Vania Regina de Assis

Níveis plasmáticos de corticosterona, testosterona e imunocompetência em Bufonídeos

Plasma levels of corticosterone, testosterone and immunocompetence in Bufonids

São Paulo

2015

Vania Regina de Assis

Níveis plasmáticos de corticosterona, testosterona e imunocompetência em Bufonídeos

Plasma levels of corticosterone, testosterone and immunocompetence in Bufonids

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: Fernando Ribeiro Gomes
Co-orientador: Carlos A. N. Iannini

São Paulo

2015

Assis, Vania Regina de.
Plasma levels of corticosterone, testosterone and immunocompetence in Bufonids / Vania Regina de Assis ; orientador Fernando Ribeiro Gomes. -- São Paulo, 2015.

134 f.

Tese (Doutorado) – Instituto de Biociências da Universidade de São Paulo. Departamento de Fisiologia.

1. Corticosterone. 2. Immune response. 3. Stress 4. Testosterone. I. Gomes,
Fernando Ribeiro. II. Universidade de São Paulo. Instituto de Biociências. Departamento de Fisiologia. III. Título.

Comissão Julgadora

_____ _____

Prof(a). Dr(a). Prof(a). Dr(a).

_____ _____

Prof(a). Dr(a). Prof(a). Dr(a).

_____

Prof(a). Dr(a).

DEDICATION

I dedicate this thesis to my **Mom**, *Clarice*, the strongest and gentlest woman that I ever met in my life; and to my **Husband**, *Jefferson*, my soulmate.

EPIGRAPH

If we knew what it was we were doing, it wouldn't be called 'research', would it?

Albert Einsten

(from p. 272 of ***Natural Capitalism***. 1999. Authors: Paul Hawken; Amory Lovins; L. Hunter Lovins. First Edition)

ACKNOWLEDGMENTS

To my professor and advisor Dr. **Fernando Ribeiro Gomes**, that always believed me, and gave me precious advices to make me keep going into the right way throughout this journey, that was my doctoral degree.

A very special thank you to my beloved husband, **Jefferson Rondolfo**, which is always supporting me in my research career, with words and acts, even when it means that we need to be apart. Stay away was hard, but be back in his arms is such amazing feeling. I love him so much.

To my best friend **Stefanny Christie Monteiro Titon**, which was always with me, pushing me up, during the hardest times at field and lab work.

To my friends **Braz Titon Jr.**, **Adriana Maria Giorgi Barsotti, Jessyca Citadini** and **Jasmine N. Dagg** for your friendship and for helping me during field and lab work. Also to **Eduardo B. Fernandes**, the lab technique, for all his help.

To my friends **Eduardo H. Moretti, Clara Aquino, Alline Lima, Christopher S. Michaelson, Meghan Kelley, Jeffrey M. Goessling** and **Judy Heenan**, for your friendship, meet each of you was a pleasure.

To professors **Carlos A. Navas Iannini, Beny Spira**, **Cristina O. M. S. Gomes**, **Eliana R. Matushima, Chelsea K. Ward** and **Mary T. Mendonça**, for assisting me with methodological advices and collaborations, which helped me to improve the implementation and discussion of this work.

To all of *Departamento de Fisiologia Geral*, especially employees **Roseli Silva Santos** and **Erika H. Takamoto de Camargo** for the work and competence, you helped a lot on this journey.

To the field assistants, **Alexandre D. de Jesus** and **Benedito Santos**, always present at collection nights in the city of São Luis do Paraitinga, and **Marcos D. Franco**, always present at collection nights in the city of Luiz Antônio.

To all, with love...

Vania Regina de Assis

(Female *Rhinella schneideri*)

The following institutions supported this study:

Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP):

Young Researcher support (2006/54699-1) conducted by FRG;

Regular grant for FRG (2013/00900-1);

Ph.D. scholarship for VRA (2011/10560-8);

BEPE scholarship for VRA (2014/05806-6)

Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPq):

PhD scholarship for VRA (140272/2011-9 GD)

Programa de Pós-graduação em Fisiologia Geral - Departamento de Fisiologia - Instituto de Biociências - USP - São Paulo.

Departamento de Fisiologia - Instituto de Biociências - USP - São Paulo.

SUMMARY:

FIGURE LIST

TABLE LIST

RESUMO GERAL

Glicocorticóides modulam a resposta imune de forma complexa em vertebrados expostos a diferentes estressores. Dado que as populações naturais têm estado expostas a uma multiplicidade de estressores, uma melhor compreensão da associação funcional entre a duração e a intensidade da resposta ao estresse, as mudanças resultantes nos níveis dos hormônios esteróides e seu impacto sobre os diferentes aspectos da imunocompetência emergem como um ponto chave para as estratégias de conservação dos vertebrados. Nós investigamos as relações entre os níveis plasmáticos de hormônios esteróides e a imunocompetência inata em anfíbios anuros, incorporando as metodologias de desafio de contenção, elevação experimental dos níveis de corticosterona por aplicação transdérmica, capacidade bactericida por espectrofotometria e o desafio imunológico com fitohemaglutinina. Nossos resultados demonstram que a capacidade bactericida plasmática (CBP) medida por espectrofotometria é um método confiável e preciso para estimar a imunocompetência de anfíbios anuros, além disso, mostramos a existência de uma grande diversidade interespecífica na CBP de anuros machos. Quando quatro diferentes espécies de Bufonídeos foram submetidas a um desafio de contenção, as respostas gerais incluíram aumento dos níveis plasmáticos de corticosterona (CORT) e da relação neutrófilo/linfócito (N:L) e diminuição dos níveis plasmáticos de testosterona (T). As respostas da CBP à contenção foram muito mais variáveis, com *R. icterica* mostrando diminuição e *R. marina* mostrando aumento dos valores de CBP. Adicionalmente, CORT e N:L tenderam a aumentar mais em resposta à contenção com restrição de movimento do que à contenção sem restrição de movimento, indicando que os sapos demostraram um aumento da resposta ao estresse quando submetidos ao estressor mais intenso. Todas as variáveis estudadas mostraram variação interespecífica. *Rhinella ornata* apresentou os maiores níveis basais de CORT quando comparado com as outras espécies, enquanto *R. ornata* e *R. icterica* mostraram os maiores

valores basais de CBP. Entretanto, as mudanças na relação N:L, nos níveis de T e na CBP, não foram correlacionadas com o aumento em CORT, dentro ou entre espécies. A aplicação transdérmica de corticosterona eficientemente simulou eventos repetidos de resposta ao estresse agudo em *Rhinella icterica*, sem alterar os parâmetros imunitários, mesmo após treze dias de tratamento. Curiosamente, o cativeiro a longo prazo não atenuou a resposta ao estresse, uma vez que estes sapos mantiveram um aumento de três vezes em CORT mesmo depois de três meses sob estas mesmas condições. Além disso, a manutenção em cativeiro a longo prazo, nas mesmas condições, aumentou a contagem total de leucócitos (TLC) e gerou uma diminuição ainda maior na CBP, sugerindo que as consequências da resposta ao estresse podem ser agravadas pelo tempo em cativeiro. Com base em nossos resultados, consideramos que uma avaliação cuidadosa é necessária para compreender a modulação da resposta imunitária pelo estresse a nível intra e interespecífico. A inclusão de diferentes segmentos da resposta imune é desejável, e a padronização da coleta de dados para todas as espécies sob o mesmo período (em geral, dentro ou fora da época reprodutiva) e mesma atividade (em geral, vocalizando ou forrageando) se faz obrigatória.

GENERAL ABSTRACT

Glucocorticoids modulate the immune response in complex ways in vertebrates exposed to different stressors. Given that natural populations have been exposed to a multitude of stressors, a better understanding of the functional association between duration and intensity of the stress response, the resulting changes in steroid hormone levels and their impact on different aspects of immunocompetence emerges as a cornerstone for vertebrate conservation strategies. We investigated the relationships between steroids levels and innate immunocompetence in anuran amphibians, incorporating the methodology of restraint challenge, experimental elevation of corticosterone levels by transdermal application, bacterial killing ability by spectrophotometry and the immune challenge with phytohemagglutinin. Our results demonstrate that the bacterial killing ability (BKA) measured by spectrophotometry is a reliable and accurate method to estimate the immunocompetence of anuran amphibians, additionally showed the existence of a large interspecific diversity in BKA from male anurans. When four different species of Bufonids were submitted to a restraint challenge, the general responses included increased in corticosterone plasma levels (CORT) and neutrophil/lymphocyte ratio (N:L) and decreased in testosterone plasma levels (T). The responses of BKA to restraint were much more variable, with *R. icterica* showing decreased and *R. marina* showing increased values. Additionally, CORT and N:L tended to increase more in response to restraint with movement restriction than to restraint without movement restriction, indicating that toads showed an increased stress response to the more intense stressor. All variables studied show interspecific variation. *Rhinella ornata* showed higher baseline CORT when compared to other species, while *R. ornata* and *R. icterica* showed the highest baseline BKA values. However, changes in N:L ratio, T levels and BKA, were not correlated to increased CORT within or between species. Transdermal application of corticosterone efficiently mimics repeated acute stress response events in *Rhinella icterica*, without changing the immune parameters even after thirteen days

of treatment. Interestingly, long-term captivity did not mitigate the stress response, since the toads maintained three fold increased CORT even after three months under these conditions. Moreover, long-term captivity in the same condition increased total leukocyte count (TLC) and generated an even greater decrease in BKA, suggesting that consequences of the stress response can be aggravated by time in captivity. Based on our results, we consider that a careful evaluation is necessary in order to understand the modulation of the immune response by stress at intra and interspecific levels. The inclusion of different segments of the immune response is desirable, and a standardized data collection for all the species under the same period (e.g. inside or outside of breeding season) and same activity (e.g. calling or foraging) is mandatory.

INTRODUÇÃO GERAL

Implicações ecológicas e evolutivas da imunocompetência

Organismos eucarióticos devem se defender continuamente da infiltração e colonização por microrganismos. Estas defesas ocorrem através de barreiras mecânicas (pele e mucosas), inibidores químicos e bioquímicos, células e fatores solúveis. As defesas imunitárias ocorrem através de mecanismos complexos e podem ser divididas em dois tipos de respostas distintas, porém inter-relacionadas: a imunidade adquirida e a imunidade inata (Tieleman et al., 2005). A imunidade adquirida requer exposição prévia a antígenos específicos, sendo esta resposta complexa e muitas vezes dependente de vários dias para ser completamente ativada. A resposta inata, por outro lado, compreende uma parcela significativa do sistema imune e atua como um mecanismo de defesa inicial contra a colonização por micro-organismos logo após a infecção, sendo rapidamente ativada e atuando de forma a eliminar ou retardar os estágios iniciais da infecção. A imunidade inata constitui-se de uma mistura de componentes humorais, tais como anticorpos naturais, complementos, proteínas de fase aguda e elementos celulares (macrófagos, neutrófilos/heterófilos e trombócitos) (Tieleman et al., 2005). Como a imunidade inata é não específica, ela não requer exposição prévia a antígenos específicos. Adicionalmente, o sistema imune inato funciona como ativador da imunidade adquirida, gerando fatores quimiotáxicos e produzindo citocinas que iniciam o desenvolvimento e a maturação de populações específicas de células T e células B (Merchant et al., 2003).

Várias linhas de evidências têm enfatizado a importância da capacidade de resposta imune como parte da evolução de diversos aspectos da história de vida dos animais. Hamilton e Zuk (1982) propuseram, por exemplo, que a preferência sexual das fêmeas de aves por machos com ornamentos estruturais mais complexos encontra-se associada à correlação entre a capacidade dos machos em resistir à carga parasitária e a condição da ornamentação. Outros estudos têm também demonstrado variação interespecífica na competência do sistema imune inato em aves

de acordo com seu ambiente. Com um exemplo, aves provenientes de ambientes com baixa incidência de parasitas, como o Ártico e ambientes marinhos, exibem defesas imunitárias menos robustas do que aves provenientes de ambientes temperados (Piersma, 1997). Complementarmente, espécies de aves com uma melhor resposta imune apresentam menores taxas metabólicas de repouso (Tieleman et al., 2005), sugerindo uma possível restrição energética para manutenção do sistema imune. Desta forma, espécies que apresentem menor custo energético de manutenção poderiam alocar proporcionalmente mais recursos para a constituição do sistema imune (Tieleman et al., 2005).

Espécies invasoras tendem a ter menor diversidade de parasitas e exibir menor prevalência de infecções do que espécies nativas (Mitchell e Power, 2003; Torchin et al., 2003) o que traz implicações da perspectiva da conservação e potencial invasivo de espécies. Lee et al., (2005) demonstraram que a resposta imune pode representar um fator importante, e geralmente ignorado, do sucesso de espécies invasoras. De acordo com este estudo, o pardal doméstico (*Passer domesticus*), uma espécie invasora altamente bem-sucedida, não apresentou nenhuma alteração da eficiência reprodutiva em resposta a um protocolo de infecção experimental, enquanto outra espécie congenérica (*Passer montanus*), uma espécie invasora bem menos agressiva, apresentou uma queda na produção de ovos de cerca de 40% em resposta ao mesmo desafio imunológico experimental (Lee et al., 2005).

Implicações ecológicas e evolutivas da imuno-modulacão por hormônios esteróides

Imuno-modulação mediada por andrógenos

O hormônio esteróide testosterona vem sendo apontado como candidato para a mediação de diversos compromissos fenotípicos em machos de vertebrados durante a estação reprodutiva (Folstald e Karter, 1992; Ketterson e Nolan, 1992; Verhulst, et al., 1999; Muehlenbein e

Bribiescas, 2005). A testosterona aumenta o sucesso reprodutivo por promover o comportamento sexual, a defesa de território, o desenvolvimento de caracteres sexuais secundários e a produção de espermatozóides, enquanto reduz simultaneamente a aptidão, por suprimir características tais como cuidado parental e função imune (Ketterson e Nolan, 1999; Folstald e Karter, 1992; Owen-Ashley et al., 2004). No que diz respeito aos efeitos imunossupressores, a testosterona promove a involução do timo e redução de outros tecidos linfóides em mamíferos, bem como da bursa de Fabricius em aves (Norton e Wira, 1977; Weinstein et al., 1984). Adicionalmente, aplicações agudas de altas doses de testosterona reduzem tanto a imunidade humoral quanto a mediada por células (Inman, 1978; Grossman, 1984; Ahmed et al., 1985), enquanto a administração sustentada de baixas doses reduz a atividade das células natural-killer e células dependentes de anticorpos, bem como a citotoxicidade mediada por células (Hou e Zheng, 1988).

