



Research paper

Acute stress, steroid plasma levels, and innate immunity in Brazilian toads

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

Departamento de Fisiologia, Instituto de Biociências, Universidade de São Paulo, Rua do Matão, trav. 14, 101, 05508-900, São Paulo, SP, Brazil

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ABSTRACT

Stress from habitat fragmentation has been shown to impact amphibian declines. Studies from a variety of vertebrates indicate that stressed animals exhibit an acute increase in circulating plasma glucocorticoid (GC) levels and consequent immunomodulation. To further explore the relationship between GCs and immunity, we subjected three species of newly captured Brazilian toads, *Rhinella ornata*, *R. icterica* and *R. schneideri* to restraint with or without movement restriction (maintenance in a moistened cloth bag vs. maintenance in a bin) for 24 h. We compared various parameters from baseline (field conditions) with values after restraint, including those associated with stress (corticosterone [CORT] plasma levels), and the neutrophil/lymphocyte ratio [N:L ratio]), potential reproduction (testosterone [T] plasma levels), and innate immunity (bacterial killing ability [BKA]). General responses to the restraint challenge (baseline vs. restraint) included increased CORT levels and N:L ratio, and decreased T levels and BKA. Additionally, CORT levels and N:L ratio tended to increase more from restraint with movement restriction than to restraint without movement restriction, indicating toads showed increased stress response to the more intense stressor. All variables showed interspecific variation at baseline conditions: *R. ornata* had higher CORT levels when compared to the other two species, while *R. icterica* had the highest BKA values. After restraint (with or without movement restriction), *R. ornata* displayed higher values for T and N:L ratio, and showed higher CORT values after restraint without movement restriction; however, the CORT values were similar among species after restraint with movement restriction. In terms of immunity, in response to restraint, BKA was different among species only after restraint with movement restriction, with *R. schneideri* showing the lowest BKA values. Our results show that restraint increases common markers of the stress response, and could reduce potential reproduction and innate immune responses in toads from all studied species. Our results also showed variation at the interspecific level, with the amplitude of change in the studied variables being consistent and more pronounced following restraint with movement restriction for the three-studied species.

1. Introduction

Amphibian populations have been in decline, which has been exacerbated in the last few decades (Stuart et al., 2004). These declines have been associated with interactions between several factors, such as climate change, habitat modification, environmental pollutants, pathogens, and invasive species that may become predators or competitors to native species (Carey et al., 1999; Hayes et al., 2010). It has been shown that habitat destruction is also a great threat to amphibian diversity (Duellman, 1999). In the common toad (*Bufo bufo*), habitat fragmentation is negatively associated with individual occurrence and body condition and positively associated with corticosterone (CORT) levels, providing evidence that habitat fragmentation represents a physiological challenge to these animals (Janin et al., 2011). The impact of habitat destruction, however, differs greatly between species (Duellman, 1999). While most anuran species disappear with forest

fragmentation, a small part of anuran diversity can benefit from this environmental alteration. The few species that benefit from human occupation are generally characterized as having numerous populations and wide geographical distribution (Duellman, 1999).

When responding to environmental challenges, even closely related species may undergo *different* physiological processes, the causes of which remain poorly understood (Bernardo et al., 2007; Tingley et al., 2009; Tomanek, 2012; Hammond et al., 2015). Glucocorticoid hormones (GCs), produced by adrenal or interrenal glands, are released in response to several stressors in all vertebrate groups (Sapolsky et al., 2000). Given that GCs are integrally involved in homeostasis and allostasis, as well as in modulating trade-offs between survival and reproduction (Angelier and Wingfield, 2013; Wingfield, 2013), monitoring these hormone levels should provide valuable indicators of response to environmental change (Hammond et al., 2015). Several physiological processes modulated by GCs, however, show a bimodal

* Corresponding author.

E-mail address: v.regina.a@gmail.com (V.R. Assis).

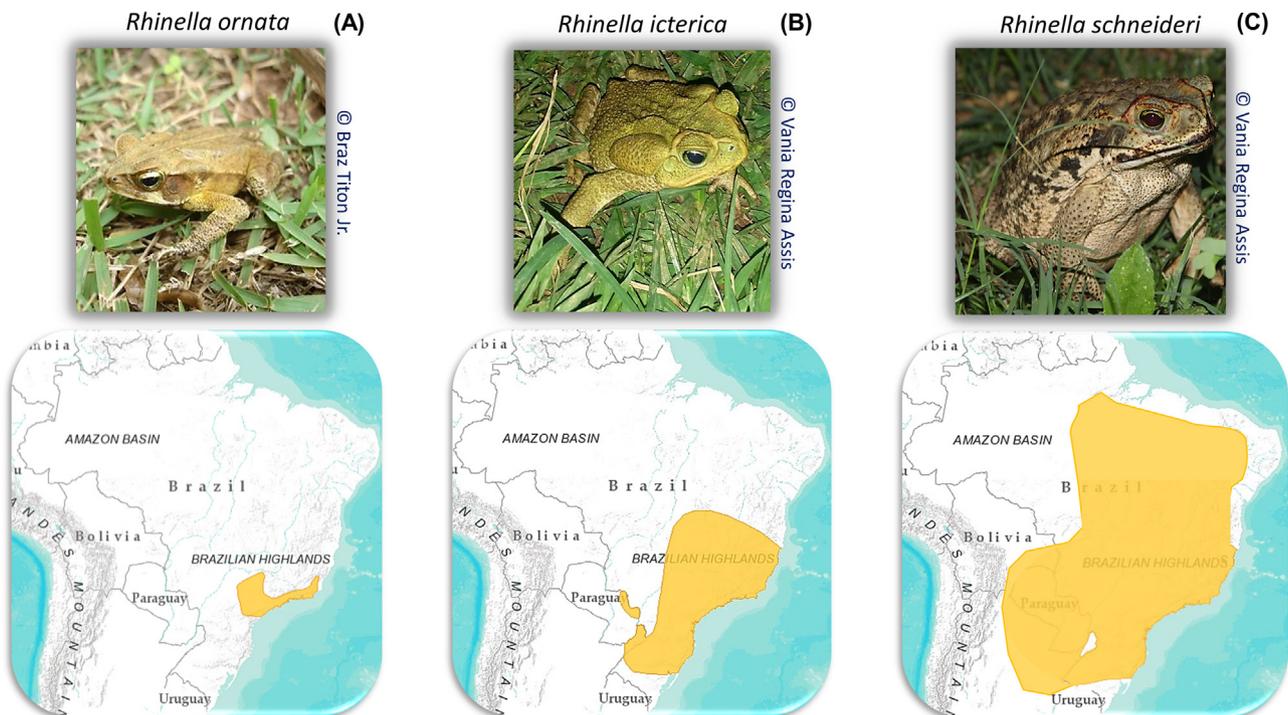


Fig. 1. *Rhinella* species studied and their geographical distribution: (A) *R. ornata*, (B) *R. icterica* and (C) *R. schneideri*. The distribution maps were generated and modified from: <http://maps.iucnredlist.org>.

pattern of response, depending on the intensity and duration of the stressor (Wiegiers and Reul, 1998; Sapolsky et al., 2000; Barriga et al., 2002; Dhabhar, 2014). Acute elevations of GCs, such as those resulting from short-term stress, may enhance immune responses (Wingfield et al., 1997; Wingfield and Romero, 2001; Dhabhar, 2009), as studies with rats have shown acute stress-induced enhancement of cell-mediated immunity (Dhabhar and McEwen 1999), as well as increased lymphocyte proliferation (Wiegiers et al. 1995). Immune suppression may also result from sustained and elevated plasma levels of GCs, as in situations of chronic stress (Sapolsky, 1992; Wingfield and Romero, 2001). The GCs immunosuppressive effects are well known and include inhibition of synthesis, release and/or efficiency of several cytokines, and other mediators that promote the immune response and inflammatory reactions (Wiegiers and Reul, 1998; Sapolsky et al., 2000).

