Adriana Maria Giorgi Barsotti

Efeitos da desidratação sobre a secreção de glicocorticoides e a função imune em sapos

Effects of dehydration on glucocorticoid secretion and immune function in toads

São Paulo

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Tese apresentada ao Instituto de Biociências da Universidade de São Paulo, para a obtenção de Título de Doutor em Ciências, na Área de Fisiologia Geral.

Orientador(a): Prof. Dr. Fernando Ribeiro Gomes

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DEDICATÓRIA

Aos meus pais, Rolando Kenworthy Barsotti e
Maria Margarida Giorgi Barsotti.
Ao meu marido e parceiro de vida, Paulo Henrique de Mello,
e aos meus filhos e companheiros, João Barsotti de Mello e
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RESUMO GERAL

A maior parte dos anfíbios apresenta alta permeabilidade hídrica e alto risco potencial de estresse hídrico. A desidratação pode representar um estressor, ativando o eixo hipotálamo- hipófise-adrenal/ interrenal (HHA/I), elevando a concentração plasmática de glicocorticoides (GC) e, consequentemente, modulando a resposta imune. Embora tenham clara implicação para o valor adaptativo, esses efeitos da desidratação sobre a imunocompetência permanecem pouco explorados na literatura, particularmente para anfíbios anuros. O objetivo dessa tese foi compreender o impacto da desidratação como estressor sobre aspectos da função imune inata em anfíbios anuros. Particularmente, foi investigado se indivíduos de *Rhinella ornata*, espécie de sapo associada a regiões mésicas e florestadas do Brasil, é capaz de ativar uma resposta de estresse quando desidratada, as consequências desse estresse hídrico sobre a imunocompetência e a capacidade de resposta a um estressor secundário. O impacto da desidratação como estressor sobre a função imune foi investigado também em populações nativas e invasoras do sapo *Sclerophrys gutturalis* da África do Sul, considerando-se as condições climáticas diferenciadas a que essas populações vivem. Brevemente, indivíduos foram submetidos à desidratação de 10% e 20% da massa corpórea padrão e tiveram amostra de sangue coletada para avaliar variáveis como a concentração plasmática de corticosterona (CORT), hematócrito (HCT), razão neutrófilo: linfócito (N:L), capacidade bactericida plasmática (CBP) e atividade fagocítica de leucócitos (AF). Em seguida, os animais foram submetidos à restrição de movimentos sob condições não-desidratantes (estressor secundário). Nossos resultados mostraram que a desidratação aumentou a CORT, HCT e N:L em sapos. O estresse de restrição secundário resultou em manutenção da concentração elevada de CORT no plasma e aumento da N:L e AF. Indivíduos de *S. gutturalis* da população invasora mostraram menor índice corpóreo, maior CBP e N:L em campo que indivíduos da população nativa. Após a submissão experimental aos estressores, sapos invasores e nativos mostraram

aumento da CORT, e os invasores mantiveram CBP comparativamente maior que os nativos. Esses resultados indicam que a desidratação é um estressor para sapos, sendo capaz de ativar o eixo HHI, aumentando a secreção de CORT e estimulando a função imunitária. Adicionalmente, os sapos invasores apresentam uma função imune inata constitutivamente elevada se comparada à dos nativos, o que poderia aumentar seu valor adaptativo no novo ambiente e favorecer o sucesso de dispersão.

Palavras-chave: Desidratação, estresse, corticosterona, imunocompetência, animais invasores.

GENERAL ABSTRACT

Among tetrapods, amphibians represent the group with the most permeable skin and the highest risk of water stress. Currently, it is known that dehydration can trigger a stress response in vertebrates, activating the hypothalamus-pituitary-adrenal/interrenal axis (HPA/I), increasing the levels of glucocorticoids (GC) and, consequently, modulating the immune response. Although they have a clear implication for the fitness, these effects of dehydration on immunocompetence remain little explored in the literature, particularly for anuran amphibians. The objective of this thesis was to understand the impact of dehydration as a stressor on aspects of innate immune function in anuran amphibians. Particularly, it was investigated whether individuals of *Rhinella ornata*, a species of toad associated with the mesic and forest regions of Brazil, is able to activate a stress response when dehydrated, the consequences of this water stress on immunocompetence and the ability to respond to a secondary stressor. The impact of dehydration as a stressor on immune function was also investigated in native and invasive populations of the toad *Sclerophrys gutturalis* in South Africa, considering the different climatic conditions in which these populations live. Briefly, individuals were subjected to dehydration of 10% and 20% of standard body mass and had a blood sample collected to assess variables such as plasma corticosterone concentration (CORT), hematocrit (HCT), neutrophil: lymphocyte ratio (N:L), bacterial killing ability (BKA) and phagocytic activity of leukocytes (PP). Then, the animals were submitted to movement restriction under non-dehydrating conditions (secondary stressor). Our results showed that dehydration increased CORT, HCT and N:L in toads. Secondary restriction stress resulted in the maintenance of a high plasma CORT concentration and an increase in N:L and PP. Individuals of *S. gutturalis* from the invasive population showed lower body index, higher BKA and N:L in the field than individuals from the native population. After experimental submission to stressors, invasive and native toads showed an increase in CORT, and the invaders maintained BKA comparatively higher than the native ones. These results indicate that dehydration is a stressor for toads, being able to activate

the HHI axis, increasing the secretion of CORT and stimulating immune function. In addition, invasive toads have a constitutively high innate immune function compared to that of natives, which could increase their fitness in the new environment and favor the success of dispersion.

Keywords: Dehydration, stress, corticosterone, immunocompetence, invasive animals.

INTRODUÇÃO GERAL

O tegumento dos vertebrados representa um compromisso evolutivo entre a necessidade de uma proteção mecânica e, ao mesmo tempo, a necessidade de trocar materiais e energia com o ambiente (Lillywhite, 2006). Embora a maior parte dos vertebrados terrestres apresentem queratinas fibrosas que fortalecem o tegumento, anfíbios possuem pouca queratina cutânea e um estrato córneo estreito (Lillywhite, 2006), caracterizando uma pele altamente permeável (Toledo & Jared, 1993). Dessa forma, comparado com outros grupos de tetrápodes, a maioria dos anfíbios apresenta alta taxa de perda evaporativa de água, o que os torna mais propensos ao risco de desidratação. Anfíbios requerem um ambiente úmido para manter as trocas gasosas, cultivar bactérias simbiontes associadas à função imune, proteger seus ovos e realizar suas atividades ecológicas (Duellman & Trueb 1994; Rollins-Smith et al. 2011; Martin & Carter, 2013; Watling & Braga, 2015), e a variação interespecífica em características do balanço hídrico encontra-se associada à disponibilidade de água no ambiente. Espécies que vivem em ambientes mais xéricos, por exemplo, mostram taxas de perda evaporativa de água mais baixas, maiores taxas de absorção de água, e melhor desempenho locomotor quando desidratados do que espécies provenientes de ambientes mésicos e florestados (Titon et al., 2010; Titon & Gomes, 2015; 2017). Em um contexto semelhante, adaptações comportamentais e fisiológicas associadas a condições ambientais mais secas já foram descritas para populações de espécies de anuros invasoras em ambientes mais xéricos que aqueles em que populações nativas são encontradas (Tingley et al., 2012; Kosmala et al., 2017, 2020; Roznik et al., 2018). Tanto o sapo cururu (*Rhinella marina*) na Austrália quanto o sapo gutural (*Sclerophrys gutturalis*) em Cape Town, na África do Sul, mostram maior tolerância à desidratação e melhor desempenho locomotor quando desidratados do que seus coespecíficos nas regiões nativas e mais úmidas (Kosmala et al., 2017; Vimercati et al., 2018, 2019). Tais características auxiliam a dispersão e aumento populacional no front de invasão.

A submissão a condições dessecantes pode desencadear uma resposta de estresse, elevando a concentração plasmática de glicocorticóides (GC) em diversas espécies de vertebrados (Moeller et al., 2017), incluindo sapos (Barsotti et al., 2019; 2021). Quando estímulos estressores são detectados na amídala e hipocampo, um sinal neuronal é enviado para o hipotálamo através das catecolaminas epinefrina e norepinefrina. As células neurossecretoras do núcleo paraventricular no hipotálamo secretam o fator liberador de corticotropina (CRF) e arginina vasopressina (AVT), que por sua vez atuam na hipófise estimulando a glândula hipofisária a secretar o hormônio adrenocorticotropico (ACTH). Existem evidências de que outros hormônios também atuem como secretagogos do ACTH, como oxitocina (OT) e mesotocina (MT), no entanto, ainda são esparsas (Romero & Wingfield, 2015). Uma vez que o ACTH foi liberado na corrente sanguínea, ele atinge as células corticosteroidogênicas e se liga aos receptores ligados na membrana estimulando a síntese de corticosteroides (Romero & Wingfield, 2015), resultando em aumento da síntese, secreção e concentração plasmática dos GC, sendo a corticosterona (CORT) o principal GC encontrado em anfíbios. A participação da CORT frente a um evento de desidratação também envolve seu papel mineralotrópico, pois esse hormônio promove a retenção e reabsorção de água na pele, bexiga e rins através do aumento de Na^+ em anfíbios (Uchiyama et al., 2014).

Os GC participam de uma resposta integrada a estressores, modulando várias funções fisiológicas e, dentre estas, as funções imunitárias vem recebendo particular atenção. Os GCs têm amplos efeitos na imunocompetência, incluindo mudanças na atividade de células natural killer, números de células T, produção de linfócitos, anticorpos e citocinas (Marketon & Glaser, 2008; Fonner et al., 2017). Dado que os GCs alteram o tráfego de leucócitos entre compartimentos, os números e proporções de leucócitos no sangue têm sido comumente utilizados como indicadores de infecção e estresse fisiológico em anfíbios e outros vertebrados (Davis et al., 2008; Campbell, 2015; Savage et al., 2016). Neutrófilos e linfócitos constituem a maioria dos leucócitos sanguíneos em anfíbios e, em

resposta a estressores (Bennett & Alspaugh, 1964; Bennett et al., 1972; Turner, 1988; Savage et al., 2016), o número de linfócitos diminui (linfopenia) enquanto o número de neutrófilos circulantes aumenta (neutrofilia), refletindo alterações na distribuição e produção dos leucócitos (Davis te al., 2008; Savage et al., 2016). Os neutrófilos são mobilizados para a corrente sanguínea e tornam-se disponíveis para recrutamento e ativação em locais de inflamação, refletindo um aumento da resposta imunitária em determinados compartimentos (Dhabhar & McEwen, 1996; Dhabhar, 2002).

É importante considerar que os GCs apresentam um efeito bifásico e complexo sobre a imunocompetência, sendo o efeito preponderante dependente de fatores como a intensidade e duração da elevação dos níveis circulantes de GCs e os tipos de receptores ativados (Dhabhar et al., 1993; 1995; Dhabhar, 2014). Majoritariamente, em resposta a estressores agudos de baixa intensidade, é observado estímulo de diversos aspectos da imunidade, como aumento da apresentação de antígenos, função celular efetora, produção de anticorpos e receptores de citocinas proinflamatórias (Dhabhar & McEwen, 1996; Dhabhar & McEwen, 1997). Em contrapartida, a elevação crônica dos GCs tem sido associada a efeitos imunossupressores, os quais incluem inibição da expressão de citocinas pró-inflamatórias e estimulação dos fatores anti-inflamatórios (Cain & Cidlowski, 2017), além de atrofia em tecidos linfóides (Sapolsky et al., 2000, Dhabhar, 2014).

Estudos com diferentes protocolos de estresse em anfíbios têm mostrado resultados diversos do papel imunomodulador realizado pela CORT (Gomes et al., 2012; Graham et al., 2012; Narayan et al., 2011; Narayan & Hero, 2014a; 2014b; Assis et al., 2015; 2017; Barsotti et al., 2017; Titon et al., 2018; 2019). A restrição de movimentos é capaz de aumentar a CORT juntamente com efeitos estimulatórios ou supressores da imunidade, variando de acordo com o tempo e intensidade do estressor, bem como do parâmetro imune avaliado. A razão N:L, por exemplo, aumenta após a restrição, mas sua resposta está associada à intensidade do estressor, sendo que o confinamento com restrição de movimentos apresenta um escopo de resposta maior quando comparada com o confinamento sem a restrição de

movimentos (Assis et al., 2015). Da mesma forma, a fagocitose é estimulada após a restrição de movimentos (Graham et al., 2012; Assis et al., 2017; Barsotti et al., *dados não publicados*). Por outro lado, embora a capacidade bactericida plasmática (CBP) também mostre estímulo e inibição em diferentes contextos, parece ser uma medida mais robusta, que mantem alta variabilidade interindividual por longos períodos e menor maleabilidade em função de estressores (Assis et al., 2015; 2017; 2019; Barsotti et al., 2017; 2019; Titon et al., 2018; 2019; 2020). Estudos mostram que uma diminuição da CBP foi observada tanto após submissão a estressores agudos quanto de longo-prazo (Graham et al., 2012; Assis et al., 2015; Titon et al., 2017; 2018; 2019).

Diante desse cenário, em que a desidratação eleva os níveis de GCs e estes atuam sobre aspectos imunitários, parece lógico esperar que o estado hídrico poderia ter considerável impacto sobre a função imune (Brush & DeNardo, 2017). Porém, a relação entre desidratação, alterações dos níveis plasmáticos de GCs e imunidade ainda é pobremente explorada. Um estudo em laboratório com o monstro-de-gila (*Heloderma suspectum*) mostrou que, após a desidratação, os animais tiveram um aumento de diferentes aspectos da imunidade inata (lise e aglutinação, CBP e perfil leucocitário), porém, sem elevação significativa dos níveis plasmáticos de corticosterona (CORT) (Moeller et al., 2017). Outro estudo, realizado com cascavel (*Crotalus atrox*), também mostrou um aumento de funções imunitárias (lise e aglutinação e atividade antimicrobiana plasmática) após a desidratação (Brush & DeNardo, 2017). Vale destacar que essas duas espécies (*H. suspectum* e *C. atrox*) experimentam em seu hábitat natural um período de seca pronunciado durante o ano (Moeller et al., 2017; Brush & DeNardo, 2017), e talvez animais adaptados a estas condições mostrem menor sensibilidade à desidratação, aumentando sua resposta imune sem elevar os níveis de GCs em resposta a esse estímulo. Por outro lado, talvez animais que habitam regiões mésicas apresentem uma resposta de estresse mais pronunciada quando desidratados, elevando os níveis de GCs e suprimindo a

imunidade, tornando o animal mais vulnerável a doenças. No entanto, estudos relacionando estado hídrico e imunidade em animais de ambientes tropicais mésicos ainda são escassos.

Rhinella ornata mostra ser um bom modelo para estudos sobre estresse hídrico em espécies de ambiente mésico, uma vez que apresenta uma distribuição geográfica associada a ambientes florestados e bordas de mata (Kloss, 1972; Jim, 1980; Baldissera et al., 2004; Titon et al., 2010). No capítulo I (*Dehydration as a stressor in toads* [*Rhinella ornata*]) e II (*Dehydration followed by a secondary stressor sustains high circulating corticosterone and improves immunity in toads*) investigamos se a desidratação é um estressor para essa espécie, sendo capaz de ativar o eixo HHI e aumentar a CORT. Adicionalmente, avaliamos a responsividade a um estressor secundário (restrição de movimentos) e aspectos da imunidade de sapos *R. ornata* exibindo diferentes níveis de desidratação.

Espécies invasoras também se mostram interessantes como sistema de observação de ajustes fisiológicos rápidos à ocupação de ambientes climaticamente distintos. No capítulo III (*Challenges of a novel range: water balance, stress and immunity in na invasive toad*) da presente tese, investigamos a resposta ao estresse e seus efeitos na imunocompetência de indivíduos nativos e invasores de *Sclerophrys gutturalis*, também apresentando diferentes níveis de desidratação.

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CAPÍTULO I – DEHYDRATION AS A STRESSOR IN TOADS (*Rhinella ornata*)

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1.1. Abstract

Availability of a humid environment is essential for amphibians to carry out their activities, and most species are characterized by low resistance to evaporative water loss. Moreover, dehydration severely compromise amphibian locomotor and foraging performance, representing a relevant selective factor modulating the evolution of its integrative phenotype. In this way, we hypothesized that dehydration is a stressor for toads, inducing a stress response comparable to that elicited by another commonly used stress protocol: restraint challenge. We evaluated changes in plasma levels of corticosterone (CORT), hematocrit (Hct) and neutrophil:lymphocyte (N:L) ratio in adult males of *Rhinella ornata*, experimentally submitted to different levels of hydration (100%, 90% and 80% of standard body mass) and to restraint challenge. Our results showed that dehydrating toads by 10% increases CORT to levels equivalent to that obtained by restraint. Moreover, toads dehydrated by 20% show a more pronounced increase in CORT, along with increased Hct and N:L ratio. In this way, we corroborated the hypothesis that dehydration triggers a pronounced stress response in *R. ornata*.

Keywords: amphibian, corticosterone, hematocrit, neutrophil:lymphocyte ratio.

1.2. Introduction

Among tetrapods, amphibians are generally characterized by highly permeable skin and comparatively higher rates of evaporative water loss (Toledo and Jared, 1993), and dehydration can affect both locomotor performance and thermal tolerance in this taxon (Titon et al, 2010; Titon and Gomes 2017; Anderson and Andrade, 2017). Some water balance traits are reported to be related with habit diversification, with arboreal anurans showing lower dehydration rates than terrestrial and aquatic ones (Wygoda, 1984; Young et al., 2005). Other traits are related with habitat diversification, with anurans inhabiting mesic environments showing lower resistance to water loss, lower rates of water uptake and higher sensitivity to dehydration when compared with anurans from xeric environments (Titon and Gomes 2015; 2017). Therefore, dehydration could represent a potential stressor for amphibians.

In response to several stressors, including dehydration (Moeller et al., 2017), the hypothalamic-pituitary-adrenal/interrenal (HPA/I) axis is activated resulting in glucocorticoids (GCs) secretion in vertebrates, with corticosterone being the main GC present in amphibians (Rollins-Smith, 2017). The activation of the HPA/I axis modulates several physiological functions, contributing to an integrative and adaptive response to short-term stressors. However, chronic activation of the HPA/I axis can have harmful effects, including reproductive inhibition and immunosuppression (Wingfield and Romero, 2011).

Along with GC changes, the neutrophil: lymphocyte (N:L) ratio increases in response to stressors in vertebrates (Davis et al., 2008). As a result of blood leukocyte redistribution following stress protocols, the increased N: L ratio have been widely used to assess stress response in many species, including toads from genus *Rhinella* (Davis et al., 2008; Assis et al., 2015; 2017; *in press*). Moreover, the N:L ratio is related with the intensity of stress response, given that *R. ornata* subjected to restraint

with movement restriction shows higher values than those restrained without movement restriction (Assis et al., *in press*).

Increased hematocrit (Hct) has also been widely used as proxy of stress response in tetrapods. Stress-driven increased Hct has been causally associated with different physiological mechanisms, including increased blood pressure; splenic contraction; stimulated release of immature erythrocytes; and increased erythropoiesis (Johnstone et al., 2015; Barsotti et al., 2017). In addition, dehydration results in extracellular fluid loss and increased Hct in several taxa, including amphibians (Hillman, 1980).