De fato, machos de diferentes grupos de vertebrados tipicamente mostram maior susceptibilidade a infecções por parasitas e uma reposta imune mais fraca frente a uma variedade de antígenos do que fêmeas (Cohn, 1979; Grossman, 1985; Alexander e Stimson, 1988). Estudos de seleção artificial realizados com mamíferos e aves vêm demonstrando que a seleção para uma alta imunocompetência diminui a ocorrência de infecções parasitárias, apresentando correlação genética negativa com níveis de testosterona e esforço reprodutivo (Mills et al., 2010), bem como com o nível de ornamentação (Verhulst et al., 1999). Desta forma, diversos estudos vêm propondo que o esforço reprodutivo dos machos poderia comprometer a sobrevivência dado o efeito imunossupressor dos andrógenos, reduzindo a resistência à infecção parasitária (Hamilton e Zuk, 1982; Folstad e Karter, 1992; Mills et al., 2010). Embora evidências de imunossupressão dependente de testosterona em vertebrados silvestres tenham sido fornecidas para um grande número de espécies (Casto *et al.*, 2001), uma meta-análise de estudos com répteis, aves e mamíferos concluiu que o efeito imunossupressor

da testosterona depende do tipo de resposta imunitária e varia consideravelmente dentro e entre grupos filogenéticos (Roberts et al., 2004). Além disso, pardais implantados com testosterona e mantidos sob condições que simulavam dias curtos, não exibiram supressão da resposta imune mediada por células, indicando que a testosterona não é obrigatoriamente imunossupressora durante todo o ano nesta espécie (Greenman et al., 2005; Martin et al., 2008).

É importante ressaltar que pelo menos parte dos efeitos imunossupressores da testosterona pode ser indireta, sendo mediada por corticosteróides (McEwen et al., 1997). Um possível mecanismo responsável pelos efeitos indiretos seria a ligação da testosterona às globulinas de ligação de corticosteróides, gerando um aumento da fração livre e ativa dos corticosteróides (Gala e Westphal, 1965; Kley et al., 1973; Bradley et al., 1980; McDonald et al., 1981; Bradley, 1987). Adicionalmente, a presença de receptores para corticosteróides e esteróides sexuais no tecido imune apoia a possibilidade dos efeitos imunossupressores diretos e indiretos (Nelson et al., 2002). Embora esta hipótese permaneça para ser testada, a existência de efeitos diretos e mediados por corticosteróides poderia adicionar uma considerável plasticidade evolutiva à conexão entre testosterona e características imunológicas (Hau, 2007).

Imuno-modulação mediada por corticosteróides

Os corticosteróides são hormônios liberados em resposta a uma vasta gama de estímulos e condições estressantes, em diversos grupos de vertebrados. Nesta situação, eles funcionam como importantes reguladores do metabolismo de carboidratos, lipídios e proteínas, além de exercer um papel fundamental na modulação e direcionamento da resposta imune no período de recuperação pós-estresse agudo (Sapolsky et al., 2000). Entretanto, a exposição a picos muito frequentes, ou a níveis cronicamente elevados destes hormônios encontram-se associados a efeitos potencialmente deletérios sobre indivíduos, incluindo diminuição das taxas de crescimento, inibição da reprodução e depressão do sistema imune (Sapolsky et al., 2000). Dado

que o estresse crônico pode prejudicar funções fisiológicas altamente relevantes para a aptidão e até diminuir a viabilidade de uma população, medidas dos níveis plasmáticos dos corticosteróides têm sido usadas como um indicador do estresse ambiental gerado a ponto de comprometer o valor adaptativo (Romero e Wikelski, 2001). Níveis altos destes hormônios no plasma têm sido encontrados em populações de animais presentes nas áreas periféricas da distribuição geográfica (Dunlap e Wingfield, 1995), em populações sujeitas a eventos climáticos cíclicos (Romero e Wikelski, 2001), bem como em populações de ambientes impactados (Wasser et al., 1997; Norris et al.,1997; Hopkins et al., 1997).

Sabe-se que o tratamento com corticosteróides inibe a síntese, liberação e/ou eficiência de várias citocinas e outros mediadores que promovem a resposta imune e as reações inflamatórias (Wiegers e Reul, 1998; Sapolsky et al., 2000). Mecanismos moleculares através dos quais os corticosteróides inibem as citocinas têm sido identificados em nível de transcrição, tradução, secreção e estabilidade do RNA mensageiro, sendo as principais consequências destes eventos moleculares a inibição da ativação das células T auxiliares tipo 1 e 2 da subpopulação CD4, e a inibição das células do sistema monócito/macrófago no sítio inflamatório (Weller et al., 1999). Os corticosteróides também causam atrofia dos tecidos linfóides, particularmente do timo, disparando a apoptose dos precursores imaturos das células T e B e das células T maduras (Sapolsky et al., 2000).

Em contraste com os efeitos imunossupressores bem conhecidos dos corticosteróides, alguns estudos vêm demonstrando que os hormônios corticosteróides têm um efeito imuno-modulador (Wilckens e DeRijk, 1997; Dhabhar, 2007; Dhabhar, 2009). De uma forma geral, em concentrações farmacológicas os corticosteróides têm efeitos imunossupressores, enquanto que em concentrações fisiológicas têm efeitos imuno-moduladores (Dhabhar 2009). Dhabhar e McEwen (1999) mostraram que os hormônios adrenais do estresse mediam um melhoramento da imunidade mediada por células na pele de ratos que é induzido por estresse agudo. A

adrenalectomia elimina este efeito de melhoramento induzido por estresse da imunidade mediada por células na pele, enquanto a administração de baixas doses de corticosterona ou adrenalina, significativamente aumentam a imunidade mediada por células na pele e produzem um aumento significativo nos números de células T nos nódulos linfáticos que drenam o local da resposta imune mediada por células (Dhabhar e McEwen, 1999). Além disso, a administração simultânea de ambos os hormônios do estresse produz um aumento maior da resposta imune mediada por células na pele dos ratos. Estes resultados demonstram que os hormônios liberados durante uma resposta de estresse agudo podem ajudar a preparar o sistema imune para potenciais desafios (em geral, ferimentos ou infecções) (Dhabhar e McEwen, 1999).

Outro estudo examinou os efeitos da corticosterona na proliferação de células T *in vitro* e demonstrou um importante mecanismo pelo qual a corticosterona pode mediar o aumento da função imune (Wiergers et al., 1995). Durante os estágios iniciais de ativação das células T, níveis baixos de corticosterona potencialmente aumentam a proliferação de linfócitos. Mais do que isso, eles mostraram que a corticosterona precisa estar presente durante o processo de ativação do receptor para células T para aumentar a resposta proliferativa (Wiergers et al., 1995). Também é importante lembrar que a inter-relação entre os sistemas endócrino e imune não é unidirecional, uma vez que citocinas, particularmente interleucina 1 (IL-1) e IL-6, são estimuladores potentes da produção de corticosteróides pela influência que exercem no hormônio liberador de corticotropina (CRH) (Besedovsky et al., 1986; Sapolsky et al., 2000). Além disso, células hipofisárias e das glândulas adrenais podem sintetizar estas citocinas (Cooke, 1999).

Inter-relações entre os eixos hipotálamo-pituitária-adrenal e hipotálamo-pituitária-gônadas

Diversos estudos, realizados principalmente com mamíferos, têm demonstrado que a ativação do eixo hipotálamo-pituitária-adrenal (HPA) em resposta a diferentes fatores estressores pode inibir diferentes pontos do funcionamento do eixo hipotálamo-pituitária-gônadas (HPG), em ambos os sexos. O aumento dos níveis de β-endorfina e do hormônio liberador de corticotropina (CRH), por exemplo, inibe a secreção do hormônio liberador de gonadotropina (GnRH) e promove uma redução da sensibilidade hipofisária ao GnRH, enquanto o aumento dos níveis de corticosteróides e prolactina promove redução da secreção do hormônio folículo estimulante (FSH) e do hormônio luteinizante (LH) (Warren, 1983; Sapolsky, 1991). Nos machos, níveis altos de corticosteróides podem também diminuir a sensibilidade testicular ao LH (Sapolsky, 2002).

Embora esta visão clássica de inibição do eixo HPG pela ativação do eixo HPA seja apoiada por diversos estudos experimentais, estudos que acompanharam a variação natural dos níveis de corticosterona e testosterona ao longo do ano em diversos vertebrados, incluindo anfíbios anuros, vem demonstrando que a interação entre estes dois eixos pode ser bem mais complexa. Segundo estes estudos, os níveis de ambos os hormônios se encontram elevados e positivamente correlacionados durante a época reprodutiva em vários vertebrados (Moore e Jessop, 2003 para revisão). De acordo com estes estudos, níveis elevados de corticosterona durante a estação reprodutiva provavelmente facilitam o comportamento reprodutivo, através da mobilização de reservas energéticas (Moore e Jessop, 2003; Assis et al., 2012). Segundo Emerson (2001), os níveis de corticosterona e testosterona estariam positivamente correlacionados com esforço vocal em anuros até o momento em que os níveis de corticosterona tornem-se elevados o bastante para disparar uma resposta de estresse, reduzindo os níveis de testosterona e inibindo a manifestação do comportamento vocal. De acordo com este modelo,

mudanças nos níveis de corticosterona poderiam ser responsáveis por determinar interrupções cíclicas da atividade reprodutiva, em que os indivíduos deixariam o coro, passando para uma atividade de forrageamento (Emerson e Hess, 2001).

Adicionalmente, estudos com mamíferos indicam que as gônadas podem também afetar o eixo HPA em todos os níveis (Malendowicz, 1994; Dallman et al., 1992), sendo estes efeitos mediados principalmente pelos esteróides sexuais (Viau, 2002), embora outros fatores gonadais também possam estar envolvidos (Kitay et al., 1966). Além disso, uma vez que as diferenças sexuais na resposta esteroidogênica ao hormônio adrenocorticotrófico (ACTH) são evidentes nas células adrenocorticais antes da maturidade sexual (Carsia et al., 1987a,b; Kocsis e Carsia, 1989), um efeito independente das gônadas (Arnold e Burgoyne, 2004) também é plausível. Em lagartos *Scelophorus undulatus*, a sensibilidade das células adrenocorticais ao ACTH não foi afetada pela orquiectomia, mas foi reduzida pelo tratamento de reposição com testosterona (Carsia et al., 2008), e a sensibilidade das células adrenocorticais de lagartos ao ACTH parece variar de acordo com a espécie, sexo e condições ambientais (Carsia et al., 2008). Estudos com células adrenocorticais derivadas de galos orquiectomizados também sugerem que a manutenção de andrógenos suprime a resposta esteroidogênica da adrenal ao ACTH (Carsia et al., 1987a, b).

Todas estas descobertas demonstram a complexidade da relação entre os sistemas endócrino e imune, e que o esforço de investigação adicional é necessário nesta área.

Anfíbios anuros como modelo para estudos das inter-relações entre hormônios esteróides e imunocompetência

Um grande declínio de populações de anfíbios vem sendo documentado em áreas de clima temperado desde o início da década de 1970 e, mais recentemente, também nos trópicos (Carey et al., 1999), mas nem todas, as populações de anfíbios têm estado em declínio em todos os seis

continentes nos quais vivem. Além da destruição do hábitat, a ocorrência de doenças infecciosas em seus hábitats relativamente intocados tem se destacado como uma das principais causas de declínio de anfíbios (Carey et al., 1999). Parte da mortalidade em massa de anfíbios ocorre em áreas geograficamente espalhadas e a pode atingir de 50 a 100% da população, sendo mais pronunciada a maiores altitudes e regiões frias. Diversos agentes infecciosos têm sido associados primariamente e/ou secundariamente ao declínio populacional de anfíbios, incluindo um fungo quitrídio parasita (Chytridiomycota; Chytridiales) (Berger et al., 1998), bactérias patogênicas como *Aeromonas hydrophilla* (Taylor et al., 1999) e iridovírus (Cunningham et al., 1996). Frequentemente, a infecção por iridovírus ocorre em áreas impactadas pelo homem, e especialmente em populações com alta densidade (Cunningham et al., 1996). Há evidências também de que *Aeromonas hydrophilla* aumente sua taxa de crescimento muitas vezes após exposição a glicocorticóides, indicando que a virulência desta bactéria patogênica pode ser modulada pela resposta ao estresse dos anfíbios (Kinney et al., 1999).

É interessante observar que, na maioria dos casos de surtos de mortes causadas por doenças infecciosas, houve relato de outras espécies de anfíbios que ocupam o mesmo hábitat, mas que não foram afetadas pela doença (Bradford et al., 1994; Carey, 1993). Estas observações levaram à uma série de questões, tais como: Existe variação interespecífica na tolerância e/ou resistência aos patógenos? Se sim, quais são os aspectos da imunidade que diferem entre as espécies? Existe uma relação causal entre sensibilidade a impactos ambientais, intensidade de ativação do eixo hipotálamo-hipófise-interrenais em resposta a estressores e imunocompetência? Estariam as diferenças interespecíficas na resposta imune associadas a diferenças nos níveis circulantes de esteróides em condições basais e pós-estresse?

