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Baseline and stress-induced changes in plasma bacterial killing ability against gram-negative bacteria are partially mediated by the complement system in *Rhinella diptycha* toads

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ABSTRACT

The plasma bacterial killing ability (BKA) is modulated by the stress response in vertebrates, including amphibians. The complement system is an effector mechanism comprised of a set of proteins present in the plasma that once activated can promote bacterial lysis. Herein, we investigated whether changes in plasma BKA as a result of the acute stress response and an immune challenge are mediated by the complement system in *Rhinella diptycha* toads. Additionally, we investigated whether the observed changes in plasma BKA are associated with changes in plasma corticosterone levels (CORT). We subjected adult male toads to a restraint or an immune challenge (with three concentrations of *Aeromonas hydrophila* heat inactivated), and then evaluated the plasma BKA against *A. hydrophila, in vitro*. We determined the complement system activity on plasma BKA, by treating the plasma (baseline, 1 h and 24 h post-restraint, and after the immune challenge) with ethylenediaminetetraacetic acid, heat, or protease. Our results showed increased CORT 1 h and 24 h after restraint and decreased plasma BKA 24 h post-restraint. The inhibitors of the complement system decreased the plasma BKA compared with untreated plasma at all times (baseline, 1 h, and 24 h after restraint), demonstrating that the plasma BKA activity is partially mediated by the complement system. The immune challenge increased CORT, with the highest values being observed in the highest bacterial concentration, compared with control. The plasma BKA was not affected by the immune challenge but was demonstrated to be partially mediated by the complement system. Our results demonstrated that restraint and the immune challenge activated the hypothalamus-pituitary-interrenal axis, by increasing plasma CORT levels in *R. diptycha*. Also, our results demonstrated the complement system is participative in the plasma BKA for baseline and post-stress situations in these toads.

1. Introduction

Amphibians have experienced an extensive population decline in recent decades (Iii et al., 2006; Hussain and Pandit, 2012; Scheele et al., 2019). This decline has been associated with several factors, such as anthropically mediated environmental changes that include climate change, deforestation, and pollutants (Rollins-Smith, 2017). In addition, infectious diseases have become established in amphibian populations, causing mass deaths in several natural populations (Becker et al., 2017). The aquatic fungus *Batrachochitrium dendrobatidis* (*Bd*), for example, is highly associated with the decline of several amphibian populations, with studies demonstrating that the infection interrupts the cutaneous transport of sodium, which leads to hyponatremia (low concentration of sodium in the blood) and heart failure, in addition to being associated with deleterious effects on the immune system (Peterson et al., 2013; Grogan et al., 2018, 2020). Peterson et al. (2013) demonstrated that elevated corticosterone (CORT, main glucocorticoid in amphibians) is associated with the chytridiomycosis anuran disease, caused by the *Bd*. This correlates with some of the deleterious effects observed during disease development, suggesting physiological processes modulated by increased CORT may be associated with the observed effects of a *Bd* outbreak in green tree-frog *Litoria caerulea*. Therefore, it is important to understand how changes in immune functions are related to increased plasma CORT, a key hormone in mediating the stress response and

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modulating the immune system in amphibians (Gomes et al., 2022).

The immune system of amphibians is composed of a set of defense mechanisms, including the complement system, which can recognize and eliminate pathogens, being an essential non-cellular mechanism of innate immunity (Rodriguez and Voyles, 2020; Ruiz and Robert, 2023). The complement system is comprised of a set of proteins, which in the absence of an infection, circulate in an inactive form. In the presence of pathogens, or antibodies bound to pathogens, the complement system is activated (Murphy and Weaver, 2017). Particular complement proteins interact with each other to form distinct pathways of complement activation, all of which promote pathogen killing, either directly or by facilitating its phagocytosis, and inducing inflammatory responses that help to fight infection (Murphy and Weaver, 2017). The complement system is activated by three main pathways: (1) The classical pathway; (2) The lectin pathway; and (3) The alternative pathway, mediated by proteins that bind to the microorganism and inactivate it (Murphy and Weaver, 2017). The complement system activation can result in three actions: (1) inflammation, which occurs when C3a acts as a chemoattractant for phagocytes to locate pathogens; (2) opsonization, where the C3b protein binds to the pathogen's cell membrane, marking it to be phagocytosed and eliminated by phagocytes; and (3) lysis of pathogenic cells.