In the present study, we tested the hypothesis that dehydration is a stressor for *R. ornata* toads, a species occurring in habitats associated with Atlantic forest (Baldissera et al., 2004), and showing high sensitivity of locomotor performance to dehydration (Titon et al., 2010). In the meantime, toads from genus *Rhinella* show increased plasma corticosterone levels (CORT) and N:L ratio in response to a standardized restraint stress protocol (Assis et al., 2015; *in press*; Gomes et al., 2012). Moreover, the magnitude of the stress response, represented by higher CORT and N:L ratio, is dependent on the intensity of the stressor (Assis et al., 2015; *in press*). Therefore, we compared physiological indicators of stress response (CORT, N:L ratio and Hct) in three groups of toads submitted to different levels of dehydration (0%, 10% and 20% of standard body mass) and one group with 100% hydrated toads submitted to restraint with movement restriction. We predicted that: 1) in response experimental dehydration, toads would increase CORT, Hct and N:L ratio; 2) increased CORT, Hct and N:L ratio would be directly proportional to the level of dehydration; 3) toads subjected to the more intense dehydration (20%) would show values of CORT and N:L ratio comparable to those restrained.

1.3. Materials and methods

1.3.1. Collection site, species studied and captivity maintenance

Adult males of *Rhinella ornata* (N = 42) were collected in São Paulo/SP, Brazil in august 2016. Individuals were kept individually in plastic boxes [20 L - 43.0 (L) × 28.5 (W) × 26.5 (H) cm] with free access to water. Each box contained foliage to maintain moisture. Box covers had holes to allow air circulation. All animals were kept inside a climatic chamber in the Department of Physiology, Institute of Biosciences, University of São Paulo for a month in a 13:11LD cycle (lights on at 06:30 h and lights off 19:30 h) at 22 ± 2°C. Toads were fed crickets (*Gryllus* sp.) and cockroaches (*Pcynoscelus* sp.) once a week. Body mass was checked weekly, and toads did not show debilitation by time in captivity. On the contrary, toads showed a significative body mass gain from 15.86g ± 0.41g to 16.76g ± 0.46g (Mean ± SE, t_{41}= -5.339, P< 0,001).

Animals were collected under a license from "Instituto Chico Mendes de conservação da Biodiversidade" (ICMBio: 29896-1). All procedures and use of biological material were approved by the Comissão de Ética no Uso de Animais (CEUA: 249/2016), of Instituto de Biociências da Universidade de São Paulo.

1.3.2. Stress of dehydration and restraint

After a month in captivity, toads were randomly divided into 4 groups: Control; Dehydration 10%; Dehydration 20% and Restraint. Standard body mass (± 0.01 g) (body mass of fully hydrated toads with empty bladders) was measured before subjecting them to the treatments. Toads from the Dehydration 10% and 20% groups had the plastic container with water and foliage removed from their boxes for approximately 12 and 24 hours, respectively, allowing toad´s to lose water until reaching these different levels of dehydration. Reduction of body mass was monitored by two weighing in an interval of 2 hours. Blood samples were collected when toads lost 10% and 20% of their standard body

mass. Thereafter, toads were immediately returned to their individual containers with free water access to allow rehydration.

Toads from Control and Restraint groups remained with available water and foliage until blood sample collection. Both groups had the blood sample taken at their standard body mass. These toads were handled and weighed as the Dehydration 10% and 20% groups in order to standardize stress due to manipulation. To perform the restraint with movement restriction, toads (100% hydrated) were placed in damp cloth sacks inside their boxes, in order to avoid dehydration, and remained under these conditions for 24 hours, as in (Assis et al., 2015; *in press*). Thereafter, a blood sample was collected.

All subjects remained in the climatic chamber under the same conditions of photoperiod and temperature during the experiment. Each group was sampled in one day, between 8:00 and 10:00 am, with a 10 min interval between individuals.

1.3.3. Blood samples

Blood samples were collected via cardiac puncture by using previously heparinized 1 mL syringes and 26G × 1/2″ needles. Only samples collected within 3 min were considered, given that CORT can be influenced by the stress of handling after 3 min (Romero and Reed, 2005). Blood samples were transferred to microcentrifuge tubes and were centrifuged (4 min at 604g), the plasma was isolated and kept in a −80 °C freezer for further CORT determination.

1.3.4. Leukocyte profile

The smear was performed with a drop of blood and 2 slides. Posteriorly, was dried for 30 min, then fixed for 20 min with methanol, stained with Giemsa solution (10%) for 15 min and observed through optical microscopy at 1000× magnification (Nikon E200, 104c). The leucocyte profile was

performed according (Campbell, 2012), and N:L ratio was calculated as the number of neutrophils divided by the number of lymphocytes on each slide.

1.3.5. Hematocrit (Hct)

The Hct was measured right after the blood samples being taken, and calculated as the proportion of red blood cells in relation to the total volume of blood after centrifuging the blood inside a microhematocrit tube (4 min at 218g).

1.3.6. CORT assay

For CORT determination, the steroid was initially extracted from 10 µl of plasma with ether according to Assis et al. (2015). CORT was measured by ELISA kits (Cayman Chemical, cat number 501320) according to the manufacturer's instructions and previous studies conducted with *Rhinella* species, including *R. ornata* (Assis et al., 2015; *in press*). The intra-assay and inter-assay variation were 6.43% and 7.40%, respectively. The sensitivity of the assays, calculated as 80% B / B0 curve value, was 51.70 pg/mL.

1.3.7. Statistical analyses

Data was initially submitted to descriptive statistics and the Shapiro-Wilk normality test. Body mass, SVL and N:L ratio showed a normal distribution when submitted to the normality test. The Hct and CORT were transformed ($Log_{10 + 1}$) to adjust to premises of parametric tests. To verify the effect of dehydration and restraint challenge on dependent variables, we used ANOVA for independent measurements, followed by tests for mean comparisons, using the Bonferroni adjustment. Statistical analyses were performed in SPSS 22 for windows.

1.4. Results

Descriptive statistics are included in Table 1.1. Both dehydration levels (10 and 20%) and restraint affected CORT, Hct and N:L ratio in *R. ornata* (Table 1.2). CORT was higher in toads from both Dehydration levels and Restraint groups, when compared to toads from the Control group (Fig. 1.1A). In addition, CORT was higher in Dehydration 20% than in Dehydration 10% toads.

The Hct in the Dehydration 20% was higher than in the Control, Dehydration 10%, and Restraint groups (Fig. 1.1B). Hct in Dehydration 10% was also higher than in Restraint group. Number of circulating neutrophils was higher in the Restraint, Dehydration 10% and Dehydration 20% groups, when compared to Control group (Table 1.1). At the same time, number of circulating lymphocytes was lower in toads from Restraint and Dehydration 20% than in toads from Control group (Table 1.1). The N:L ratio was also higher in the Restraint and Dehydration 20% groups, when compared to Control group (Fig 1.1C).

Table 1.1. Descriptive statistics of the hematocrit, plasma corticosterone levels, lymphocyte, neutrophil and neutrophil: lymphocyte ratio analyzed in the Control group and after dehydration of 10% and 20% and restriction movement for *Rhinella ornata*.

Variables	Group	N	Mean	SD	95% Confidence Interval	
					Lower Bound	Upper Bound
Hct (%)	Control	10	24.40	8.17	1.29	1.44
	Dehydration 10%	10	32.73	8.43	1.42	1.58
	Dehydration 20%	10	45.64	4.44	1.58	1.74
	Restriction	4	19.75	5.18	1.16	1.41
CORT (ng/mL)	Control	10	12.27	8.43	0.76	1.25
	Dehydration 10%	10	80.92	97.43	1.39	1.89
	Dehydration 20%	8	282.35	149.56	2.09	2.64
	Restriction	4	87.49	71.76	1.45	2.24
NEU (%)	Control	6	9.00	5.22	0.24	17.76
	Dehydration 10%	4	30.75	10.94	20.03	41.47
	Dehydration 20%	9	32.22	6.92	25.07	39.37
	Restriction	4	34.25	19.29	23.53	44.97
LYM (%)	Control	6	80.00	8.30	68.92	91.08
	Dehydration 10%	4	61.25	10.91	47.68	74.82
	Dehydration 20%	9	50.56	11.91	41.51	59.60
	Restriction	4	54.50	21.30	40.93	68.07
N:L	Control	6	0.12	0.07	-0.12	0.35
	Dehydration 10%	4	0.54	0.26	0.25	0.82
	Dehydration 20%	9	0.67	0.22	0.48	0.86
	Restriction	4	0.78	0.53	0.49	1.07

The control group consisted of 100% hydrated and unrestricted individuals; individuals in the Dehydration groups were deprived of water until they lost 10% and 20% of their body mass; the Restriction group was composed of subjects 100% hydrated and submitted to the stress of restriction of movement for a period of 24 hours. **Abbreviations: N:** number of individuals; **SD:** standard deviation; **Hct:** hematocrit; **CORT:** Plasma corticosterone level; **NEU:** neutrophil; **LYM:** lymphocyte; **N:L:** neutrophil: lymphocyte ratio.

Table 1.2. Statistical summary of ANOVA for independent measurements of the of the hematocrit, plasma corticosterone levels, lymphocyte, neutrophil and neutrophil: lymphocyte ratio (N:L) analyzed in the Control group and after dehydration of 10% and 20% and restriction movement in *Rhinella ornata*.

Variable	Source	Type III SS	DF	MS	F	Sig.
Hct (%)	Corrected Model	0.570	3	0.190	12.784	< **0.001**
	Intercept	60.103	1	60.103	4045.646	< **0.001**
	Group	0.570	3	0.190	12.784	< **0.001**
	Error	0.431	29	0.015		
	Total	72.841	33			
	Corrected Total	1.001	32			
CORT (ng/mL)	Corrected Model	8.378	3	2.793	19.175	< **0.001**
	Intercept	81.583	1	81.583	560.152	< **0.001**
	Group	8.378	3	2.793	19.175	< **0.001**
	Error	4.078	28	0.146		
	Total	99.241	32			
	Corrected Total	12.456	31			
NEU (%)	Corrected Model	2443.379	3	814.460	7.757	**0.001**
	Intercept	14506.921	1	14506.921	138.157	< **0.001**
	Group	2443.379	3	814.46	7.757	**0.001**
	Error	1995.056	19	105.003		
	Total	20300.000	23			
	Corrected Total	4438.435	22			
LYM (%)	Corrected Model	3315.941	3	1105.314	6.571	**0.003**
	Intercept	77999.691	1	77999.691	463.707	< **0.001**
	Group	3315.941	3	1105.314	6.571	**0.003**
	Error	3195.972	19	168.209		
	Total	91486.000	23			
	Corrected Total	6511.913	22			
N:L	Corrected Model	1.447	3	0.482	6.396	**0.004**
	Intercept	5.701	1	5.701	75.62	< **0.001**
	Group	1.447	3	0.482	6.396	**0.004**
	Error	1.432	19	0.075		
	Total	9.154	23			
	Corrected Total	2.879	22			

The control group consisted of 100% hydrated and unrestricted individuals; individuals in the Dehydration groups were deprived of water until they lost 10% and 20% of their body mass; the Restriction group was composed of subjects 100% hydrated and submitted to the stress of restriction of movement for a period of 24 hours. **Abbreviations: SS:** sum of squares; **DF:** degrees of freedom; **MS:** mean square; **Hct:** hematocrit; **CORT:** Plasma corticosterone level; **NEU:** neutrophil; **LYM:** lymphocyte; **N:L:** neutrophil: lymphocyte ratio.

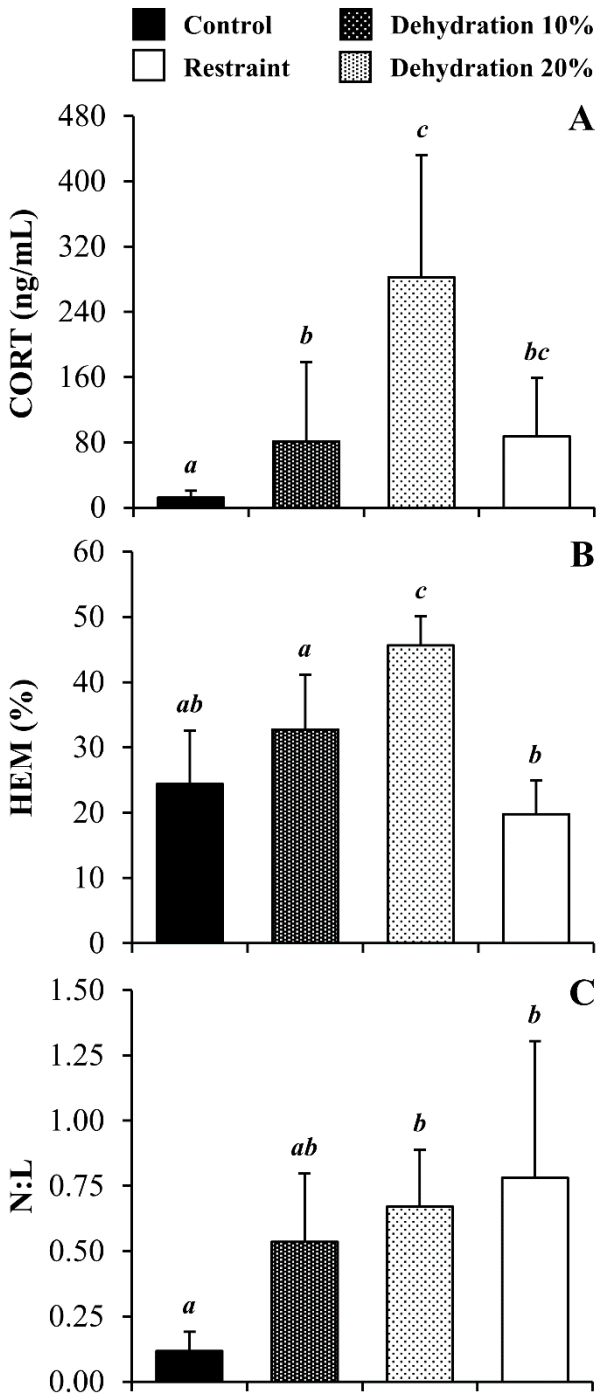

Figure 1.1. Physiological variables associated to control group and in response to dehydration (10 and 20%) and movement restriction challenge in *Rhinella ornata*. A. Plasma levels of corticosterone (CORT) B. Hematocrit (Hct) C. Neutrophil: Lymphocyte (N:L) ratio in the Control group after 10% and 20% dehydration and movement restriction. Data are represented as mean ± SD. Different letters represent a significant difference Bonferroni post-hoc tests ($P \leq 0.05$).

1.5. Discussion

Toads experimentally submitted to dehydration corresponding to 10% of standard body mass and to restraint showed 6.7 and 7-fold higher CORT, respectively, than hydrated and nonrestrained toads. The CORT values observed in *R. ornata* evidence a stress response to moderate dehydration comparable to those observed in previous studies using the same protocol of restraint, a protocol commonly used to promote acute stress response in *Rhinella* species, including *R. ornata* (Assis et al., 2015; *in press*; Gomes et al., 2012; Graham et al., 2012). Moreover, dehydration by 20% resulted in 23-fold increase in CORT when compared hydrated and non-restraint toads, evidencing that an intense dehydration in *R. ornata* can lead to a more intense stress response.

An elevation of GC plasma levels has been previously documented in response to dehydration for other tetrapods, with a large interspecific variation. Rats, birds and lizards, for example, showed an increase in GCs when exposed to dehydration stress (Arnhold et al., 2007; Cain and Lien, 1985; Moeller et al., 2017). Previous studies have shown that dehydration affects amphibian locomotor performance and thermal tolerance (Titon et al., 2010; Anderson and Andrade, 2017; Titon and Gomes, 2017), increases body fluid concentration (hematocrit, hemoglobin content, plasma osmolarity and urea concentration) (Ruibal 1962, Shoemaker 1964, Ruibal et al., 1969; Shoemaker et al., 1969; Shoemaker and Nagy, 1977; Degani et al., 1984, Jørgensen, 1997; Lillywhite, 2006; Anderson et al., 2017), decreases the mass of visceral organs the rates of evaporative water loss (Anderson et al., 2017). However, to date there are few studies showing the relationship of dehydration with GCs levels anurans. Jessop et al. (2013) have shown large daily variation in corticosterone plasma levels and hydric state in invasive toads (*Rhinella marina*) from the Australian desert during hot-dry season. These toads exhibit increased corticosterone plasma levels as their hydration level begins to decrease, which coincides with the period of highest temperature of the day (Jessop et al., 2013). In the same study, the authors showed that individuals of *R. marina* treated with adrenocorticotropic hormone exhibited

higher of corticosterone plasma levels and greater evaporative water loss when compared to individuals treated with dexamethasone and control individuals (Jessop et al., 2013).

In accordance to our predictions, *R. ornata* showed increased CORT in response to dehydration. Previous studies have emphasized that *R. ornata* is characterized by high sensitivity to dehydration. Dixo et al. (2009) showed that genetic diversity of *R. ornata* is lower in small fragments compared to medium or large fragments connected to large forest areas. These results suggest these toads do not cross open environments, characterized by high risk of dehydration, in order to migrate between fragments (Dixo et al., 2009). Accordingly, when compared to other species from genus *Rhinella, R. ornata* exhibits greater sensitivity of locomotor performance to dehydration and lower rates of water absorption when dehydrated (Titon et al., 2010). The sharp rise in CORT observed in response to dehydration in our study demonstrates that dehydration can represent a stressful condition for these toads, which could impair relevant activities such as foraging, predator escape, and breeding. Additional studies are necessary to test for adaptational patterns in the magnitude of stress response to dehydration in *Rhinella* inhabiting habitats characterized by different abiotic characteristics.

In response to dehydration, anurans increase the secretion of arginine vasotocin (AVT) and angiotensin II (Ang II). AVT increases the permeability of the epithelium to water and stimulates water and Na^+ uptake in the bladder and skin (Uchiyama and Konno, 2006). The Ang II facilitates water-seeking behavior, promoting cutaneous rehydration (Hillyard et al., 1998), and stimulates the adrenal cortex to increase aldosterone (ALD) synthesis in vertebrates, included amphibians (Konno et al., 2005). Complementarily, AVT can directly stimulate ACTH, CORT and ALD secretion (Greenberg and Verbalis, 2006; Arnhold et al., 2007; Romero and Wingfield, 2015) in different vertebrates. In amphibians, ALD and CORT present mineralotropic actions, increasing Na^+ transport in the skin, bladder and kidneys, promoting water retention and reabsorption (Uchiyama et al., 2014). Given that CORT participates on the integrated physiological response of anurans to dehydration, it is possible

that increased CORT due to dehydration in *R. ornata* contributes to protection against possible deleterious effects caused by the loss of extracellular fluid volume. Furthermore, since dehydration leads to a loss of extracellular fluid volume (Cheuvront and Kenefick, 2014), the increased Hct in 20% dehydrated toads could be, at least in part, due to extracellular dehydration. On the other hand, the adrenocortical response to dehydration activates the glucocorticoid receptors, which in turn may decrease the production of genes and proteins responsible for the increased absorption of Na^+ in the kidneys and bladder, interfering in the osmotic balance (Rogerson and Fuller, 2000; McCormick and Bradshaw, 2006; Jessop et al., 2013). Thus, these contrasting points of view show that more studies need to be performed to better elucidate the relationship and mechanisms involved in dehydration and stress.