Em relação a estas questões, Gomes et al., (2012) compararam a resposta imune inata e os níveis de corticosterona basais e pós-estresse de contenção, de três espécies de sapos Bufonídeos (*Rhinella icterica*, *R. ornata* e *R. schneideri*), caracterizadas por diferentes graus

de dependência de ambientes florestados. Segundo este estudo, *R. ornata*, a espécie com maior grau de dependência de ambientes florestados, apresenta os maiores níveis basais e pós-estresse de contenção de corticosterona, enquanto *R. schneideri*, a espécie com distribuição geográfica mais associada a áreas naturalmente abertas ou desmatadas, apresenta a maior capacidade bactericida plasmática (CBP). Os autores também encontraram uma associação negativa interespecífica entre CBP e os níveis basais de corticosterona nestes sapos, sugerindo que a menor imunocompetência das espécies de ambientes florestados poderia estar associada ao efeito imunossupressor da corticosterona, reduzindo suas chances de ocupar áreas naturalmente abertas ou desmatadas. Também é interessante notar que os níveis basais de corticosterona em *R. ornata* são equivalentes aos encontrados em *R. schneideri* após estresse experimental de contenção (Gomes et al., 2012). Estes resultados preliminares motivaram a ampliação do estudo da relação entre níveis de esteróides e imunocompetência inata no gênero *Rhinella*, incorporando a metodologia de CBP por espectrofotometria, a elevação experimental dos níveis hormonais de corticosterona via administração transdérmica, e o desafio imunológico com PHA.

Referências Bibliográficas

Ahmed, S.A., Dauphinée, M.J. & Talal, N. 1985. Effects of short-term administration of sex hormones on normal and autoinmune mice. *J. Immunol.* 134, 204-210.

Alexander, J. & Stimson, W.H. 1988. Sex hormones and the course of parasistic infection. *Parasitol. Today.* 4, 189-193.

Arnold, A.P. & Burgoyne, P.S. 2004. Are XX and XY brain cells intrinsically different? *Trends Endocrinol. Metab.* 15, 6-11.

Assis, V.R., Navas, C.A., Mendonça, M.T. & Gomes, F.R. 2012. Vocal and territorial behavior in the Smith frog (*Hypsiboas faber*): Relationships with plasma levels of corticosterone and testosterone. *Comp. Biochem. Physiol.* 163, 265-271.

Berger, L., Speare, R., Daszak, P., Green, D.E., Cunningham, A.A., Goggin, C.L., Slocombe, R., Ragan, M.A., Hyatt, A.D., McDonald, K.R., Hines, H.B., Lips, K.R., Marantelli, G. & Parkes, H. 1998. Chytridiomycosis causes amphibian mortality associated with population declines in the rainforests of Australia and Central America. *Proc. Natl. Acad. Sci.* 95, 9031-9036.

Besedovsky, H., del Rey. A., Sorkin, E. & Dinarello, C.A. 1986. Immunoregulatory feedback between interleukin-1 and glucocorticoid hormones. *Science* 238, 522-524.

Bradford, D.F., Graber, D.M. & Tabatabai, F. 1994. Population declines of the native frog, *Rana muscosa*, in Sequoia and Kings Canyon National Parks, California. *Southwestern Nat.* 39, 323-327.

Bradley, A.J. 1987. Stress and mortality in the red-tailed phascogale, *Phascogale calura* (Marsupialia: Dasyuridae). *Gen. Comp. Endocrinol.* 67, 85-100.

Bradley, A.J., McDonald, L.R. & Lee, A.K. 1980. Stress and mortality in a small marsupial (*Antechinus stuartii*, Macleay). *Gen. Comp. Endocrinol.* 40, 188-200.

Carey, C. 1993. Hypothesis concerning the causes of the disappearance of boreal toads from the mountains of Colorado. *Cons. Biol.* 7, 355-362.

Carey, C., Cohen, N. & Rollins-Smith, L. 1999. Amphibian declines: an immunological perspective**.** *Develop. Comp. Immunol.* 23, 459-472.

Carsia, R.V., McIlroy, P.J., Cox, R.M., Barret, M. & John-Alder, H.B. 2008. Gonadal modulation of in vitro steroidogenic properties of dispersed adrenocortical cells from Sceloporus lizards**.** *Gen. Comp. Endocrinol.* 158, 202-210.

Carsia, R.V., Morin, M.E., Rosen, H.D. & Weber, H. 1987a. Ontogenic corticosteroidogenesis of the domestic fowl: response of isolated adrenocortical cells. *Proc. Soc. Exp. Biol. Med.* 184, 436-445.

Carsia, R.V., Reisch, N.M., Fennel, M.J. & Weber, H. 1987b. Adrenocortical function of the domestic fowl: effects of orchiectomy and androgen replacement. *Proc. Soc. Exp. Biol. Med.* 185, 223-232.

Casto, J.M., Nolan, V.Jr. & Ketterson, E.D. 2001. Steroid hormones and immune function: Experimental studies in wild and captive dark-eyed juncos (*Junco hyemalis*). *Am. Nat.* 157, 408-420.

Cohn, D.A. 1979. Sensitivity to androgen: a possible factor in sex differences in the immune response. *Clin. Exper. Immunol.* 38, 218-227.

Cooke, A. 1999. Regulação da resposta imune. In: *Imunologia.* Roitt, I., Brostoff, J. & Male, D. (eds), pp. 171-185.

Cunningham, A.A., Langton, T.E.S., Bennett, P.M., Lewin, J.F., Drury, S.E.N., Gough, R.E. & MacGregor, S.K. 1996. Pathological and microbiological findings from incidents of unusual mortality of the common frog (*Rana temporaria*). *Phil. Trans. R. Soc. Lond. B.* 351, 1539-1557.

Dallman, M.F., Akana, S.F., Scribner, K.A., Bradbury, M.J., Walker, C.D., Strack, A.M. & Cascio, C.S. 1992. Stress, feedback and facilitation in the hypothalamo-pituitary-adrenal axis. *J. Neuroendocrinol.* 4, 517-526.

Dhabhar FS & McEwen BS. 1999. Enhancing versus suppressive effects of stress hormones on skin immune function. *Proc. Natl. Acad. Sci.* 96, 1059–1064.

Dhabhar FS & McEwen BS. 2007. Bidirectional effects of stress on immune function: possible explanations for salubrious as well as harmful effects; in Ader R (ed): *Psychoneuroimmunology*, ed 4. San Diego, Elsevier, pp 723–760.

Dhabhar, F.S. 2009. Enhancing versus suppressive effects of stress on immune function: Implications for immunoprotection and immunopathology. *Neuroimmunomodulation.* 16, 300–317.

Dunlap, K.D. & Wingfield, J.C. 1995. External and internal influences on indices of physiological stress. I. Seasonal and population variation in adrenocortical secretion of free-living lizards, *Sceloporus occidentalis*. *J. Exp. Zool.* 271, 36-46.

Emerson, S.B. & Hess, D.L. 2001. Glucocorticoids, androgens, testis mass, and the energetics of vocalization in breeding male frogs. *Horm. Behav.* 39, 59-69.

Emerson, S.B. 2001. Male advertisement calls: behavioral variation and physiological processes. In: *Anuran Communication*, M.J. Ryan (ed.). Smithsonian Institution Press.

Folstad, I. & Karter, A.J. 1992. Parasites, bright males, and the immunocompetence handicap. *Am. Nat.* 139, 603-622.

Gala, R.R. & Westphal, U. 1965. Corticosteroid binding globulin in the rat: studies on the sex difference. *Endocrinology.* 77, 841-851.

Gomes, F.R., Oliveira, R.V., Assis, V.R., Titon Jr., B., Moretti, E.H., & Mendonça, M.T. 2012. Interspecific Variation in Innate Immune Defenses and Stress Response of Toads from Botucatu (São Paulo, Brazil). *South Amer. J. Herpet.* 7, 1-8.

Greenman, C. G., Martin, L. B. & Hau, M. 2005 Reproductive state, but not testosterone, reduces immune function in male house sparrows (*Passer domesticus*). *Physiol. Biochem. Zool.* 78, 60–68.

Grossman, C.J. 1984. Regulation of the immune system by sex steroids. *Endocr. Rev.* 5, 435-455.

Grossman, C.J. 1985. Interactions between the gonadal steroids and the immune system. *Science.* 227, 257-261.

Hamilton, W.D. & Zuk, M. 1982. Heritable true fitness and bright birds: a role for parasites? *Science.* 218, 384-387.

Hau, M. 2007. Regulation of male traits by testosterone: implications for the evolution of vertebrate life histories. *BioEssays.* 29, 133-144.

Hopkins, W.A., Mendonça, M.T. & Congdon, J.D. 1997. Increased circulating levels of testosterone and corticosterone in southern toads, *Bufo terrestris*, exposed to coal combustion wastes. *Comp. Biochem. Physiol.* 122C, 191-196.

Hou, J. & Zheng, W.F. 1988. Effect of sex hormones on NK and ADCC activity of mice. Int. J. Immunopharm. 10, 15-22.

Inman, R.D. 1978. Immunologic sex differences and the female predominance in systemic lupus erythematosus. *Arthritis Rheum.* 21, 849-852.

Ketterson, E.D. & Nolan, V.Jr. 1992. Hormones and life histories: an integrative approach. *Am. Nat.* 140, S33-S62.

Ketterson, E.D. & Nolan, V.Jr. 1999. Adaptation, exaptation, and constraint: A hormonal perspective. *Am. Nat.* 154, S4-S25.

Kinney, K.S., Austin, C.E., Morton, D.S. & Sonnenfield, G. 1999. Catecholamine enhancement of *Aeromonas hydrophila* growth. *Microb. Pathog.* 26, 85-91.

Kitay, J.I., Coyne, M.D., Nelson, R. & Newsom, W. 1966. Relation of the testis to adrenal enzyme activity and adrenal corticosterone production in the rat. *Endocrinol.* 78, 1061-1066.

Kley, H.K., Herrmann, J., Morgner, K.D. & Kruskemper, H.I. 1973. Effects of testosterone oenanthate on plasma concentrations of thyroxine, cortisol, testosterone and hormone binding proteins in patients with hypogonadism. *Horm. Metab. Res.* 5, 271-274.

Kocsis, J.F. & Carsia, R.V. 1989. Steroidogenic properties of isolated Turkey adrenocortical cells. *Dom. Anim. Endocrinol.* 6, 121-131.

Lee, K.A., Martin II, L.B. & Wikelski, M.C. 2005. Responding to inflammatory challenges is less costly for a successful avian invader, the house sparrow (*Passer domesticus*), than its less-invader congener. *Oecologia.* 145, 244-251.

Malendowicz, L.K., 1994. Cytophysiology of the mammalian adrenal cortex as related to sex, gonadectomy and gonadal hormones. Wydawnictwo Poznanskiego Towarzystwa Przyjaciół Nauk, Poznan, Poland.

Martin, L. B., Weil, Z. M., & Nelson, R. J. 2008. Seasonal changes in vertebrate immune activity: mediation by physiological trade-offs. *Philos. Trans. R. Soc. London Ser. B. 363*, 321-339.

McDonald, I.R., Lee, A.K., Bradley, A.J. & Than, K.A. 1981. Endocrine changes in dasyurid marsupiais with differing mortality patterns.*Gen. Comp. Endocrinol.* 11, 292-301.

McEwen, B.S., Biron, C.A., Brunson, K.W., Bulloch, K. & Chambers, W.H. 1997. The role of adrenocorticoids as modulators of immune function in health and disease: Neural, endocrine and immune interactions. *Brain Res. Rev.* 23, 79-133.

Merchant, M.E., Roche, C., Elsey, R.M. & Prudhomme, J. 2003. Antibacterial properties of serum from the American alligator (*Alligator mississippiensis*). *Comp. Biochem. Physiol.* 136, 505-513.

Mills, S.C., Grapputo, A., Jokinen, I., Koskela, E., Mappes, T. & Poikonen, T. 2010. Fitness trade-offs mediated by immunosuppression costs in a small mammal. *Evol.* 64, 166-179.

Mitchell, C.E. & Power, A.G. 2003. Release of invasive plants from fungal and viral pathogens. *Nat.* 421, 625-627.

Moore, I.T. & Jessop, T.S. 2003. Stress, reproduction, and adrenocortical modulation in amphibians and reptiles. *Horm. Behav.* 43, 39-47.

Muehlenbein, M.P. & Bribiescas, R.G. 2005. Testosterone-mediated immune functions and male life histories. *Am. J. Human. Biol.* 17, 527-558.

Nelson, R.J., Demas, G.E., Klein, S.K. & Kriegsfeld, L.J. 2002. *Seasonal patterns of stress, immune function and disease.* Cambridge: Cambridge Univ. Press.

Norris, D.O., Felt, S.B., Woodling, J.D. & Dores, R.M. 1997. Immunocytochemical and histological differences in the interregnal axis of feral brown trout, *Salmo trutta*, in metal-contaminated waters. *Gen. Comp. Endocrinol.* 108, 343-351.

Norton, J. & Wira, C.R. 1977. Dose-related effects of the sex hormones and cortisol on the growth of the bursa of fabricius in chick embryos. *J. Ster. Biochem.* 8, 985-987.

Owen-Ashley, N.T., Hasselquist, D. & Wingfield, J.C. 2004. Androgens and the immunocompetence handicap hypothesis: Unraveling direct and indirect pathways of immunosuppression in song sparrows. *Am. Nat.* 164, 490-505.

Piersma, T. 1997. Do global patterns of habitat use and migration co-evolve with relative investments in immunocompetence due to spatial variation in parasite pressure? *Oikos* 80, 623-631.

Roberts, M.L., Buchanan, K.L. & Evans, M.R. 2004. Testing the immunocompetence handicap hypothesis: a review of the evidence. *Anim. Behav.* 68, 227-239.

Romero, L.M. & Wikelski, M. 2001. Corticosterone level predict survival probalities of Galápagos marine iguanas during El Niño events. *PNAS* 98, 7366-7370.

Sapolsky, R.M. 1991. Testicular function, social rank and personality of wild babbons. *Psychoneuroendocrinology***.** 16, 281-293.

Sapolsky, R.M. 2002. Endocrinology of the stress-response. Pp. 409-450. *In*: *Behavioral Endocrinology,* 2º edition. Becker, J.B., Breedlove, S.M., Crews, D. e M.M. McCarthy (eds.). The MIT Press.

Sapolsky, R.M., Romero, L.M. & Muck, A.U. 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocrinol. Rev.* 21, 55-89.

Taylor, S.K., Williams, E.S., Thorne, E.T., Mills, K.W. & Withers, D.I. 1999. Causes of mortality of the Wyoming toad (*Bufo baxteri*). *J. Wildlife Diseases.* 35, 49-57.