As in other vertebrates, the amphibian immune system is responsible for surveillance, and elimination of possible threats to the organism and is modulated in response to stressors (Rollins-Smith, 2001; Ruiz and Robert, 2023). The stress response involves endocrine signals that modulate most aspects of physiology, including immune functions (Sapolsky, 2002). The mobilization of energetic substrates also occurs during the stress response. Glucocorticoids are steroid hormones that modulate lipid, protein, and carbohydrate metabolism and are essential in mediating the stress response (Sapolsky, 2002). In amphibians, the increase in plasma CORT levels is one of the most studied aspects of the stress response and the associated immunomodulation (Moore and Jessop, 2003; Graham et al., 2012; Assis et al., 2020; Gomes et al., 2022).

Several studies with amphibians have investigated immune-endocrine interactions following short-term stressors. Restraint stress and immune challenges are commonly used in these studies. Both have been shown to modulate plasma CORT levels, as well as cellular and non-cellular aspects of the immune system (Moore and Jessop, 2003; Graham et al., 2012; Assis et al., 2020; Gomes et al., 2022; Lima et al., 2022). The immune challenge with lipopolysaccharide (LPS; component of the gram-negative bacterial cell wall) induces an inflammatory response, leading to increased gene expression of pro-inflammatory cytokines (Gardner et al., 2018; Floreste et al., 2022, 2023; Garcia Neto et al., 2022). Additionally, LPS responses trigger the increase in plasma CORT and plasma bacterial killing ability (BKA) in *Rhinella* species (Gardner et al., 2018; Ferreira et al., 2021; Titon Junior et al., 2021; Bastos et al., 2022; Floreste et al., 2022, 2023; Garcia Neto et al., 2022). Specifically, the plasma BKA can be up (Bakewell et al., 2021) or downregulated (Graham et al., 2012; Assis et al., 2019; Vasconcelos-Teixeira et al., 2022) by the short-term stress response in some anurans. The plasma BKA is an effective mechanism against bacterial pathogens, being comprised of a set of proteins present in the plasma, including the complement system, lysozymes, natural antibodies, and acute phase proteins, among others (Rodriguez and Voyles, 2020). It is an effector mechanism that once activated can promote bacterial lysis (Rodriguez and Voyles, 2020). However, although the plasma BKA is often used in several studies with amphibians, there is no information about which components of the plasma BKA are being modulated by the stress response, and also if the complement system has an essential participation in the plasma BKA in amphibians (Rodriguez and Voyles, 2020). Thus, understanding which components of the immune system are modulated in response to stressors and whether these changes are associated with plasma CORT concentrations can help to better understand the endocrine-immune interactions in this group.

A recent study with box turtles (*Terrapene carolina* and *T. ornata*) showed both species presented a robust plasma BKA against gram-negative and gram-positive bacteria after capture, which was considerably reduced after the treatment with general complement system inhibitors (heat, protease, and EDTA) (Adamovicz et al., 2020). Their results indicate the underlying mechanism is mediated by the complement system. Herein, we tested the hypothesis that the complement system is partially responsible for baseline and stress-induced changes in the plasma BKA against a gram-negative bacterium (*Aeromonas hydrophila*) in an amphibian species (*Rhinella diptycha*). Additionally, we intend to determine whether the changes observed in the plasma BKA and possibly the complement system, are correlated with plasma CORT levels. We predicted that (1) changes in plasma BKA will follow restraint and the immune challenge, with great participation of the complement system both, at baseline and post-stress and immune challenge; and (2) the greater the increase in CORT (due to restraint and immune challenge) the more intense the changes in the plasma BKA and complement system.

2. Materials and methods

2.1. Experiment 1 (restraint)

Adult males of *Rhinella diptycha* were collected in September 2021 in Botucatu-SP (22°46′59.9"S 48°28′28.1"W). The animals were located by visual inspection and collected while foraging ($n = 20$). Immediately after individual visualization, the toad was collected, and a blood sample (200ul) was taken by cardiac puncture (0 h). Thereafter, the animal was subjected to restraint stress with movement restriction (placed in a damp cloth bag and closed with a string, then kept in an individual plastic bin (4.3 l – 29 × 18 × 15 cm L × W × H)), close to the site of capture for 24 h, according to Assis et al. (2019). The animals were sampled again 1 h and 24 h after the beginning of the restraint (repeated measurements). After the second blood collection, the animals had their body mass (+/− 0.00 g) and snout-vent-length (+/− 0.00 mm) measured and were returned to the site of capture.