Additionally, our results showed an increase in the N:L ratio in response dehydration and restraint. Increased N:L ratio results from exposure to stressors (e.g. restraint, captivity) and to GC treatments (e.g. transdermal application, hormone reposition in adrenalectomized animals) in several vertebrates (Davis et al., 2008; Dhabbar, 2002), including toads (Assis et al., 2015; *in press*). Previous studies with rodents have shown that lymphocytes are directed from bloodstream to other body compartments, including skin and lymph nodes, in response to stressors (Dhabbar, 2002). Meanwhile, neutrophils enter the bloodstream, a response that makes them available for recruitment and activation at sites of inflammation (Dhabbar, 2002). Therefore, the N:L ratio change is a result of this cell redistribution following a stress protocol. Assis *et al.* (2015; *in press*) found increased N:L ratio after restraint with movement restriction in individuals of *R. icterica* and *R. ornata* but not in individuals subjected to a less stressfull restraint without movement restriction. Similarly, in this study, individuals of *R. ornata* showed a more pronounced increase in N:L ratio when subjected to the higher dehydration level. These results are in accordance with the positive relation between N:L ratio and the magnitude

of stressors (Davis et al., 2008; Assis et al., 2015; *in press*), corroborating N:L ratio might be an indicative of intensity of stress response.

1.6. Conclusions

Our results show that dehydration is a pervasive stressor for *R. ornata*. Dehydrated toads by 10% of the standard body mass showed increased CORT similar to those obtained through restraint, a widely used protocol to elicit stress response in vertebrates. Moreover, toads dehydrated by 20% displayed a much more pronounced increase in CORT response, accompanied by elevated Hct and N:L ratio. Our results corroborate the large impact of dehydration on physiological processes of this toad from mesic Atlantic forest (Titon et al., 2010; Titon and Gomes, 2017). Future studies investigating the relation between hydric state and corticosterone plasma levels across different seasons will be necessary to understand the ecological relevance of these results.

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CAPÍTULO II - DEHYDRATION FOLLOWED BY A SECONDARY STRESSOR SUSTAINS HIGH CIRCULATING CORTICOSTERONE AND IMPROVES IMMUNITY IN TOADS

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2.1. Abstract

Amphibians have been suffering a populational decline due mainly to habitat loss, which alters the abiotic conditions of the microhabitat and exposes them to a greater risk of desiccation. In this context, understanding how dehydration can modulate the hypothalamus-pituitary-interrenal axis (HPI) and the immune response is an imperative question to predict how stressors can affect amphibian species. We explored dehydration as a stressor for toads, and evaluated the responsiveness to a secondary stressor, by measuring changes in corticosterone plasma levels (CORT) and immune aspects of *Rhinella ornata* exhibiting different levels of dehydration. Individuals of *R. ornata* were dehydrated mild and severely, then, they were submitted to restraint stress challenge for 1 and 24 hours. Our results show that dehydration increases hematocrit (HCT) and CORT in *R. ornata* toads. Besides, restraint stress challenge induced an acute stress response in fully-hydrated. Otherwise, restraint stress challenge in previously dehydrated toads did not induce an additional increase in CORT, but those toads sustained elevated CORT up to 24h of restraint, and this response reflected an increase in immunity, increasing the neutrophil: lymphocyte ratio (N:L) and the phagocytic activity (PP) in these animals. Our results shed light on the implications of the interaction of acute stressors on the immune physiology of anurans, showing that dehydration followed by movement restriction promoted sustained high CORT along with immune redistribution, and improving innate cellular immune function. However, we encourage studies on this interaction in animals under chronic stress and at different stages of life history.

Keywords: Amphibians; Stress response; Hydration levels; Glucocorticoids; Immunocompetence.

2.2. Introduction

Increased activation of the hypothalamic-pituitary-adrenal/interrenal axis (HPA/I) occurs in response to different stressors in vertebrates, resulting in enhanced secretion of cortisol/ corticosterone in the bloodstream (Vera et al., 2017). Glucocorticoids (GC) modulate different body functions, such as reproduction and the immune response (Saposlsky, 2002). While the response to acute stressors is considered adaptive, as it promotes survival during fight or flight response and has beneficial effects such as temporary suppression of reproduction, increased foraging activity, gluconeogenesis and immunoenhancing (Wingfield et al.,1997; Sapolsky et al., 2000; Wingfield and Romero, 2011; Sapolsky, 2002; Dhabhar, 2014), the response to chronic stressors is more commonly associated with deleterious effects, such as inhibition of reproduction and immunosuppression (Wingfield and Romero, 2011; Sapolsky, 2002).

Most studies on stress have focused mainly on the deleterious consequences of chronic stressors by showing that frequent activation of the HPA/I axis can compromise fitness (Sapolsky et al., 2000; Dhabhar, 2014). Nonetheless, the relationship between HPA/I activation and its effects, including immunomodulation, are complex and dependent on the intensity and duration of the stress response (Graham et al., 2012; Assis et al., 2019; Titon et al., 2019). While chronic stressors or chronically elevated glucocorticoid are generally related to a decrease in both immune cell proliferation and proinflammatory cytokine production, acute stressors or acutely increases in glucocorticoids are related to immunostimulatory effects, such as increased cellular function and inflammatory responses (Dhabhar et al., 1997; Dhabhar, 2014). For example, the phagocytosis and non-cellular immunity can be enhanced after acute intense stressors, whilst the inflammatory response, measured as swelling followed by an antigen, and wound healing, can be impaired following chronic stressors in mammals and anurans (Dhabhar and McEwen, 1997; Assis et al., 2017; Falso et al., 2015). However, an acute stressor can also have immunosuppressive effects, depending on its intensity. Toads, for example, show

decreased in bacterial killing ability (BKA) when subjected to an acute high intensity stressor and not immunoestimulatory effects, more commonly found in the face of less-intense short-term stressors (Assis et al., 2019).

Furthermore, exposure to chronic stressors may alter the responsiveness of the HPA/I axis to secondary acute stressors (Dallman, 1993; Marti et al., 1994; Rich and Romero, 2005). Animals living under chronic and/ or intense acute stress can enter a state of allostatic overload when subjected to a secondary stressor, affecting their ability to mount an additional rapid response to the new stressor, thus compromising their survival (Romero & Wingfield, 2015). For example, brown trout living in a contaminated environment were not able to increase corticosteroids secretion after capture and handling, showing that animals living under chronic stress lose their ability to mount a response to an acute stressor (Norris et al., 1997; 1999; Romero & Wingfield, 2015). Thus, allostatic overload resulting from interacting stressors may result in imunossupression and increased susceptibility to pathogens.

Dehydration activates the HPA/I axis, culminating in increased glucocorticoid secretion in some vertebrates (Moeller et al., 2017; Brischoux et al., 2020), including toads (Barsotti et al., 2019; 2021). Corticosterone (CORT), the main glucocorticoid in non-mammalian vertebrates, such as amphibians, reptiles, and birds) shows mineralocorticoid effects in different vertebrates (Duggan and Lofts, 1978; Dauphin-Villemant and Xavier, 1987; Bentley, 2002; McCormick and Bradshaw, 2006; Duopué et al., 2014), participating in physiological and behavioral adjustments in the face of dehydration (Dupoué et al., 2014). Many anthropic environmental changes have been linked to increased dry periods, lower availability of humid microenvironments, and less dewy nights (Seebacher and Alford, 2002; Walvoord, 2003). Such changes may, in turn, result in fewer nights with environmental conditions that allow foraging and reproduction activities for anurans (Moore and Gatten, 1989; Preest and Pough, 1989, 2003; Rogowitz et al., 1999; Walvoord, 2003; Titon et al., 2010;

Tracy et al., 2014). Although anurans can maintain their hydrological status in drier meteorological conditions by selecting wet microenvironments as refuges (Zug and Zug, 1979; Schwarzkopf and Alford, 1996; Seebacher and Alford, 2002), under the conditions of low availability of adequate refuges found in many anthropized environments, anurans can quickly overheat, dehydrate, and die (Florance et al., 2011; Jessop et al., 2013). Thus, the response to dehydration deserves more attention today because it may represent an important stressor for anurans living in anthropized environments, promoting increased CORT and, consequently, modulating immunocompetence and altering their ability to respond to secondary acute stressors (Rich & Romero, 2005).

Knowing that both dehydration and movement restriction are stressors for toads (Assis et al., 2015; 2019; Barsotti et al., 2019; 2021), the aim of the present study was assessing the responsiveness to a secondary stressor (movement restraint challenge) after dehydrating toads at different levels, as well as its immunomodulatory effects. We tested the hypothesis that the toads showing more severe dehydration, the lower the responsiveness to the secondary stressor (restraint challenge). Considering that the immunomodulatory effects of the stress response are complex and dependent on stress duration and intensity, we opted to not employ directional predictions on the immunomodulatory effects of the restraint challenge exposure of toads previously submitted to dehydration. To test our hypotheses, we compared physiological indicators of the stress response (CORT and N:L ratio), hydration state (HCT) and immune function (plasma bacterial killing ability [BKA] and phagocytosis activity of blood leukocytes [PP]) in three groups of toads submitted to different hydration levels [fully hydrated, mild dehydration (10%) and severe dehydration (20%)] and subsequently subjected to restraint stress challenge for 1 and 24 hours.

2.3. Material and methods

2.3.1. Study site, species included and captive maintenance

Adult males of *R. ornata* (N = 23) were collected at the Institute of Biosciences of the University of São Paulo, in the state of São Paulo, SP, Brazil. (23º 33' 55" S, 46º 43' 51" W) in August 2018. Individuals were kept individually in plastic containers [20 L; 43.0(L) × 28.5(W) × 26.5(H) cm] with free access to water. Each container had foliage to maintain moisture. Container lids had holes to allow air circulation. All animals were kept inside a climatic chamber (Eletrolab, São Paulo, São Paulo, Brazil) at the Department of Physiology, Institute of Biosciences, University of São Paulo for a week in a 13:11/L:D cycle (lights on at 06:30 a.m. and lights off at 07:30 p.m.) at 22 ± 2°C. Toads were fed with crickets (*Gryllus* sp.) and cockroaches (*Pcynoscelus* sp.) once a week.

Animals were collected under a license from "Instituto Chico Mendes de conservação da Biodiversidade" (ICMBio: 29896-1). All procedures and use of biological material were approved by the Comissão de Ética no Uso de Animais (CEUA: 249/2016), of Instituto de Biociências da Universidade de São Paulo.

2.3.2. Dehydration and restraint challenge

After a week in captivity, the animals were randomly divided into 3 groups with different dehydration levels: Fully hydrated (100% hydrated), mild dehydration (10% dehydrated) and, severe dehydration (20% dehydrated). Standard body mass (±0.01 g; body mass of fully hydrated toads with empty bladders) was measured before subjecting them to the treatments. The animals in the fully hydrated group remained with access to water throughout data collection. The mild dehydration and severe dehydration groups had the foliage removed from their maintenance containers and were deprived of access to water, which allowed a loss of evaporative water. The reduction in hydration level was estimated by the reduction of body mass through time. Body mass was periodically monitored

until the desired mild dehydration (10%) and severe dehydration (20%) reduction in body mass of the individuals, which occurred in a period of 24 and 48 hours, respectively. Toads from the fully hydrated group were handled and weighed as the animals in the mild and severe dehydration groups to control for handling stress. Toads from all groups had blood samples collected and were subsequently subjected to restraint with movement restriction for 24 hours according to Barsotti et al. (2019). Blood samples were collected when the individuals reached the intended hydration level and after movement restriction challenge (1 and 24h).

To perform the restraint stress challenge, toads were individually placed in damp cloth bags (to restrict movements) inside their maintenance containers and had blood samples collected after 1 hour and 24 hours in these conditions (Assis et al., 2015; 2019; Barsotti et al., 2019; 2021).

To verify whether dehydrated toads rehydrated during the restriction challenge, five individuals were dehydrated at 10% and 20% from their standard mass (in the same way as previously described) and subsequently, submitted the restriction challenge in damp cloth bags for a period of 24 hours. After that time, the toads' body mass was recorded and their hydration levels calculated.

2.3.3. Blood collection

Blood samples (about 80-100 µl) were collected by cardiac puncture with 1ml syringes and previously heparinized 26Gx1 / 2 "needles at time 0, 1, and 24h after the movement restriction. Only samples collected within 3 min were considered, given that CORT can be influenced by the stress of handling after 3 min (Romero & Reed, 2005). Each blood sample was immediately transferred in duplicate to a microcentrifuge tube, one aliquot (40-50 µl) was used to perform the phagocytosis assay, N:L ratio, and HTC, and the remaining aliquot was centrifuged to isolate the plasma (4 min at 604 g). Plasma samples were stored at -80°C to further evaluate plasma bacterial killing ability and plasma corticosterone levels.

2.3.4. Hematocrit (HCT)

The HCT was calculated as the proportion of red blood cells in relation to the total volume of blood after centrifuging blood samples in a microhematocrit centrifuge (4 min at 218 g).

2.3.5. Neutrophil: Lymphocyte (N:L) ratio

A drop of blood was used to perform a blood smear. The slide was stained with Giemsa solution (10%) and observed under an optical microscope (100X objective - Nikon E200, 104c). One hundred leukocytes were counted and classified based on morphology as neutrophils, lymphocytes, eosinophils, basophils, and monocytes on each slide (Campbell, 2012). The N:L ratio was calculated as the number of neutrophils divided by the number of lymphocytes.

2.3.6. Plasma bacterial killing ability (BKA)

The plasma BKA assay was conducted according to Assis et al. (2013) with slight modifications for *Aeromonas hydrophila* (IOC/FDA 110–36), donated by the Oswaldo Cruz Foundation (FIOCRUZ; RJ) (Detailed modifications are in the Supplementary File). Briefly, plasma samples (10 µl) diluted in amphibian Ringer's solution (190 µl) were mixed with 10 µL of bacteria working solution (2.5×10^7 microorganisms ml^{-1}). Positive controls consisted of 10µl of bacteria working solution in 200 µl of Ringer's solution, and negative control contained 210 µl of Ringer's solution. All samples and controls were incubated for 60 min at 37°C. After the incubation period, 500 µl of TSB were added to each sample. The bacterial suspensions were thoroughly mixed and transferred in duplicate (300 µl) to a 96-wells microplate. The microplate was incubated at 37 °C for 1 h, and thereafter the optical density of the samples was measured hourly in a 96-well microplate spectrophotometer (595 nm), totaling four readings. The plasma BKA was evaluated at the beginning of the bacterial exponential growth phase and calculated according to the formula: 1 - (optical density of sample / optical density of positive

control), which represents the proportion of killed microorganisms in the samples compared to the positive control.

2.3.7. Plasma dilution quantification

To verify whether the BKA was altered due to increased protein concentration as a result of reduced blood volume after dehydration, we performed a plasma dilution test according to Brusch and DeNardo (2017). We randomly selected plasma from 10 individuals in the Dehydration 10% and 20% groups and used plasma aliquots from individuals at two times: fully hydrated and dehydrated. Osmolality was measured by using an osmometer (Vapro®Vapor Pressure Osmometer Model 5600, Wescor Inc.). The aliquot of dehydrated plasma was diluted with nanopure water in order to achieve the same osmolarity of the hydrated animal's aliquot. BKA assays were performed with concentrated and diluted samples from dehydrated toads. The t-test for paired samples showed that BKA did not differ between concentrated and diluted samples (t = -0.117; df = 9; P = 0.910).

2.3.8. Phagocytosis assay and imaging flow cytometry

Phagocytic activity of blood leukocytes was evaluated as described by Titon et al. (2019). Cell viability was determined by Trypan blue exclusion method in a hemocytometer. The analysis of phagocytosis was performed by adding 100 ul of Zymosan-CFSE (Sigma, St Louis, Mo, USA) suspension (1 x 10^6 particles) in 1000 ul of APBS containing 2 x 10^5 cells (neutrophils and monocytes). Samples were incubated for 60 min, under agitation at 25°C, and then washed with 2 ml of 6 mM of ethylenediamine tetra acetic acid (EDTA) solution, in order to stop phagocytosis and to wash off the excess of free zymosan particles. After centrifugation (4°C, 259 g, 7 min), pellets were resuspended in 200 ul of cold (4°C) paraformaldehyde (1%) for cell fixation. After 1 hour, 500 ul of APBS was added,

samples were centrifuged (4°C, 259 g, 7 min) and pellets were resuspended in 100 ul of APBS for flow cytometry.

Cells were analyzed on an image flow cytometer (AMNIS Flowsight imaging flow cytometer Merck-Millipore, German) interfaced with a DELL computer with 10,000 events collected using the 488 nm laser at a 20x magnification, through INSPIRE software. Data analysis was performed using IDEAS analysis software (EMD Millipore) version 6.1 for windows. After gating on side scatter *vs*. brightfield plot, phagocytosis of peritoneal cells was measured by phagocytosis percentage (PP), which represents the percentage of cells that engulfed at least one zymosan particle.

2.3.9. Corticosterone plasma levels

Steroid hormones were initially extracted with ether according to Assis et al. (2015; 2019). Plasma CORT levels were determined using EIA kits (CORT number 501320; Cayman Chemical), according to the manufacturer's instructions and previous studies conducted with *Rhinella* toads, including *R. ornata* species (Assis et al., 2019; Barsotti et al., 2019; Titon et al., 2019). The mean values for intra and inter-assay variation were 12.66% and 10.15% respectively. The sensitivity of the assays was 42 pg/ml.

2.3.10. Statistical analysis

Descriptive statistics were performed for all variables (mean and standard deviation) and the Shapiro-Wilk normality test and Levene´s test for homogeneity of variance were performed to evaluate parametric test assumptions. Therefore, some variables were transformed to fit the assumptions: CORT (fifth root), N:L ratio ($\log_{10}$), BKA (arcsine), and PP (ninth root). Unstandardized residuals from a linear regression of body mass as a function of SVL were saved and used as body index (BI). A set of mixed ANCOVAs was performed to evaluate the possible effects of body mass and BI as covariates.

In these ANCOVAs, physiological variables were considered as dependent variables, dehydration (fully hydrated, mild dehydration , and severe dehydration) were considered as between-subject factor and movement restriction duration (0h, 1h, and 24h) were considered as within-subject factors. When a covariate effect was not present, the covariate was eliminated from the model. All analyses were followed by means comparison tests using Bonferroni adjustment. Additionally, the difference in BKA between concentrated and diluted samples was evaluated by student t-test for paired samples. To find out if the toads rehydrated during movement restriction, a t-test for paired samples was performed comparing the standard body mass and the mass after movement restriction for previously dehydrated toads at 10% and 20%. Statistical analyses were performed in SPSS 22 for Windows (IBM Corp., Armonk, NY).