Tieleman, B.I., Williams, J.B., Ricklefs, R.E. & Klasing, K.C. 2005. Constitutive innate immunity is a component of the pace-of-life syndrome in tropical birds. *Proc. R. Soc. B* 272, 1715-1720.

Torchin, M.E., Lafferty, K.D., Dobson, A.P., Mckenzie, V.J. & Kuris, A.M. 2003. Introduced species and their missing parasites. *Nat.* 421, 628-630.

Verhulst, S., Dieleman, S.J. & Parmentier, H.K. 1999. A tradeoff between immunocompetence and sexual ornamentation in domestic fowl. *Proc. Natl. Acad. Sci.* 96, 4478-4481.

Viau, V. 2002. Functional cross-talk between the hypothalamic-pituitary-gonadal and adrenal axes. *J. Neuroendocrinol***.** 14, 506-513.

Warren, M. 1983. Effects of undernutrition on reproductive function in the human. *Endocrinol. Rev.* 4, 363-377.

Wasser, S.K., Bevis, K., King,G. & Hanson, E. 1997. Nonivasive physiological masures of disturbance in the Northern Spotted Owl. *Cons. Biol.* 11, 1019-1022.

Weinstein, Y., Ran, S. & Segal, S. 1984. Sex-associated differences in the regulation of immune responses controlled by the MHC of the mouse. *J. Immunol.* 132, 656-661.

Weller, I., Lockwood, C.M. & Chandra, R.K. 1999. Imunodeficências secundárias. In: *Imunologia.* Roitt, I., Brostoff, J. & Male, D. (eds), pp. 285-292.

Wiegers, G.J., Labeur, M.S., Stec, I.E., Klinkert, W.E., Holsboe, R.F. & Reul, J.M. 1995. Glucocorticoids accelerate anti-T-cell receptor-induced T-cell growth. *J. Immunol.* 155, 1893– 1902

Wiegers, G.J. & Reul, J.M.H.M. 1998. Induction of cytokine receptors by glococorticoids; functional and pathological significance. *Trends Pharmacol. Sci.* 19, 317-321.

Wilckens, T. & DeRijk, R. 1997. Glucocorticoids and immune function: unknown dimensions and new frontiers. *Immunol. Today.* 18: 418–424.

CHAPTER 1 – ANTIMICROBIAL CAPACITY OF PLASMA FROM ANURANS OF THE ATLANTIC FOREST

Vania Regina de Assis[1,*], Stefanny Christie Monteiro Titon[1], Adriana Maria Giorgi Barsotti[1], Beny Spira[2], Fernando Ribeiro Gomes[1]

[1] Departamento de Fisiologia, Instituto de Biociências, Universidade de São Paulo, Rua do Matão, trav. 14, 321, 05508-900, São Paulo, SP/Brazil

[2] Departamento de Microbiologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, Av. Prof. Lineu Prestes, 1374, Ed. Biomédicas II, 05508-900, São Paulo, SP/Brazil

* Corresponding author. Tel.: +55 11 3091 7522; fax: +55 11 3091 8095

E-mail address: v.regina.a@gmail.com

This chapter is a published paper: Assis, V.R., Titon, S.C.M., Barsotti, A.M.G., Spira, B. & Gomes, F.R. 2013. Antimicrobial Capacity of Plasma from Anurans of the Atlantic Forest. ***South American Journal of Herpetology.*** *8*(3), 155–160. doi:10.2994/SAJH-D-13-00007.1

1.1. Abstract

The viability and interpretation of techniques for the evaluation of immunocompetence of animals in their natural environment has been largely debated. One of these methods is based on testing the antimicrobial capacity of the blood and/or plasma, *in vitro,* which could rapidly and effectively assess the immunological conditions of natural populations. We tested the applicability of the antimicrobial capacity of plasma (ACP) assay in anuran amphibians from the Atlantic forest. The assay was performed by measuring both the turbidity (in a spectrophotometer) and the colony forming units (CFU) of the remaining bacteria (*Escherichia coli*) following exposure to the amphibian plasma. Although both assays were correlated, the ACP assay by spectrophotometry showed 10 times lower intra-assay variation. We also found interspecific variation in the ACP, as well as the maintenance of ACP values in males from the same population, collected in different breeding seasons. Thus, the estimation of ACP by spectrophotometry provides a convenient and accurate method to evaluate the innate immunocompetence in comparative and ecophysiological studies of anuran amphibians.

Keywords: Amphibians, Anura, Ecological immunology, Frogs, Immunity, Immunocompetence, Toads

1.2. Introduction

The immunocompetence of an individual can be defined as the ability to respond appropriately following exposure to a pathogen (Demas et al., 2011). The activation of the immune system results in fitness costs, including reducing current and future reproductive potential, and reduced rates of growth and survival (Norris and Evans, 2000; Lee and Klasing, 2004; Verhulst et al., 2005, Hasselquist and Nilsson, 2012). Additionally, the vertebrate immune system consists of many components that interact, each one with a specific value of protection and metabolic costs inherent of production and utilization (Millet et al., 2007). The emerging field of ecological immunology seeks to understand the diversity in the composition and action of the immune system of animals and to correlate this diversity with aspects of the ecology and life history under an evolutionary approach (Norris and Evans, 2000; Ricklefs and Wikelski, 2002; Verhulst et al., 2005).

One of the challenges of this emerging field is the determination of biologically relevant and viable techniques for assessing immunocompetence of animals in their natural environment. One proposal that has been considered suitable for rapid and effective assessment of the immune conditions of natural populations is the evaluation of the antimicrobial activity of the blood/plasma *in vitro*. Usually this test involves mixing the blood/plasma with a standard number of microorganisms, followed by incubation of the mixture to allow the blood/plasma to kill the microorganisms, and quantification of the remaining microorganisms. The antimicrobial activity represents a combination of several mechanisms of the innate immune system, such as the antimicrobial activity of humoral proteins (when the test is conducted only with plasma) and phagocytic activity of leukocytes (when the test is performed with blood), representing a general and integrative estimate of the innate immunocompetence (Matson et al., 2006; Millet et al., 2007). An additional advantage of this assay is the utilization of non-species-

specific reagents, which makes it a useful tool to assess the innate immunity of a wide range of species (Millet et al., 2007).

Studies conducted with different vertebrates, but mainly in birds, have demonstrated that the antimicrobial capacity of the blood/plasma varies among individuals within a population (Tieleman et al., 2005) as well as between species (Merchant et al., 2006; Millet et al., 2007), being reproducible (Tieleman et al., 2010). Additionally, the variability in antimicrobial capacity is associated with different aspects of physiology and life history, such that it could be a useful tool to understand the evolutionary divergence of the immune system and its trade-offs with other biological traits (Millet et al., 2007; Lee et al., 2008). Studies with birds have also shown that the antimicrobial capacity of blood is reduced in response to a stress protocol by restriction (birds were kept in small fabric bags for 60 min – Matson et al., 2006), and has a negative correlation with corticosterone plasma levels (Millet et al., 2007).

Amongst the few and recent studies with anuran amphibians, Graham and collaborators (2012) verified an increase in corticosterone plasma levels and a decrease in antimicrobial capacity of plasma (ACP) from *Rhinella marina* after application of a stress protocol by restriction [toads were kept in labeled cloth bags, moistened, placed into buckets (approximately 8 bags per bucket) for the night – Graham et al., 2012]. Gomes and collaborators (2012) found a negative relationship between ACP and corticosterone plasma levels in three species of *Rhinella* with different degrees of dependence on forested habitats. According to this study, *R. ornata*, the species with a higher degree of dependence on forested habitats, showed the highest baseline and post-stress corticosterone plasma levels (after 18h in captivity, toads were individually kept in small wet fabric bags for 60 min – Gomes et al., 2012), while *R. schneideri*, the species with geographical distribution more associated to naturally open or deforested areas, presented the highest ACP. These results point to a possible compromise between the function of the hypothalamic-pituitary-interrenal axis and innate

immunocompetence, which could be an important determinant in the ability to occupy areas modified by humans (Gomes et al., 2012).

The majority of ecoimmunological studies which measured the antimicrobial activity of blood or plasma analyzed the interaction of blood/plasma with microorganisms (usually bacteria), followed by plating on agar-filled petri dishes and subsequent counting of colony forming units (CFU). An alternative method consists in reading the optical density of blood/plasma samples inoculated with microorganisms (Merchant et al., 2003; Liebl and Martin, 2009). This methodology presents a number of advantages, including reduction of the volume and the processing time of the samples, elimination of the need of manual counting and, mainly, a drastic reduction of intra-assay variation (Liebl and Martin, 2009). Considering the advantages of using spectrophotometry to determine the ACP, we tested the applicability of this methodology for assessing the ACP of anuran amphibians. Tests were performed aiming to: 1) evaluate if the methodology used for birds (Liebl and Martin, 2009) could be employed to amphibians adjusting, if necessary, the quantities of plasma and bacteria used; and 2) make a comparative analysis of the ACP test by spectrophotometry and by CFU counting, as in Gomes et al. (2012). Additionally, we tested the existence of interspecific variation in ACP of male anurans during calling activity, using the spectrophotometric analysis.

1.3. Materials and methods

1.3.1. Animals and study site

The plasma of males from six different species were used*:* *Hypsiboas albopunctatus* (*N*=13), *H. faber* (*N*=24), *Rhinella icterica* (*N*=46), *R. jimi* (*N*=4), *R. ornata* (*N*=18), and *Scinax fuscovarius* (N=5). Three *R. icterica* and all *R. jimi* individuals were kept in an animal facility and were used in other studies as well. The plasma from these animals was used only for the standardization tests, and were not included in the analysis of interspecific variation. *S.*

fuscovarius and *R. ornata* specimens were collected in dependencies of the Biosciences Institute at the University of São Paulo (23º 33' 45" S, 46º 43' 40" W); *R. icterica* and *H. albopunctatus* were collected around the city of São Luiz do Paraitinga (23º 13' 2" S, 45º 18' 38" W), and *H. faber* were collected in the Botanical Garden of São Paulo (23º 38' 08" S, 46º 38' 00" W). All these localities are in the state of São Paulo, Brazil. Eleven individuals of *R. ornata* were collected in 2011 and 7 individuals in 2012. The specimens were collected with the authorization of IBAMA (process 17895-1) and from the Ethics Committee on the use of Vertebrate Animals in Experimentation (protocol 053/2008).

1.3.2. Blood collection

Animals were located by visual inspection, captured and bled (about 100 – 150 µL) via cardiac puncture with 1 mL syringes and needles 26Gx1/2" previously heparinized. Only blood samples collected within 3 min after the capture of the animals were considered for analysis. This restriction is due to the fact that after 3 min of capture, corticosterone plasma levels can be affected by the stress of capture and handling (Romero and Reed, 2005), and increased corticosterone may reduce ACP (Millet et al., 2007).

1.3.3. Processing of blood samples

All blood samples were labeled and kept on ice until being transferred to microcentrifuge tubes for plasma separation (4 min at 3000 rpm) in the same night. The separated plasma was pipetted off into cryotubes and kept in liquid nitrogen and/or stored at -80ºC for later analysis.

1.3.4. Growth of bacteria

Pellets of non-pathogenic *Escherichia coli* (MicrobioLogics, #24311 – ATCC 8739) were resuspended in 1 mL sterile PBS. Then, 100 µL of this solution were transferred to 5 mL TSB

(Tryptic Soy Broth), and incubated overnight at 37ºC. On the next day, bacteria concentration was measured in a spectrophotometer (Spectra Max 250). Serial dilutions in sterile amphibian Ringer solution were performed to obtain the working concentration of 10^6 bacteria mL^{-1}.

1.3.5. Antimicrobial capacity of plasma (ACP) assay by spectrophotometry

Plasma samples diluted (1: 20) in Ringer's solution (10 µL plasma: 190 µL Ringer) were mixed with 10 µL of *E. coli* working solution (~10^4 microorganisms) followed by incubation for 60 min at 37ºC. The positive control consisted of 10 µL of *E. coli* working solution in 200 µL of Ringer's solution (no plasma), and the negative control contained only 210 µL of Ringer's solution. All samples were incubated under the same conditions.

After the incubation period, 500 µL of TSB were added to each sample. The bacterial suspensions were thoroughly mixed and 300 µL of each one were transferred (in duplicates) to a 96 wells microplate. The microplate was incubated at 37 ºC for 2 hours, and from then on the optical density of the samples was hourly measured in a plate spectrophotometer (wavelength 600 nm), totaling 4 readings. The ACP was 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. The antimicrobial capacity was evaluated at the beginning of the bacterial exponential growth phase.

In addition, the ACP measured by spectrophotometry was tested in 16 individuals of *R. icterica*, by using 5 µL, 8 µL and 10 µL of plasma diluted in the Ringer's solution (1: 20) to 10 µL of bacteria (~10^4 microorganisms) to evaluate differences in results according to the amount of plasma. And 10 individuals of *R. icterica* were also used to compare ACP after 30 min, 60 min and 90 min for detecting differences in results according to the interaction time between 10 µL diluted plasma and 10 µL (~10^4 microorganisms) suspension of *E. coli.*

1.3.6. Antimicrobial capacity of plasma (ACP) assay by CFU counting

The procedure for ACP assay by CFU counting was essentially the same as described in item *2.6*, except that after 60 min of incubation of *E. coli* and plasma, 100 µL of each sample were plated in duplicate on Petri dishes containing TSB medium. Plates were incubated at 37ºC for 14h and the CFU were counted. The ACP was calculated according to the formula: *1 - (number of CFUs of the sample plate / number of CFUs in the positive control plate).*

1.3.7. Statistical analyses

The data were initially submitted to descriptive analyses and the Shapiro-Wilk test of normality. Given the absence of data normality, the equivalence of ACP determined by spectrophotometry and CFU counting was tested by non-parametric correlation (*Spearman*). The intra-assay variability of ACP determined by the two methods was assessed by calculating the coefficient of variation between duplicates. The effect of different plasma dilutions and different incubation times was analyzed by the Friedman test. The existence of interspecific variation in ACP was performed by the Kruskal-Wallis test, excluding *H. albopunctatus*, since individuals of this species showed no ACP. Comparison of the ACP from individuals of *R. ornata* collected in different years was performed using the Mann-Whitney test. All analyzes are performed using SPSS version 17.