2.2. Experiment 2 (immune challenge)

Adult males of *Rhinella diptycha* ($n = 40$) were collected in September 2021 in the same location as experiment 1. The animals were transported to the laboratory and kept individually inside plastic boxes [20 l— 43.0 × 28.5 × 26.5 cm] for 15 days, with free access to water. Light and temperature conditions were maintained at DL 13:11 (lights on at 5:40 am and off at 6:40 pm) and 21 ± 2 °C.

To perform the immune challenge, we used *A. hydrophila* since it is a bacterium with the pathogenic potential to cause disease in amphibians under natural conditions (Rollins-Smith, 2017). The bacteria *A. hydrophila* was heat-inactivated (boiled) before injections. After 10 days in captivity, all the animals had their body mass and snout-vent-length measured. The individuals were divided into 4 groups with body mass and snout-vent-length equally distributed among them. The 4 groups were established: Saline: animals submitted to injection with saline solution (300ul of saline solution for amphibians) ($n = 10$); A1: animals injected with a concentration of 3×10^7 of *Aeromonas hydrophila* bacteria diluted in 300ul of saline solution (n = 10); A2: animals injected with a concentration of 3×10^8 of *A. hydrophila* diluted in 300ul of saline solution (n = 10); A3: animals injected with a concentration of 3×10^9 of *A. hydrophila* diluted in 300ul of saline solution (n = 10).

On the fifteenth day in the laboratory, the animals received an intraperitoneal injection according to the concentrations described above, according to the protocol by (Robert et al., 2014). The injections were performed at night; 20 min after the lights were turned off, using red lights. The animals were randomly injected following the sequence: Saline, A1, A2, and A3. All animals were injected within a period of 1:30 h. The animals were sampled by cardiac puncture (600ul of blood) 6 h

after the injection, according to Titon Junior et al. (2021) and Floreste et al. (2022). After blood sampling, the animals were decapitated, and the organs were collected for other projects.

2.3. Blood sampling

In both experiments, at each determined time (Restraint: (field) 0 h, 1 h, and 24 h and Immune challenge: 6 h after saline or bacteria injection) blood samples were collected by cardiac puncture with 1 ml heparinized syringes and 26Gx1/2′ needles. Only samples collected within a three-minute interval were considered to avoid manipulation influence on plasma corticosterone levels (Romero and Reed, 2005). Blood samples were centrifuged (604 *g*, 4 min) and then the plasma was isolated. Plasma samples were stored in a freezer (−80 °C) for measurement of plasma CORT concentrations and determination of plasma BKA.

The animal capture and all procedures performed were carried out under the permanent license of the Chico Mendes Institute for Biodiversity Conservation (ICMBio, process number 29896–1) and approval of the Ethics Committee of the Institute of Biosciences of the University of São Paulo (CEUA, protocol 371/2020).

2.4. Bacterial killing ability (BKA)

The plasma bacterial killing ability (BKA) assay was performed according to (Assis et al., 2013; Moretti et al., 2019). Briefly, plasma samples were diluted in Ringer's solution (10 μl plasma: 190 μl Ringer), and to this dilution was added 10 μl of *A. hydrophila* working solution (~ 2.5×10^5 microorganisms). As a positive control for the assay, 10 μl of *A. hydrophila* working solution was diluted in 200 μl of Ringer's solution, and as a negative control, 210 μl of Ringer's solution was used. All samples and controls were incubated for 1 h at 25 °C. After the incubation period, 500 μl of tryptone soy broth was added to each sample, and 300 μl of each was transferred (in duplicate) to a 96-well microplate. The microplate was incubated at 37 °C for 1 h, and then the optical density of the samples was measured every hour in a plate spectrophotometer (wavelength of 595 nm), totaling 4 readings. The plasma BKA against a gram-negative bacterium was calculated at the beginning of the exponential phase of bacterial growth according to the formula: 1 - (optical density of the sample/optical density of the positive control), which represents the proportion of dead microorganisms in the samples compared to the positive control.

Complement proteins are susceptible to degradation or inhibition by multiple mechanisms *in vitro*, including degradation by proteases, heat denaturation, and inhibition by cation chelators, including EDTA (Soltis et al., 1979; Nilsson and Ekdahl, 2012; Adamovicz et al., 2020). We followed the protocol of Adamovicz et al. (2020). Plasma was treated (1) with 20 mM EDTA, (2) thermally at 56 °C for 30 min, or (3) with 50 U of protease, before incubation with bacteria for 1 h. After the plasma treatment, we followed the same protocol for plasma BKA. Then, the optical density of the samples was measured every hour in a plate spectrophotometer (wavelength 595 nm) to determine the bacterial density. We compared intact plasma samples with samples treated independently with EDTA, heat, and protease.

