by *Alaria* sp. Trematode Cercariae. J Parasitol 91:686–8.

Berger S, Martin LB, Wikelski M, Romero LM, Kalko EKV, Vitousek MN, Rödl T. 2005. Corticosterone suppresses immune activity in territorial Galápagos marine iguanas during reproduction. Horm Behav 47:419–29.

Billig ST, Weber RN, Zimmerman LM, Wilcoxen TE. 2020. Effects of elevated corticosterone on humoral innate and antibody-mediated immunity in southern leopard frog (*Lithobates sphenocephalus*) tadpoles. J Exp Zool A Ecol Integr Physiol 333:756–66.

Bliley JM, Woodley SK. 2012. The effects of repeated handling and corticosterone treatment on behavior in an amphibian (Ocoee salamander: *Desmognathus ocoee*). Physiol Behav 105:1132–9.

Boes M, Prodeus AP, Schmidt T, Carroll MC, Chen J. 1998. A critical role of natural immunoglobulin M in immediate defense against systemic bacterial infection. J Exp Med 188:2381–6.

Bonneaud C, Faivre B, Sorci G. 2003. Assessing the cost of mounting an immune response. Am Nat 161:367–79.

Burnstein KL, Bellingham DL, Jewell CM, Powell-Oliver FE, Cidlowski JA. 1991. Autoregulation of glucocorticoid receptor gene expression. Steroids 56:52–8.

Cain DW, Cidlowski JA. 2017. Immune regulation by glucocorticoids. Nat Rev Immunol 17:233–47.

Carlton ED, Demas GE, French SS. 2012. Leptin, a neuroendocrine mediator of immune responses, inflammation, and sickness behaviors. Horm Behav 62:272–9.

Carroll MC. 2004. The complement system in regulation of adaptive immunity. Nat Immunol 5:981–6.

Cassettari BO, Madelaire CB, Gomes FR. 2022. Elevated corticosterone levels are associated with increased immunocompetence in male toads, both when calling and under experimental conditions. Horm Behav 137:105083.

Claunch NM, Bartoszek IA, Tillis S, Stacy NI, Ossiboff RJ, Oakey S, Schoenle LA, Wellehan JFX, Romagosa CM. 2022. Comparative biochemistry and physiology, part A physiological effects of capture and short-term captivity in an invasive snake species, the Burmese python (*Python bivittatus*) in Florida. Comp Biochem Physiol A: Mol Integr Physiol 267:111162.

Claunch NM, Frazier JA, Escallón C, Vernasco BJ, Moore IT, Taylor EN. 2017. Physiological and behavioral effects of exogenous corticosterone in a free-ranging ectotherm. Gen Comp Endocrinol 248:87–96.

Conway-Campbell BL, McKenna MA, Wiles CC, Atkinson HC, de Kloet ER, Lightman SL. 2007. Proteasome-dependent down-regulation of activated nuclear hippocampal glucocorticoid receptors determines dynamic responses to corticosterone. Endocrinology 148:5470–7.

Crespi EJ, Rissler LJ, Mattheus NM, Engbrecht K, Duncan SI, Seaborn T, Hall EM, Peterson JD, Brunner JL. 2015. Geophysiology of wood frogs: landscape patterns of prevalence of disease and circulating hormone concentrations across the Eastern Range. Integr Comp Biol 55:602–17.

Crespi EJ, Williams TD, Jessop TS, Delehanty B. 2013. Life history and the ecology of stress: how do glucocorticoid hormones influence life-history variation in animals? Funct Ecol 27:93–106.

Dagher Z, Xu S, Negoro PE, Khan NS, Feldman MB, Reedy JL, Tam JM, Sykes DB, Mansour MK. 2018. Fluorescent tracking of yeast division clarifies the essential role of spleen tyrosine kinase in the intracellular control of candida glabrata in macrophages. Front Immunol 9:1058.

Davis AK, Maerz JC. 2010. Effects of exogenous corticosterone on circulating leukocytes of a salamander (*Ambystoma talpoideum*) with unusually abundant eosinophils. Int J Zool 2010:1–8.