Regarding immunosuppression, several studies have also proposed that male reproductive effort could compromise survival through immunosuppressive effects of androgens, reducing the resistance to parasitic infection (Hamilton and Zuk, 1982; Folstad and Karter, 1992; Mills et al., 2010). Although evidence of testosterone-dependent immunosuppression in wild vertebrates has been provided for several species (Casto et al., 2001), a meta-analysis of studies on reptiles, birds and mammals concluded that the immunosuppressive effect of testosterone (T) depends on the type of immune response and varies considerably within and between phylogenetic groups (Roberts et al., 2004). Furthermore, studies in a diversity of vertebrate species have shown that stressors typically suppress T levels, sometimes quite rapidly (Greenberg and Wingfield, 1987; Deviche et al., 2010; Deviche et al., 2012; Deviche et al., 2014). Reduction of T levels has been reported after induced acute stress in reptiles (reviewed in Tokarz and Summers, 2011), and after a restraint challenge and toe-clipping in toads (Narayan et al., 2011b; Narayan et al., 2012). Therefore, increased CORT associated with changes in T may play an important role in acute stress-induced immunomodulatory effects.

In a comparative study conducted with three species of Brazilian toads from genus *Rhinella* (*Rhinella ornata*, *R. icterica* and *R. schneideri*), Gomes et al. (2012) found a negative relationship between innate

immunity (bacterial killing ability [BKA]) and CORT levels. According to this study, *R. ornata*, the species with higher degree of dependence on forested habitats, showed highest baseline and post-stress CORT levels, while *R. schneideri*, the species with geographical distribution more associated to naturally open or deforested areas, presented highest BKA. These results point to a possible compromise between plasma GC levels and innate immunity in toads, which could be an important determinant to the interspecific variance in ability to occupy disturbed areas (Gomes et al., 2012). To further explore the relationship between immunity and steroid hormone levels in these toads, we evaluated physiological parameters associated with stress responses: CORT levels and neutrophil/lymphocyte ratio (N:L ratio), reproductive potential (T), and innate immunity (BKA), at baseline (field conditions) and post-restraint (24 h under captivity) under two different conditions: with and without movement restriction. We predicted that at the intraspecific level: 1) Restraint without movement restriction would represent a low-intensity short-term stressor, elevating CORT levels, N:L ratio, and BKA, while decreasing T levels when compared with baseline conditions in all three studied species; and 2) Restraint with movement restriction would represent a higher-intensity short-term stressor, eliciting higher increases in CORT levels and N:L ratio while triggering decreased BKA and T levels compared to restrained toads without movement restriction. We also conducted a preliminary assessment of interspecific differences in stress physiology mediating innate immune responses. At the interspecific level, we predicted that: 1) Baseline CORT levels and N:L ratio should be higher in *R. ornata*, the species with distribution more restricted to forested environments, followed by *R. icterica* and *R. schneideri*; 2) Baseline innate immune response should vary in the opposite direction, with *R. schneideri*, the species with widespread distribution, showing the highest BKA values followed by *R. icterica* and *R. ornata*, and 3) Post-restraint, *R. ornata* should have higher CORT levels and N:L ratio and *R. schneideri* should have higher BKA.

2. Materials and methods

2.1. Species and studied sites

We collected data for three different species of *Rhinella*, which differ in their geographical distribution and susceptibility to deforestation (Fig. 1): 1) *R. ornata*: a species from the *Rhinella crucifer* group (Baldissera et al., 2004), with a geographical distribution restricted to forested habitat associated with the Atlantic Rainforest in Brazil (Fig. 1A); 2) *R. icterica*: a species belonging to the *Rhinella marina* group (Maciel et al., 2010), which although also has a distribution associated with the Atlantic Rainforest in Brazil, can be easily found in naturally open or impacted areas (Fig. 1B); and 3) *R. schneideri*: a species that also belongs to the *Rhinella marina* group (Maciel et al., 2010) that has a wide geographical distribution and is found in open areas in the Atlantic Rainforest and Cerrado in Brazil (Fig. 1C).

Rhinella icterica males were collected in January 2013 ($N = 20$), in the city of São Luiz do Paraitinga – SP/Brazil ($23^{\circ} 13'23''\text{S}$, $45^{\circ} 18'38''\text{W}$) and males of *R. schneideri* were collected in February 2013 ($N = 20$) in the city of Luiz Antônio – SP/Brazil ($21^{\circ} 30'23''\text{S}$, $47^{\circ} 50'38''\text{W}$). *Rhinella ornata* males were collected in August 2014 ($N = 19$) in the city of Botucatu – SP/Brazil ($22^{\circ} 53' 09''\text{S}$, $48^{\circ} 26' 42''\text{W}$). Males from all species were collected within their reproductive season, but only males of *R. ornata* were calling at the time of collection.

Collections were performed under authorization from Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio, process 17895-1) and 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 (Protocol 142/2011).

2.2. Collecting and processing blood samples

Animals were located by visual inspection, captured, and bled in the field (approximately 150–200 μl of blood) via cardiac puncture with 1 ml syringes and needles 26Gx1/2" previously heparinized. Blood samples were kept for analysis only if collection was performed within 3 min after animal capture, to avoid any influence of the stress of capture and handling on hormone levels (Romero and Reed, 2005).

All blood samples kept on ice until they were divided into two aliquots on the same night. One was used to obtain blood slides (for analysis of leukocyte profile), and the other was centrifuged to isolate plasma (4 min at 3000 rpm). Plasma samples (ranging from 100 to 150 μl) from all species were stored in cryovials, frozen in liquid nitrogen, and then transferred to a -80°C freezer until analysis of CORT, T, and BKA.

2.3. Physiological variables

2.3.1. Leukocyte profile

A drop of blood was used to perform each blood smear slide. Two slides were made for each animal, with one being stained with Giemsa solution (10%) and observed under an optical microscope (100 \times objective, using oil immersion – Nikon E200, 104c). For differential leukocyte counts, 100 leukocytes were counted on each slide, and classified based on morphology as neutrophils, lymphocytes, eosinophils, basophils, or monocytes (Campbell, 2006). The N:L ratio were obtained from counts of each slide.

2.3.2. Bacterial killing ability (BKA)

Assessment of BKA was performed according to the methods of 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 *E. coli* working solution ($\sim 10^4$ 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 the incubation period, 500 μl of tryptic soy broth (TSB) were added to each sample. The bacterial suspensions were thoroughly mixed and 300 μl of each were transferred (in duplicates) to a 96 wells microplate. The microplate was incubated at 37°C for 2 h, and thereafter the optical density of the samples was measured hourly in a plate spectrophotometer (wavelength 600 nm), for a total of 4 readings. The BKA was calculated according to the formula: $1 - (\text{optical density of sample} / \text{optical density of positive control})$, which represents the proportion of killed microorganisms in the samples compared to the positive control. The bacterial killing ability was evaluated at the beginning of the bacterial exponential growth phase.

2.3.3. Hormonal assay

Plasma samples were extracted with ether following the methods of Mendonça et al. (1996) and Assis et al. (2017). Briefly, 3 ml of ether was added to 10 μl of each sample, vortexed for 30 s, and centrifuged (4°C , 9 min, at 1800 rpm). The samples were then allowed to settle at -80°C for 7 min, and the liquid phase was transferred to another tube. These tubes were kept in a laminar flow hood at room temperature ($20 \pm 2^{\circ}\text{C}$), until evaporation of all ether (approximately 24 h). The samples were resuspended in EIA buffer, and CORT and T were assayed using EIA kits (CORT number 500655; T number 582701, Cayman Chemical), according to the manufacturer's instructions.

We estimated intra-assay variation for CORT to be 6.40% and for T to be 4.47%. Inter-assay variation was estimated using the average of four intermediate values from the standard curve (recommended by the kit instructions) and was 10.59% for CORT and 11.16% for T. Sensitivity of the assays were 31.25 pg/ml for CORT and 10.89 pg/ml for T.

2.4. Restraint with and without movement restriction

Immediately after blood collection in the field, the same toads were randomly placed in two groups: 1) restraint without movement restriction in individual plastic bins (4.3 L – $29 \times 18 \times 15$ cm L \times W \times H), or 2) restraint with movement restriction by placing individuals into moistened cloth bags in the individual plastic bins. Toads in both groups remained under the described conditions for 24 h. The bins were fitted with lids with holes to allow air circulation. Toads were exposed to the natural light cycle and temperatures compatible with their natural thermal regime. At 24 h, all toads were bled again to reassess the same physiological variables measured at baseline (CORT levels, N:L ratio, BKA and T levels). Upon termination of this experimental protocol, toads were measured (snout vent-length, 0.01 mm), weighed (body mass, 0.01 g), and returned to their collection point at night.