2.5. Plasma corticosterone (CORT) levels

Steroid hormones were initially extracted with ether and determined by ELISA commercial kits (CORT #501320; T #582701 Cayman Chemical), according to the manufacturer's instructions and previous studies conducted with this same species (Titon et al., 2018; Titon Junior et al., 2021; Vasconcelos-Teixeira et al., 2022). For the restraint data, inter and intra-assay variation was 6.34% and 5.28%, respectively, and the sensitivity of the assay was 23.68 pg/ml. For the immune challenge, inter and intra-assay variation was 7.73% and 7.45%, respectively, and the sensitivity of the assays was 33.03 pg/ml.

2.6. Statistical analysis

All variables were subjected to the Shapiro-Wilk normality test and Levine's homogeneity tests. All variables showed normality and homogeneity, except for plasma CORT levels in experiment 2 (immune challenge). Parametric and non-parametric tests were evaluated by analyses of variance (ANOVA) or the Kruskal-Wallis test. For the investigation of the restraint, the CORT plasma levels were used as a dependent variable, and the restraint (0, 1, and 24 h) as a factor in a mixed ANOVA. Plasma BKA was used as a dependent variable where treatment with plasma inhibitors (untreated plasma, EDTA, heat, or protease) was used as a within-subject factor, and restraint (0, 1, and 24 h) was used as a between-subject factor. For the immune challenge, plasma CORT levels were used as a dependent variable and the immune challenge (saline, A1, A2, and A3) as a factor in a Kruskal Wallis test. Plasma BKA, in turn, was used as a dependent variable, and treatment with plasma inhibitors (untreated plasma, EDTA, heat, and protease) and immune challenge (saline, A1, A2, and A3) were used as factors in an ANOVA. When relevant, the analysis was followed by multiple pairwise comparisons using Tukey (for ANOVAs) or Bonferroni (for Kruskal-Wallis) adjustment. Pearson or Spearman correlations were used to test correlations between variables. All statistical tests were performed using IBM SPSS 26 for Windows. The final sample size varied within the experiments and variables since there was insufficient plasma to run all the assays. The sample size for the restraint was: CORT $n = 12$ and BKA $n = 17$; and for the immune challenge: CORT Saline $n = 7$, A1 $n = 8$, A2 $n = 10$, A3 $n = 9$, and BKA Saline n = 9, A1 n = 10, A2 n = 9 and A3 $n = 10$. The sample size is also included in the figures and tables in the supplementary materials.

3. Results

3.1. Experiment 1: Restraint

Restraint affected plasma CORT levels ($F_{2,22} = 21.247$; $P < 0.001$). The plasma CORT levels are higher at 1 h and 24 h after the restraint when compared with the time 0 h (Fig. 1A; $P \leq 0.002$).

The restraint affected the plasma BKA ($F_{2,30} = 17.927$; $P < 0.001$) so that the plasma BKA was reduced 24 h after the restraint (Fig. 2A; $P \leq$