1.4. Results

The ACP of 10 µl plasma from *R. icterica* diluted in Ringer's solution (1: 20) in the presence of ~10^4 bacteria was significantly higher than when 8 µL or 5 µL plasma were used ($S = 10.20$, $P = 0.006$; Figure 1.1A). The length of incubation also affected the ACP ($S = 15.20$, $P \leq 0.001$), being lower at 30 min than at 60 or 90 min (Figure 1.1B).

The ACP values determined by spectrophotometry and by CFU counting were equivalent (*rho* = 0.406, *P* = 0.012; Figure 1.2), and the coefficient of variation of the former was 10 times lower ($CV_{Turbidity}$ = 0.03; CV_{CFU} = 0.30).

Male frogs during peak calling activity showed interspecific variation of ACP determined via spectrophotometry [H (3) = 19.20, $P \leq 0.001$; Figure 1.3]. Moreover, male *R. ornata* collected during calling activity in the years 2011 and 2012 did not differ in ACP level (U = 36.50, *P* = 0.853; Figure 1.4).

1.5. Discussion

The tests performed in this study validate the method of determination of ACP for anuran amphibians by spectrophotometry, modified from Liebl and Martin (2009). Additionally, we confirmed the equivalence of determinations of ACP via spectrophotometry and CFU counting as in Gomes et al. (2012). However, as pointed out by Liebl and Martin (2009) in a study conducted with birds, the determination of ACP by spectrophotometry has a number of advantages, such as reduced sample volumes, reduced time and costs of sample processing, and a substantial lower variation between sample duplicates. Our results also demonstrate the existence of a large interspecific diversity in ACP from male anurans during calling activity, as well as similar values for calling male ACP from the same species collected in different years (*R. ornata*).

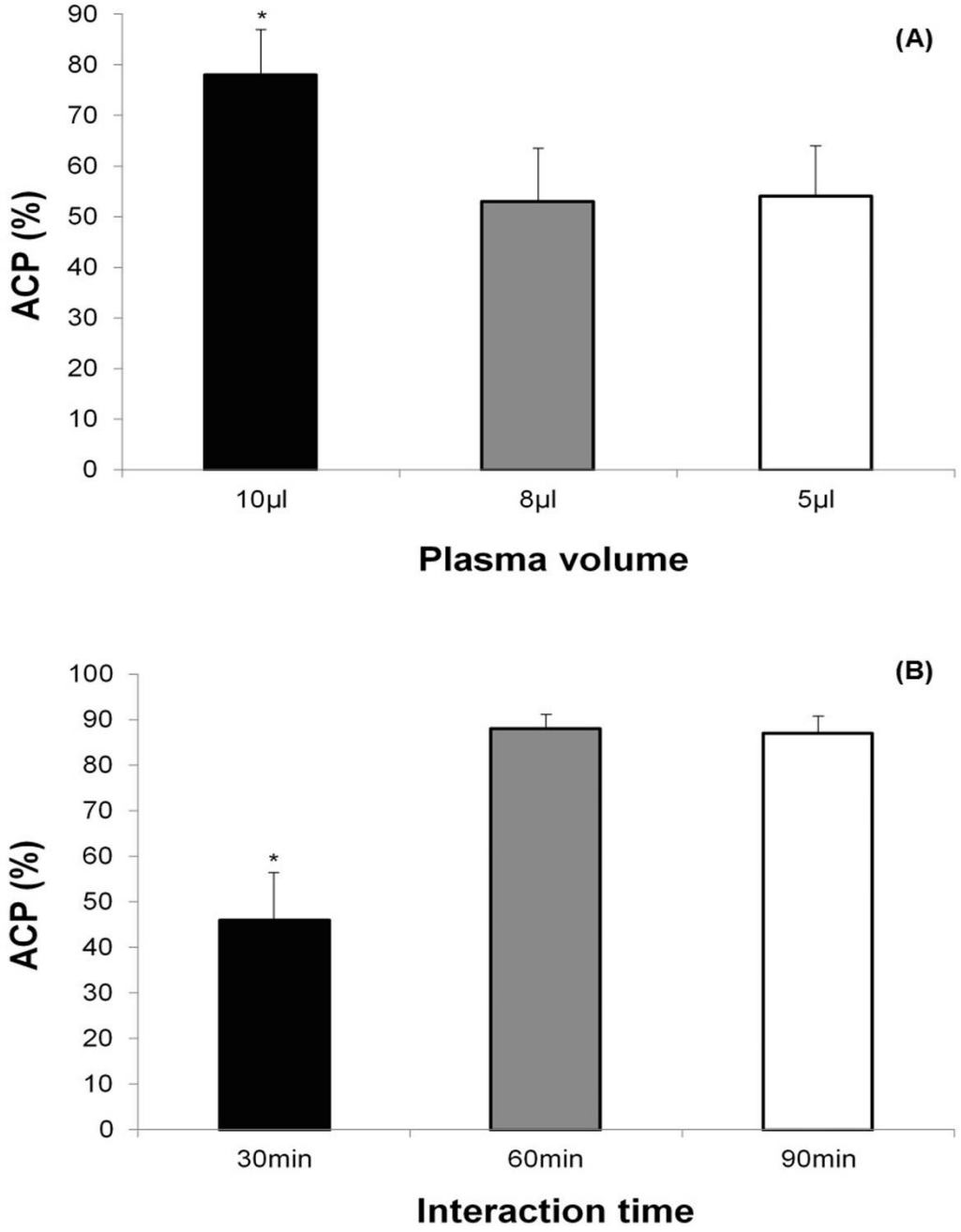

Figure 1. 1. Differences in antimicrobial capacity of plasma (ACP) for *R. icterica* according to: **(A)** Plasma volume used in the assay ($P = 0.006$). The number of individuals tested was the same in all cases (N=16). **(B)** Kinetics of ACP with a mixture of 10 µL of diluted plasma and 10 µL of an *E. coli* suspension (~10^4 microorganisms) ($P \leq 0.001$). The number of individuals tested was the same in all cases (N=10). The bars represent mean ± standard error.

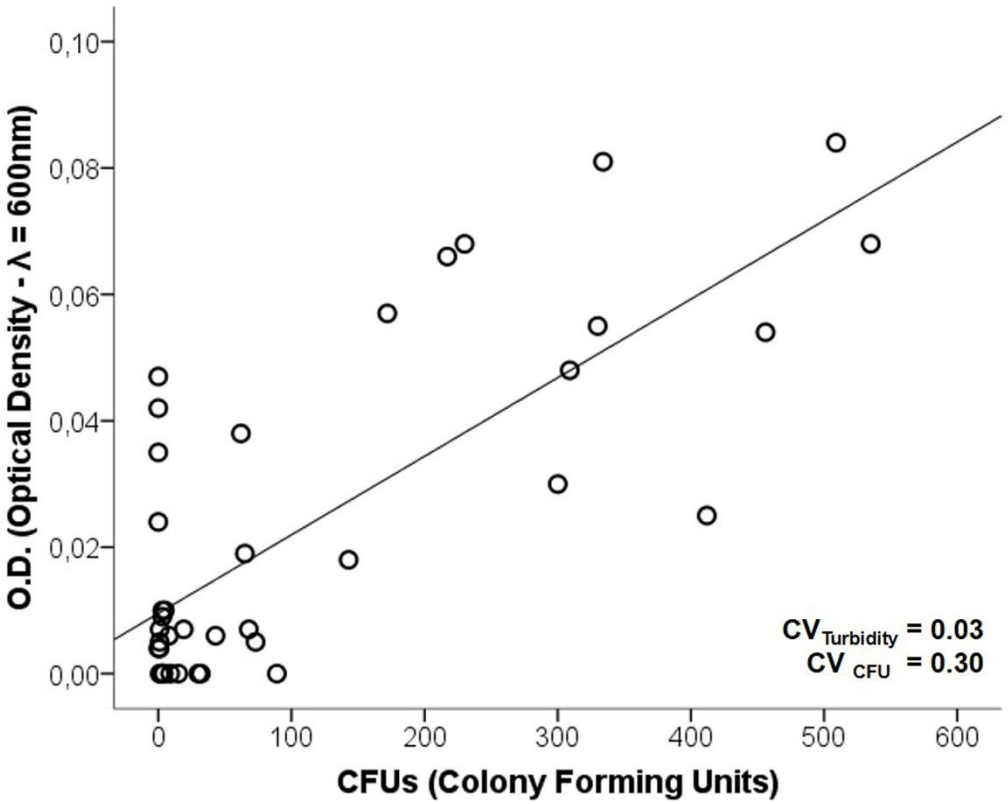

Figure 1. 2. Antimicrobial capacity of plasma (ACP) determined by spectrophotometry (y-axis) and by CFU counting (x-axis). The results of both methods are positively correlated (*rho* = 0.406, *P* = 0.012). However, the variability in the spectrophotometry method is ten times lower than by CFU counting.

The use of plate spectrophotometry for determining ACP enabled the sample volume reduction used from 7.5 µL, as in Gomes et al. (2012), to 5 µL of plasma by duplicate. Although this reduction in sample volume seems small, it allows performing measurements on a much wider range of anuran species, characterized by reduced body size. Even greater reductions in sample plasma volumes, such as the 1.5 µL per replica employed by Liebl and Martin (2009), might be possible by using a Nanodrop spectrophotometer, further expanding the diversity of frog species for which these tests can reliably be done.

Male anuran amphibians exhibit interspecific variation in ACP during their vocal activity. Among the species studied, *H. albopunctatus* was particularly interesting because it showed null ACP against *E. coli*. The absence of antimicrobial activity was maintained even when the

proportion between plasma volume and diluted *E. coli* (~10^4 microorganisms) was 10 times higher than that used in the present study (results not shown). It is possible that individuals from this species have a greater investment in the production and maintenance of other components of the immune system, such as blood cell components and antimicrobial peptides synthesized by granular glands in the dermis (Rollins-Smith, 2009). The second species tested with lower ACP also belongs to the genus *Hypsiboas*, suggesting the possibility of phylogenetic signal for this trait in anurans (Blomberg et al., 2003). However, inclusion of a greater number of species is required to test this hypothesis.

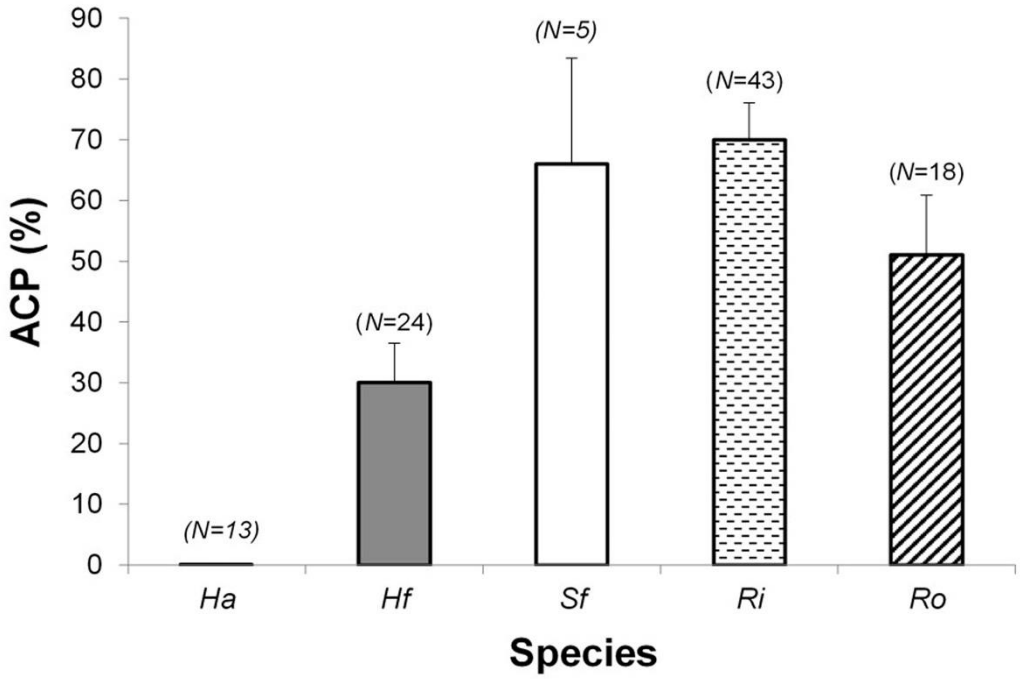

Figure 1. 3. Anuran interspecific variation of the antimicrobial capacity of plasma (ACP) ($P \leq 0.001$). The bars represent the mean ± standard error with *N* in parentheses (Abbreviations – *Ha: Hypsiboas albopunctatus; Hf: Hypsiboas faber; Sf: Scinax fuscovarius; Ri: Rhinella icterica; Ro: Rhinella ornata*).

In contrast, ACP remained constant when calling males from the same population of *R. ornata* were evaluated in two consecutive breeding seasons. It is also interesting to note that individuals of *R. ornata* collected for this study showed ACP values smaller than those of *R. icterica*, a result similar to that observed by Gomes et al. (2012) with other populations of these species, and measured by CFU counting. These results suggest the existence of interspecific differences in ACP of male anurans. Accordingly, studies have shown the existence of substantial variation in the antimicrobial capacity and other aspects of the immune response associated with different stages of life history, energy demand and nutritional status (Norris and Evans, 2000; Crommenacker et al., 2010, Moore et al., 2011, Beechler et al., 2012). Thus, it is important to emphasize that inter-population and interspecific comparisons should be restricted to samples collected in the same life-history conditions, for example, during the breeding season.