2.4. Results

Descriptive statistics are shown in Table 2.1. Neither body mass nor BI presented a significant effect as covariates for all dependent variables ($P \geq 0.214$, Table 2S1). HCT and CORT were affected by an interaction between dehydration and restraint stress ($F_{4,38} = 5.469$, $P = 0.001$, $F_{4,40} = 6.010$, $P = 0.001$, respectively; Table 2.2). HCT was higher in severely dehydrated toads when compared to the fully hydrated ones ($P = 0.004$). For mildly dehydrated toads, HTC decreased after 24 hours of restraint ($P \leq 0.005$, Fig 2.1A). For severely dehydrated animals, HCT progressively decreased 1h and 24h under the restraint challenge ($P \leq 0.023$, Fig. 2.1A). Dehydration increased CORT ($P = 0.006$, Fig. 2.1B), but restraint challenge increased CORT only on fully hydrated toads ($P \leq 0.001$, Figure 2.1B). NLR and PP were affected by restraint ($F_{1.6,30.8} = 16.075$, $P < 0.001$, $F_{2,32} = 9.912$, $P < 0.001$, respectively, Table 2.2). The N:L ratio increased in all hydration levels after 1h under restraint ($P \leq 0.001$, Fig. 2.1C) and decreased after 24h under restraint on toads from mild dehydration group ($P = 0.013$, Figure 2.1C). PP increased after 24h under restraint in toads from mild and severe dehydration

groups ($P \leq 0.025$, Figure 2.2A). Plasma BKA was not affected by dehydration and restraint ($F_{4,38} = 0.297$, $P = 0.878$, Table 2, Figure 2.2B).

Toads previously dehydrated at 10% and 20% showed mass gain, indicating that they were able to rehydrate during the restriction challenge in damp cloth bags ($t = 3.006$; $df = 4$; $P = 0.04$ and $t = 3.641$; $df = 4$; $P = 0.02$, respectively). Descriptive statistics are shown in table 2.3.

Table 2.1. Descriptive statistic, presented as mean ± standard deviation, of physiological and morphological variables for *Rhinella ornata* in different hydration levels (fully hydrated, mild dehydration and severe dehydration) submitted to different movement restriction duration (before/0h, 1h and 24h movement restriction).

Variable	Dehydration	Restraint Duration					
		0h	N	1h	N	24h	N
HCT (%)	Fully hydrated	22.80 ± 9.65	7	24.39 ± 4.04	7	23.57 ± 5.55	7
	Mild Dehydration	31.83 ± 8.68	7	28.49 ± 4.81	8	18.60 ± 5.66	8
	Severe Dehydration	38.19 ± 5.23	8	28.69 ± 3.96	8	19.80 ± 3.01	8
CORT (ng/ml)	Fully hydrated	4.65 ± 3.55	7	104.05 ± 95.37	7	33.83 ± 22.66	7
	Mild Dehydration	54.50 ± 45.71	8	46.54 ± 27.14	8	49.60 ± 34.63	8
	Severe Dehydration	96.45 ± 59.96	8	95.12 ± 53.90	8	70.32 ± 27.65	8
N:L	Fully hydrated	0.14 ± 0.05	7	0.45 ± 0.32	7	0.85 ± 0.59	7
	Mild Dehydration	0.43 ± 0.20	8	1.51 ± 0.70	8	0.67 ± 0.24	8
	Severe Dehydration	0.56 ± 0.39	8	1.14 ± 0.62	8	0.98 ± 0.69	8
PP (%)	Fully hydrated	3.59 ± 2.61	6	3.11 ± 1.82	6	3.82 ± 3.22	7
	Mild Dehydration	1.61 ± 0.61	7	3.90 ± 1.90	8	4.80 ± 2.54	8
	Severe Dehydration	2.57 ± 1.90	8	4.52 ± 1.91	8	4.43 ± 1.64	7
BKA (%)	Fully hydrated	57.4 ± 44.2	7	62.4 ± 36.7	7	56.4 ± 43.5	7
	Mild Dehydration	38.6 ± 43.5	8	19.8 ± 31.6	8	42.0 ± 45.2	8
	Severe Dehydration	46.3 ± 45.0	7	49.3 ± 42.6	8	52.3 ± 43.9	8
BM (g)	Fully hydrated	13.95 ± 1.66	7	-		-	
	Mild Dehydration	14.41 ± 2.19	8	-		-	
	Severe Dehydration	15.62 ± 2.26	8	-		-	
SVL (mm)	Fully hydrated	57.20 ± 2.66	7	-		-	
	Mild Dehydration	57.29 ± 2.30	8	-		-	
	Severe Dehydration	59.13 ± 2.46	8	-		-	

Abbreviation as follow: **HCT:** Hematocrit; **CORT:** corticosterone plasma levels; **N:L:** Neutrophil: Lymphocyte ratio; **BKA:** bacterial killing ability; **PP:** phagocytosis percentage; **BM:** body mass; **SVL:** snout-vent length.

Table 2.2. Effect of dehydration and restraint as stressors in *Rhinella ornata*. Hormonal and immunological variables were used as dependent variables, dehydration (fully hydrated, dehydration 10% and dehydration 20%) was considered as between subject factor and movement restriction duration (before/0h, 1h and 24h movement restriction) were considered as within subject factors.

Variable	Source	Type III SS	DF	MS	F	P
HCT	Intercept	45775.681	1	45775.681	1361.810	**0.000**
	Dehydration	316.482	2	158.241	4.708	**0.022**
	Error (Dehydration)	638.663	19	33.614		
	Restraint Duration	1198.274	2	599.137	16.963	**0.000**
	Restraint Duration*Dehydration	772.699	4	193.175	5.469	**0.001**
	Error (Restraint Duration)	1342.180	38	35.321		
CORT	Intercept	301.514	1	301.514	1068.638	**0.000**
	Dehydration	3.107	2	1.554	5.506	**0.012**
	Error (Dehydration)	5.643	20	0.282		
	Restraint Duration	1.502	2	0.751	7.587	**0.002**
	Restraint Duration*Dehydration	2.380	4	0.595	6.010	**0.001**
	Error (Restraint Duration)	3.959	40	0.099		
N:L	Intercept	5.455	1	5.455	24.063	**0.000**
	Dehydration	0.335	2	0.167	0.739	0.491
	Error (Dehydration)	4.307	19	0.227		
	Restraint Duration[a]	2.391	1.6	1.473	16.075	**0.000**
	Restraint Duration*Dehydration[a]	0.275	3.2	0.085	0.924	0.447
	Error (Restraint Duration)[a]	2.826	30.8	0.092		
PP	Intercept	70.351	1	70.351	13379.014	**0.000**
	Dehydration	0.006	2	0.003	0.604	0.559
	Error (Dehydration)	0.084	16	0.005		
	Restraint Duration	0.086	2	0.043	9.912	**0.000**
	Restraint Duration*Dehydration	0.028	4	0.007	1.599	0.199
	Error (Restraint Duration)	0.139	32	0.004		
BKA	Intercept	21.684	1	21.684	39.139	**0.000**
	Dehydration	1.371	2	0.685	1.237	0.313
	Error (Dehydration)	10.527	19	0.554		
	Restraint Duration	0.166	2	0.083	0.447	0.643
	Restraint Duration*Dehydration	0.221	4	0.055	0.297	0.878
	Error (Restraint Duration)	7.067	38	0.186		

Abbreviation as follow: **Type III SS:** Type III sum of squares; **DF:** Degrees of freedom; **MS:** Mean square; **HCT:** Hematocrit; **CORT:** corticosterone plasma levels; **N:L:** Neutrophil: Lymphocyte ratio; **BKA:** bacterial killing ability; **PP:** phagocytosis percentage; **a:** Huynh-Feldt degree of freedom correction ($\varepsilon = 0.812$). Variables with *P* significant $\leq$ 0.05 are highlighted in bold.

Table 2.3. Descriptive statistics of standard mass*, desired mass, post-restriction mass, hydration level and rehydration rates for *Rhinella ornata* toads dehydrated at 10% and 20% and subjected to 24 hours to the restriction challenge in wet cloth bags.

	Variable		N	Minimum	Maximum	Mean±SD
Dehydration 10%	Standard Mass	(g)	5	13.09	28.23	17.06±6.38
	Desired Mass	(g)	5	11.78	25.41	15.35±5.74
	Post- restriction Mass	(g)	5	12.53	27.8	16.54±6.38
	Hydration Level	(%)	5	93.95	100.53	96.79±2.66
	Rehydration Rates	($g.h^{-1}$)	5	0.02	0.09	0.05±0.03
		($\%.h^{-1}$)	5	0.13	0.45	0.28±0.12
Dehydration 20%	Standard Mass	(g)	5	14.24	29.35	18.23±6.39
	Desired Mass	(g)	5	11.39	23.48	14.58±5.11
	Post- restriction Mass	(g)	5	13.43	27.38	17.26±5.85
	Hydration Level	(%)	5	93.29	97.56	94.90±1.71
	Rehydration Rates	($g.h^{-1}$)	5	0.08	0.16	0.11±0.03
		($\%.h^{-1}$)	5	0.55	0.77	0.63±0.09

*Standard Mass: 100% hydrated individuals with empty bladders; SD: standard deviation.

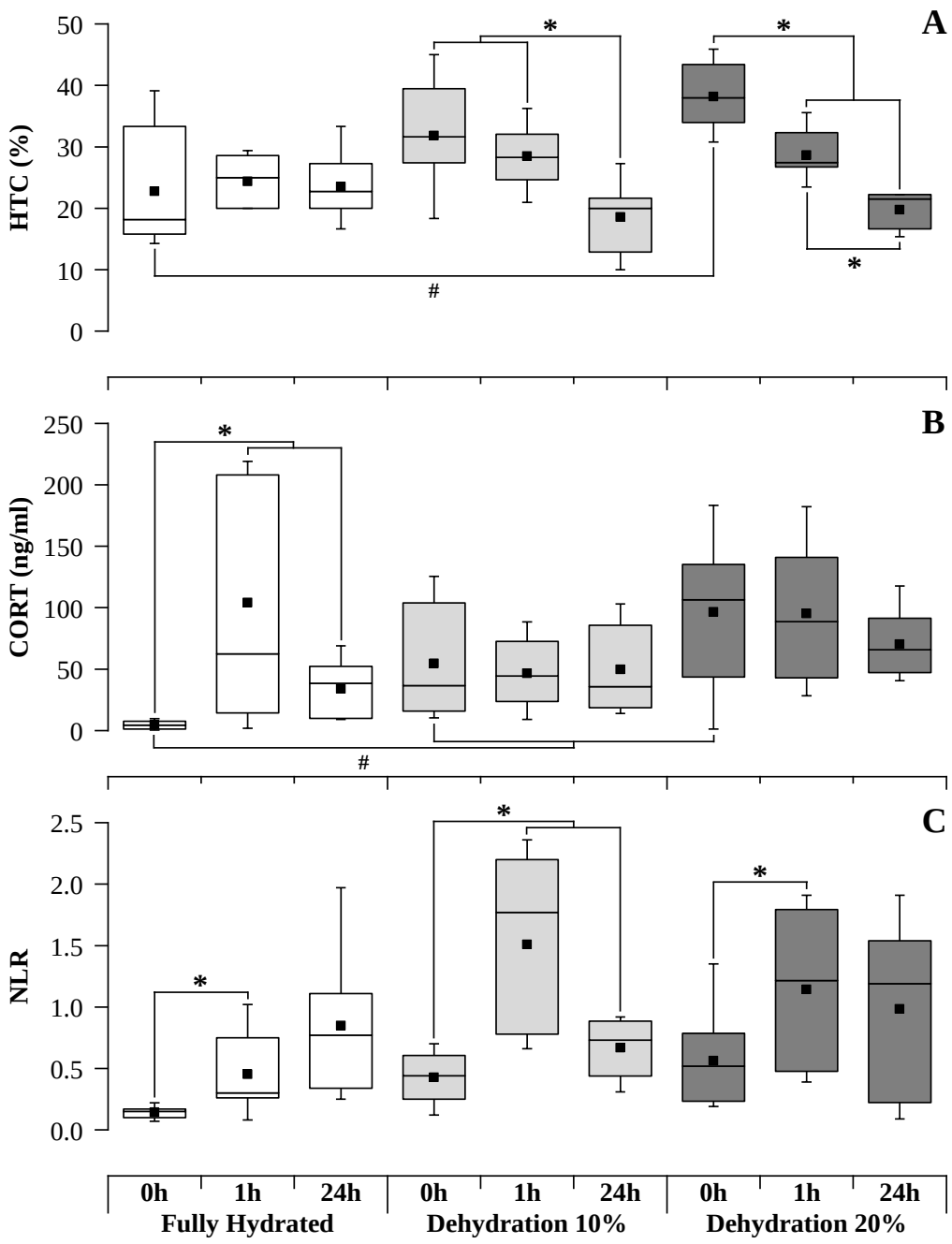

Figure 2.1. Effect of dehydration and movement restriction as stressors in *Rhinella ornata*. Toads were submitted to different levels of dehydration (fully hydrated, mild dehydration and severe dehydration) and different restraint duration (before/0h, 1h and 24h movement restriction). **A:** hematocrit (HCT); **B:** corticosterone plasma levels (CORT); **C:** Neutrophil: Lymphocyte ratio (N:L). Boxplot inside lines indicate medians; lower and upper borders represent 1st and 3rd quartiles, respectively; black squares indicate means; whiskers represents upper and lower limits of 1.5 times inter-quartile range. Asterisk (*) represents significant differences ($P \leq 0.05$) within dehydration levels and hashtag ($^{\#}$) within restraint duration. Bonferroni adjustments were used for all pairwise comparisons.

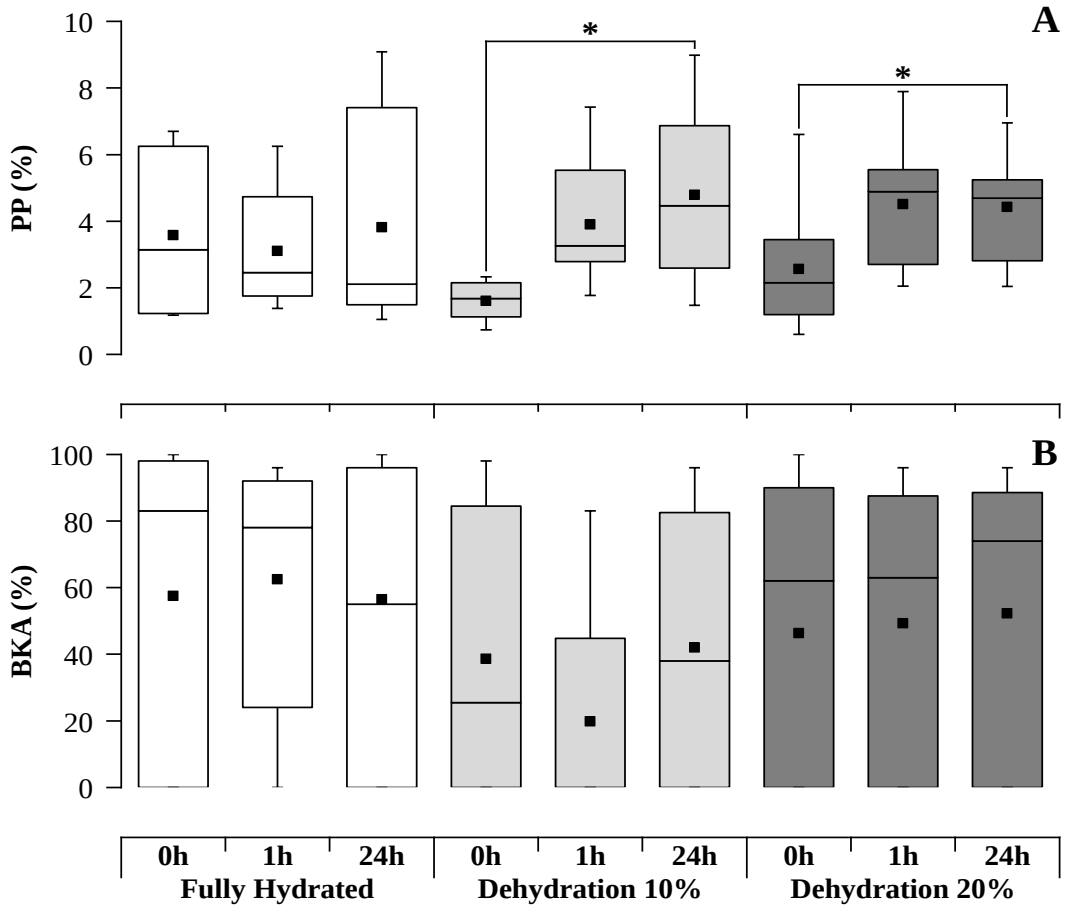

Figure 2.2. Effect of dehydration and movement restriction as stressors in *Rhinella ornata*. Toads were submitted to different levels of dehydration (fully hydrated, mild dehydration and severe dehydration) and different restraint duration (before/0h, 1h and 24h movement restriction). **A:** bacterial killing ability (BKA); **B:** phagocytosis percentage (PP). Boxplot inside lines indicate medians; lower and upper borders represent 1st and 3rd quartiles, respectively; black squares indicate means; whiskers represents upper and lower limits of 1.5 times inter-quartile range. Asterisk (*) represents significant differences ($P \leq 0.05$) within dehydration levels and hashtag ($^{\#}$) within restraint duration. Bonferroni adjustments were used for all pairwise comparisons.

2.5. Discussion

CORT increased in *R. ornata* toads submitted to mild and severe dehydration, and in response to restraint challenge in fully hydrated individuals. N:L ratio also increased in response to restraint stress in hydrated and dehydrated toads. These results corroborate previous studies showing that

dehydration and restraint are stressors for these animals, affecting immune cell distribution between body compartments (Assis et al., 2015; 2019; Barsotti et al., 2019; 2021). However, hydrated toads responded to restraint with an acute elevation of CORT which peaked 1h after restriction and tended to decrease within 24h, while dehydrated toads showed a sustained elevation in CORT when submitted to 24h of restraint. Interestingly, restraint increased PP only in dehydrated toads, demonstrating that the consecutive submission to these acute stressors resulted in immune-stimulatory effects in these toads.

As expected, individuals of *R. ornata* submitted to dehydration showed increased HCT, particularly evident at higher dehydration levels (Barsotti et al., 2019). This result corroborates studies with several groups of vertebrates, including amphibians (Hillman, 1980; Cheuvront and Kenefick, 2014; Barsotti et al., 2019; 2021). In this study, we also observed a decrease in HCT in the dehydrated toads after restraint. This was probably due to the fact that the restraint challenge was conducted on wet cloth bags, allowing rehydration of these toads, as we can proved in the test performed.

Increased GC levels in response to stress protocols can be observed in several vertebrates (Glavin et al., 1994; Matson et al., 2006; Malisch et al., 2008), including amphibians subjected to restraint challenge and dehydration (Assis et al., 2015; 2019; Barsotti et al., 2019; 2021). Increased CORT was also found after dehydration in *R. ornata*, with a more pronounced response observed in toads from the severe dehydration group. These results corroborate previous observations by Barsotti et al., (2019), showing that dehydration represents a pervasive stressor for these toads from the mesic Brazilian Atlantic forest, with activation of the HPI axis proportional to dehydration levels. It is important to consider in this context that glucocorticoids shown important mineralotropic effects in vertebrates (Agarwal and Mirshahi, 1999; de Kloet et al., 2000; Thunhorst et al., 2007; Shelat et al., 1999; Liu et al., 2010; Vera et al., 2017). Anuran amphibians show increased secretion of arginine vasotocin (AVT) and angiotensin II (Ang II) after dehydration and, in several vertebrates, AVT can

stimulate the secretion of adrenocorticotropic hormone (ACTH), CORT and aldosterone (ALD) (Arnhold et al., 2007; Greenberg & Verbalis, 2006; Romero & Wingfield, 2015). In amphibians, CORT increases the absorption of Na^+ in the skin, intestine and urinary bladder (McCormick and Bradshaw, 2006), promoting water retention and reabsorption (Uchiyama et al., 2014). Additionally, CORT also acts in behavioral responses to dehydration, increasing the water search in dehydrated toads (Madelaire et al., 2020). This integrated physiological response to CORT and other hormones in dehydrated anurans, possibly protects these animals against possible deleterious effects caused by the loss of extracellular fluid volume.