2.5. Statistical analysis

Data were initially analyzed with descriptive statistics and Shapiro-Wilk normality test. Variables showed absence of normality and therefore were transformed to fit the prerequisites of parametric tests: 1) BKA – arccosine; 2) N:L ratio – $\log_{10}(N + 1)$; 3) CORT – $\log_{10}(N + 1)$; and 4) T – $\log_{10}(N + 1)$. Any outliers identified from a Z-score test were deleted: One measure from *R. ornata* (N:L ratio: $Z = 3.82$); 2 from *R. icterica* (BKA: $Z = -3.58$; N:L ratio: $Z = 3.00$) and 2 from *R. schneideri* (BKA: $Z = -3.125$; N:L ratio: $Z = 15.33$).

Analyses of covariance (ANCOVA) were used for intraspecific comparisons, with physiological traits as dependent variables, with restraint challenge (baseline or restraint) and group (with or without movement restriction) as factors, and body mass as covariable. When variables were not affected by body mass, a set of mixed analyses of variance (ANOVA) were used, with physiological measurements treated as dependent variables, and restraint challenge (baseline and restraint) and group (with or without movement restriction) treated as factors, followed, when appropriate, by multiple pair-wise comparison tests

(Bonferroni). The mixed ANOVA allows the analyses of both inter (independent) and intra (repeated) subject variation in two or more groups. Since baseline T levels of *R. ornata* were extremely high and could not be determined (details in Section 3), a *t*-test for independent samples was used to investigate differences between the groups (with or without movement restriction) on T levels after restraint for this species.

For the interspecific comparisons, ANCOVAs were used, with physiological traits treated as dependent variables, species as a factor, and body mass as covariable for baseline and each specific restraint group (with and without movement restriction). The variables not affected by body mass were subjected to a set of independent ANOVAs, with physiological traits treated as dependent variables and species treated as a factor for each condition: baseline and restraint (with and without movement restriction), followed, when appropriate, by multiple pair-wise comparison tests (Bonferroni).

To test for correlations between the same variables at different conditions, or correlations between different variables at same conditions, parametric correlation tests (Pearson) were used. We also calculated the magnitude of CORT levels, T levels, and N:L ratio changes due to the restraint challenge as a difference between post-restraint and baseline values (by subtracting baseline from post-restrained values, inside each group: with and without movement restriction), as in Claunch et al. (2017), and included these variables in the correlation analyses. All analyzes were performed using IBM SPSS Statistics 22.

3. Results

3.1. Intraspecific comparisons

3.1.1. *Rhinella ornata*

Descriptive statistics for body measures and all physiological variables in *R. ornata* are shown in Table S1. Body mass did not affect any of the investigated variables under any circumstance (body mass or the interaction body mass * group [with and without movement restriction]; $P \geq 0.083$).

Restraint challenge affected only N:L ratio in *R. ornata* (Table 1). Although baseline CORT tended to be higher, there were no statistical differences between baseline and restraint groups (with or without movement restriction) in CORT levels (Fig. 2A). Toads with higher baseline CORT levels showed the highest decrease in CORT levels after restraint ($r = -0.843$; $P \leq 0.001$). Baseline T levels were exceptionally high in this species and we were able to determine T levels for only four samples (114.70 ± 17.66 ng/ml; mean \pm standard deviation), preventing comparisons with post-treatment levels. After 24 h under restraint, *R. ornata* displayed detectable T levels, and showed no differences between groups ($t_{17} = -0.764$, $P = 0.455$; Fig. 2B).

Compared to the baseline values, there was a trend of increased N:L ratio after restraint without movement restriction (Fig. 2C). Toads submitted to restraint with movement restriction showed a four-fold increase in the N:L ratio compared to baseline values, and individuals without movement restriction tended to have higher ratios (Fig. 2C). BKA was similar in all tested groups (Fig. 2D). There was a positive correlation between CORT and BKA after restraint ($r = 0.532$; $P = 0.023$).

3.1.2. *Rhinella icterica*

Descriptive statistics of all variables for *R. icterica* are shown in Table S2. Baseline N:L ratio ($F_{1,14} = 4.890$, $P = 0.044$) and BKA ($F_{1,15} = 20.118$, $P \leq 0.001$) were affected by body mass, with larger toads having the lowest N:L ratio, and the highest BKA values. BKA was also affected by the interaction body mass * group: with and without movement restriction ($F_{1,15} = 7.613$, $P = 0.015$), with toads with lower body masses under restraint and movement restriction displaying the lowest BKA, and individuals with higher body mass restrained without movement restriction showing the highest BKA values. However, body

Table 1

Restraint challenge effects on physiological variables of *Rhinella ornata* tested through a set of mixed ANOVAs, with BKA, N:L ratio and CORT plasma levels as dependent variables, and restraint challenge (baseline or restraint) and group (with and without movement restriction) as factors.

Variable	Source	Type III SS	DF	MS	F	P
BKA	Intercept	143681.430	1	143681.430	151.063	0.000
	Group	1341.513	1	1341.513	1.410	0.252
	Error (Group)	15218.158	16	951.135		
	Restraint challenge	150.389	1	150.389	0.162	0.692
	Restraint challenge * Group	1389.550	1	1389.550	1.500	0.238
	Error (Restraint challenge)	14817.584	16	926.099		
N:L	Intercept	2.169	1	2.169	87.034	0.000
	Group	0.065	1	0.065	2.590	0.128
	Error (Group)	0.374	15	0.025		
	Restraint challenge	0.266	1	0.266	13.713	0.002
	Restraint challenge * Group	0.049	1	0.049	2.523	0.133
	Error (Restraint challenge)	0.291	15	0.019		
CORT	Intercept	79.813	1	79.813	295.673	0.000
	Group	0.019	1	0.019	0.071	0.793
	Error (Group)	4.319	16	0.270		
	Restraint challenge	0.122	1	0.122	0.755	0.398
	Restraint challenge * Group	0.132	1	0.132	0.821	0.378
	Error (Restraint challenge)	2.578	16	0.161		

BKA: Bacterial killing ability; N:L: Neutrophil/lymphocyte ratio; CORT: Corticosterone; Restraint challenge: Baseline or restraint; Group: With or without movement restriction; Type III SS: Type III sum of squares; DF: Degrees of freedom; MS: Mean square. Variables with P significant < 0.05 are highlighted in bold.

mass did not differ between groups ($t_{18} = 0.497$, $P = 0.625$).

The restraint challenge affected all physiological variables measured in *R. icterica* (Table 2). CORT levels increased 7.5 and 3 times relative to baseline values, following restraint with and without movement restriction, respectively (Fig. 3A). Additionally, toads under movement restriction showed a 2.5-fold greater CORT increase than individuals without movement restriction after restraint (Fig. 3A). When compared to baseline values, T levels decreased 4- and 8-fold relative to baseline values, following restraint with and without movement restriction, respectively (Fig. 3B). Toads with higher baseline T displayed the most accentuated decrease in T in response to the restraint challenge ($r = -0.945$; $P \leq 0.001$).

There were no significant differences in N:L ratio between toads submitted to restraint without movement restriction and baseline values, despite the trend of higher N:L ratio in restrained toads (Fig. 3C). Restraint with movement restriction increased N:L ratio 4 times compared to baseline, and individuals submitted to restraint with movement restriction tended to show higher N:L ratio than individuals without movement restriction (Fig. 3C). BKA decreased after restraint with movement restriction in relation to the baseline values, with toads having the higher magnitude of increase in CORT also showing the lowest BKA values ($r = -0.686$; $P = 0.041$). No significant changes in BKA were observed after restraint without movement restriction when compared to baseline (Fig. 3D).