Davis AK, Maney DL. 2018. The use of glucocorticoid hormones or leucocyte profiles to measure stress in vertebrates: what's the difference? Methods Ecol Evol 9:1556–68.

Davis AK, Maney DL, Maerz JC. 2008. The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. Funct Ecol 22:760–72.

Demas GE. 2004. The energetics of immunity: a neuroendocrine link between energy balance and immune function. Horm Behav 45:173–80.

Demas GE, Adamo SA, French SS. 2011. Neuroendocrine-immune crosstalk in vertebrates and invertebrates: implications for host defence. Funct Ecol 25:29–39.

Desmet SJ, De Bosscher K. 2017. Glucocorticoid receptors: finding the middle ground. J Clin Invest 127:1136–45.

Deviche P, Gao S, Davies S, Sharp PJ, Dawson A. 2012. Rapid stress-induced inhibition of plasma testosterone in free-ranging male rufous-winged sparrows, *Peucaea carpalis*: characterization, time course, and recovery. Gen Comp Endocrinol 177:1–8.

Dhabhar FS. 2014. Effects of stress on immune function: the good, the bad, and the beautiful. Immunol Res 58: 193–210.

Dong Q, Salva A, Sottas CM, Niu E, Holmes M, Hardy MP. 2004. Rapid glucocorticoid mediation of suppressed testosterone biosynthesis in male mice subjected to immobilization stress. J Androl 25:973–81.

DuRant SE, Romero LM, Talent LG, Hopkins WA. 2008. Effect of exogenous corticosterone on respiration in a reptile. Gen Comp Endocrinol 156:126–33.

Falso PG, Noble CA, Diaz JM, Hayes TB. 2015. The effect of long-term corticosterone treatment on blood cell differentials and function in laboratory and wild-caught amphibian models. Gen Comp Endocrinol 212:73–83.

Fischer CP, Romero LM. 2019. Chronic captivity stress in wild animals is highly species-specific. Conserv Physiol 7:1–38.

Folstad I, Karter AJ. 1992. Parasites, bright males, and the immunocompetence handicap. Am Nat 139:603–22.

Fonner C, Patel S, Boord S, Venesky M, Woodley S. 2017. Effects of corticosterone on infection and disease in salamanders exposed to the amphibian fungal pathogen *Batrachochytrium dendrobatidis*. Dis Aquat Organ 123:159–71.

Foo YZ, Nakagawa S, Rhodes G, Simmons LW. 2017. The effects of sex hormones on immune function: a meta-analysis. Biol Rev 92:551–71.

Friday B, Holzheuser C, Lips KR, Longo A V. 2020. Preparing for invasion: assessing risk of infection by chytrid fungi in southeastern plethodontid salamanders. J Exp Zool A Ecol Integr Physiol 333:829–40.

Froese JMW, Smits JEG, Wickstrom ML. 2005. Evaluation of two methods for measuring nonspecific immunity in tiger salamanders (*Ambystoma tigrinum*). J Wildl Dis 41:209–17.

Garcia Neto PG, Titon SCM, de Assis VR, Muxel SM, Titon B, Jr, Ferreira LF, Markus RP, Gomes FR, Fernandes PACM. 2022. Immune and endocrine responses of cururu toads (*Rhinella icterica*) in their natural habitat after LPS stimulation. Comp Biochem Physiol A Mol Integr Physiol 269:111213.

Gardner S, de Assis VR, Zhao H, Gomes FR, Peatman E, Mendonça MT. 2018. Differential gene expression to an LPS challenge in relation to exogenous corticosterone in the invasive cane toad (*Rhinella marina*). Dev Comp Immunol 88: 114–23.

Gastón MS, Pereyra LC, Vaira M. 2019. Artificial light at night and captivity induces differential effects on leukocyte profile, body condition, and erythrocyte size of a diurnal toad. J Exp Zool A Ecol Integr Physiol 331:93–102.

Godbey WT. 2022. Voyage into the cell. In: Biotechnology and its Applications. Massachusetts (MA): Elsevier. p. 11–46.