Restraint challenge also increased CORT in fully hydrated *R. ornata* toads, with a more pronounced CORT response at 1h than at 24h under restraint. This pattern of temporal variation in CORT levels suggests hydrated toads showed an intense acute stress response at the onset of the stimulus applied. Otherwise, dehydrated toads maintained high CORT levels for a longer time of restraint stress (24h). Mildly dehydrated toads tended to reduce the activity of the HPI axis at the end of the restraint, while severely dehydrated toads maintained constantly high CORT throughout the 24h of restraint. However, no further increase in CORT was observed in response to restraint when toads were previously dehydrated. Similar results were recently described for birds, in which dehydration increased CORT but no further increment in GC levels was observed when dehydrated birds were subjected to 30 minutes of restraint stress (Brischoux et al., 2020). It is possible that, although restriction in damp cloth bags allowed rehydration along time toads remained restrained, the stressors summed up and interacted, extending the time the HPI axis was highly stimulated.

Besides, the brief CORT peak observed in fully hydrated toads subjected to restraint came with a prolonged increase in N:L ratio, but no changes in PP. Otherwise, the extended period of stimulated HPI axis by restraining toads previously dehydrated allowed immunostimulatory effects, evidenced by increased PP along with increased N:L ratio. Previous studies have shown that animals subjected to

chronic stressors in the wild tend to reduce the intensity of response to acute secondary stressors, evidencing exhaustion or allostatic overload, compromising the efficiency of several functions and fitness (Norris et al., 1997; Wingfield and Romero, 2015). Otherwise, acutely increased GC levels following acute stressors has been more frequently described as beneficial, with associated augmented physiological functions, such as immune reactivity (Breuner et al., 2008; Dhabhar, 2014). Restraint stress in previously dehydrated toads keeps elevated CORT levels. Moreover, this CORT increase showed an immunostimulatory effect in these individuals, as previously observed in different contexts and phylogenetic groups (Barriga et al., 2001; Martin, 2009; Dhabhar, 2014; Hopkins and DuRant, 2011; Tort, 2011; Titon et al., 2019). This effect possibly prepares organisms for a better immune response to injury and infection, commonly associated with stress contexts (Dhabhar, 2014).

Fully hydrated individuals increased N:L ratio following restraint stress, with the amplitude of the response being positively associated with the restraint stress duration. Additionally, 1h-restraint stress increased the N:L ratio for all groups independently of the hydration level. Several studies show neutrophilia following exposure to different types of stressors in various taxa (Davis et al., 2008), including amphibians submitted to different stressors, such as restraint stress and dehydration (Assis et al., 2015; 2019; Barsotti et al., 2019; 2021). Stress-induced leukocyte redistribution is associated with lymphocytes being directed from circulation to other compartments of the body, which explains the lymphopenia, together with neutrophils entering the bloodstream and becoming available to assist in the potential inflammation process (Davis et al., 2008; Dhabhar, 2014). Interestingly, mildly dehydrated individuals showed similar N:L ratio values (0.43) to those fully hydrated after 1h- restraint stress (0.45), and severely dehydrated individuals showed even higher values (0.56), a pattern previously described for *R. ornata* (Barsotti et al., 2019). These results corroborate previous observations that stress-induced increases in the N:L ratio are often positively correlated with the intensity of the stressor in anurans (Assis et al., 2015; 2019; Barsotti et al., 2019; Titon et al., 2019).

Several studies have shown increased phagocytic capacity associated with the increased CORT in vertebrates submitted to acute stressors (Forner et al., 1995; Rodriguez et al., 2001; Barriga et al., 2001; Graham et al., 2012). Improved immune response (e.g. agglutination, lysis, and BKA) associated with increased CORT has been also previously described after dehydration in lizards (Moeller et al., 2017). Phagocytic activity did not respond to dehydration in *R. ornata* toads. In contrast, PP increased after 24 hours of restraint in both groups of dehydrated toads. It would be interesting to evaluate a group of toads submitted to dehydration for different periods without restraint stress to verify if the immunostimulatory effect observed in the present study might be also observed after dehydration for longer periods. It is also important to consider that stress-induced effects on immune cell distribution and function may be due to other mediators, such as catecholamines (Reviewed in: Dhabhar, 2014). Therefore, increased PP and N:L ratio following restraint stress in the dehydrated *R. ornata* individuals might have been promoted not only by increased CORT, but also by the interaction of CORT with other stress mediators, such as increased catecholamines (Benschop et al., 1996; Roi and Rai, 2004).

Rhinella ornata showed no differences in BKA after dehydration and restraint stress challenge. Plasma BKA's main contributor is the complement system, an important component of innate immunity (Merchant et al., 2003; French and Neuman-Lee, 2012) and it is a target of immunomodulation by the HPI axis (Dhabhar & McEwen, 1997; Sapolsky et al., 2000). Several studies have shown that after acute or chronic stressors the BKA-associated proteins can be regulated in birds, reptiles, mammals and amphibians (Buehler et al., 2008; French et al., 2010; Zylberberg, 2015; Hernández-Arciga et al., 2018). In anurans, toad species of the genus *Rhinella* have shown different patterns of response. Whereas *R. marina* and *R. icterica* decreased BKA following 24h- restraint stress (Graham et al. 2012; Assis et al., 2019), and *R. diptycha* decreased BKA after 30 days in captivity (Titon et al., 2017), *R. ornata* did not show BKA alterations, either after acute or mid-term stressors (Assis et al., 2019; Titon et al., 2019). In accordance, our results showed the absence of changes

following dehydration and restraint stress, which might be related to lower responsiveness of this immune variable to acute and short-term stress in *R. ornata* than other congeneric species (Assis et al., 2019; Titon et al., 2019). Given the observed differences in the immune variables (BKA and PP), it is possible that stress-induced immunomodulation can be selective, and variable between phylogenetically related species (Graham et al., 2012; Titon et al., 2019).

Given that amphibians have been experiencing a remarkable population decline, mainly due to habitat loss, climate change and infectious diseases (Rollins-Smith, 2017), understanding how dehydration can modulate the HPI and, consequently the immune response, is important to understand how stressors can affect amphibian populations in a changing world. Our results demonstrate that acute dehydration increased CORT plasma levels, which remained elevated without increment after a secondary stressor (restraint challenge). Moreover, dehydration followed by restraint stress promoted immune redistribution and improved cellular innate immune function (increased N:L ratio and PP). However, deleterious effects over anuran's immune functions could be observed under long-term stress (Titon et al., 2018; 2019), which might compromise individual survival. Our results shed light on the implications of interacting acute stressors on the anuran immune physiology. We encourage further studies on the interaction of dehydration and the response to a secondary stressor in animals under chronic stress and at different stages of life history.

2.6. Acknowledgement

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2.8. Supplementary file

Supplementary materials for

Dehydration followed by a secondary stressor sustains high circulating corticosterone and improves immunity in toads

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<u>This file includes:</u>

Materials and methods for temperature growth of *Aeromonas hydrophila*.

Figure S1

Table S1

Materials and methods for temperature growth of *Aeromonas hydrophila*.

In order to conducted the bacterial killing assay with *Aeromonas hydrophila* bacterium, we performed the assay according to Assis et al. (2013) with slight modifications for the *A. hydrophila* (IOC/FDA 110-36), donated by the Oswaldo Cruz Foundation (FIOCRUZ; RJ). A sample of stock cultured *A. hydrophila* (bacteria + tryptic soy broth [TSB] + glycerol at -80 °C) were resuspended in 5mL sterile TSB and incubated overnight at 37°C. In the next day, serial dilutions of A. hydrophila at 10^4 bacteria/mL and 10^5 bacteria/mL concentrations were incubated for 7h and measured hourly in a plate spectrophotometer (wavelength: 595nm).

Our results showed the *A. hydrophila* strain used in this study showed higher growth at 37 °C than at 28 °C at both 10^4 bacteria/mL and 10^5 bacteria/mL concentrations (Figure S1). In this way, we opted to conduct our BKA tests at 37°C.

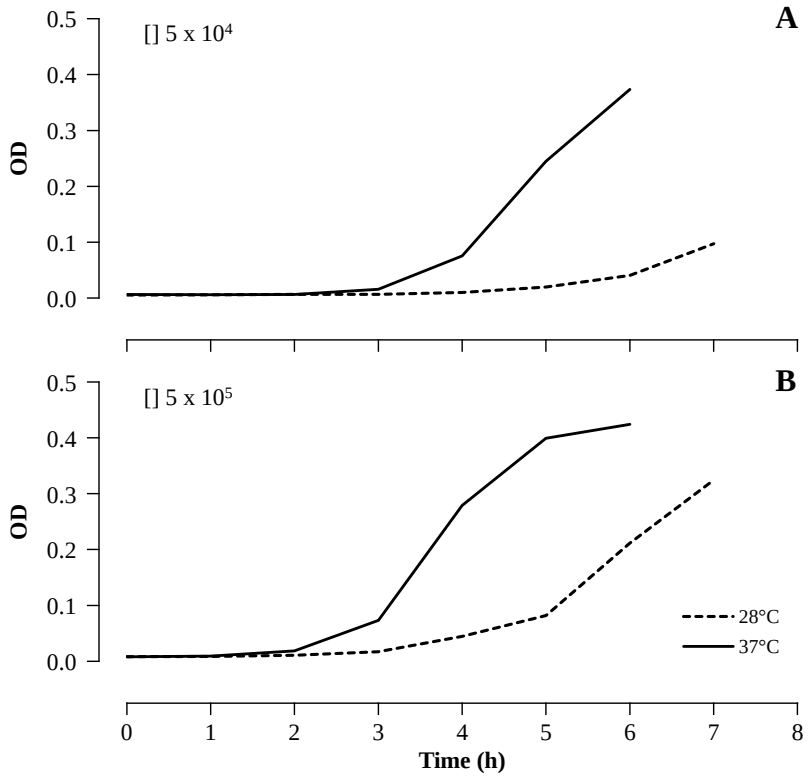

Figure 2S1: *Aeromonas hydrophila* growth at 28°C and 37°C. A: *A. hydrophila* at 5 x 10^4 bacteria/mL; **B:** *A. hydrophila* at 5 x 10^5 bacteria/mL.

Table 2S1: Effect of body mass and body index as covariates in *Rhinella ornata*. Hormonal and immunological variables were used as dependent variables, body mas and body index as covariates. Dehydration (fully hydrated, dehydration 10% and dehydration 20%) were considered as between subject factor and movement restriction duration (before /0h, 1h and 24h movement restriction) were considered as within subject factors.

Variable	Source	Type III SS	DF	MS	F	P
HTC	Body Mass	12.101	1	12.101	0.348	0.563
	Error	626.563	18	34.809		
	Body Index	4.686	1	4.686	0.133	0.720
	Error	633.977	18	35.221		
CORT	Body Mass	0.452	1	0.452	1.656	0.214
	Error	5.190	19	0.273		
	Body Index	0.020	1	0.020	0.067	0.799
	Error	5.623	19	0.296		
N:L	Body Mass	0.333	1	0.333	1.508	0.235
	Error	3.974	18	0.221		
	Body Index	0.295	1	0.295	1.326	0.265
	Error	4.012	18	0.223		
PP	Body Mass	0.005	1	0.005	0.929	0.350
	Error	0.079	15	0.005		
	Body Index	0.007	1	0.007	1.443	0.248
	Error	0.077	15	0.005		
BKA	Body Mass	0.118	1	0.118	0.204	0.657
	Error	10.408	18	0.578		
	Body Index	0.376	1	0.376	0.666	0.425
	Error	10.151	18	0.564		

Abbreviation as follow: **Type III SS:** Type III sum of squares; **DF:** Degrees of freedom; **MS:** Mean square; **HTC:** Hematocrit; **CORT:** corticosterone plasma levels; **N:L:** Neutrophil: Lymphocyte ratio; **BKA:** bacterial killing ability; **PP:** phagocytosis percentage. Variables with *P* significant ≤ 0.05 are highlighted in bold.

CAPÍTULO III - CHALLENGES OF A NOVEL RANGE: WATER BALANCE, STRESS AND IMMUNITY IN AN INVASIVE TOAD

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3.1. Abstract

Species introduced by human activities can alter the normal functioning of ecosystems promoting negative impacts on native biodiversity, as they can rapidly expand their population size, demonstrating phenotypic plasticity and possible adaptive capacity to novel environments. Twenty years ago, the guttural toad, *Sclerophrys gutturalis*, was introduced to a peri-urban area of Cape Town, with cooler and drier climatic characteristics than its native source population, Durban, South Africa. Our goal was to understand the phenotypic changes, in terms of physiology and immunity, of populations in native and novel environments. We evaluated body index (BI), field hydration level, plasma corticosterone levels (CORT), proportion of neutrophils: lymphocytes (N: L), plasma bacterial killing ability (BKA), and hematocrit (HTC) in the field, and after standardized stressors (dehydration and movement restriction) in males from the native and invasive populations. Toads from the invasive population presented lower BI and tended to show a lower field hydration state, which is consistent with living in the drier environmental conditions of Cape Town. Additionally, invasive toads also showed higher BKA and N:L ratio under field conditions. After exposure to stressors, invasive animals presented higher BKA than the natives. Individuals from both populations showed increased CORT after dehydration, an intense stressor for these animals. The highest BKA and N:L ratio in the field and after submission to stressors in the laboratory shows that the invasive population has a phenotype that might increase their fitness, leading to adaptive responses in the novel environment and, thus, favoring successful dispersion and population increase.

Keywords: invasive species, dehydration, corticosterone, immunocompetence.

3.2. Introduction

Species introduced through human activities are present in many ecosystems and some of these species may become invasive and rapidly expand their populations (Torchin et al., 2003; Seebacher and Franklin, 2011). A high invasive potential is mainly due to their ability to produce integrated phenotypes reconciling pressures from many selective agents allowing for greater expansion efficiency (Reznick and Ghalambor, 2001; Lee, 2002; Sax et al., 2007, Jessop et al., 2013). Due to this rapid evolution and population increase, invasive species are currently recognized as one of the main threats to vertebrate populations (Mooney and Hobbs, 2000; Strauss et al., 2006) as they exploit the same resources used by local populations (Altieri et al., 2010), thus causing biodiversity losses (Pimentel, 2014). For this reason, invasive species provide an excellent model for investigating how organisms mitigate the various challenges associated with rapid environmental change.

To understand how these species adapt to environmental changes in novel habitats, it is necessary to integrate information on physiological mechanisms and processes that regulate and integrate the various phenotypic characteristics optimizing the fitness of individuals (Wingfield, 2008; Chown et al., 2010; Martin et al., 2011; Cohen et al., 2012; Jessop et al., 2013). Some environmental challenges, as potential stressors, can activate the hypothalamic-pituitary-adrenal/interrenal (HPA/I) axis in vertebrates, resulting increased levels of plasma glucocorticoids (GCs), such as corticosterone (CORT). CORT is a metabolic hormone, and its secretion is increased in response to higher energy demands associated with environmental challenges, such as limited resources and/ or divergent climatic conditions (Bonier et al., 2009). Additionally, it can modulate several functions, such as reproduction, behavior, immunocompetence, and growth (Sapolsky et al., 2000; Dhabhar, 2014). The acute activation of the HPA/I axis results in an integrative and adaptive response to short-term stressors (Wingfield et al., 1997; Sapolsky et al., 2000; Wingfield and Romero, 2001; Sapolsky, 2002). For example, GCs are known to be immunomodulatory hormones and have bimodal effects on the immune

system. Short-term increased GC secretion in response to acute stressors is associated with imunnoenhancing effects, including increased presentation of antigens, effector cell function and production of antibodies and cytokines (Dhabhar and McEwen, 1996; Dhabhar, 1998; Dhabhar and McEwen, 1997; Madelaire et al., 2017). These immunoenhancing effects increase immune surveillance in the face of stressors, preparing the immune system for a rapid response to an immune challenge, such as injury or infection (Dhabhar et al., 1994, 1995, 1996; Dhabhar and McEwen, 1996; Dhabhar, 1998). On the other hand, long-term increase of GC secretion in response to chronic stressors is usually associated with immunosuppressive effects (Dhabhar and McEwen, 1997; Dhabhar, 2014; Titon et al., 2018), such as suppression of maturation, differentiation and proliferation of immune cells (Sternberg, 2006; Martin, 2009) in addition to triggering apoptosis in T and B cells (Sapolsky et al., 2000) and reduction of anti-inflammatory effects (Dhabhar, 2014). Additionally, exposure to chronic stressors can alter the ability of the HPI axis to respond to acute secondary stressors (Rich and Romero, 2005). In the wild, animals may be subjected to chronic and/or intense acute stressors, and additional stressors may lead to allostatic overload, affecting their ability to mount a rapid response and compromising their survival (Romero and Wingfield, 2015).

When compared with other tetrapods, amphibians have comparatively higher rates of transcutaneous evaporation (Toledo and Jared, 1993), and one of the challenges in a new environment may be the maintenance of adequate hydration levels. Dehydration affects locomotor performance and its underlying physiological determinants (e.g. Vimercati et al., 2018), which may impair ecologically relevant behaviors such as breeding, foraging, and escape from predators (Moore and Gatten, 1989; Titon and Gomes, 2017). Accordingly, dehydration represents a potent stressor for toads, activating the HPI axis and increasing CORT secretion (Barsotti et al., 2019), which may affect immune function, as observed in other ectothermic vertebrates (Moeller et al., 2013; 2017). Despite the general physiological characteristics assumed in the literature as potentially restrictive to the spread of

originally mesic amphibians to more xeric novel environments, recent studies have described fast and integrated behavioral and physiological adaptations of invasive amphibians to unfavorable drier conditions (Tingley et al., 2012; Kosmala et al., 2017; 2020; Roznik et al., 2018). Cane toads (*Rhinella marina*) from the invasion front in the Australian desert, for example, show greater tolerance to dehydration and better locomotor performance when dehydrated than their native conspecifics (Kosmala et al., 2017). In addition, cane toads from drier areas in Australia show higher rates of water uptake than those from more mesic areas (Tingley et al., 2012). Moreover, the behavioral flexibility associated with shelter selection and adjustments of daily movement rates according to ambient moisture has been key for invasive cane toads to successfully colonize arid zones in Australia (Tingley and Shine, 2011).

Likewise, an invasive population of guttural toads (*Sclerophrys gutturalis*) has recently established (2000) in Cape Town, in a climate significantly drier and colder than that of the native source area of Durban, South Africa (Telford et al., 2019; Measey et al., 2020). Previous studies have shown that these invasive toads outperformed those from the native range in locomotor trials when dehydrated and were more efficient in minimizing rates of water loss through postural adjustments (Vimercati et al., 2018; 2019). However, the interaction between response to stress and functions modulated by this response, such as immunocompetence, has not yet been explored in this invasive population of the guttural toad. Despite an eradication program, the species is still spreading across the invaded area, in a small peri-urban area of Cape Town (Measey et al., 2017; Davies et al., 2020). Therefore, it is important to study the adjustments in the physiological characteristics and life history of invasive guttural toads, in order to predict its potential for propagation within Cape Town. In addition, by comparing the invasive population with the native population, we can clarify information about the possible phenomena that may increase the fitness of this species in the invaded area.