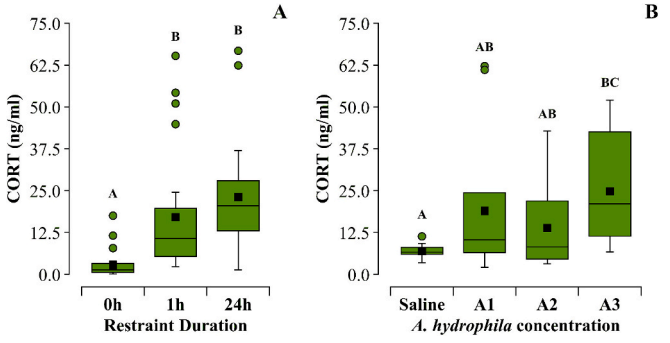

Fig. 1. Effect of restraint and the immune challenge with heat-inactivated *Aeromonas hydrophila* bacteria on plasma corticosterone (CORT) levels in *Rhinella diptycha*. **A:** On the vertical axis, the plasma corticosterone levels, on the vertical axis, restraint duration (0, 1, and 24 h, $n = 12$). Capital letters represent Tukey comparisons between restraint duration, with different letters indicating differences where $P < 0.05$. **B:** On the vertical axis the plasma corticosterone levels, on the vertical axis immune challenge groups, Saline ($n = 7$), A1: 3×10^7 ($n = 8$), A2: 3×10^8 ($n = 10$), A3: 3×10^9 ($n = 9$). Capital letters represent Bonferroni comparisons between immune challenge groups, with different letters indicating differences where $P < 0.05$. Boxplot inside lines indicates medians, lower and upper borders represent 1st and 3rd quartiles, respectively, black squares indicate means, whiskers represent upper and lower limits of 1.5 times inter-quartile range, and circles represent data outside this range.

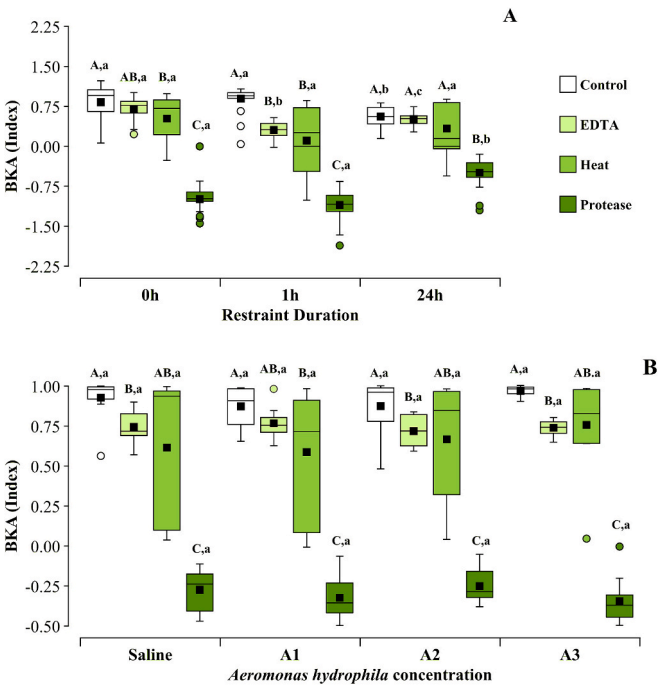

Fig. 2. Effects of restraint, the immune challenge with heat-inactivated *Aeromonas hydrophila* bacteria, and treatment with complement system inhibitors (EDTA, heat, protease) on plasma bacterial killing ability (BKA) in *Rhinella diptycha*. A: On the vertical axis, indices of plasma bacterial killing ability, on the vertical axis, restraint duration, with the types of treatments being differentiated by standards ($n = 17$). Lowercase letters represent Tukey comparisons between restraint groups (0, 1, and 24 h), with different letters indicating differences where $P < 0.05$. Capital letters represent Tukey comparisons within treatment groups (control, EDTA, hear, and protease), with different letters indicating differences where $P < 0.05$. **B:** On the vertical axis indices of plasma bacterial killing ability, on the vertical axis immune challenge groups, Saline (n = 9), A1: 3×10^7 (n = 10), A2: 3×10^8 (n = 9), A3: 3×10^9 (n = 10). Lowercase letters represent Tukey comparisons between immune challenge groups (Saline, A1, A2, and A3), with different letters indicating differences where $P < 0.05$. Capital letters represent Tukey comparisons within treatment groups (control, EDTA, heat, and protease), with different letters indicating differences where $P < 0.05$. Boxplot inside lines indicates medians, lower and upper borders represent 1st and 3rd quartiles, respectively, black squares indicate means, whiskers represent upper and lower limits of 1.5 times inter-quartile range, and circles represent data outside this range. The BKA indices represent the proportion of dead microorganisms in the samples compared to the positive control.

0.007). Treatments with complement system inhibitors affected plasma BKA at baseline (0 h) and post-restraint ($F_{1.3,19,49} = 175.973$; $P < 0.001$, Greenhouse-Geisser DF correction $\varepsilon = 0.433$; (Fig. 2A). Treatment with heat, and protease decreased the plasma BKA at baseline (0 h) and 1 h post-restraint when compared with untreated plasma ($P \leq 0.001$). Only protease treatment reduced plasma BKA 24 h post-restraint, compared with untreated plasma ($P \leq 0.001$).

Descriptive statistics and detailed statistical analysis are available as supplementary material (Tables S1-S4). No correlations were found between CORT and BKA at any circumstance 0, 1, or 24 h restraint ($P \geq 0.166$).