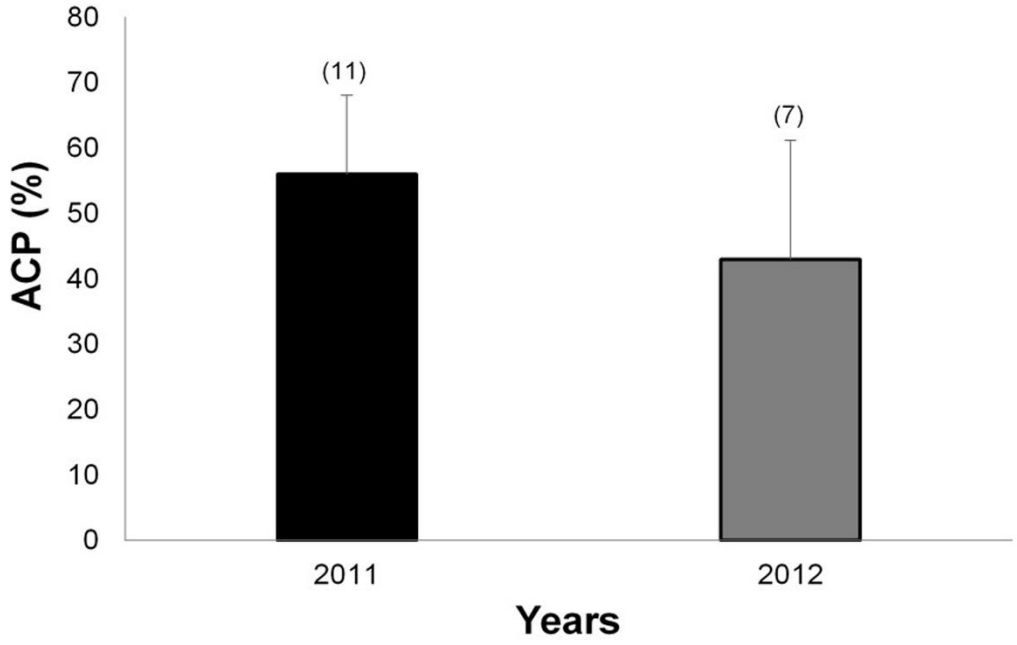

Figure 1. 4. Antimicrobial capacity of plasma (ACP) for males of *Rhinella ornata* collected during calling activity in different years, showing no difference between years (P = 0.853). The bars represent the mean ± standard error with N in parentheses.

We conclude that the measurement of ACP by spectrophotometry is a reliable and accurate method to estimate the immunocompetence of anuran amphibians that can be used in comparative and ecophysiological studies. Additional tests including other strains of microorganisms, the use of blood samples, as well as antimicrobial peptides synthesized by granular glands in the dermis can provide a more complete evaluation for the antimicrobial activity of these animals. The implications of ecology and life history on the ACP interspecific variation also remain to be investigated in anuran amphibians.

1.6. Acknowledgments

We would like to thank the people who assisted us during fieldwork, especially Braz Titon Junior. This research was funded by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) through grants JP-2006/54699-1 to F.R.G. and a Ph.D scholarship-2011/10560-8 to V.R.A.; Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) through a Ph.D scholarship-140272/2011-9 to V.R.A. and a master's scholarship to A.M.G.B.; and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) through a master's scholarship for S.C.M.T.

1.7. References

Beechler, B. R., H. Broughton, A. Bell, V. O. Ezenwa, and A. E. Jolles. 2012. Innate immunity in free-ranging African Buffalo (*Syncerus caffer*): Associations with parasite infection and white blood cell counts. *Physiological and Biochemical Zoology*. 85: 255-264.

Blomberg, S. P., T. Garland, and A. R. Ives. 2003. Testing for phylogenetic signal in comparative data: behavioral traits are more labile. *Evolution.* 57: 717-745.

Crommenacker, J., N. P. C. Horrocks, M. A. Versteegh, J. Komdeur, B. I. Tieleman, and K. D. Matson. 2010. Effects of immune supplementation and immune challenge on oxidative status and physiology in a model bird: implications for ecologists. *Journal of Experimental Biology.* 213: 3527-3535.

Demas, G. E., D. A. Zysling, B. R. Beechler, M. P. Muehlenbein, and S. S. French. 2011. Beyond phytohaemagglutinin: assessing vertebrate immune function across ecological contexts. *The Journal of animal ecology*. *80:* 710-30.

Gomes, F. R., R. V. Oliveira, V. R. Assis, B. Titon Jr., E. H. Moretti, and M. T. Mendonça. 2012. Interspecific Variation in Innate Immune Defenses and Stress Response of Toads from Botucatu (São Paulo, Brazil). *South American Journal of Herpetology.* 7: 1-8.

Graham, S. P., C. Kelehear, G. P. Brown, and R. Shine. 2012. Corticosterone-immune interactions during captive stress in invading Australian cane toads (*Rhinella marina*). *Hormones and Behavior.* 62: 146-153.

Hasselquist, D. and J. A. Nilsson. 2012. Physiological mechanisms mediating costs of immune responses: what can we learn from studies of birds? *Animal Behavior*. 83: 1303-1312.

Lee, K. A. and K. C. Klasing. 2004. A role for immunology in invasion biology. *Trends in Ecology & Evolution.* 19: 523-529.

Lee, K. A., M. Wikelski, W. D. Robinson, T. R. Robinson, and K. C. Klasing. 2008. Constitutive immune defenses correlate with life-history variables in tropical birds. *Journal of Animal Ecology.* 77: 356-363.

Liebl, A. L. and L. B. Martin. 2009. Simple quantification of blood and plasma antimicrobial capacity using spectrophotometry. *Functional Ecology.* 23: 1091-1096.

Matson, K.D., B. I. Tieleman, and K. C. Klassing. 2006. Capture stress and the bactericidal competence of blood and plasma in five species of tropical birds. *Physiology Biochemistry and Zoology.* 79: 556-564.

Merchant, M. E., C. Roche, R. M. Elsey, and J. Prudhomme. 2003. Antibacterial properties of serum from the American alligator (*Alligator mississippiensis*). *Comparative Biochemistry and Physiology B.* 136: 505-513.

Merchant, M. E., K. Mills, N. Leger, E. Jerkins, K. A. Vliet, and N. McDaniel. 2006. Comparisons of innate immune activity of all known living crocodylian species. *Comparative Biochemistry and Physiology B.* 143: 133-137.

Millet, S., J. Bennett, K. A. Lee, M. Hau, and K. C. Klassing. 2007. Quantifying and comparing constitutive immunity across avian species. *Developmental & Comparative Immunology.* 31: 188-201.

Moore, M. S., J. D. Reichard, T. D. Murtha, B. Zahedi, R. M. Fallier, and T. H. Kunz. 2011. Specific alterations in complement protein activity of Little Brown Myotis (*Myotis lucifugus*) hibernating in white-nose syndrome affected sites. *PLoS ONE.* 6: e27430.

Norris, K. and M. R. Evans. 2000. Ecological immunology: life history trade-offs and immune defense in birds. *Behavioral Ecology.* 11: 19-26.

Ricklefs, R. E. and M. Wikelski. 2002. The physiology/life-history nexus. *Trends in Ecology & Evolution.* 17: 462-8.

Rollins-Smith, L. A. 2009. The role of amphibian antimicrobial peptides in protection of amphibians from pathogens linked to global amphibian declines. *Biochimica et Biophysica Acta.* 1788: 1593-1599.

Romero, L. M. and J. M. Reed. 2005. Collecting baseline corticosterone samples in the field: is under 3 min good enough? *Comparative and Biochemistry Physiology A.* 140: 73-79.

Tieleman, B.I., J.B. Williams, R.E. Ricklefs, and K.C. Klasing. 2005. Constitutive innate immunity is a component of the pace-of-life syndrome in tropical birds *Proceedings of The Royal Society B.* 272: 1715-1720.

Tieleman, B. I., E. Croese, B. Helm, and M. A. Versteegh. 2010. Repeatability and individual correlates of microbicidal capacity of bird blood. *Comparative and Biochemistry Physiology A.* 156: 537-540.

Verhulst, S., B. Riedstra, and P. Wiersma. 2005. Brood size and immunity costs in zebra finches *Taeniopygia guttata*. *Journal of Avian Biology.* 36: 22-30.

CHAPTER 2 – RESTRAINT CHALLENGE, PLASMA CORTICOSTERONE LEVELS, AND IMMUNE PARAMETERS IN NATIVE BRAZILIAN TOADS AND INVASIVE CANE TOADS FROM FLORIDA/USA

Vania Regina de Assis[1*], Stefanny Christie Monteiro Titon[1], Christopher S. Michaelson[2], Mary T. Mendonça[2], Fernando Ribeiro Gomes[1]

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

[2]Department of Biological Sciences, 331, Funchess Hall, Auburn University, 36849, Auburn, AL, USA

* Corresponding author. Tel.: +55 11 3091 7522; fax: +55 11 3091 8095

E-mail address: v.regina.a@gmail.com

2.1. Abstract

Previous studies in a variety of vertebrates indicate that stressed animals exhibit an acute increase in circulating plasma glucocorticoid levels and a consequent modulation of immune response. Little is known about physiological reactions to stressors in amphibians and the consequences of elevation of glucocorticoids on their immune response. In order to further explore the relationship between glucocorticoids and immune response, we subjected newly captured toads to a restraint challenge without or with movement restriction (maintenance in a bin vs. maintenance in a moistened bag) for 24h. We compared baseline (under field conditions) and stressed (restrained conditions) variables associated to stress (corticosterone plasma levels [CORT] and the neutrophil/lymphocyte ratio [N:L]), potential reproduction (testosterone plasma levels [T]) and immune response (bacterial killing ability [BKA]) on three species of Brazilian toads (*Rhinella icterica, R. ornata* and *R. schneideri*) and two populations of the invasive cane toad (*R. marina*) from Florida/USA. General responses to restraint challenge included increased CORT and N:L ratio, and decreased T levels. The responses of BKA to restraint were much more variable, with *R. icterica* showing decreased and *R. marina* showing increased values. Additionally, CORT and N:L tended to increase more in response to restraint with movement restriction than to restraint without movement restriction, indicating that toads showed an increased stress response to the more intense stressor. All variables studied show interspecific variation. *Rhinella ornata* showed higher baseline CORT when compared to other species, while *Rhinella ornata* and *R. icterica* showed the highest baseline BKA values. However, changes in N:L ratio, T levels and BKA, were not correlated to CORT within or between species. Based on our results, we consider that a careful evaluation is necessary in order to understand the interrelationship between immune system and its modulation by stress at intra and interspecific levels. The inclusion of different segments of the immune response is

desirable, and a standardized data collection for all the species under the same period (e.g. inside or outside of breeding season) and same activity (e.g. calling or foraging) is mandatory.

Keywords: Stress, Corticosterone, Immune response, Restraint, Bacterial killing ability, Testosterone

2.2. Introduction

The amphibian populations have been in decline, which have been aggravated in the last few decades. The main causes of this decline are interactions between several factors, such as climate change, habitat modification, environmental pollutants, pathogens, and the invasive species that may become predators or competitors to native species (Carey et al., 1999; Hayes et al., 2010). It is known that habitat destruction is a great threat to amphibian diversity (Duellman, 1999). In the common toad (*Bufo bufo*), habitat fragmentation is negatively associated to the individual occurrence and body condition and positively associated to corticosterone levels (CORT), evidencing that habitat fragmentation represents a physiological challenge to these animals (Janin et al., 2011). However, the impact of habitat destruction differ greatly between species (Duellman, 1999). While most of the anuran species disappear when the forest is fragmented, a small part of anuran diversity can be even benefited by this environmental alteration. These few species benefited by human occupation are generally characterized by numerous populations and wide geographical distribution (Duellman, 1999).

Responses to environmental challenges may vary significantly, even among ecologically similar and closely related species (Hammond et al., 2015). Although, the reasons for this variation remain poorly understood, physiological processes probably play an integral role in the generation of such differences (Bernardo et al., 2007; Tingley et al., 2009; Tomanek, 2012; Hammond et al., 2015). Since glucocorticoids are integrally involved in homeostasis and allostasis and in moderating trade-offs between survival and reproduction (Angelier and Wingfield, 2013; Wingfield, 2013), they should provide valuable indicators of response to environmental change (Hammond et al., 2015). Gomes et al. (2012) found a negative relationship between bacterial killing ability (BKA) and CORT when three species of toads are compared. According to the study, *R. ornata*, the species with a higher degree of dependence on forested habitats, showed highest baseline and post-stress CORT, while *R. schneideri*, the

species with geographical distribution more associated to naturally open or deforested areas, presented highest BKA. These results point to a possible compromise between the glucocorticoid plasma levels and innate immunocompetence, which could be an important determinant to the interspecific variance in ability to occupy disturbed areas (Gomes et al., 2012).

It is known that glucocorticoid hormones are produced by adrenal or interrenal glands, and that they are released in response to several stressors in all vertebrate groups (Sapolsky et al., 2000). In the short-term stress, glucocorticoids may have beneficial effects in the immune system (Wingfield et al., 1997; Romero and Wingfield, 2001). However, in situations of long-term stress, sustained and elevated plasma levels of glucocorticoids may have negative consequences (Sapolsky, 1992; Wingfield and Romero, 2001). The immunosuppressive effects are well known and include inhibition of synthesis, release and/or efficiency of several cytokines and other mediators that promote the immune response and inflammatory reactions (Wiegers and Reul, 1998; Sapolsky et al., 2000). In contrast, some studies show that glucocorticoid hormones also have immunomodulatory effects (Wilckens and DeRijk, 1997; Dhabhar, 2007; Dhabhar, 2009). Glucocorticoids generally show immunosuppressive effects at pharmacological concentrations, whereas it may even increase the immune response at physiologic concentrations. Dhabhar and McEwen (1999) showed that adrenal stress hormones mediate acute stress-induced enhancement of skin cell-mediated immunity in rats. Additionally, Wiergers et al. (1995) showed that, during the early stages of T-cell activation, low levels of corticosterone potently enhance lymphocyte proliferation. Moreover, corticosterone needs to be present during the process of T-cell receptor activation to enhance the proliferative response (Wiergers et al., 1995).