Goessling JM, Kennedy H, Mendonça MT, Wilson AE. 2015. A meta-analysis of plasma corticosterone and heterophil: lymphocyte ratios — is there conservation of physiological stress responses over time? Funct Ecol 29:1189–96.

Graham SP, Kelehear C, Brown GP, Shine R. 2012. Corticosterone–immune interactions during captive stress in invading Australian cane toads (*Rhinella marina*). Horm Behav 62:146–53.

Hanssen SA, Hasselquist D, Folstad I, Erikstad KE. 2004. Costs of immunity: immune responsiveness reduces survival in a vertebrate. Proc R Soc Lond B Biol Sci 271:925–30.

Hopkins WA, DuRant SE. 2011. Innate immunity and stress physiology of eastern hellbenders (*Cryptobranchus alleganiensis*) from two stream reaches with differing habitat quality. Gen Comp Endocrinol 174:107–15.

Hudson SB, Lidgard AD, French SS. 2020. Glucocorticoids, energy metabolites, and immunity vary across allostatic states for plateau side-blotched lizards (*Uta stansburiana uniformis*) residing in a heterogeneous thermal environment. J Exp Zool A Ecol Integr Physiol 333:732–43.

Jessop TS, Dempster T, Letnic M, Webb JK. 2014. Interplay among nocturnal activity, melatonin, corticosterone and performance in the invasive cane toad (*Rhinella marinus*). Gen Comp Endocrinol 206:43–50.

Kaiser K, Devito J, Jones CG, Marentes A, Perez R, Umeh L, Weickum RM, McGovern KE, Wilson EH, Saltzman W. 2015. Reproductive and immune effects of chronic corticosterone treatment in male white's treefrogs, *Litoria caerulea*. Conserv Physiol 3:cov022.

Koutsos EA, Klasing KC. 2014. Factors modulating the avian immune system. In: Avian immunology. Masachussetts (MA): Elsevier. pp. 299–313.

Liebl AL, Martin LB. 2013. Stress hormone receptors change as range expansion progresses in house sparrows. Biol Lett 9:20130181.

Lima AS, Ferreira L de F, Silva DP, Gomes FR, Titon SCM. 2020. Thermal sensitivity of bullfrog's immune response kept at different temperatures. J Exp Zool A Ecol Integr Physiol 333:767–78.

McCormick GL, Langkilde T. 2014. Immune responses of eastern fence lizards (*Sceloporus undulatus*) to repeated acute elevation of corticosterone. Gen Comp Endocrinol 204:135–40.

McCormick GL, Shea K, Langkilde T. 2015. How do duration, frequency, and intensity of exogenous CORT elevation affect immune outcomes of stress? Gen Comp Endocrinol 222: 81–7.

Maciel NM, Collevatti RG, Colli GR, Schwartz EF. 2010. Late Miocene diversification and phylogenetic relationships of the huge toads in the *Rhinella marina* (Linnaeus, 1758) species group (Anura: Bufonidae). Mol Phylogenet Evol 57: 787–97.

Madelaire CB, Cassettari B de O, Gomes FR. 2019. Immunomodulation by testosterone and corticosterone in toads: experimental evidences from transdermal application. Gen Comp Endocrinol 273:227–35.

Madelaire CB, Gomes FR. 2016. Breeding under unpredictable conditions: annual variation in gonadal maturation, energetic reserves and plasma levels of androgens and corticosterone in anurans from the Brazilian semi-arid. Gen Comp Endocrinol 228:9–16.

Martin LB, Scheuerlein A, Wikelski M. 2003. Immune activity elevates energy expenditure of house sparrows: a link between direct and indirect costs? Proc R Soc Lond B Biol Sci 270:153–8.

Meylan S, Haussy C, Voituron Y. 2010. Physiological actions of corticosterone and its modulation by an immune challenge in reptiles. Gen Comp Endocrinol 169:158–66.

Moore MC, Thompson CW, Marler CA. 1991. Reciprocal changes in corticosterone and testosterone levels following acute and chronic handling stress in the tree lizard, *Urosaurus ornatus*. Gen Comp Endocrinol 81:217–26.