3.1.3. *Rhinella schneideri*

Descriptive statistics for all investigated variables in *R. schneideri* are shown in Table S3. Baseline body mass affected CORT ($F_{1,16} = 7.490$, $P = 0.015$), as toads with higher CORT values possessed the lowest

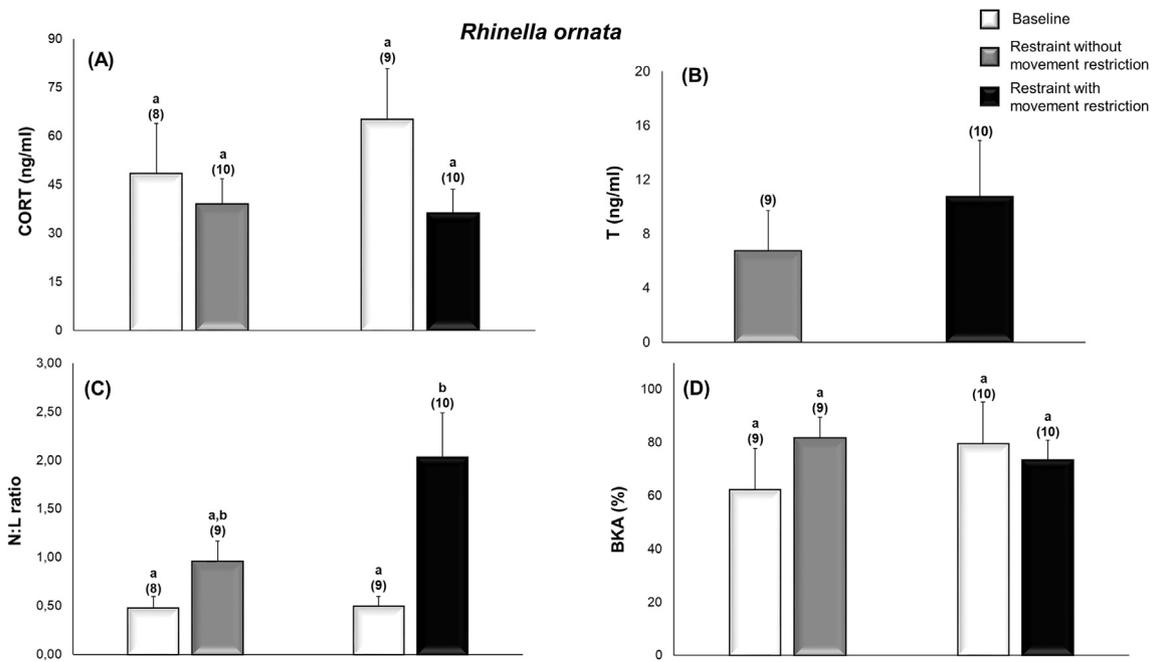


Fig. 2. Comparison of physiological variables at baseline and after 24 h of the restraint challenge in *Rhinella ornata*. The bars represent the mean \pm standard error with *N* in parentheses. (A) Corticosterone (CORT) plasma levels. (B) Neutrophil/lymphocyte ratio (N:L ratio). (C) Bacterial killing ability of plasma (BKA). (D) Testosterone (T) plasma levels. Letters above the bars represent statistical differences for a mixed ANOVA followed by tests for multiple comparisons with Bonferroni adjustment, with different letters representing statistical difference with $P \leq 0.05$.

body mass; however, body mass did not differ between groups with or without movement restriction ($t_{18} = 0.632$, $P = 0.535$).

All physiological variables were affected by the restraint challenge in *R. schneideri*, except BKA (Table 3). CORT levels increased 11- and 5-fold after restraint with or without movement restriction relative to the baseline values, respectively (Fig. 4A). Toads submitted to restraint with movement restriction also showed a trend of higher CORT values than individuals without movement restriction (Fig. 4A). When compared to the baseline values, T levels were 3.5- and 3-fold lower in animals submitted to restraint with or without movement restriction, respectively (Fig. 4B). Toads with the highest baseline T values presented the most pronounced decrease in T following restraint ($r = -0.974$; $P < 0.001$).

The restraint challenge increased N:L ratio, resulting in values 2-fold higher in toads submitted to restraint without movement restriction compared to baseline values (Fig. 4C). There were no differences in BKA in response to the restraint challenge (baseline vs. restraint) or between groups (with vs. without movement restriction) (Fig. 4D). Toads with the lowest N:L ratio at baseline tended to display the highest amplitude of response after restraint ($r = -0.422$; $P < 0.072$).

3.2. Interspecific comparisons

3.2.1. Baseline values

Baseline CORT differed among species (Table S4), and was affected by body mass ($F_{1,52} = 5.769$; $P = 0.020$), with a negative interspecific correlation between CORT and body mass ($r = -0.996$; $P \leq 0.001$). *Rhinella ornata* displayed baseline CORT levels 13.5- and 25.5-fold higher than *R. icterica* and *R. schneideri*, respectively (Fig. 5A). T levels were not affected by body mass ($F_{1,34} = 2.088$; $P = 0.158$), but differed between species ($t_{36} = 3.147$, $P = 0.003$), with *R. icterica* showing 3.5-fold higher T values than *R. schneideri* in the field (Fig. 5B).

The N:L ratio also differed among species (Table S4), and were affected by body mass and the interaction body mass * species ($P \leq 0.016$), with *R. ornata* showing 2.5-fold higher values than *R. icterica* and 4-fold higher values than *R. schneideri* (Fig. 5C). Baseline BKA differed between species (Table S4) with *R. icterica* showing 20% higher

BKA than the other two species (Fig. 5D).

3.2.2. Restraint (24 h under captivity)

• Without movement restriction

CORT differed among species (Table S5) and was affected by body mass ($F_{1,23} = 4.745$; $P = 0.040$) after restraint without movement restriction. *Rhinella ornata* showed CORT levels 2.5- and 3-fold higher than *R. icterica* and *R. schneideri*, respectively (Fig. 6A). T levels differed between species (Table S5) and was not affected by body mass ($F_{1,21} = 1.876$; $P = 0.185$), with *R. ornata* showing 5-fold higher values than the other species (Fig. 6B).

The N:L ratio also differed among species (Table S5) and was not affected by body mass ($F_{1,22} = 1.004$; $P = 0.327$). *Rhinella ornata* showed N:L ratio 3-fold and 3.5-fold higher values than *R. icterica* and *R. schneideri*, respectively (Fig. 6C). BKA did not differ among species (Table S5; Fig. 6D) but was affected by the interaction body mass * species after restraint without movement restriction ($F_{2,22} = 5.230$; $P = 0.014$), with *R. schneideri*, the species showing the highest body mass, displaying the lowest BKA values.

• With movement restriction

CORT did not differ among species (Table S6; Fig. 7A) and was not affected by body mass after restraint with movement restriction ($F_{1,24} = 0.153$; $P = 0.699$). T levels were affected by body mass ($F_{1,23} = 5.917$; $P = 0.023$) and differed among species (Table S6), with *R. ornata* showing 6.7- and 14.7-fold higher values than *R. icterica* and *R. schneideri*, respectively (Fig. 7B).

N:L ratio were not affected by body mass ($F_{1,23} = 0.427$; $P = 0.520$), and differed among species (Table S6), with *R. ornata* showing 3- and 10.5-fold higher values than *R. icterica* and *R. schneideri*, respectively (Fig. 7C). BKA also differed among species (Table S6) and was not affected by body mass ($F_{1,23} = 0.263$; $P = 0.613$), with *R. schneideri* showing 23% lower BKA values than *R. icterica* after restraint with movement restriction (Fig. 7D).

Table 2

Restraint challenge effects on physiological variables of *Rhinella icterica* tested through a set of mixed ANOVAs, with BKA, N:L ratio and CORT and T plasma levels as dependent variables, and restraint challenge (baseline or restraint) and group (with and without movement restriction) as factors.