Several studies have also proposed that male reproductive effort could compromise survival through immunosuppressive effects of androgens, reducing the resistance to parasitic

infection (Hamilton and Zuk, 1982; Folstad and Karter, 1992; Mills et al., 2010). Although evidences of testosterone-dependent immunosuppression in wild vertebrates have been provided for several species (Casto et al., 2001), a meta-analysis of studies on reptiles, birds and mammals concluded that the immunosuppressive effect of testosterone depends on the type of immune response and varies considerably within and between phylogenetic groups (Roberts et al., 2004). Additionally, house sparrows implanted with testosterone and held in short days showed no suppression of cell-mediated immune activity, indicating that testosterone is not obligatorily immunosuppressive year-round in this species (Greenman et al., 2005; Martin et al., 2008). Furthermore, studies in a diversity of vertebrate species have shown that stressors typically suppress testosterone levels (T), sometimes quite rapidly (Greenberg and Wingfield, 1987; Deviche et al., 2010, Deviche et al., 2012; Deviche et al., 2014). Reduction on T levels has been reported after induced acute stress in reptiles (reviewed in Tokarz and Summers, 2011), and after a restraint challenge and toe-clipping in toads (Narayan et al., 2011b; Narayan et al., 2012).

The comparison of stress and immune responses between native and invasive populations have been intensively investigated (Tompkins et al., 2003; Lee and Klasing, 2004; Lee, 2006; Martin et al., 2010). There are several reasons to predict differences in immune system between invasive and native populations. Invasive species tend to have lower diversity of parasites and display less prevalence of infections than native species (Mitchell and Power, 2003; Torchin et al., 2003). When invasive species are expanding into novel areas, they may be exposed to altered pathogen pressures, which may invoke altered immune investment (Lee and Klasing 2004; Horrocks et al., 2011; Llewellyn et al., 2012; Brown et al., 2014). Alternatively, there is a potential energetic trade-off between immunocompetence and dispersal rates. Selection of rapidly dispersing phenotypes might restrict energetic investment on the immune responses (Philips et al., 2008; Norris and Evans, 2000). Moreover, such restricted investment on the

immune responses might be focused on one division of the immune system (innate or adaptive) over the other, increasing susceptibility to some pathogens while improving defense against others (Norris and Evans, 2000).

We conducted a comparative study on immune and stress responses on natural and invasive populaitons of several species of toads (genus *Rhinella*) that differ in geographic distribution and susceptibility to deforestation. We included in this study three species of Brazilian toads (*Rhinella icterica, R. ornata* and *R. schneideri*) and two populations of the invasive cane toad species (*R. marina*) from Florida/USA. Physiological variables associated to stress (CORT and the neutrophil/lymphocyte ratio [N:L]), reproductive potential (testosterone plasma levels [T]) and immune response (BKA) were compared at field condtions (baseline) and after 24 hours of restraint challenge under two different conditions: with and without movement restriction. We predicted that: 1) Baseline CORT levels and N:L ratio should be higher in *R. ornata*, the species with distribution more restricted to forested environments, followed by *R. icterica, R. schneideri* and *R. marina;* 2) Baseline immune response should vary in the opposite direction, with the invasive populations of *R. marina*, the species with widespread distribution, showing the higher values followed by *R. schneideri, R. icterica* and *R. ornata;* 3) After 24h of restraint challenge, CORT levels and N:L ratio should be higher, while BKA and T levels should be lower for all the four species tested. Additionally, animals in restraint with movement restriction should have higher CORT levels and N:L ratio, and lower BKA and T levels when they are compared with restrained animals without movement restriction; and (4) the species showing higher baseline CORT should be those displaying lower response to the restraint challenge.

2.3. Materials and methods

2.3.1. Species studied

In this study, we collected data for four different species of *Rhinella*, which differ in their geographical distribution and susceptibility to deforestation (Figure 2.1):

1) *R. ornata*: a species belonging to the *Rhinella crucifer* group (Baldissera Jr. et al., 2004), with geographical distribution associated to Atlantic Rainforest in Brazil, restricted to forested habitats;

2) *R. icterica:* a species belonging to the *Rhinella marina* group (Maciel et al., 2010), which also has its distribution associated to Atlantic Rainforest in Brazil, but can be easily found in naturally open or impacted areas;

3) *R. schneideri:* also belonging to the *Rhinella marina* group (Maciel et al., 2010), with wide geographical distribution, being found in open areas in the Atlantic Rainforest and Cerrado in Brazil; and

4) *Rhinella marina*: a species also belonging to the *Rhinella marina* group (Maciel et al., 2010) with geographical distribution associated to Amazon rainforest in Brazil, where they are native, and a high invasive potential in places where they were introduced, including the Australian desert (Llewellyn et al., 2012; Brown et al., 2011a,b; Graham et al., 2012). At the United States, they were introduced at South Florida, and more recently, they might be found at even greater latitudes.

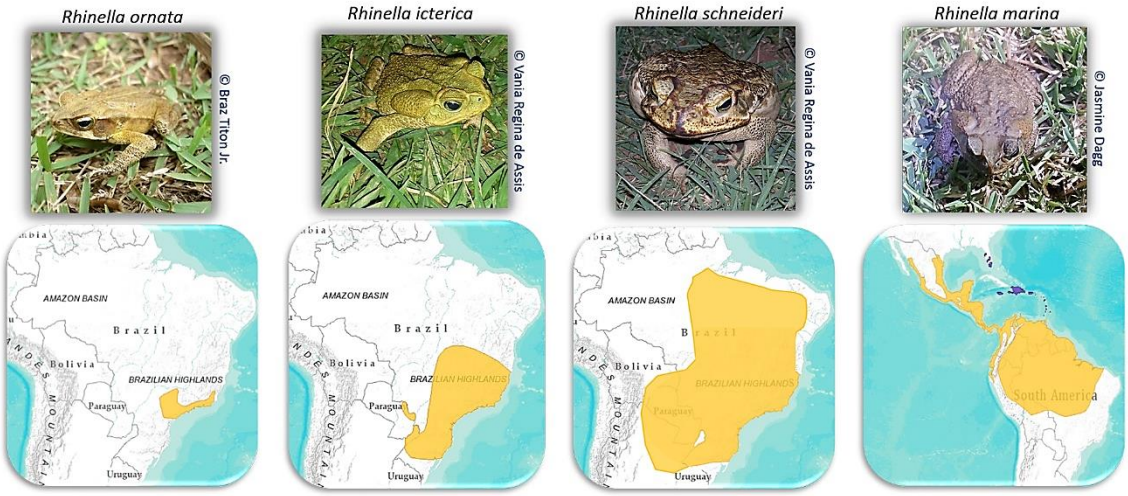

Figure 2. 1. Rhinella species studied and their geographical distribution. In places where the species are native, the distribution's color is yellow, and where the species were introduced, the distribution's color is purple. The graphs were generated and modified from: http://maps.iucnredlist.org

2.3.2. Study sites

Males of the species *R. icterica* were collected in January 2013 (*N* = 20), in the city of São Luiz do Paraitinga – SP/Brazil (23 ° 13'23 "S, 45 ° 18'38" WO) and males of the species *R. schneideri* were collected in February 2013 (*N* = 20) in the city of Luiz Antônio – SP/Brazil (21 ° 30'23 "S, 47 ° 50'38" WO). Males of the species *R. marina* were collected in July 2014 (*N* = 16), in the city of New Port Richey – FL/USA (28°14'56" N, 82°43'4" WO). Another *R. marina* males (*N* = 18) were collected in September in the city of Miami Springs – FL/USA (25°49'11" N, 80°17'28" WO). The animals of these three species were collected during the breeding season, but were not calling.

Males of the species *R. ornata* were collected in August 2014 (*N* = 19) in the city of Botucatu – SP/Brazil (22° 53 ' 09 "S, 48° 26 ' 42" WO). The animals of this species were collected during the reproductive season, and they were calling.

All the animals were used to compare the baseline values of hormone levels and immunocompetence with the values after 24h of acute stress in captivity (restraint challenge), with and without movement restriction.

The collections in Brazil were performed under authorization from Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA, process 17895-1) and laboratory procedures were performed under the approval of the Comissão de Ética no Uso de Animais (CEUA) do Instituto de Biociências da Universidade de São Paulo (CEUA - n° 142/2011). Fieldwork in the United States was done in collaboration with Dr. Mary T. Mendonça, and all the animals were collected under the permission approved on IACUC for Florida cane toad work. *PRN: 2013-233.*

2.3.3. Collecting and processing blood samples

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

All blood samples were identified and kept on ice until they were divided into two aliquots on the same night. One of these aliquots was used for blood smear (for further analysis of leukocyte profile), the other aliquot was centrifuged to isolate the plasma (4 min at 3000 rpm). Plasma samples (a range 100-150 µl) from all species were stored in cryogenic tubes, and kept in liquid nitrogen until they could be transferred to a -80°C freezer, for posterior corticosterone assays and analyses of bacterial killing ability. Additionally, for the species *R. icterica* and *R. schneideri*, we also conducted testosterone assays.

2.3.4. Analysis of blood parameters

a) Leukocyte profile

A drop of blood (about 2 µl) was used to perform each blood smear slide. Two slides were made for each animal and, subsequently, one of these slides was stained with Giemsa solution (10%) and observed under an optical microscope (100X objective, using oil immersion - Nikon E200, 104c). For differential leukocyte counts, 100 leukocytes were counted on each slide, and classified based on morphology as neutrophils, lymphocytes, eosinophils, basophils, and monocytes (Campbell, 2006). Based on the leukocyte profile, the ratio between neutrophils and lymphocytes (N:L) was calculated.

b) Bacterial killing ability (BKA)

This assay was conducted according to Assis et al. (2013). Briefly, plasma samples diluted (1: 20) in Ringer's solution (10 µl plasma: 190 µl Ringer) were mixed with 10 µl of *E. coli* working solution (~10^4 microorganisms). Positive controls consisted of 10 µl of *E. coli* working solution in 200 µl of Ringer's solution, and negative control contained 210 µl of Ringer's solution. All samples and controls were incubated for 30 min at 37ºC. After the incubation period, 500 µl of tryptic soy broth (TSB) were added to each sample. The bacterial suspensions were thoroughly mixed and 300 µl of each one were transferred (in duplicates) to a 96 wells microplate. The microplate was incubated at 37ºC for 2 hours, and thereafter the optical density of the samples was measured hourly in a plate spectrophotometer (wavelength 600 nm), totaling 4 readings. The BKA was 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. The bacterial killing ability was evaluated at the beginning of the bacterial exponential growth phase.

c) Hormonal assay

Plasma samples were extracted with ether according to Mendonça et al. (1996). Briefly, 3 ml of ether was added to 10 µl of each sample, and then vortexed for 30 seconds and centrifuged (4°C, 9 min, at 1800 rpm). Next, the samples were allowed to decant in -80ºC freezer for 7 min and the liquid phase was transferred to another tube. These tubes were kept in laminar flow hood at room temperature (20 ± 2°C), until all of the ether had evaporated (approximatelly 24h). The samples were resuspended in EIA buffer and CORT and T were assayed using EIA kits (CORT number 500655; T number 582701, Cayman Chemical), according to the manufacturer's instructions.

We estimated intra-assay variation for CORT to be 7.89% and for T to be 4.47% and inter-assay variation was estimated using the average of four intermediate values from the standard curve (as recommended by the kit instructions) and it was 10.88% for CORT and 11.16% for T. Sensitivity of the assays were 30.00pg/ml for CORT and 6.00pg/ml for T.

2.3.5. Restraint challenge with and without movement restriction

Immediately after blood collection in the field, individuals were randomly placed in two groups. In one of them, the toads were directly put into the individual plastic containers (restraint without movement restriction) for 24h. In the other group, toads were put within moistened cloth bags (in Brazil) or plastic bags with water (in USA), and then in the individual plastic containers (restraint with movement restriction), where they remained for 24h. The lids of the containers had holes to allow air circulation. The plastic containers in Brazil [4.3L – 29 (L) x 17.8 (W) x 14.6 (H) cm] were 4 times bigger than those that we used at USA [1L].

Animals were exposed to the natural light cycle and temperatures compatible with its natural thermal regime. At the end of 24h, the individuals were bled again to assess measures of immunocompetence, CORT and T. Upon termination of this experimental protocol, animals

were measured (mm), weighed (0.01g) and returned to their collection point at night in Brazil. For *R. marina*, since they are invasive in the USA, they were taken to the laboratory at Auburn University, where they were euthanized by an overdose with Orajel® (Benzocaine 20%).

2.3.6. Statistical analysis

The data were initially analyzed with descriptive statistics and Shapiro-Wilk normality test. Some variables showed absence of normality and were transformed to fit the prerequisites of parametric tests: 1) BKA – arccosine; 2) N:L – $\log_{10}(N+1)$; 3) CORT – $\log_{10}(N+1)$; and 4) T – $\log_{10}(N+1)$. The Z-score test was used to identify outliers, and based on the results of this test, some data were deleted: One data from *R. ornata* (N:L: $Z = 3.82$); 2 data from *R. icterica* (BKA: $Z = -3.58$; N:L: $Z = 3.00$) and 2 data from *R. schneideri* (BKA: $Z = -3.125$; N:L: $Z = 15.33$). Paired t-tests were used to compare the variables in the field (baseline) and after 24h of restraint with and without movement restriction in each species. To compare the restraint groups (with and without movement restriction), in each species, were used independent t-tests. For interspecific analyses, we used ANCOVAs where species was included as a factor and body mass as a covariate. When the ANCOVAs presented species as a significant factor, they were followed by tests for mean multiple comparisons with the Bonferroni adjustment. To test the correlations between the same variables at different conditions, or the correlations between different variables at same conditions, were used parametric correlations (Pearson). All analyzes were performed using IBM SPSS Statistics 22.

2.4. Results

2.4.1. By species

a) Rhinella ornata

When compared to the baseline values, the N:L ratio was two times (t = -3.117, df = 7, P = 0.017) and four times (t = -2.971, df = 8, P = 0.018) higher in animals after 24h of restraint challenge without and with movement restriction, respectively (Figure 2.2; Table 2.1). Additionally, after the restraint challenge with movement restriction, there was a positive correlation between N:L ratio and CORT (r = 0.748; P = 0.021), despite no increase in CORT. Also, there was a positive correlation between CORT and BKA after the restraint challenge with movement restriction (r = 0.862; P = 0.003).

The variables did not differ in toads restrained with and without movement restriction (t = -1.686, df = 16, $P \geq 0.111$; Table 2.1).