In the present study, we aim to compare the stress response and its effects in immunocompetence of native and invasive populations of *S. gutturalis*. We hypothesize that invasive individuals of *S. gutturalis* are under chronic stress in Cape Town's drier environment, and thus show lower immunocompetence and a reduced ability to respond to an acute stressor when compared to individuals from native populations in the more humid region of Durban. To test our hypothesis, we compared blood samples collected in the field and after experimental manipulation in the laboratory of male individuals from invasive and native populations. The experiment consisted of dehydrating animals by 10% or 20%, followed by movement restriction for one hour. Blood samples were collected before and after dehydration, and after restriction. We measured body index and hydration state in the field, hematocrit (HTC) as an estimate of hydration levels, corticosterone plasma levels (CORT) and neutrophil: lymphocyte (N:L) ratio as estimates of stress, and bacterial killing ability (BKA) as an estimate of immunocompetence. According to our hypotheses and experimental design employed we predicted that: 1) individuals of *S. gutturalis* from the invasive population will display higher CORT and N:L ratio, lower hydration state and lower BKA in the field when compared to the native population; 2) after submission to dehydration and movement restriction stress, toads from both populations will present increased CORT and N:L ratio, and lower BKA; 3) individuals from the native population will have higher CORT, N:L ratio and BKA after both dehydration and movement restriction.

3.3. Material and methods

3.3.1. Study species, collection localities and maintenance in captivity

Male *Sclerophrys gutturalis* were collected in Cape Town (87 m a.s.l., 34°01′S, 18°25′E) and Durban (75 m a.s.l., 29°47′S, 31°01′E), corresponding to invasive and native populations, respectively (see Telford et al., 2019).

In Cape Town, 25 invasive individuals were collected in the peri-urban Constantia region with prior permission from CapeNature (Permit No.: CN44-31-7259) and with the assistance of the Nature Conservation Corporation. The collections were carried out in private residences, where toads were found close to or immersed in bodies of water (e.g. fountains, ponds, and swimming pools: see Vimercati et al., 2017), between 17 and 27 January 2019 [Average temperature, dew point, and relative humidity for the collection days: 20.2ºC, 13.5ºC and 65.8%, respectively (Source: Weather Underground website, available at https://www.wunderground.com/dashboard/pws/IWCAPECO2)].

A total of 20 male *Sclerophrys gutturalis* individuals from the native population were collected in a similar peri-urban area of Durban North with prior permission from KZN Wildlife (Permit No.: 0P 4353/2018), between 16 and 23 February 2019 [Average temperature, dew point, and relative humidity for the collection days: 25.5ºC, 21.8ºC and 81.1%, respectively (Source: Weather Underground website, available at https://www.wunderground.com/dashboard/pws/IKWAZULU12)]. The collections were carried out on residential streets, some private residences, and a golf course.

The animals were collected at both sites during the reproductive period of the species (Vimercati et al. 2019). They were located by visual inspection, captured by hand and a blood sample was collected in the field (details below). Subsequently, animals were weighed using a portable balance (±0.01 g, WTB 2000, Radwag, Radom, Poland) and placed individually in pots with water overnight to rehydrate. After rehydration, individuals had their bladders emptied by pressure in the ventrum (Titon and Gomes, 2017), and were then weighed again. Body mass of 100% hydrated individuals with empty bladders were considered as the standard mass (Ruibal, 1962; Titon and Gomes, 2017). Field hydration state was calculated as the field mass divided by the standard mass after rehydration (Preest and Pough, 1989; Preest et al., 1992; Tracy et al., 2014). Animals of both populations were brought to Stellenbosch University and kept individually in plastic containers (43.0 (W) x 28.5 (L) x 26.5 (H) cm) with foliage, and water and crickets (*Acheta domesticus*) were provided *ad libitum*. The containers and water were

checked daily, and the boxes cleaned when needed. Animals were kept in a climatic chamber with constant temperature (24°C ± 1°C), relative humidity (40% ± 1%) and 13L:11D light regime. In order to allow for the recovery of blood volume, all individuals were kept in these conditions for at least one week before being submitted to stress by dehydration and movement restriction. After the experiments were finished, we measured animals' snout-vent length (SVL) in order to calculate the body index (BI), which was calculated as unstandardized residuals of a linear regression of standard mass as a function of SVL.

3.3.2. Collection of blood samples

Blood collection was performed by cardiac puncture with 1 mL syringes and previously heparinized 26Gx1 / 2 " needles. Only blood samples collected within 3 min after the capture of the animal were considered in order to avoid possible influences of capture and manipulation (Romero and Reed, 2005; Tylan et al., 2020). The individuals were sampled once at the time of collection and twice in the experiment, for each sample, approximately 100μL of blood was drawn. The minimal interval between the date of collection and the experiment in the laboratory was 1 week, enough time for the animals to recover the blood, since amphibians, especially terrestrial ones, have a high capacity to regulate blood volume in the face of dehydration and hemorrhage (Hillman, 2018). These samples were used to evaluate the corticosterone plasma levels (CORT), neutrophil:lymphocyte ratio (N: L ratio), bacterial killing ability (BKA) and hematocrit (HTC) collected in the field and after subjection to stressors in the laboratory.

All blood samples were identified and kept on ice until divided into three aliquots. One of these aliquots was used to mount two blood smear slides for leukocyte profile (≈ 4 μL), another for hematocrit (HTC) (≈ 5 μL) measurement and a third one was transferred to Eppendorf tubes and centrifuged (4 min at 604 g) for plasma separation. Plasma samples were kept on ice until transferred

to the -80 °C freezer the same night. 5-10 μL of plasma were used for hormonal and BKA analysis, respectively.

3.3.3. Dehydration and movement restriction

The animals were randomly divided into 3 groups with different levels of hydration: control (100% hydrated), 10% and 20% dehydration groups. An ANOVA test with Bonferroni adjustment was performed to assess whether there was a statistical difference between groups with respect to mass. The test showed that there was no difference in mass between groups for both populations (native population: $P = 0.441$; $F = 0.850$; invasive population: $P = 0.665$; $F = 0.281$). The animals in the control group remained with access to water throughout the period of data collection. The toads from the 10% and 20% dehydration groups were weighed in the laboratory again when fully hydrated and with empty bladders. They were then deprived of food two days before the experiment and of access to water the day before the experiment until the time of blood sampling, which allowed for natural evaporative water loss. To increase the rate of water loss for the animals from the 20% dehydration group, we replaced the plastic top of the box by a mesh. Both groups lost the desired mass in a period of 24 hours. The next morning the toads were weighed to check if they reached the mass corresponding to the level of dehydration desired and then had a blood sample collected. If the toads had not reached the desired mass, we would place them closer to the fan to dehydrate more quickly and reach the desired mass. Toads had their body mass reduced through cutaneous water loss by 10% or 20% of the initial body mass; for example, a toad with an initial mass of 100 g achieves a dehydration percentage of 10% or 20% with a mass of 90 g and 80 g, respectively. Once reaching the target mass, individuals had samples of blood collected. Toads from the control group were handled and weighed as those from the 10% and 20% dehydration groups in order to control for handling stress and, later, they were submitted to movement restriction stress.

To perform the movement restriction stress test, toads from all groups were placed in wet cloth bags inside their individual maintenance containers and remained in these conditions for a period of 24 hours, following Assis et al. (2015). After this period, blood samples were collected to evaluate the variables: BKA, N:L ratio, CORT and HTC.

The collections and the laboratory experiment were authorized by the Research Ethics Committee: Animal Care and Use of Stellenbosch University (reference number #ACU-2019-8839).

3.3.4. Bacterial Killing Ability (BKA)

To perform this test, we followed the protocol of Assis et al. (2013). Briefly, plasma samples were diluted in amphibian Ringer's solution (10 µL plasma: 190 µL Ringer's) and then mixed with a 10 µL *Aeromonas hydrophila* working solution ($1x10^5$ microorganisms mL^{-1}, *A. hydrophila*; IOC/ FDA 110- 36). Positive controls consisted of 10 µL bacteria working solution in 200 µL Ringer's solution, and negative controls contained 210 µL Ringer's solution. Both controls and samples were incubated at 37 ºC, optimal temperature for bacterial growth, for 60 minutes. We chose to use the optimal growth temperature of *A. hydrophila* instead of an ecologically relevant temperature for *S. gutturalis* because there is interspecific variation in temperatures that maximize BKA in bufonids (Moretti et al., 2019), and we did not access the thermal sensitivity curve of BKA for *S. gutturalis* previously to this study. In addition, most studies involving interaction between plasma and bacteria use the optimal growth temperature for the bacteria and this allows us to make comparisons with other studies. After incubation, 500 µL of TSB (Tryptic Soy Broth) was added to each sample, vortexed and pipetted (300 µL from each sample), in duplicate, into a 96-well plate. The microplate was incubated at 37 °C for 1 h, and thereafter the optical density of the samples was measured hourly in a plate spectrophotometer (Thermo, model Multiskan EX) at a wavelength of 595 nm, totaling four readings. The plasma BKA was evaluated at the beginning of the bacterial exponential growth phase and calculated

according to the formula: 1 – [optical density of sample/ optical density of positive control (Assis et al., 2013)]. The optical density of the samples is the average of the two duplicates. The result of this division is the proportion of microorganisms that the toads' plasma was able to kill when compared to the proportion of microorganisms in the positive control.

3.3.5. Leukocyte profile

One drop of blood (≈ 2 μL) was used to make two blood smear slides. One of them was stained with Giemsa's solution (10%) to perform differential counting leukocyte types. In an optical microscope with a magnification of 100X, one hundred leukocytes were counted on each slide and classified into neutrophils, lymphocytes, eosinophils, basophils and monocytes for each animal (Campbell, 2012).

3.3.6. Hormone dosage

Steroid hormones were initially extracted with ether according to Assis et al. (2015; 2019). Plasma CORT levels were determined using EIA kits (CORT number 501320; Cayman Chemical), according to the manufacturer's instructions and previous studies conducted with toads from the genus *Rhinella* (Assis et al., 2019; Titon et al., 2018; Madelaire et al., 2019; Barsotti et al., 2019). The intra- and inter-assay variation were 11.22% and 3.35%, respectively. The sensitivity of the assays, calculated as 80% B/B0 curve value, was 22.84 pg.

Validation of the use of the corticosterone assay kit from Cayman Chemicals (cat number 501320; Cayman Chemical, Ann Arbor, Michigan) for *S. gutturalis* was conducted with a parallelism test, including baseline. Pooled plasma samples leftover (25 μL of 10 subjects) were initially extracted with 5 mL of ethyl ether and followed the same procedures mentioned above (according to Assis et al.,

2015, 2019). At the end, these pooled samples were resuspended and diluted in EIA buffer. The top standard of the corticosterone kit and the pooled plasma samples were used for a serial dilution (neat, 1:2, 1:4; 1:8, 1:16, 1:32, 1:64 and 1:128) and assayed on the same plate. The standard and sample corticosterone concentration were plotted as a function of Binding/Total Binding, and the 50% binding point was considered indicative of the best dilution factor to run the samples. The standard and sample curves were parallel, not crossing each other (Fig. 1), corroborating the functionality of the assay for guttural toads. The best dilution factor for baseline pooled plasma samples from *S. gutturalis* corresponds to 1:2 (Fig. 1).

3.3.7. Hematocrit (HTC)

The HTC was measured shortly after blood samples were collected and calculated as the ratio of blood cells to total blood volume after centrifugation of blood within a microhematocrit tube (4 min at 218 g).

3.3.8. Statistical Analysis

Data were initially submitted to descriptive statistics and the Shapiro-Wilk normality test. HTC ($\log_{10+1}$), BKA (ACOS), N:L ratio (square root) and CORT ($\log_{10+1}$) were transformed to adjust to the premise for parametric tests. Differences in field variables between invasive and native populations were verified by t-tests. A set of mixed ANCOVAs were performed to evaluate possible effects of body index as a covariate for the variables of the experiment. The body index was calculated as unstandardized residuals of a linear regression of standard mass as a function of snout-vent-length and were considered as a proxy of body condition to both populations.

In these ANCOVAs, physiological variables were considered as dependent variables, dehydration (control, 10% dehydration and 20% dehydration) and population (native and invasive)

were considered as a between subject factor and restraint was considered as a within subject factor following a full factorial model. When the covariate effect was not present, the covariate was eliminated from the model. The analysis was followed by a means comparison test, using the Bonferroni adjustment. Statistical analyses were performed in SPSS 22 for Windows (IBM Corp., Armonk, NY).

3.4. Results

3.4.1. Comparison between invasive and native population of baseline samples

Individuals from Cape Town (invasive population) and Durban (native population) differed in baseline variables of field mass, standard body mass, body index, BKA and N:L ratio (Table 3.1). The field mass recorded at the time of collection (t_{41} = -3.859, P < 0.001) and the body condition (t_{34} = -6.632P < 0.001, Fig. 3.1 A) were higher for the native population compared to the invasive population. In addition, the standard body mass was higher in individuals from the native population (t_{39} = -4.767, P< 0.001). Although not significant, there was a tendency of higher field hydration state for individuals from the native population (t_{39} = -1.795, P = 0.080; Fig. 3.1 B). Individuals from the native population showed lower BKA and N: L compared to those from the invasive population (t_{40} = -5.525, P < 0.001, t_{39} = 2.316, P = 0.026; Figs. 3.1 C and 3.1 D, respectively). CORT did not differ between populations ($t_{20.182}$ = -0.811, P = 0.427, Fig. 3.1 E).

Table 3.1. Descriptive statistics and statistical analysis (t-test) comparing field data (body mass, rehydrated mass, dehydration level in field, snout-vent length, body index, bacterial killing ability, neutrophil: lymphocyte ratio and corticosterone plasma levels) of invasive and native *Sclerophrys gutturalis* populations. Due to technical issues, not all variables were measured for all individuals.

Variables	**Population**	**N**	**Mean±SD**	**t**	**DF**	***P-value***
Field Mass (g)	Invasive	23	31.810±9.029	-3.859	41	< **0.001**
	Native	20	43.983±11.633			
Standard Body Mass (g)	Invasive	21	30.800±8.904	-4.767	39	< **0.001**
	Native	20	45.606±10.928			
Field Hydration State (%)	Invasive	21	-1.606±10.365	-1.795	39	0.080
	Native	20	3.815±8.868			
SVL (mm)	Invasive	25	73.336±7.296	-0.389	38	0.699
	Native	15	74.267±7.363			
Body Condition (g.mm-3)	Invasive	21	-5.162±5.075	-6.632	34	< **0.001**
	Native	15	7.227±6.110			
BKA Field (%)	Invasive	23	80.855±19.818	-5.525	40	< **0.001**
	Native	19	40.300±31.252			
N:L Ratio Field	Invasive	25	0.488±0.208	2.316	39	**0.026**
	Native	20	0.345±0.201			
CORT (ng/mL)	Invasive	12	0.154±0.095	-0.811	20.182	0.427
	Native	13	0.159±0.101			

SD: Standard deviation; **DF:** degrees of freedom; **SVL:** Snout-Vent Length; **BKA:** Bacterial Killing Ability; **N:L Ratio:** Neutrophil: Lymphocyte Ratio; **CORT:** Corticosterone plasma levels.

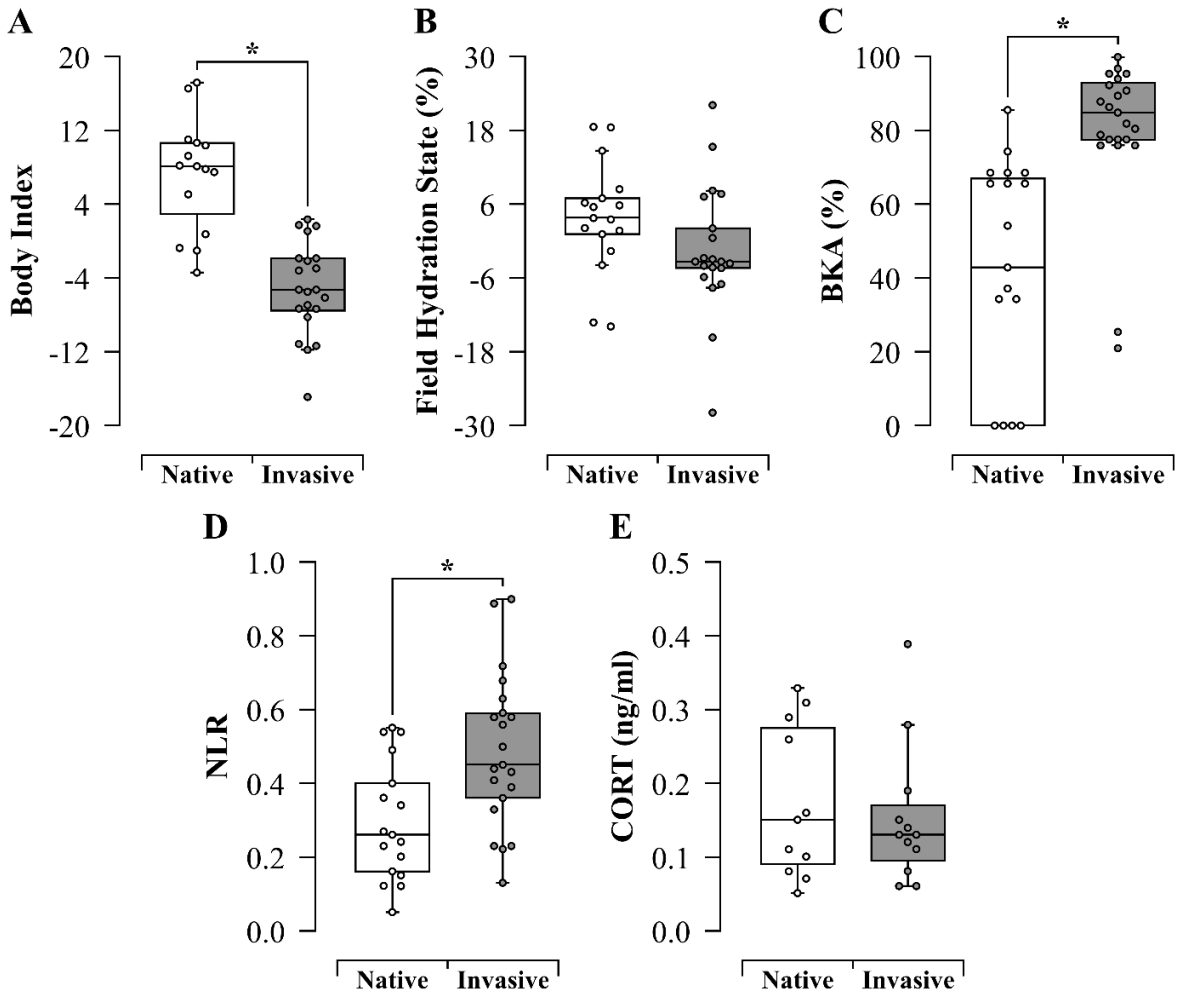

Figure 3.1. Variables collected in the field from the invasive (Cape Town) and native (Durban) populations of males of *Sclerophrys gutturalis*. Body index (**A**); Field Hydration State (**B**); Bacterial Killing Ability (**C**); Neutrophil: Lymphocyte Ratio (**D**); Corticosterone Plasma Levels (**E**). Boxplot inside lines indicate medians, lower and upper borders represent 1st and 3rd quartiles, respectively, whiskers represents upper and lower limits of 1.5 times inter-quartile range and circles represent individual data points. Asterisk (*****) represents mean multiple comparisons with Bonferroni adjustment significant differences ($P \leq 0.05$).