Variable	Source	Type III SS	DF	MS	F	P
BKA	Intercept	215615.305	1	215615.305	335.908	0.000
	Group	359.348	1	359.348	0.560	0.465
	Error (Group)	10912.098	17	641.888		
	Restraint challenge	2096.091	1	2096.091	6.694	0.019
	Restraint challenge * Group	86.541	1	86.541	0.276	0.606
	Error (Restraint challenge)	5323.375	17	313.140		
	N:L	Intercept	0.514	1	0.514	76.295
Group		0.011	1	0.011	1.586	0.226
Error (Group)		0.108	16	0.007		
Restraint challenge		0.066	1	0.066	11.140	0.004
Restraint challenge * Group		0.029	1	0.029	4.887	0.042
Error (Restraint challenge)		0.095	16	0.006		
CORT		Intercept	35.438	1	35.438	391.431
	Group	0.281	1	0.281	3.099	0.095
	Error (Group)	1.630	18	0.091		
	Restraint challenge	3.335	1	3.335	40.221	0.000
	Restraint challenge * Group	0.203	1	0.203	2.449	0.135
	Error (Restraint challenge)	1.493	18	0.083		
	T	Intercept	11.196	1	11.196	181.608
Group		0.000	1	0.000	0.005	0.946
Error (Group)		0.925	15	0.062		
Restraint challenge		1.503	1	1.503	18.730	0.001
Restraint challenge * Group		0.001	1	0.001	0.010	0.920
Error (Restraint challenge)		1.203	15	0.080		

BKA: Bacterial killing ability; N:L: Neutrophil/lymphocyte ratio; CORT: Corticosterone; T: Testosterone; Restraint challenge: Baseline or restraint; Group: With or without movement restriction; Type III SS: Type III sum of squares; DF: Degrees of freedom; MS: Mean square. Variables with *P* significant < 0.05 are highlighted in bold.

4. Discussion

4.1. Intraspecific effects

Toads showed a general response pattern following restraint, which included increased physiological indicators of stress, represented by CORT levels and N:L ratio, and decreased physiological variables related to reproduction and innate immune response, represented by T levels and BKA, respectively.

According to our predictions, when compared to baseline, *R. icterica* and *R. schneideri* showed higher CORT levels following restraint, corroborating previous studies with anurans (Narayan et al., 2011a; Narayan et al., 2012; Gomes et al., 2012; Graham et al., 2012). Additionally, CORT tended to increase in response to restraint with movement restriction, compared to restraint without movement restriction. This difference was statistically significant for *R. icterica* and although not significant, the same pattern was observed for *R. schneideri*. These results suggest that toads are able to respond differently to the intensity of the stressor (Assis et al., 2015). Interestingly, *R. ornata* was the only species not showing an increase in CORT levels in response to the restraint challenge. Although all toads were collected within breeding season, *R. ornata* was the only species in which males were

collected during calling activity. CORT levels are generally high during reproductive season among vertebrates, including anurans (Moore and Jessop, 2003; Assis et al., 2012), which most likely facilitates reproductive behavior through mobilization of energy reserves (Moore and Jessop, 2003; Landys et al., 2006; Carr, 2011). Given that calling activity entail high energetic expenditure (e.g. Pough et al., 1992; Wells, 2001), CORT levels are high and positively correlated with calling rate in anurans (Emerson and Hess, 1996; Leary et al., 2005; Assis et al., 2012). By sustaining high calling rates, individuals of *R. ornata* most likely already had high levels of CORT, similar to those that would have otherwise been elicited by the restraint stress protocol.

N:L ratio were higher than values characteristic of baseline conditions for all the species following restraint. This is in accordance with patterns reported by other studies, which include increased N:L ratio following restraint stress in macaques (Morrow-Tesch et al., 1993), captivity stress in salamanders (Davis and Maerz, 2008) and lizards (Seddon and Klukowski, 2012); and following exogenous administration of corticosterone in salamanders (Davis and Maerz, 2010). The leukocyte profile is altered by stress and can be directly related to the plasma levels of stress hormones. Specifically, changes in leukocyte profile caused by stress or by treatment with GCs include increased numbers of circulating neutrophils and decreased numbers of circulating lymphocytes (Davis et al., 2008). Twenty-four hours of restraint without movement restriction increased CORT levels, while restraint with movement restriction elevated CORT further, as well as increased N:L ratio in *R. icterica*. Although toads generally responded to restraint with increased CORT and N:L ratio, supporting our hypotheses and previous interpretations from other authors, these variables were not correlated in any of the three studied species. Interestingly, *R. ornata* responded to the restraint challenge by increasing N:L ratio, although CORT did not differ from the values from field conditions. Therefore, it is possible that individuals of *R. ornata* already possessed elevated CORT levels in the field (baseline) and, when restrained, showed the common response to the more intense stressor for the other species, increased N:L ratio. GC plasma levels have been used as a proxy of stress levels in natural populations (Dunlap and Wingfield, 1995; Hopkins et al., 1997; Norris et al., 1997; Janin et al., 2011). Additionally, some authors assume that changes in CORT directly cause a proportional change in N:L ratio, and N:L ratio could consequently be used as an indication of CORT changes over time (Davis et al., 2008). In this study, although N:L ratio increased in response to restraint, we found no correlation with CORT, which has been observed in free-living birds (Vleck et al., 2000; Muller et al., 2011). We do, however, agree with Muller et al. (2011) that N:L ratio and CORT can be used together to provide a comprehensive picture about the stress condition of animals.

All three species, *R. icterica*, *R. ornata* and *R. schneideri*, showed a reduction of T after restraint. For *R. ornata*, baseline T levels were exceptionally high, being determined for only four individuals. The low number of individuals that had their T levels measured under baseline conditions did not allow formal statistical comparisons, but provided some evidence that restraint challenge also reduces T levels in this species, as levels were lower and detectable by the hormone kit used after restraint. Additionally, *R. ornata* and *R. icterica*, the species with higher T levels, showed a higher decrease of T under stress conditions, which has been described previously for birds (Deviche et al., 2012; Deviche et al., 2014). Those authors suggested that stressed birds keep a minimum T level necessary for maintenance of T-dependent behavioral and morphological sexual characteristics and/or physiological functions, particularly avoiding the negative feedback on gonadotropin secretion (Deviche et al., 2014). In our study and in both studies with birds (Deviche et al., 2012; Deviche et al., 2014), changes in T were not correlated with those observed in CORT, suggesting that T reduction in response to stressors is not simply a function of increased CORT secretion (Deviche et al., 2012). Studies among diverse of vertebrate species have shown that stressors typically suppress T, sometimes quite

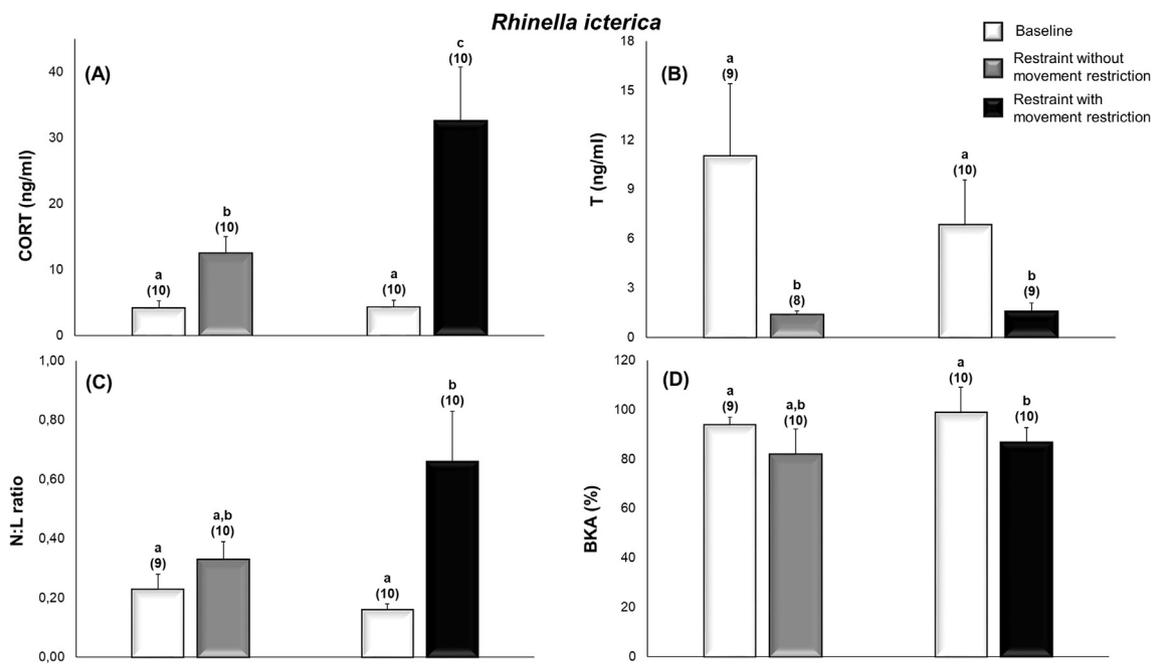


Fig. 3. Comparison of physiological variables at baseline and after 24 h of the restraint challenge in *Rhinella icterica*. The bars represent the mean \pm standard error with *N* in parentheses. (A) Corticosterone (CORT) plasma levels. (B) Neutrophil/lymphocyte ratio (N:L ratio). (C) Bacterial killing ability of plasma (BKA). (D) Testosterone (T) plasma levels. Letters above the bars represent statistical differences for a mixed ANOVA followed by tests for multiple comparisons with Bonferroni adjustment, with different letters representing statistical difference with $P \leq 0.05$.