Morales H, Robert J. 2008. In vivo and in vitro techniques for comparative study of antiviral T-cell responses in the amphibian *Xenopus*. Biol Proced Online 10:1–8.

Morales HD, Abramowitz L, Gertz J, Sowa J, Vogel A, Robert J. 2010. Innate immune responses and permissiveness to ranavirus infection of peritoneal leukocytes in the frog *Xenopus laevis*. J Virol 84:4912–22.

Moretti EH, Ortega Chinchilla JE, Marques FS, Fernandes PACM, Gomes FR. 2018. Behavioral fever decreases metabolic response to lipopolysaccharide in yellow *Cururu* toads (*Rhinella icterica*). Physiol Behav 191:73–81.

Moretti EH, Titon SCM, Titon B, Jr, Marques FS, Gomes FR. 2019. Thermal sensitivity of innate immune response in three species of *Rhinella *toads. Comp Biochem Physiol A: Mol Integr Physiol 237:110542.

Müller C, Jenni-Eiermann S, Jenni L. 2011. Heterophils/lymphocytes-ratio and circulating corticosterone do not indicate the same stress imposed on Eurasian kestrel nestlings. Funct Ecol 25:566–76.

Narayan EJ, Hero J-M, Cockrem JF. 2012. Inverse urinary corticosterone and testosterone metabolite responses to different durations of restraint in the cane toad (*Rhinella marina*). Gen Comp Endocrinol 179:345–9.

Neuman-Lee LA, Stokes AN, Greenfield S, Hopkins GR, Brodie ED, French SS. 2015. The role of corticosterone and toxicity in the antipredator behavior of the rough-skinned newt (*Taricha granulosa*). Gen Comp Endocrinol 213:59–64.

Neuman-Lee LA, Bobby Fokidis H, Spence AR, Van der Walt M, Smith GD, Durham S, French SS. 2015. Food restriction and chronic stress alter energy use and affect immunity in an infrequent feeder. Funct Ecol 29:1453–62.

Ochsenbein AF, Fehr T, Lutz C, Suter M, Brombacher F, Hengartner H, Zinkernagel RM. 1999. Control of early viral and bacterial distribution and disease by natural antibodies. Science 286:2156–9.

Park Y, Abihssira-García IS, Thalmann S, Wiegertjes GF, Barreda DR, Olsvik PA, Kiron V. 2020. Imaging flow cytometry protocols for examining phagocytosis of microplastics and bioparticles by immune cells of aquatic animals. Front Immunol 11:203.

Prado DMA, Gomes FR, Madelaire CB. 2021. Effects of corticosterone treatment and wound healing on reproductive traits of American bullfrogs. J Exp Zool A Ecol Integr Physiol 335:275–85.

Ricklin D, Hajishengallis G, Yang K, Lambris JD. 2010. Complement: a key system for immune surveillance and homeostasis. Nat Immunol 11:785–97.

Roberts ML, Buchanan KL, Evans MR. 2004. Testing the immunocompetence handicap hypothesis: a review of the evidence. Anim Behav 68:227–39.

Romero LM, Reed JM. 2005. Collecting baseline corticosterone samples in the field: is under 3 min good enough? Comp Biochem Physiol A: Mol Integr Physiol 140:73–9.

Sapolsky RM, Romero ML, Munck AU. 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr Rev 21:55–89.

Schulte-Hostedde AI, Zinner B, Millar JS, Hickling GJ. 2005. Restitution of mass–size residuals: validating body condition indices. Ecology 86:155–63.

Seddon RJ, Klukowski M. 2012. Influence of stressor duration on leukocyte and hormonal responses in male southeastern five-lined skinks (*Plestiodon inexpectatus*). J Exp Zool A Ecol Integr Physiol 317:499–510.

Spence AR, French SS, Hopkins GR, Durso AM, Hudson SB, Smith GD, Neuman-Lee LA. 2020. Long-term monitoring of two snake species reveals immune–endocrine interactions and the importance of ecological context. J Exp Zool A Ecol Integr Physiol 333:744–55.