3.4.2. Experimental dehydration and movement restriction: comparison between invasive and native populations

Mixed ANOVA shows significant difference between populations for HTC and BKA ($F_{1,29}$ = 8.018, P = 0.008, $F_{1,34}$ = 142.202, P < 0.001, respectively, Tables 3.2 and 3.3). N:L ratio was affected

by restraint, dehydration and an interaction between populations and restraint ($F_{1, 33}$ = 87.737, P < 0.001, $F_{2,33}$ = 4.739, P = 0.016, $F_{1,33}$ = 10.117, P = 0.003, respectively, Table 3.3). Individuals from the native population showed lower BKA than individuals from the invasive population both after dehydration and after movement restriction (control group: P < 0.001, 10% dehydration group: P < 0.001, 20% dehydration group after dehydration: P = 0.010, 20% dehydration group after restriction of movement: P < 0.001, Fig. 3.2). In addition, within the invasive population, toads dehydrated by 20% increased BKA compared to the control group (P = 0.034, Fig. 3.2). The populations had no changes in the N: L ratio after dehydration. Nonetheless, individuals from the invasive population showed increased N:L ratio after movement restriction in control, 10% and 20% dehydration groups (P < 0.001; Fig. 3.3). Individuals from the native population also showed higher N:L ratio after movement restriction in control and 10% dehydration groups (P = 0.001; P = 0.012, respectively), but not in the 20% dehydration group (P = 0.417, Fig. 3.3).

Toads from native and invasive populations did not differ in CORT in response to the treatments. CORT was higher in invasive toads dehydrated by 10% and 20% when compared with the control group (P = 0.002 and P = 0.007, respectively, Fig. 3.4). Invasive toads dehydrated by 10% presented higher CORT than those from the control group after movement restriction (P = 0.019, Fig. 3.4). In parallel, CORT was higher in native toads dehydrated by 20% when compared to the control group (P = 0.017, Fig. 3.4). Invasive and native toads showed lower CORT after movement restriction when compared to those measured after dehydration at 20% (P = 0.005 and P = 0.022, respectively, Fig. 3.4). The results also showed an interaction between restriction and group for CORT (P = 0.003, Table 3.3).

Native toads showed higher HTC than invasive toads after movement restriction in control and 20% dehydration groups (P = 0.030 and P = 0.046, respectively; Fig. 3.5).

Table 3.2. Descriptive statistics of hematocrit, bacterial killing ability, neutrophil: lymphocyte ratio and corticosterone plasma levels analyzed after the experiment of dehydration stress and movement restriction for *Sclerophrys gutturalis* invasive and native populations.

Variable	Restriction	Population	Group	N	Mean	SD
HTC (%)	Pre	Invasive	Control	6	19.028	9.402
			Dehydration 10%	7	27.701	7.443
			Dehydration 20%	8	26.976	11.662
		Native	Control	3	25.740	3.942
			Dehydration 10%	6	42.666	10.665
			Dehydration 20%	6	47.023	9.724
	Post	Invasive	Control	6	18.631	7.251
			Dehydration 10%	7	22.648	3.057
			Dehydration 20%	8	19.795	13.680
		Native	Control	3	30.553	4.809
			Dehydration 10%	6	32.110	13.029
			Dehydration 20%	6	25.945	9.977
BKA (%)	Pre	Invasive	Control	8	77.795	24.020
			Dehydration 10%	7	69.611	19.498
			Dehydration 20%	8	81.196	9.976
		Native	Control	5	10.968	14.873
			Dehydration 10%	6	16.458	10.134
			Dehydration 20%	6	22.223	7.410
	Post	Invasive	Control	8	76.302	8.903
			Dehydration 10%	7	69.185	29.308
			Dehydration 20%	8	71.245	25.467
		Native	Control	5	24.516	13.573
			Dehydration 10%	6	18.085	10.120
			Dehydration 20%	6	20.833	11.053
N:L Ratio	Pre	Invasive	Control	7	0.391	0.191
			Dehydration 10%	9	0.492	0.240
			Dehydration 20%	8	0.301	0.307
		Native	Control	5	0.480	0.204
			Dehydration 10%	6	0.748	0.310
			Dehydration 20%	6	0.546	0.202
	Post	Invasive	Control	7	1.444	0.601
			Dehydration 10%	9	1.202	0.371
			Dehydration 20%	8	0.953	0.302
		Native	Control	5	1.094	0.249
			Dehydration 10%	6	1.253	0.632
			Dehydration 20%	6	0.665	0.275

CORT (ng/mL)	Pre	Invasive	Control	7	0.226	0.121
			Dehydration 10%	9	0.900	0.505
			Dehydration 20%	8	0.820	0.252
		Native	Control	4	0.368	0.315
			Dehydration 10%	6	0.841	0.329
			Dehydration 20%	6	1.037	0.385
	Post	Invasive	Control	7	0.379	0.267
			Dehydration 10%	9	0.778	0.333
			Dehydration 20%	8	0.495	0.223
		Native	Control	4	0.535	0.034
			Dehydration 10%	6	0.593	0.306
			Dehydration 20%	6	0.737	0.275

SD: Standard Deviation; **HTC:** Hematocrit; **BKA:** Bacterial Killing Ability; **N:L Ratio:** Neutrophil: Lymphocyte Ratio; **CORT:** Corticosterone plasma levels.

Table 3.3. Statistical analysis of mixed ANOVA, for repeated and independent measurements at two different times (for the control group: pre- and post-restriction, for the 10% and 20% dehydration groups: dehydration and post-restriction), as well as interaction between treatment and group.

Variable	Source	Type III SS	DF	MS	F	P
HTC	Intercept	128.843	1	128.843	4768.653	< **0.001**
	Restraint	0.007	1	0.007	0.387	0.539
	Population	0.217	1	0.217	8.018	**0.008**
	Group	0.116	2	0.058	2.152	0.134
	Restraint*Population	0.002	1	0.002	0.131	0.720
	Restraint*Group	0.027	2	0.013	0.786	0.465
	Restraint*Population*Group	0.069	2	0.034	1.999	0.154
	Population*Group	0.059	2	0.030	1.095	0.348
	Error (Restraint)	0.497	29	0.017		
	Error	0.784	29	0.027		
BKA	Intercept	274526.693	1	274526.693	1321.536	< **0.001**
	Restraint	719.941	1	719.941	3.898	0.057
	Population	29540.097	1	29540.097	142.202	< **0.001**
	Group	581.098	2	290.549	1.399	0.261
	Restraint*Population	221.591	1	221.591	1.200	0.281
	Restraint*Group	1.834	2	0.917	0.005	0.995
	Restraint*Population*Group	305.449	2	152.724	0.827	0.446
	Population*Group	1041.507	2	520.754	2.507	0.096
	Error (Restraint)	6279.394	34	184.688		
	Error	7062.926	34	207.733		
N:L Ratio	Intercept	56.426	1	56.426	1260.811	< **0.001**
	Restraint	2.196	1	2.196	87.737	< **0.001**
	Population	0.001	1	0.001	0.032	0.858
	Group	0.424	2	0.212	4.739	**0.016**
	Restraint*Population	0.253	1	0.253	10.117	**0.003**
	Restraint*Group	0.143	2	0.072	2.866	0.071
	Restraint*Population*Group	0.053	2	0.026	1.054	0.360
	Population*Group	0.023	2	0.011	0.255	0.777
	Error (Restraint)	0.826	33	0.025		
	Error	1.477	33	0.045		
CORT	Intercept	30.924	1	30.924	207.239	< **0.001**
	Restraint	0.237	1	0.237	5.013	**0.032**
	Population	0.137	1	0.137	0.919	0.344
	Group	2.369	2	1.185	7.939	**0.001**
	Restraint*Population	0.004	1	0.004	0.086	0.770

Restraint*Group	0.679	2	0.340	7.182	**0.003**
Restraint*Population*Group	0.024	2	0.012	0.254	0.777
Population*Group	0.472	2	0.236	1.580	0.221
Error (Restraint)	1.608	34	0.047		
Error	5.073	34	0.149		

SS: Sum of Squares; **DF:** degrees of freedom; **MS:** Mean Square; **HTC:** Hematocrit; **BKA:** Bacterial Killing Ability; **N:L Ratio:** Neutrophil: Lymphocyte Ratio; **CORT:** Corticosterone plasma levels.

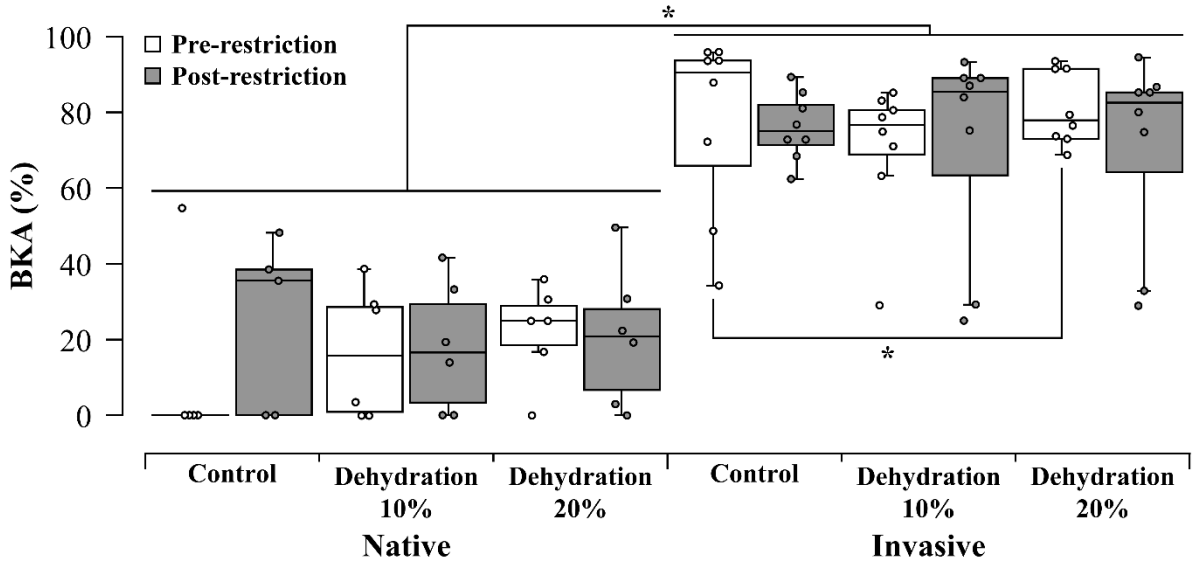

Figure 3.2. Bacterial Killing Ability against *Aeromonas hydrophila* of *Sclerophrys gutturalis* males of the invasive and native population in two distinct moments (for the control group: pre- and post-restriction, for the 10% and 20% dehydration groups: dehydration and post-restriction. Boxplot inside lines indicate medians, lower and upper borders represent 1st and 3rd quartiles, respectively, whiskers represents upper and lower limits of 1.5 times inter-quartile range and circles represent individual data points. Asterisk (*****) represents mean multiple comparisons with Bonferroni adjustment significant differences ($P \leq 0.05$).

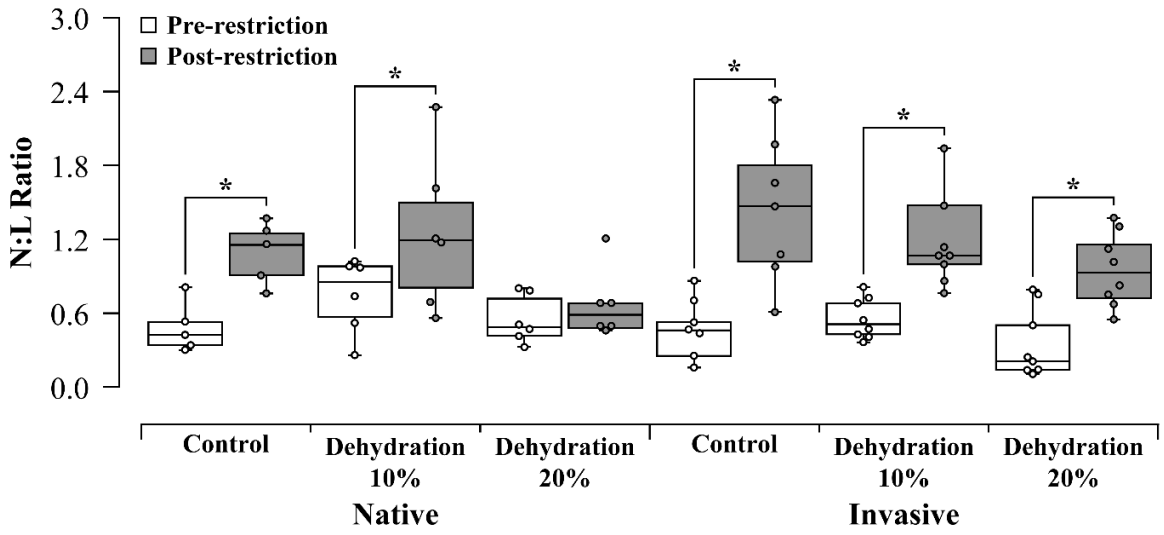

Figure 3.3. Neutrophil: Lymphocyte ratio of *Sclerophrys gutturalis* males of the invasive and native population in two distinct moments (for the control group: pre- and post-restriction, for the 10% and 20% dehydration groups: dehydration and post-restriction). Boxplot inside lines indicate medians, lower and upper borders represent 1st and 3rd quartiles, respectively, whiskers represents upper and lower limits of 1.5 times inter-quartile range and circles represent individual data points. Asterisk (*****) represents mean multiple comparisons with Bonferroni adjustment significant differences ($P \leq 0.05$).

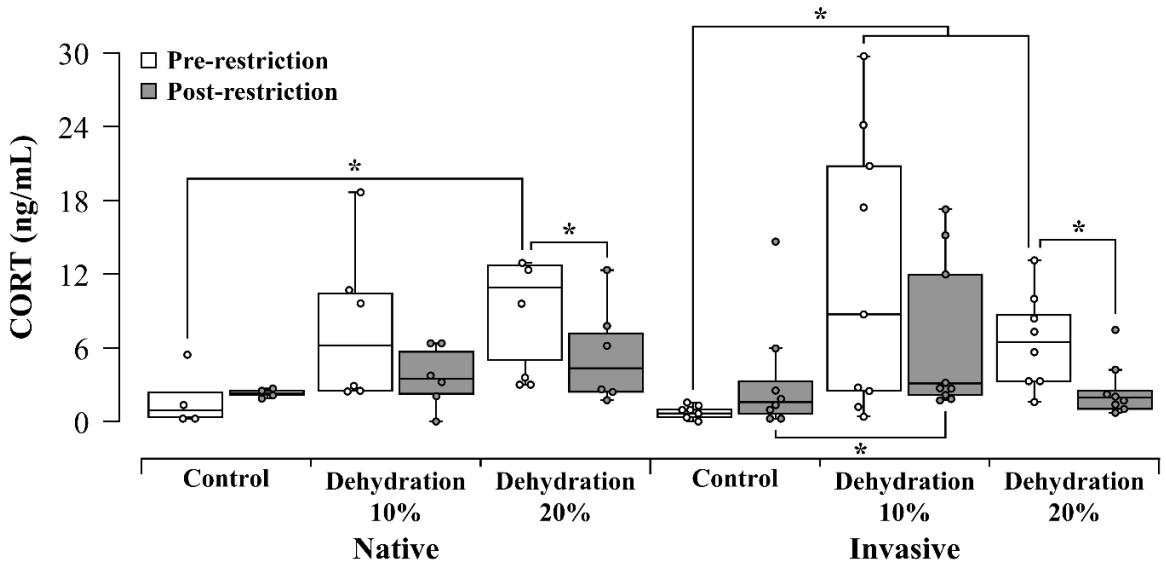

Figure 3.4. Corticosterone plasma levels of *Sclerophrys gutturalis* males of the invasive and native population in two distinct moments (for the control group: pre- and post-restriction, for the 10% and 20% dehydration groups: dehydration and post-restriction). Boxplot inside lines indicate medians, lower and upper borders represent 1st and 3rd quartiles, respectively, whiskers represents upper and lower limits of 1.5 times inter-quartile range and circles represent individual data points. Asterisk (*****) represents mean multiple comparisons with Bonferroni adjustment significant differences ($P \leq 0.05$).

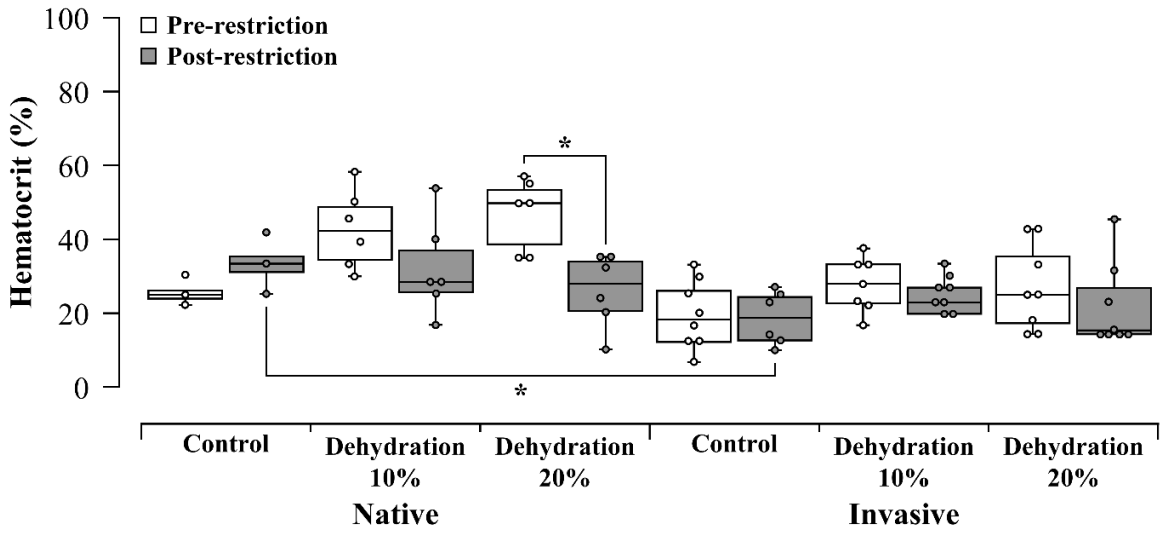

Figure 3.5. Hematocrit of *Sclerophrys gutturalis* males of the invasive and native population in two distinct moments (for the control group: pre- and post-restriction, for the 10% and 20% dehydration groups: dehydration and post-restriction). Boxplot inside lines indicate medians, lower and upper borders represent 1st and 3rd quartiles, respectively, whiskers represents upper and lower limits of 1.5 times inter-quartile range and circles represent individual data points. Asterisk (*****) represents mean multiple comparisons with Bonferroni adjustment significant differences ($P \leq 0.05$).