rapidly (Greenberg and Wingfield, 1987; Moore et al., 1991; Jones and Bell, 2004; Deviche et al., 2010; Tokarz and Summers, 2011; Deviche et al., 2012; Deviche et al., 2014), including in anurans (Narayan et al., 2011b; Narayan et al., 2012). Multiple physiological mechanisms can potentially explain the transition between positive to negative relationships between CORT and T, including rapid direct inhibition of testicular function by GCs (Dong et al., 2004; Hardy et al., 2005; Hu et al., 2008; Martin and Tremblay, 2008), and an acceleration of T clearance through interactions of CORT with plasma corticosterone-binding globulin (Deviche et al., 2001). More studies in anurans are necessary to determine which mechanisms are involved in how stress and CORT influence changes in T.

Rhinella icterica had decreased BKA in response to restraint challenge. *Rhinella ornata* and *R. schneideri* also tended to have decreased BKA after restraint with movement restriction. Similarly, studies using a restraint stress protocol in birds showed a reduction in BKA (Matson et al., 2006) and a negative correlation with CORT (Millet et al., 2007). Previous studies with anurans, using restraint stress protocol (Graham et al., 2012) and long-term captivity (Assis et al., 2015; Titon et al., 2017), also reported declines in BKA, but no significant correlations between CORT and BKA. We found a negative correlation between BKA and the amplitude of increase in CORT after restraint without movement restriction only in *R. icterica*. Contrary to these results, *R. ornata* showed a positive correlation between CORT and BKA in response to restraint challenge. Interestingly, a positive relationship between stress following restraint and BKA was found in a salamander (*Cryptobranchus alleganiensis*), showing that BKA may increase after an acute stress (Hopkins and DuRant, 2011). We know that BKA differs between species (Assis et al., 2013), and different patterns of response to the same stress protocol are completely plausible. It is also possible that correlations between CORT and immunity could interact with T in toads, as has already found for anurans (Titon et al., 2017) and birds (Davies et al., 2016).

4.2. Interspecific comparisons

Males of *R. ornata* showed higher baseline CORT levels than the

other species. As already discussed, *R. ornata* was the only species calling in the field during the nights of data collection, despite all the species were collected within the breeding season. Since anuran males show correlated variation in calling performance and CORT (Moore et al., 2005; Assis et al., 2012), and even phylogenetically close species can vary dramatically in calling performance (Bevier et al., 2008), the observed interspecific variation in baseline CORT might reflect, at least in part, differences associated with calling behavior. Additionally, there was a negative correlation between baseline CORT and body mass in our study. In birds, variation in baseline CORT among species was inversely related to body mass and length of the breeding season (Hau et al., 2010). Individuals of smaller species have higher mass-specific metabolic rates (Calder, 1996), and given that some studies have found that GCs can increase metabolic rate (DuRant et al., 2008; Preest and Cree, 2008; Wack et al., 2012), it is possible that the allometric association of CORT in toads have energetic implications.

It is interesting to note that *R. ornata* also tended to have the highest CORT levels after restraint, while the lowest CORT levels were found in *R. schneideri*. Interestingly, *R. schneideri*, the species with the lowest baseline CORT levels, showed the most pronounced post-restraint increase in CORT levels. This trend for higher amplitude of acute stress response in individuals with lower baseline CORT has been previously reported at intraspecific level for snakes (Claunch et al., 2017). These results suggest that more generalist species, which normally occupy and persist in naturally open and/or disturbed areas, might adjust more efficiently to these stressful conditions by maintaining lower baseline CORT levels (Gomes et al., 2012). To formally test a hypothesis on these lines, a comparative study including a larger number of species should be conducted, with results analyzed using phylogenetic statistical methods (Rezende and Diniz-Filho, 2012).

Rhinella ornata showed higher N:L ratio at baseline and after restraint conditions than the other species. As previously discussed, changes in CORT have been functionally associated with altered numbers of neutrophils and lymphocytes in circulation (Davis et al., 2008; Seddon and Klukowski, 2012). Although we observed no significant correlation between N:L ratio and CORT levels, there was a strong trend for species with higher CORT following restraint to also show higher

Table 3

Restraint challenge effects on physiological variables of *Rhinella schneideri* tested through a set of mixed ANOVAs, with BKA, N:L ratio, CORT and T plasma levels as dependent variables, and restraint challenge (baseline or restraint) and group (with and without movement restriction) as factors.

Variable	Source	Type III SS	DF	MS	F	P
BKA	Intercept	87992.404	1	87992.404	406.370	0.000
	Group	153.120	1	153.120	0.707	0.412
	Error (Group)	3681.056	17	216.533		
	Restraint challenge	55.571	1	55.571	0.458	0.508
	Restraint	390.100	1	390.100	3.215	0.091
	challenge * Group					
	Error (Restraint challenge)	2062.422	17	121.319		
N:L	Intercept	0.170	1	0.170	129.594	0.000
	Group	0.002	1	0.002	1.876	0.189
	Error (Group)	0.022	17	0.001		
	Restraint challenge	0.010	1	0.010	11.146	0.004
	Restraint	0.002	1	0.002	2.905	0.107
	challenge * Group					
	Error (Restraint challenge)	0.015	17	0.001		
CORT	Intercept	23.119	1	23.119	188.044	0.000
	Group	0.251	1	0.251	2.043	0.170
	Error (Group)	2.213	18	0.123		
	Restraint challenge	4.180	1	4.180	30.834	0.000
	Restraint	0.378	1	0.378	2.791	0.112
	challenge * Group					
	Error (Restraint challenge)	2.440	18	0.136		
T	Intercept	5.182	1	5.182	107.629	0.000
	Group	0.098	1	0.098	2.030	0.172
	Error (Group)	0.818	17	0.048		
	Restraint challenge	0.439	1	0.439	14.770	0.001
	Restraint	0.016	1	0.016	0.544	0.471
	challenge * Group					
	Error (Restraint challenge)	0.506	17	0.030		

BKA: Bacterial killing ability; N:L: Neutrophil/lymphocyte ratio; CORT: Corticosterone; T: Testosterone; Restraint challenge: Baseline or restraint; Group: With or without movement restriction; Type III SS: Type III sum of squares; DF: Degrees of freedom; MS: Mean square. Variables with *P* significant < 0.05 are highlighted in bold.

N:L ratio under the same conditions, reinforcing pattern previously observed by other authors at the intraspecific level (Vleck et al., 2000; Müller et al., 2011). Moreover, the three studied species showed increased N:L ratio following the restraint challenge, even without CORT changes for *R. ornata*. Our results also demonstrated that the amplitude of N:L ratio increase could be associated with the intensity of the stressor, with *R. icterica* and *R. ornata* displaying higher increases in N:L ratio in response to restraint with movement restriction than without movement restriction.