3.2. Experiment 2: Immune challenge

The immune challenge affected plasma CORT levels ($H_{(3)} = 13.179$; $\chi 2 = 13.511$; $P = 0.004$). Plasma CORT increased according to the bacterial concentration, being statistically different only in animals from the A3 group compared to the saline group (Fig. 1B; $P = 0.003$).

Saline plasma BKA was not affected by the immune challenge ($F_{3,33} = 0.459$; $P = 0.713$). Plasma BKA was diminished by complement system inhibitors ($F_{1.34,44.2} = 279.690$; $P \leq 0.001$, Greenhouse-Geisser DF correction $\varepsilon = 0.447$). EDTA treatment decreased plasma BKA in the saline, A2, and A3 groups, whereas heat treatment significantly decreased plasma BKA only in the A1 group, while protease eliminated plasma BKA in all groups (Fig. 2B; $P \leq 0.003$).

Descriptive statistics and detailed statistical analysis are available as supplementary material (Tables S1, S2, and S5). Plasma BKA was correlated with CORT in the saline group ($r_{(6)} = 0.950$; $P = 0.004$). No other correlations were found between CORT and BKA in the immune challenge ($P \geq 0.195$).

4. Discussion

4.1. Experiment 1: Restraint

Our results showed that restraint increased plasma CORT levels 1 h and 24 h post-restraint, corroborating that the restraint protocol represents a stressor for this species as observed in previous studies with anurans, including several species of the genus *Rhinella* (Graham et al., 2012; Assis et al., 2019; Barsotti et al., 2019; Titon et al., 2021, 2022a; Lima et al., 2022; Vasconcelos-Teixeira et al., 2022). Interestingly, CORT levels were similar at 1 h and 24 h after restraint in *R. diptycha*. Studies showing temporal patterns of the stress effects on CORT secretion are scarce. A study carried out with toads *R. marina* demonstrated a gradual increase in plasma CORT levels with maximum values being observed 6 h–8 h after restraint (Narayan et al., 2013). Another study, with *R. icterica,* demonstrated that prolonged exposure to restraint for 48 h showed the maximum values for plasma CORT 1 h post-restraint when compared with baseline (Titon et al., 2022a). In this study, we did not observe any difference in plasma CORT comparing 1- and 24-h post-restraint, suggesting that the animals maintained HPI axis activation throughout the 24 h. In fact, due to the slow metabolism, ectotherms may endure chronic CORT elevation for longer periods than endotherms (Landys et al., 2006).

The endocrine mediators of the stress response can promote immunosuppression (Sapolsky et al., 2000; Assis et al., 2015), and such effects may have influenced the plasma BKA decrease 24 h post-restraint in *R. diptycha*. Also, the plasma BKA was reduced only after 24 h post-restraint when compared with 0 and 1 h in *R. diptycha*, as previously observed in this same (Vasconcelos-Teixeira et al., 2022) and other anuran species (Graham et al., 2012; Assis et al., 2015; Lima et al., 2022). Although changes in immune function, including plasma BKA, are reported and expected in amphibians subjected to stressors, the effector mechanisms of the immune system related to these changes are little explored. In this study, we also demonstrated a within-treatment effect, with baseline and 1 h post-restraint plasma BKA against a gram-negative bacterium being significantly reduced after treatment with EDTA, protease, and heat, indicating that the underlying mechanism may be mediated partially by the complement system and possibly other proteins such as natural antibodies or acute phase proteins. High doses of glucocorticoids are capable of inhibiting activation of the alternative complement system pathway in mammals, for example (Packard and Weiler, 1983), which could explain why our post-restraint toads (with higher plasma CORT levels) showed lower plasma BKA. On the other hand, new studies suggest that cortisol up-regulates the alternative complement cascade pathway in the catfish (*Ictalurus punctatus*) and in the rainbow trout (*Oncorhynchus mykiss*) (Yona and Gordon, 2007; Jiang et al., 2020). Also, catfish (*Ictalurus punctatus*) submitted to a stress protocol and subsequent immune challenge showed increased lysozyme activity (Small and Bilodeau, 2005). In accordance, our results demonstrate that the complement system is responsible for ~50% of BKA, but something else, maybe lysozyme activity, was responsible for the other 50%. Altogether, these results help to illustrate that the endocrine mediators of the stress response effects in the complement system and maybe other mediators might be consistent with its effects in

the inflammatory response, with complex actions that range from immunostimulatory to immunosuppressive effects (Elenkov and Chrousos, 2002). Future studies are necessary to determine glucocorticoid effects on the complement system and possibly other serum proteins in anurans.