Stacy NI, Hollinger C, Arnold JE, Cray C, Pendl H, Nelson PJ, Harvey JW. 2022. Left shift and toxic change in heterophils and neutrophils of non-mammalian vertebrates: a comparative review, image atlas, and practical considerations. Vet Clin Pathol 51:18–44.

Thomas JR, Woodley SK. 2015. Treatment with corticosterone delays cutaneous wound healing in male and female salamanders. Gen Comp Endocrinol 216:33–8.

Titon SCM, Titon B Jr, Cobo de Figueiredo A, Rangel Floreste F, Siqueira Lima A, Cunha Cyrino J, Ribeiro Gomes F. 2022a. Plasma steroids and immune measures vary with restraint duration in a toad (*Rhinella icterica*). Gen Comp Endocrinol 318:113987.

Titon SCM, de Assis VR, Junior BT, Kinker GS, Queiroz Hazarbassanov NGT de, Lima AS, Oliveira Massoco C de, Fernandes PA, Gomes FR, Markus RP. 2022b. Optimizing studies of phagocytic activity by flowsight cytometry in amphibians. South Am J Herpetol 23:58–66.

Titon SCM, de Assis VR, Titon B Jr, Cassettari B de O, Fernandes PACM, Gomes FR. 2017. Captivity effects on immune response and steroid plasma levels of a Brazilian toad (*Rhinella schneideri*). J Exp Zool A Ecol Integr Physiol 327: 127–38.

Titon SCM, de Assis VR, Titon B, Barsotti AMG, Flanagan SP, Gomes FR. 2016. Calling rate, corticosterone plasma levels and immunocompetence of Hypsiboas albopunctatus. Comp Biochem Physiol A: Mol Integr Physiol 201:53–60.

Titon SCM, Titon B, Jr, de Assis VR, Kinker GS, Fernandes PACM, Gomes FR. 2018. Interplay among steroids, body condition and immunity in response to long-term captivity in toads. Sci Rep 8:17168.

Titon S, Titon B, Jr, Gomes F, de Assis VR. 2021a. Short-term stressors and corticosterone effects on immunity in male toads (*Rhinella icterica*): a neuroimmune-endocrine approach. Brain Behav Immun 13:100230.

Titon SCM, Titon B, Jr, de Assis VR, Vasconcelos-Teixeira R, Garcia Neto PG, Lima AS, Ferreira LDF, Fernandes PA, Gomes FR, Markus RP. 2021b. Hormonal daily variation co-varies with immunity in captive male bullfrogs (*Lithobates catesbeianus*). Gen Comp Endocrinol 303: 113702.

Titon SCM, Titon B, Jr, Barsotti AMG, Gomes FR, de Assis VR. 2019. Time-related immunomodulation by stressors and corticosterone transdermal application in toads. PLoS One 14:e0222856.

Viney ME, Riley EM, Buchanan KL. 2005. Optimal immune responses: immunocompetence revisited. Trends Ecol Evol 20:665–9.

Wack CL, DuRant SE, Hopkins WA, Lovern MB, Feldhoff RC, Woodley SK. 2012. Elevated plasma corticosterone increases metabolic rate in a terrestrial salamander. Comp Biochem Physiol A: Mol Integr Physiol 161:153–8.

Wack CL, Lovern MB, Woodley SK. 2010. Transdermal delivery of corticosterone in terrestrial amphibians. Gen Comp Endocrinol 169:269–75.

Wallace AD, Cao Y, Chandramouleeswaran S, Cidlowski JA. 2010. Lysine 419 targets human glucocorticoid receptor for proteasomal degradation. Steroids 75:1016–23.

Webster JI, Tonelli L, Sternberg EM. 2002. Neuroendocrine regulation of immunity. Annu Rev Immunol 20:125–63.

Desprat JL, Lengagne T, Dumet A, Desouhant E, Mondy N. 2015. Immunocompetence handicap hypothesis in tree frog: trade-off between sexual signals and immunity? Behav Ecol 26:1138–46.