Rhinella ornata showed higher T than *R. icterica* and *R. schneideri* after restraint (with and without movement restriction). Although we were unable to run an ANOVA to compare T for these 3 species under baseline conditions, the 4 individuals of *R. ornata* that had T measured under baseline conditions also showed mean values 13 and 46 times higher than those measured for *R. icterica* and *R. schneideri*, respectively. T levels can show huge variation within the breeding season (Zerani et al., 1991; Houck and Woodley, 1995; Canosa and Ceballos, 2002; Assis et al., 2012). Moreover, high androgen levels are associated with displays of sexual behavior among breeding frog species (Emerson and Hess, 2001; Leary et al., 2005), with studies showing a positive correlation between calling behavior and T levels in anurans (Moore et al., 2005; Assis et al., 2012). Thus, as for CORT, the difference in T at baseline, might be related to calling behavior implications on T levels. The restraint challenge decreased T in the three-studied species, with the species with the highest baseline T levels (*R. ornata*) showing the

highest decrease after restraint (with and without movement restriction), followed by *R. icterica* and *R. schneideri*. Furthermore, as there was no correlation between T and CORT after the restraint challenge for any of the studied species, our results reinforce that the decrease in T levels in response to a stress protocol is not a simple function of increase in CORT levels.

Regarding immunity, *R. icterica* showed the highest baseline BKA values. This result was not expected, given that in the previous comparative study by Gomes et al. (2012), *R. schneideri* showed the highest baseline BKA values and lowest baseline CORT levels when compared to the other two species of *Rhinella*. Our initial hypothesis was that the species with lower levels of CORT should also have higher BKA, indicating lower immunosuppressive potential. However, we know that CORT has a complex immunomodulatory effect, and GC levels and the immune response vary greatly even at intraspecific level, depending on environmental conditions and time of life cycle (Norris and Evans, 2000; Lee and Klasing, 2004; Verhulst et al., 2005; Lee, 2006; Romero, 2002; Lattin and Romero, 2013; Wingfield and Romero, 2001). Following restraint with movement restriction, BKA differed only between *R. icterica* and *R. schneideri*, again with *R. icterica* showing higher BKA. The complex patterns of variation at intraspecific level complicate interspecific comparison. Sampling more species with data analyzed under a phylogenetic statistical framework, and sampling all species closer in time (e.g. same month) could help to better discern the sources of variation at intra and interspecific levels. Furthermore, the inclusion of different segments of the immune response (e.g. humoral, cellular mediated, and inflammatory responses) may be very informative, since the investment in each segment of immune response can differ within or between species.

5. Conclusions

Twenty-four hours of restraint promoted a stress response in different species of toads (*Rhinella*) that included increased CORT levels and N:L ratio, and decreased T levels and BKA. *Rhinella ornata* was the only species not showing increased CORT levels in response to the restraint challenge, possibly due to already experiencing high CORT levels related to vocal activity. Moreover, CORT levels and N:L ratio tended to increase more in response to restraint with movement restriction than to restraint without movement restriction, relative to baseline values, suggesting that these toads are able to respond differently to the intensity of the stressor applied. *Rhinella icterica* showed a reduction in BKA, with *R. ornata* and *R. schneideri* also displaying a trend to decrease BKA after restraint with movement restriction, indicating that acute stress response can promote decreased innate immune response in toads.

Species of *Rhinella* differ in all physiological variables measured. *Rhinella ornata* showed higher CORT levels, T levels and N:L ratio when compared to other species, possibly due to calling behavior in the field for this species. *Rhinella icterica* showed higher BKA than the other two studied species, with differences among species being more evident at baseline and after restraint with movement restriction than after restraint without movement restriction. Our results also suggest that more generalist species, which normally occupy and persist in naturally open and/or disturbed areas, might adjust more efficiently to stressful conditions by maintaining lower baseline and post stress CORT levels. However, interspecific comparisons in GC plasma levels and the immune response are difficult to establish, since these physiological variables vary greatly even at intraspecific level, depending on environmental conditions and time of life cycle. In this way, it is necessary to standardize data collection for all the species compared under the same period (e.g. inside or outside of breeding season) and same activity (e.g. calling or foraging), given that vocal activity is a source of great variability in hormonal levels and probably on immune response.

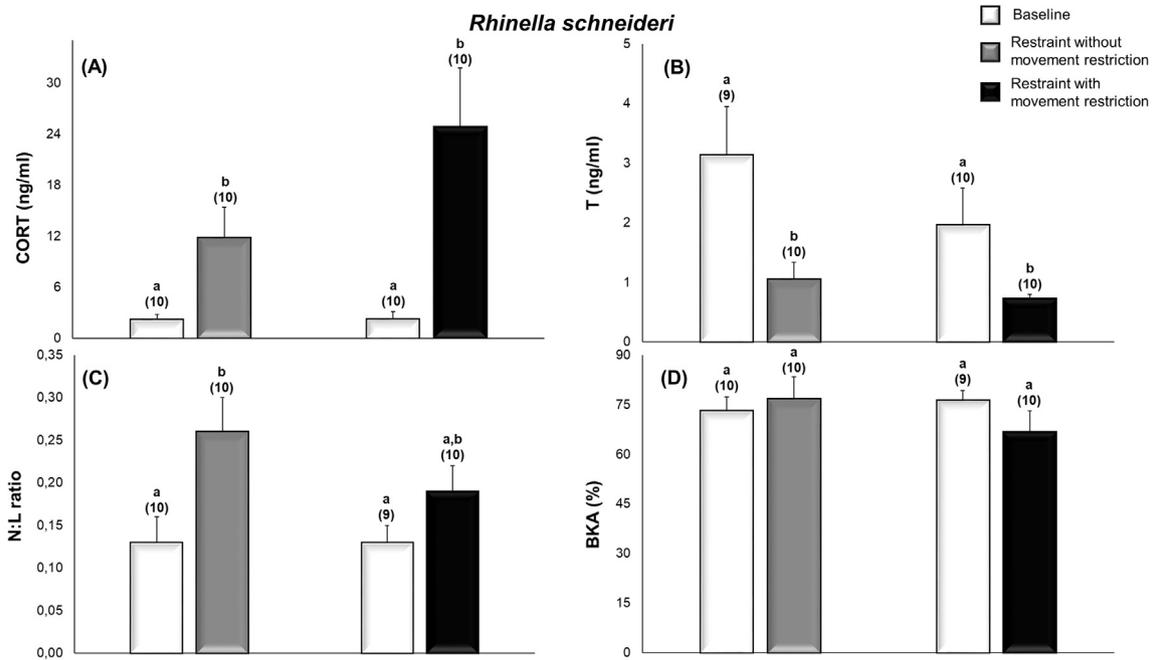


Fig. 4. Comparison of physiological variables at baseline and after 24 h of the restraint challenge in *Rhinella schneideri*. The bars represent the mean \pm standard error with *N* in parentheses. (A) Corticosterone (CORT) plasma levels. (B) Neutrophil/lymphocyte ratio (N:L ratio). (C) Bacterial killing ability of plasma (BKA). (D) Testosterone (T) plasma levels. Letters above the bars represent statistical differences for a mixed ANOVA followed by tests for multiple comparisons with Bonferroni adjustment, with different letters representing statistical difference with $P \leq 0.05$.