Although we observed changes in plasma CORT and plasma BKA in response to restraint, no correlation between these two variables was observed under restraint conditions. Some studies report a positive correlation between CORT and plasma BKA in *Rhinella* species submitted to 24 h restraint (Assis et al., 2019). On the other hand, some studies showed that CORT and plasma BKA were not correlated 1 and 24 h after restraint in *R. diptycha* (Assis et al., 2019; Vasconcelos-Teixeira et al., 2022). It is possible that plasma BKA modulation by CORT may change over time, and/or may be related to other endocrine mediators and not just CORT in *R. diptycha* (Titon et al., 2018), a pattern yet to be determined in anurans under short-term stress conditions.

It is worth mentioning that the restraint collection is a standard protocol to perform with repeated measures (Assis et al., 2019; Titon et al., 2019; Titon et al., 2021, 2022b; Vasconcelos-Teixeira et al., 2022). However, this method lacks control with the repeated blood sampling from the toads. It is important to have an unrestrained frog to collect blood samples from to compare how repeated cardiac punctures influenced the hormonal and immune collected metrics. We intended to perform a test to understand how repeated blood sampling could influence our variables, but at this point, it was not possible, mostly by the fact that we collected the animals in their natural habitat.

4.2. Experiment 2: Immune challenge

As a response to the immune challenge, the production of cytokines (*e.g.*, tumor necrosis factor - α, interleukin (IL)–1, and IL-6) activate the HPI axis, consequently, increasing plasma CORT levels (Elenkov and Chrousos, 2002; Sapolsky, 2002). Despite not measuring cytokine release, the increase in plasma CORT in response to an immune challenge with lipopolysaccharide has recently been reported in amphibians (Titon et al., 2021, 2022b; Titon Junior et al., 2021; Bastos et al., 2022). In this study, plasma CORT levels increased following the immune challenge with *A. hydrophila* in *R. diptycha*. Moreover, the increase in CORT was observed to be associated with the concentration of the injected pathogen, with the highest values being observed in individuals injected with the highest bacterial concentration (Garcia Neto et al., 2024). In a study with snakes (*Sistrurus miliarius*), infected individuals with the fungus *Ophidiomyces ophiodiicola*, severely symptomatic demonstrated elevated CORT plasma levels compared with moderated infected and non-infected ones (Lind et al., 2018). In birds, (*Haemorhous mexicanus*) individuals infected with a bacterial pathogen (*Mycoplasma gallisepticum*) showed that the greater the disease severity the highest the CORT plasma levels (Love et al., 2016). In amphibians, Gabor et al. (2015) described tadpoles of *Alytes obstetricians* infected with a hypervirulent *Bd* lineage had significantly higher CORT than those infected with a hypovirulent one showing that more aggressive infections lead to increased CORT release rates (Gabor et al., 2015). Therefore, there is evidence that pathogen load/concentration and/or disease severity can influence the production of hormones such as CORT in distinct vertebrates.

Regarding the immune activation in response to a pathogen, the immune response is a complex process that involves the activation of several pathways and molecules (Cain and Cidlowski, 2017). In accordance, changes in the immune response, such as phagocytosis activity of immune cells, and plasma BKA, among others, following an immune challenge have been described in amphibians (Garcia Neto et al., 2020; Titon Junior et al., 2021; Floreste et al., 2022; Titon et al., 2022b). The immune challenge with lipopolysaccharide, for example, has been described to increase plasma BKA within the first 6 h post-injection in the same species of this study, *R. diptycha* (Titon Junior et al., 2021). However, our results did not demonstrate changes in plasma BKA in response to the immune challenge with *A. hydrophila*, a gram-negative bacterium that possesses lipopolysaccharide in its cell wall structure (Howard and Buckley, 1985). In fishes (*Oreochromis niloticus),* the mRNA expression for proteins from the complement system occurs 12 h post peritoneum bacterial (*Streptococcus agalactiae*) injection (Chen et al., 2018). It is possible that the use of a complete pathogen can take a little longer to activate several pathways in the transcriptional molecules modulating the complement system and other proteins, such as the acute phase proteins, responsible for the plasma BKA, which remains to be tested in amphibians. Moreover, complement proteins can signal leukocyte clearance of pathogens, and leukocytes can also activate the complement proteins (Murphy and Weaver, 2017). Since leukocytes are absent in our assay, the complement system was not fully activated. Assays including whole blood would be interesting for future investigations.