3.5. Discussion

Guttural toads, *Sclerophrys gutturalis*, from the invasive population had a lower body index and standard body mass, and higher BKA and N: L ratio, compared to individuals from the native population under field conditions. These results are consistent with previous studies showing that the challenges of invasion can directly impact body condition of invasive animals (Brown et al., 2011; Vimercati et al., 2019). Cape Town is characterized by a Mediterranean climate with limited water availability in summer, compared to the native region of these invasive guttural toads, which could reduce the amount and diversity of arthropod prey for anurans (Vimercati et al., 2019). They are probably also restricted in hours of foraging activity, given the increased need to remain longer in microenvironments in contact with water. Additionally, the invasive population of *S. gutturalis* had lower field body mass and tended to show a lower field hydration state than those from the native

range. Vimercati et al. (2018) compared the same two populations of *S. gutturalis* and found similar results, along with a positive relationship between hydration level and relative humidity. These results in conjunction suggest that these invasive toads live under stressful conditions of higher dehydration potential.

The invasive population of *S. gutturalis* had higher BKA and N: L ratio compare to the native population. Some studies have shown a lower immune response of toads on the invasion front compared to longer established populations (Brown et al., 2007; Brown and Shine, 2014; Llewellyn et al., 2012). However, the literature has shown diverse and contradictory data related to immunocompetence of invasive organisms. These organisms are exposed to new biotic and abiotic conditions, which exert novel selective pressures (Müller-Schärer et al., 2004; Carroll 2008; White and Perkins, 2012; Brown et al., 2015), and favor the emergence of phenotypes that increase dispersal (Travis and Dytham 2002; Shine et al., 2011; Brown et al., 2015; Courant et al., 2019) among other traits. Thus, it follows that diminished immune function could make invasive toads more susceptible to new pathogens, decreasing their survival and invasion success. Similar to our results, Brown et al. (2014) showed that offspring of invasive cane toads (*Rhinella marina*) in Australia have higher BKA, higher numbers of neutrophils and phagocytic activity on the invasion front when compared to offspring of toads from long-established populations bred under standard conditions. These results suggest genetically based shifts in immune response compromised by long-distance dispersal in free-ranging toads (Brown et al., 2014). Additionally, studies with shore crabs and birds show greater non-specific immune function of invasive individuals when compared to native ones (Keogh et al., 2017; Moller and Cassey, 2004). Still, according to Brown et al. (2014), the baseline levels of complement and neutrophils have been reinforced in cane toads on the invasion front, while the costs associated with its activation decreased. Thus, it is possible that invasive animals, such as *S. gutturalis*, tend to have increased constitutive immunity associated with increased surveillance. This would ensure a

faster response to immunological challenges, reducing the scope of immune activation after infection and its costs associated with fever, anorexia, decreased locomotor activity, growth rates (Klasing and Korver, 1997; Spurlock, 1997; Lee and Klasing, 2004), and potentially dangerous systemic inflammatory response (Janeway et al., 2001; Klein and Nelson, 1999; Lee and Klasing, 2004). It is unknown whether such responses are exaggerated in the invasive population as novel climates differ from the original, or if they are, at least in part, genetically determined in guttural toads. These hypotheses remain to be tested.

Blood leukocyte numbers and proportions have also been commonly used as indicators of physiological stress in vertebrates, including toads from the genus *Rhinella* (Davis et al., 2008; Campbell, 2015; Savage et al., 2016; Assis et al., 2015; 2017; 2019; Barsotti et al., 2017; 2019). In response to various stressors, including dehydration and malnutrition, the number of circulating neutrophils increases while the number of lymphocytes decreases, reflecting changes in leukocyte distribution and production (Davis et al., 2008; Savage et al., 2016; Barsotti et al., 2019). Moreover, increased N: L ratio has been shown to reflect more accurately chronic stress than increased glucocorticoid plasma levels (Davis et al., 2008; Swan and Hickman, 2014; Hickman, 2017). Thus, it is possible that anurans from the invading population in different environmental conditions and facing new challenges, may be more stressed, reflecting the neutrophilia and lymphopenia observed in our results.

Contrary to what we expected, toads from the invasive population show higher BKA both after dehydration and restriction protocols. Interestingly, the difference between populations regarding field BKA was maintained after captivity and all the experimental protocols. These results show that BKA's response was robust to exposure to acute stressors, at least within the period analyzed, suggesting an increase in constitutive immunity in the invasive population.

Moreover, dehydrating toads by 20% increased CORT and BKA, which may indicate an immunostimulatory effect of CORT. Accordingly, increased CORT along with different immune variables after submission to an acute stressor has been repeatedly observed in anurans (Assis et al., 2015; 2017; 2019; Titon et al., 2018; 2019; Barsotti et al., 2017; 2019). Dhabhar et al. (1996) showed that acute stress results in a rapid reduction in circulating leukocyte numbers associated with a redistribution of blood leukocytes to other organs, tending to increase the immune response in some body compartments. An acute response to stressors is also associated with increased antigen presentation, effector cell function, antibody production and cytokines expression (Dhabhar and McEwen, 1997). Additionally, in response to short-term stressors and acute increase in glucocorticoids, there is an increase in activity of the complement system (Dhabhar and McEwen, 1996; 1997; Dhabhar, 1998), which is also consistent with higher BKA response in dehydrated toads.

According to our predictions, dehydration activated the HPI axis culminating in increased CORT, mainly when toads were dehydrated by 20%. This increase in CORT indicates that dehydration is a stressor for *S. gutturalis*, as has already been concluded for other vertebrate species (Arnhold, et al., 2007; Cain and Lien, 1985; Moeller et al., 2017), including toads from genus *Rhinella* (Barsotti et al., 2019). Since CORT participates in the integrated physiological response of anurans to dehydration, due to its mineralotropic actions (Broillet et al., 1993; Chen et al., 1998; Vera et al., 2017), it is possible that the increase of CORT observed in *S. gutturalis* may contribute to protection against possible deleterious effects caused by the loss of volume of extracellular fluid. Moreover, glucocorticoids potentiate the effects of angiotensin II (ANG II) mediating drinking behavior in mammals (Ganesan and Sumners, 1989; Sumners et al., 1991; Takei, 2000), and can similarly stimulate water seeking-behavior in anurans. Interestingly, hydrated individuals of *S. gutturalis* who had their CORT artificially increased, through transdermal application, showed an increase in water-seeking behavior (Madelaire et al., 2020). Considering that invasive toads tended to show lower hydration levels than native toads

under field conditions, this higher efficiency in finding water due to increased CORT when dehydrated might favor the dispersal of the invasive Cape Town population.

Movement restriction represented a stressor for *S. gutturalis*, increasing the N: L ratio for both populations. Changes in leukocyte numbers and proportions in the blood are often used as indicators of infection and physiological stress in amphibians and other vertebrates (Assis et al., 2015; 2017; 2019; Barsotti et al., 2017; 2019; Davis et al., 2008; Campbell, 2015; Savage et al., 2016). Neutrophils and lymphocytes constitute the majority of blood leukocytes in amphibians (Savage et al., 2016) and the number of neutrophils increases and the lymphocyte decreases in the face of stressors, reflecting changes in leukocyte distribution and production (Davis, 2001; Savage et al., 2016). Assis et al. (2015) showed increased N: L ratio after restraint with movement restriction in *R. icterica*, but not in individuals subjected to less stressful restraint without movement restriction. Additionally, Barsotti et al. (2019) showed that *R. ornata* subjected to 20% dehydration showed a more pronounced increase in N: L than those subjected to 10% dehydration.

CORT was not increased after 24 h of movement restriction in hydrated native and invasive toads, in contrast with previous observations on other anuran species (Assis et al., 2015; 2019; Barsotti et al., 2019). Given that the N: L ratio increased after movement restriction in these toads, it is probable that a peak of increased CORT in response to this treatment occurred earlier than the time at which the blood sample was collected. Moreover, it is interesting to observe that, both invasive and native toads that were dehydrated by 20% and posteriorly submitted to movement restriction, CORT values after movement restriction were lower than those measured after intense dehydration. Given that movement restriction was performed in damp cloth bags, toads may have rehydrated and reduced CORT during the period of movement restriction. Rehydration up to almost 100% has been previously observed in another toad (*Rhinella ornata*) under the same experimental conditions (Table 6- supp. file). Even so, these results suggest that movement restriction was not a stressor as intense as dehydration for this

species. Likewise, intense dehydration is a more potent stressor than movement restriction in *R. ornata* (Barsotti et al., 2019), probably the only other anuran species from which there is data available on these two stressors.

In summary, invasive toads show lower standard field body mass, lower body index and tended to show lower hydration status in the field, which is consistent with life in the novel drier environmental conditions of Cape Town. Invading toads also showed higher N: L and BKA ratio in the field, compared to those from the native population, suggesting that invasive toads face stressful conditions and invest comparatively more in non-expensive components of their immune system. It is interesting to observe the persistence of inter-populational differences in BKA, even after submission to acute stress (dehydration and movement restriction). Although these traits have favored the permanence and dispersion of *S. gutturalis* in the invasive range, it is important to emphasize that we have investigated short-term phenotypic differences between these populations. An analysis of genetic diversity suggests this invasive population is not derived from founder effects (Telford et al., 2019). These differences might be, at least in part, due to plastic responses to current environmental differences during the breeding season (Vimercati et al., 2019), or fast adaptive responses after 20 years of their introduction in Cape Town (Vimercati et al., 2018). Additional studies, including rearing individuals form both populations under common garden conditions, would be necessary to understand the causes of this phenotypic divergence.

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3.8. Supplementary file

Supplementary materials for

Challenges of a novel range: water balance, stress and immunity in an invasive toad

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Figure 4S1

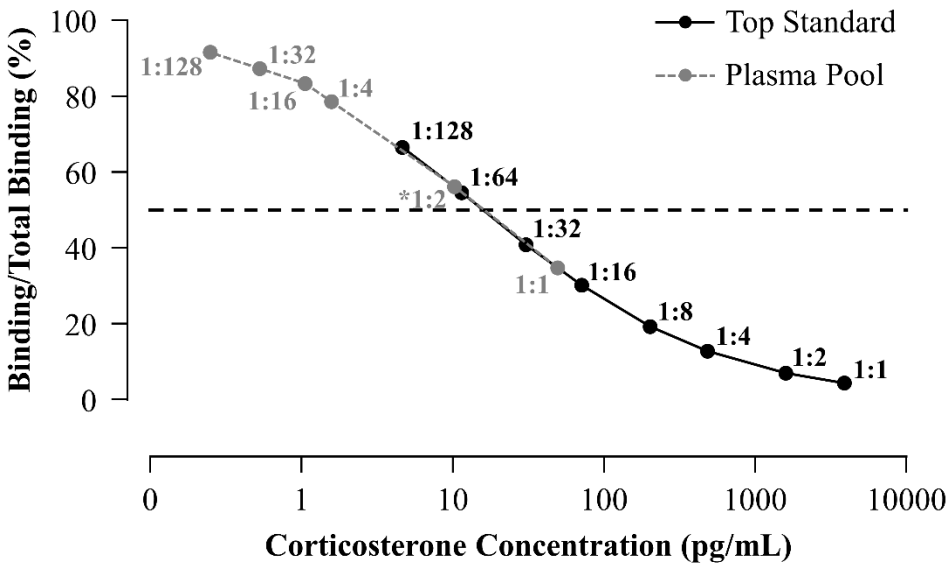

Figure 3S1. Parallelism curve. Binding displacement curves of serially diluted *Sclerophrys gutturalis* pooled plasma at baseline conditions, against the corticosterone standard used in the corticosterone enzyme-immunoassay. The y-axis shows the % Hormone Binding/Total Binding measured at 412 nm. The 50% binding point is denoted using a dashed line, which determined dilution factors for the extracted plasma samples. The asterisk (*) represents the best dilution factor.

CONCLUSÕES GERAIS

A exposição das duas espécies de sapos estudadas, *R. ornata* e *S. gutturalis*, a diferentes níveis de desidratação (10 % e 20% da massa corpórea padrão) resultou em aumento correspondente da concentração plasmática de corticosterona (CORT). Esse resultado foi observado inclusive para indivíduos da população invasora de *S. gutturalis*, os quais demonstram ser mais resistentes ao estresse hídrico. Dessa forma, a desidratação representa um estressor para esses anuros, com intensidade de resposta variável e dependente de intensidade do estressor.

A exposição de indivíduos de *R. ornata* à restrição de movimentos (em condições não desidratantes) após a desidratação resultou na manutenção da concentração elevada de CORT plasmática e refletiu em aumento da razão neutrófilo: linfócito (N:L) e atividade fagocítica (AF). Sendo assim, a submissão desses sapos a dois estressores agudos de forma consecutiva gerou uma reposta de estresse sustentada e um efeito imunoestimulador. Vale ressaltar que esses resultados são pertinentes à submissão consecutiva a dois estressores agudos e que, talvez, a submissão inicial a um estressor crônico antes do agudo secundário pudesse gerar resultados diferentes, como uma imunossupressão.

Com relação a *S. gutturalis*, indivíduos da população invasora mostraram maior capacidade bactericida plasmática (CBP) e N:L em campo, e mantiveram a CBP maior após a submissão aos estressores (desidratação seguida de restrição de movimentos) quando comparados com indivíduos da população nativa. Esses resultados indicam que sapos invasores apresentam funções imunitárias constitutivamente elevadas, o que poderia conferir vantagens de ajustes às condições climáticas prevalentes no ambiente recentemente ocupado.

Dado que as populações de anfíbios vêm sofrendo um declínio acentuado, principalmente devido às mudanças climáticas, fragmentação e perda de habitat, as quais expõem os anfíbios a um maior risco de dessecação, entender como a desidratação pode modular a resposta a estressores e a

imunocompetência se tornou uma questão imprescindível a ser investigada. Estudos futuros, envolvendo a desidratação e exposição a estressores crônicos, bem como estudos comparativos de animais de diferentes biomas, seriam interessantes para dar continuidade ao entendimento dessas relações.

ANEXO 1 – CERTIFICADO DE APROVAÇÃO DO COMITÊ DE ÉTICA NO USO DE ANIMAIS (CEUA IB/USP)

USP Universidade de São Paulo
Instituto de Biociências

www.ib.usp.br

CERTIFIED

We certify that the proposal entitled "Water stress and immune response in frogs (Rhinella ornata) inhabiting fragments of Atlantic Forest", registered under No. 249/2016 (Proc. 16.1.182.41.6), under the responsibility of Prof. Dr. Fernando Ribeiro Gomes and with the participation of the collaborator Adriana Maria Giorgi Barsotti (IB / USP), which involves the use of animals belonging to the Chordata filipe, sub-vertebrate Vertebrata (except humans), for the purposes of scientific research is in agreement with the provisions of Law No. 11,794, dated October 08, 2008, of Decree No. 6.899, of July 15, 2009 and with the regulations issued by the National Council for the Control of Animal Experimentation (CONCEA), and was approved by the Ethics Committee at Use of Animals - CEUA of the Institute of Biosciences of the University of São Paulo at a meeting on September 12, 2016.

Duration of the authorization: 12/09/2016 to 11/09/2018
Purpose: Scientific Research
SISBIO request or authorization number: 29896-1
Activities: capture, specimen collection and marking
Species / Taxonomic Groups: Rhinella ornata (toad-cururuzinho)
Location of activities: Preserved and fragmented Atlantic Forest, in Ibiúna - SP and São Luis do Paraitinga - SP
CEUA suggests that dead specimens be offered for storage in some Amphibian Collection.

Addendum

The Ethics Committee on the Use of Animals - CEUA of the Institute of Biosciences of the University of São Paulo, on 10/31/2018 approved the inclusion of 30 individuals of Sclerophrys gutturalisas, males; extension of the validity period until 04/30/2019 and inclusion of collection site in Cape Town and Durban, South Africa.

NOTE: All the items needed for the positive recommendation of this study were considered. Therefore, the handling would be in accordance with the standards applied in Brazil. It is not within the jurisdiction of CEUA of IB-USP and CONCEA itself to evaluate the suitability of the project according to animal use regulations under experimentation in South Africa.

OBS .: Any change and / or intercurrence must be communicated to CEUA-IB.

Prof. Dr. Pedro Augusto Carlos Magno Fernandes
Coordinator of the Committee on Ethics in the Use of Animals

Rua do Matão • Travessa 14 • nº 321 • Cidade Universitária • São Paulo • SP • CEP 05508-090 • Brasil

ANEXO 2 – CERTIFICATE OF APPROVAL OF THE RESEARCH ETHICS COMMITTEE: ANIMAL CARE AND USE (STELLENBOSCH UNIVERSITY)

UNIVERSITEIT
STELLENBOSCH
UNIVERSITY

Approved with Stipulations

18 January 2019

PI: Miss Adriana Barsotti

REC: ACU Reference #: ACU-2019-8839

Title: Challenges of invasion front: Water balance, water seeking behavior, stress and immunity in Sclerophrys gutturalis

Dear Miss Adriana Barsotti

Your Response to Modifications, with reference number ACU-2019-8839 was reviewed on 17 January 2019 by the Research Ethics Committee: Animal Care and Use via committee review procedures and was approved on condition that the following stipulations are adhered to:

1. A monitoring sheet must be kept in order to constantly monitor and record the well-being of the captured animals. In a note to the committee please explain what will happen to those individuals that does not eat?
2. The applicant is requested justify why only male animals will be targeted.
3. Euthanasia: The applicant is requested to provide more clarity on the issues listed below:

   3.1 Did the previous studies subject this species to dehydration?

   3.2 Did they check waterproofing of the skin?

   3.3 Did they pith any frogs?

   IP Injection should be less stressful than taking blood directly from the heart when frogs are turned over. Why not inject directly into the heart if IP is regarded as stressful? The committee is concerned that the dehydrated frogs will take longer to be affected by MS222.

General Stipulations:

SAVC Authorisation: The applicant must submit proof of adherence to the SAVC rules related to conducting veterinary procedures on animals.

Permits: The applicant must also submit copies of permits once issued by the relevant conservation authority.

Applicants are reminded that they are expected to comply with accepted standards for the use of animals in research and teaching as reflected in the South African National Standards 10386: 2008. The SANS 10386: 2008 document is available on the Division for Research Developments website www.sun.ac.za/research.

As provided for in the Veterinary and Para-Veterinary Professions Act, 1982. It is the principal investigator's responsibility to ensure that all study participants are registered with or have been authorised by the South African Veterinary Council (SAVC) to perform the procedures on animals, or will be performing the procedures under the direct and continuous supervision of a SAVC-registered veterinary professional or SAVC-registered para-veterinary professional, who are acting within the scope of practice for their profession.

Please remember to use your REC: ACU reference number: # ACU-2019-8839 on any documents or correspondence with the REC: ACU concerning your research protocol.

If you have any questions or need further help, please contact the REC: ACU office at 021 808 9003.

Visit the Division for Research Developments website www.sun.ac.za/research for documentation on REC: ACU policy and procedures.

Sincerely,

Mr Winston Beukes

Coordinator: Research Ethics (Animal Care and Use)