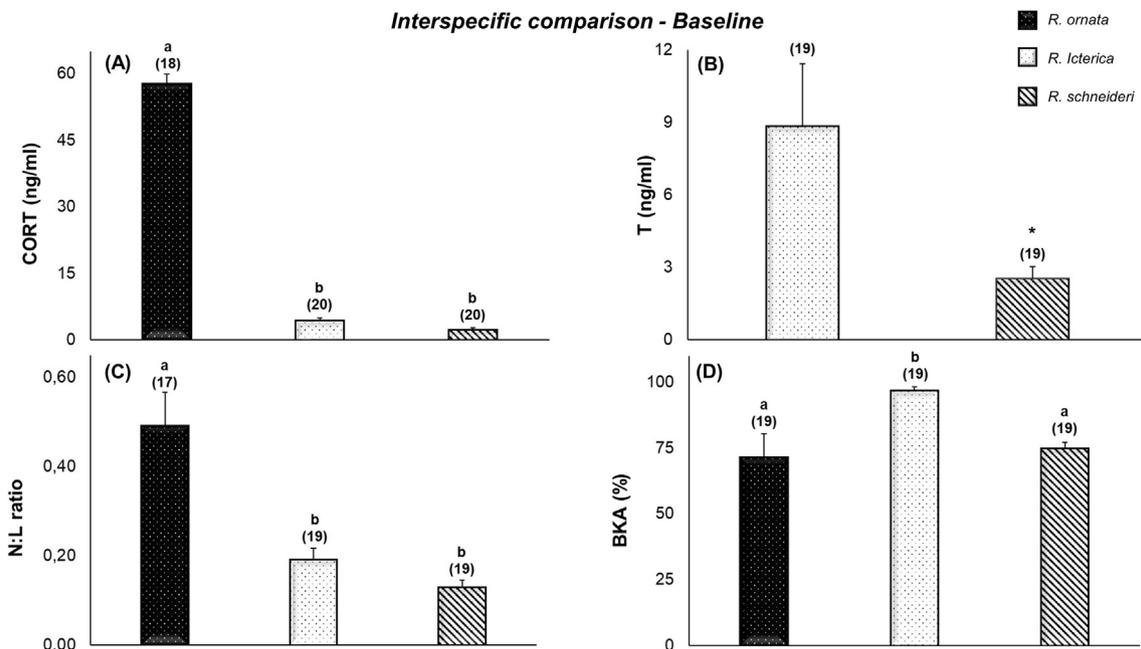


Fig. 5. Comparison of physiological variables at baseline in the three *Rhinella* species (*R. ornata*, *R. ictERICA* and *R. schneideri*). The bars represent the mean \pm standard error with *N* in parentheses. (A) Corticosterone (CORT) plasma levels. (B) Neutrophil/lymphocyte ratio (N:L ratio). (C) Bacterial killing ability of plasma (BKA). (D) Testosterone (T) plasma levels. Letters above the bars represent statistical differences for a univariate ANOVA followed by tests for multiple comparisons with Bonferroni adjustment, with different letters representing statistical difference with $P \leq 0.05$. The asterisk in (B) represents *t*-test statistical differences with $P \leq 0.05$.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.ygcen.2018.05.008>.

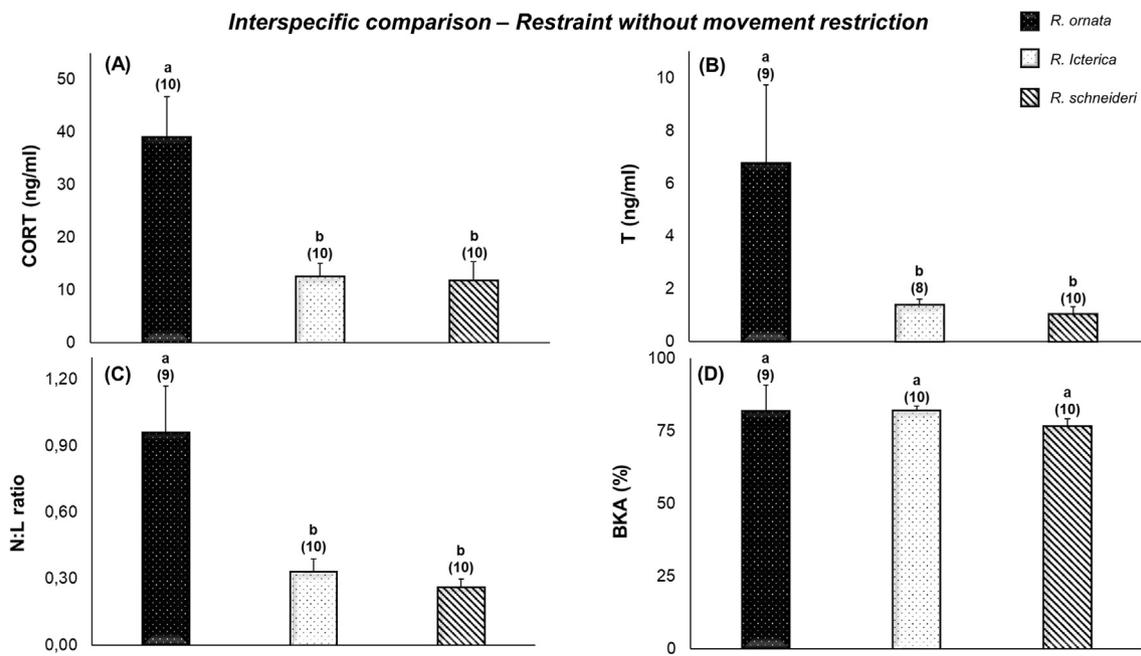


Fig. 6. Comparison of physiological variables after 24 h of restraint challenge without movement restriction in the three *Rhinella* species (*R. ornata*, *R. ictERICA* and *R. schneideri*). The bars represent the mean \pm standard error with *N* in parentheses. (A) Corticosterone (CORT) plasma levels. (B) Neutrophil/lymphocyte (N:L) ratio. (C) Bacterial killing ability of plasma (BKA). (D) Testosterone (T) plasma levels. Letters above the bars represent statistical differences for a univariate ANOVA followed by tests for multiple comparisons with Bonferroni adjustment, with different letters representing statistical difference with $P \leq 0.05$.

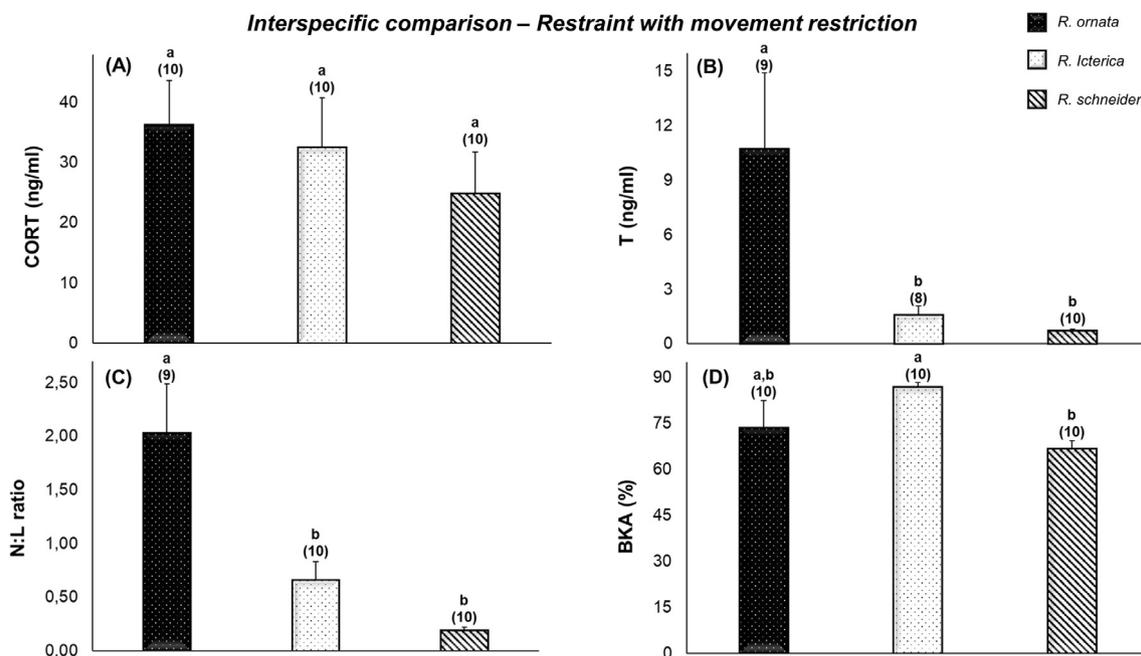


Fig. 7. Comparison of physiological variables after 24 h of restraint challenge with movement restriction in the three *Rhinella* species (*R. ornata*, *R. ictERICA* and *R. schneideri*). The bars represent the mean \pm standard error with *N* in parentheses. (A) Corticosterone (CORT) plasma levels. (B) Neutrophil/lymphocyte ratio (N:L ratio). (C) Bacterial killing ability of plasma (BKA). (D) Testosterone (T) plasma levels. Letters above the bars represent statistical differences for a univariate ANOVA followed by tests for multiple comparisons with Bonferroni adjustment, with different letters representing statistical difference with $P \leq 0.05$.

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