4.3. Plasma bacterial killing ability and the inhibitors of the complement system treatments

Plasma BKA against a gram-negative bacterium from both restraint and immune challenge experiments showed an overall within-treatment effect with plasma BKA from restraint and the immune challenge being reduced following treatments (EDTA, heat, and protease) in different proportions. These results demonstrate the complement system and other proteins, such as lysozymes and natural antibodies can influence the plasma BKA in baseline and post-stress conditions in *R. diptycha*. Interestingly, our results demonstrated that the complement system is important for the plasma BKA against a gram-negative bacterium 24 h after restraint, since it was significantly decreased after heat and EDTA treatments. Similar results have been shown in other vertebrate models. In humans, for example, EDTA treatment inhibits the classical, lectin, and alternative complement pathways (Schenkein, 1988; Petersen et al., 2001). Similarly, caiman (*Caiman latirostris*) serum complement system activity is inhibited by EDTA (50 mM) and heat (56 °C, 30 min; (Siroski et al., 2010)), while heat (56 °C, 30 min) and proteases treatments completely abolished serum complement system activity in rattlesnake (*Crotalus viridis*; (Baker and Merchant, 2018)). In this way, our results contribute to better illustrating that the complement system of anurans is comparable in structure and function to those of mammals and other ectotherms, suggesting the complement system is highly conserved across taxa. Otherwise, for the immune challenge, heat decreased plasma BKA only in A1 and EDTA showed a slight decrease in plasma BKA in A2 and A3, demonstrating the complement system has little participation in the plasma BKA against a gram-negative bacterium in individuals facing a possible infection.

The most expressive plasma BKA inhibitor was protease treatment to both restraint and immune challenge experiments, in which plasma BKA was eliminated (plasma BKA index <0, Fig. 2). This result agrees with previous studies in which protease treatment in two turtle species (*Chelydra serpentina* and *Macrochelys temminckii*) eliminated plasma antibacterial activity (Baker et al., 2019). In both cases, these results confirm that the molecules acting in the plasma are proteinaceous (Baker et al., 2019). The mechanisms involved in the plasma BKA are all passive of deactivation by proteases (Matson et al., 2006; Millet et al., 2007). They represent a combination of several humoral proteins, involving the complement system, natural antibodies, lysozymes acute phase proteins antimicrobial proteins, among others (Matson et al., 2006; Millet et al., 2007). Our data suggests that complement is responsible for ~50% of BKA activity (Control is 100% killing, whereas heat and/or EDTA bring BKA down to ~60% killing). However, the majority of BKA activity is from some innate immune effector that is deactivated by protease, demonstrating that other protein components have important functional aspects in the plasma BKA against gram-negative bacteria in *R. diptycha* toads. However, the contribution of specific protein systems for the plasma BKA against the gram-negative bacterium *A. hydrophila*, remains to be determined in anurans.

5. Conclusions

Our results showed that the restraint and the immune challenge increased the plasma CORT levels, demonstrating activation of the HPI axis in *R. diptycha*. Plasma BKA against gram-negative bacterium was decreased by restraint after 24 h, demonstrating the restraint-induced immunosuppressive effect in these animals. However, we did not observe changes in plasma BKA against gram-negative bacteria following the immune challenge, at least 6 h after the bacterial injection. Additionally, the plasma BKA from both experiments was reduced following the treatments that inhibit the complement system and other proteins such as natural antibodies, acute phase proteins, and lysozymes, demonstrating its influence on the plasma BKA against gram-negative bacterium of the studied animals both in baseline and post-stress conditions.

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Stefany Antunes de Oliveira Rosa: Writing – original draft, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Braz Titon Junior:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Aymam Cobo de Figueiredo:** Writing – original draft, Methodology. **Alan Siqueira Lima:** Writing – review & editing, Methodology. **Fernando Ribeiro Gomes:** Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Stefanny Christie Monteiro Titon:** Writing – original draft, Supervision, Project administration, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors have no competing interests to declare.

Data availability

The full data to this article can be found online at: https://data.mendeley.com/datasets/nn48gc24cn/1

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Appendix A. Supplementary data

Supplementary tables to this article can be found online at https://doi.org/10.1016/j.cbpa.2024.111701.

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