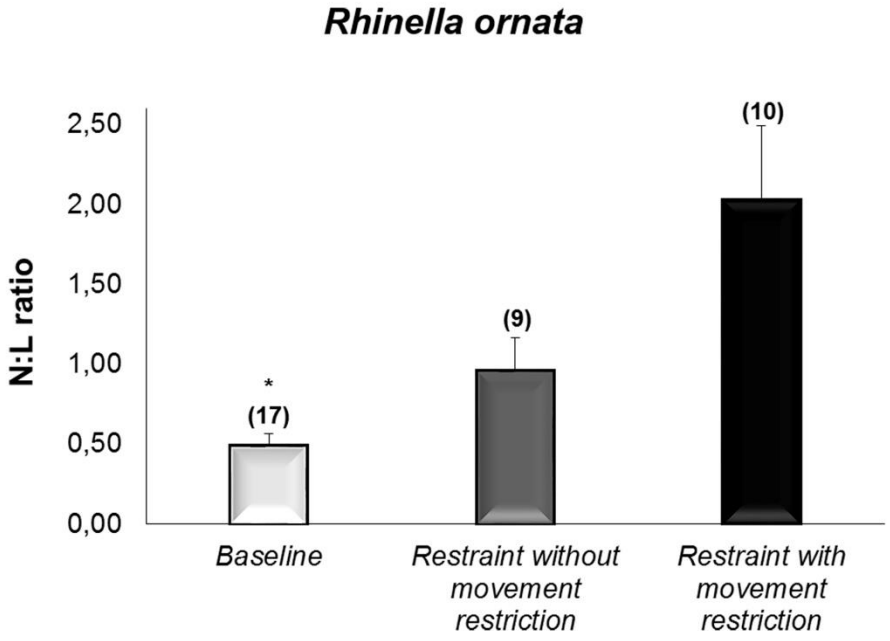

Figure 2. 2. Comparison on Neutrophil/ Lymphocyte ratio at baseline and after 24h of the restraint challenge in *R. ornata*. The bars represent the mean ± standard error with *N* in parentheses. $^{*}P \leq 0.05$.

Table 2. 1. Descriptive statistics of blood parameters, body mass and snout-vent-length for individuals of *R. ornata* under field conditions and after restraint challenge with and without movement restriction.

		Parameter	N	Minimum	Maximum	Mean ± SD
FIELD		BKA (%)	19	0.00	100.00	71.42 ± 39.29
		N:L	17	0.09	1.23	0.49 ± 0.31
		CORT (ng/ml)	18	4.58	285.28	115.87 ± 93.39
		Body Mass (g)	19	11.49	19.63	15.83 ± 2.40
		SVL (mm)	19	53.95	65.15	59.18 ± 3.42
AFTER 24H OF RESTRAINT CHALLANGE	**Without movement restriction**	BKA (%)	9	0.27	1.00	82.77 ± 28.32
		N:L	9	0.38	1.97	0.96 ± 0.62
		CORT (ng/ml)	9	23.00	131.20	66.74 ± 35.64
		Body Mass (g)	9	11.49	18.93	14.63 ± 2.35
		SVL (mm)	9	53.95	63.60	57.22 ± 3.18
	With movement restriction	BKA (%)	10	0.00	100.00	73.50 ± 39.88
		N:L	10	0.15	5.14	2,03 ± 1.46
		CORT (ng/ml)	10	2.95	155.46	92,85 ± 48.17
		Body Mass (g)	10	14.14	19.63	16,91 ± 1.98
		SVL (mm)	10	55.96	65.15	60,94 ± 2.68

BKA: Bacterial killing ability; **N:L**: Neutrophil/ Lymphocyte ratio; **CORT**: Corticosterone plasma levels; **SVL:** Snout-vent-length.

b) Rhinella icterica

After 24h of restraint challenge, CORT levels increased 3.5 times (t = -3.419, df = 9, P = 0.008) and 9 times (t = -4.987, df = 8, P = 0.001) relative to the baseline values, respectively without and with movement restriction (Table 2.2; Figure 2.3A). Individuals with movement restriction showed 2.5 times higher values of CORT (t = -2.096, df = 18, P = 0.050) than individuals without movement restriction (Figure 2.3A; Table 2.2)

When compared to the baseline values, the N:L ratio was 3.5 times higher (t = -3.194, df = 8, P = 0.013) in animals after 24h of restraint challenge with movement restriction (Figure 2.3B; Table 2.2). Individuals with movement restriction also showed 2 times higher values (t = -1.795, df = 17, P = 0.045 [1-tailed]) than individuals without movement restriction (Figure 2.3B; Table 2.2).

After 24h of restraint challenge with movement restriction, the BKA decreased 10% (t = 2.069, df = 9, P = 0.035 [1-tailed]) in relation to the baseline values (Figure 2.3C; Table 2.2). There was no correlation between BKA and CORT in any circumstance (r = -0.595; $P \geq 0.091$).

Table 2. 2. Descriptive statistics of blood parameters, body mass and snout-vent-length for individuals of *R. icterica* under field conditions and after restraint challenge with and without movement restriction.

		Parameter	N	Minimum	Maximum	Mean ± SD
FIELD		BKA (%)	19	81.00	100.00	96.63 ± 6.92
		N:L	19	0.08	0.53	0.19 ± 0.11
		CORT (ng/ml)	20	0.76	23.80	7.71 ± 6.12
		T (ng/ml)	19	0.97	42.32	8.85 ± 11.27
		Body Mass (g)	20	44.20	188.60	119.88 ± 38.66
		SVL (mm)	19	81.89	131.80	112.72 ± 11.93
AFTER 24H OF RESTRAINT CHALLENGE	**Without movement restriction**	BKA (%)	10	0.00	100.00	82.00 ± 32.08
		N:L	10	0.12	0.65	0.33 ± 0.18
		CORT (ng/ml)	10	4.59	90.66	27.35 ± 23.97
		T (ng/ml)	8	0077	2.30	1.39 ± 0.65
		Body Mass (g)	10	44.20	188.60	124.26 ± 42.95
		SVL (mm)	10	81.89	131.80	114.96 ± 14.67
	With movement restriction	BKA (%)	10	45.00	100.00	86.80 ± 18.62
		N:L	10	0.12	1.81	0.66 ± 0.53
		CORT (ng/ml)	10	7.39	165.22	66.40 ± 48.85
		T (ng/ml)	9	0.36	5.38	1.59 ± 1.52
		Body Mass (g)	10	67.20	188.35	115.50 ± 35.61
		SVL (mm)	9	98.67	126.78	110.23 ± 8.07

BKA: Bacterial killing ability; **N:L**: Neutrophil/ Lymphocyte ratio; **CORT**: Corticosterone plasma levels; **T:** Testosterone plasma levels; **SVL:** Snout-vent-length.

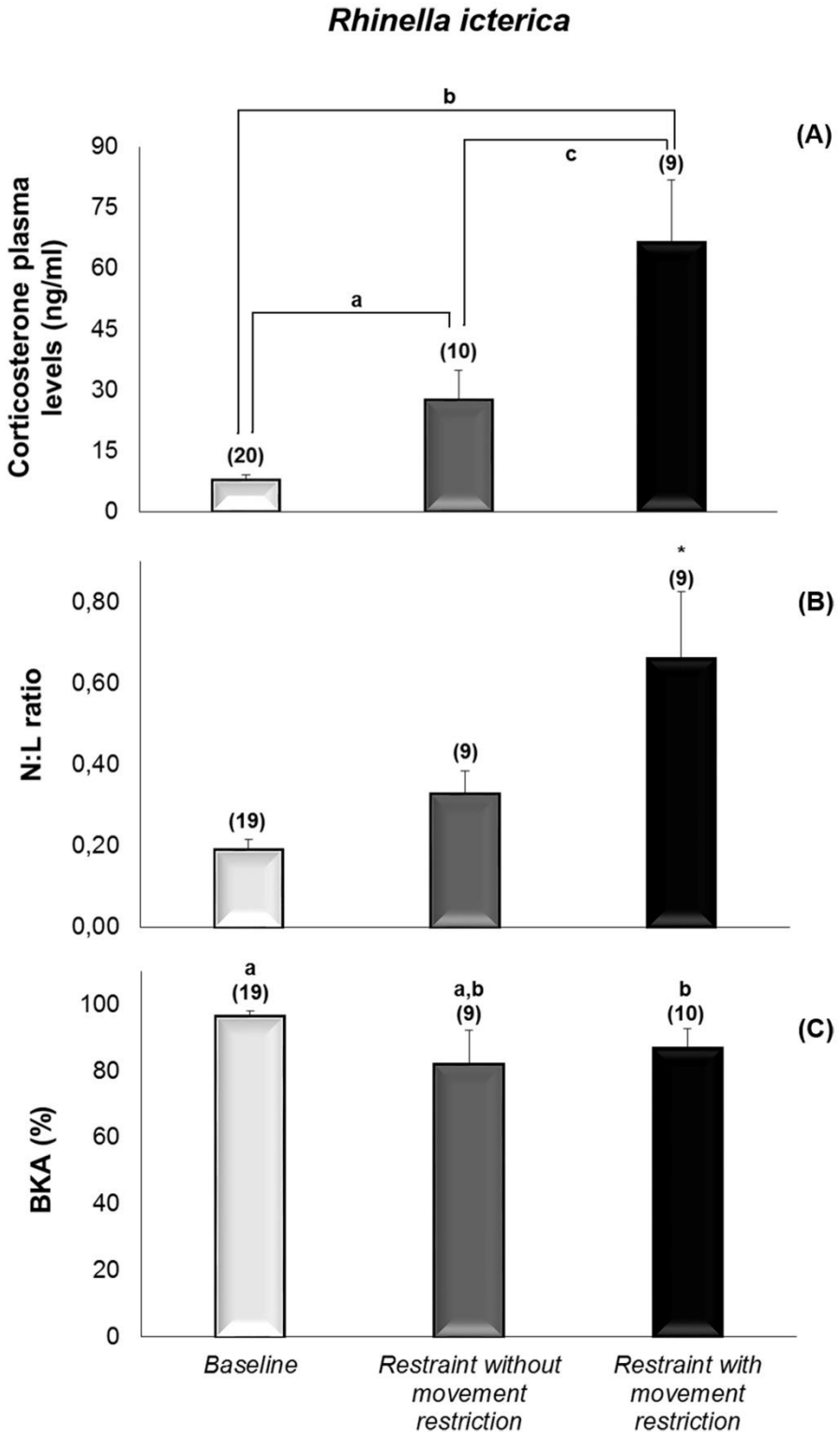

Figure 2. 3. Comparison on blood parameters at baseline and after 24h of the restraint challenge in *R. icterica*. The bars represent the mean ± standard error with *N* in parentheses. **(A)** Corticosterone plasma levels: $^{a}P \leq 0.01$; $^{b}P \leq 0.001$; $^{c}P \leq 0.05$. **(B)** Neutrophil/ Lymphocyte ratio (N:L): $^{*}P \leq 0.05$. **(C)** Bacterial killing ability of plasma (BKA): $^{a}P = 0.035$ (1-tailed).

When compared to the baseline values, the T levels were 6 times lower (t = 2.925, df = 8, $P \leq 0.019$) in both restrained groups (Figure 2.4; Table 2.2). There was no correlation between T and CORT in any moment (r = -0.467; $P \geq 0.205$).

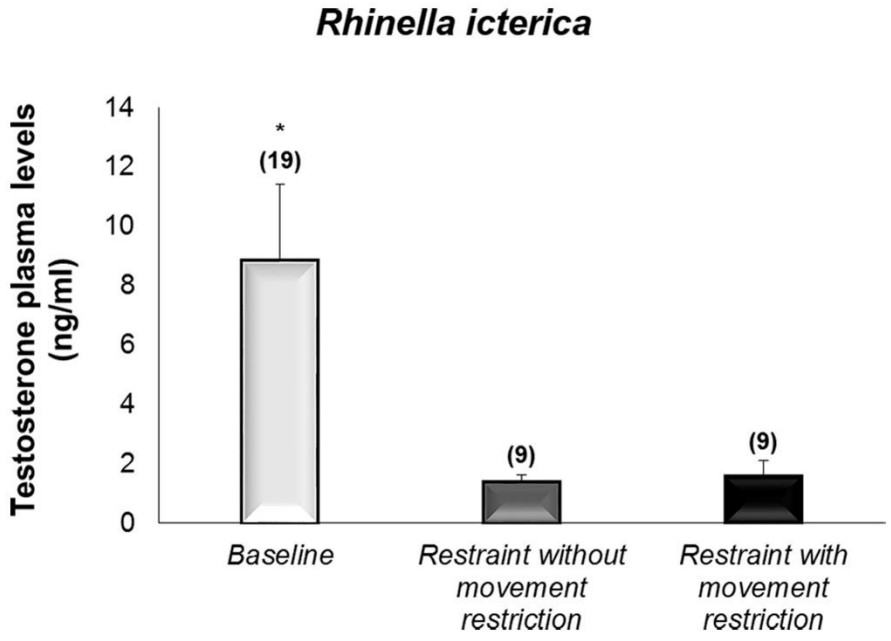

Figure 2. 4. Comparison on testosterone plasma levels at baseline and after 24h of the restraint challenge in *R. icterica*. The bars represent the mean ± standard error with *N* in parentheses. $^{*}P \leq 0.05$.

c) Rhinella schneideri

In *R. schneideri,* after 24h of restraint challenge, CORT levels increased 5 times (t = -2.224, df = 9, P = 0.050) and 11 times (t = -5.735, df = 9, $P \leq 0.0001$) relative to the baseline values, respectively without and with movement restriction. Individuals with movement restriction also showed two times higher values of CORT (t = -1.864, df = 18, P = 0.040 [1-tailed]) than individuals without movement restriction (Figure 2.5A; Table 2.3).

For the N:L ratio, the values were 2 times higher (t = -3.361, df = 9, P = 0.008) in restrained animals without movement restriction compared to baseline values (Figure 2.5B; Table 2.3). After the restraint challenge with movement restriction, *R. schneideri* showed a

positive correlation between N:L ratio and CORT (r = 0.639; P = 0.047). There were no differences in BKA with treatments (t = 1.696, df = 8, $P \geq 0.128$; Table 2.3), and BKA did not correlate with CORT in any circumstance (r = 0.268; $P \geq 0.455$).

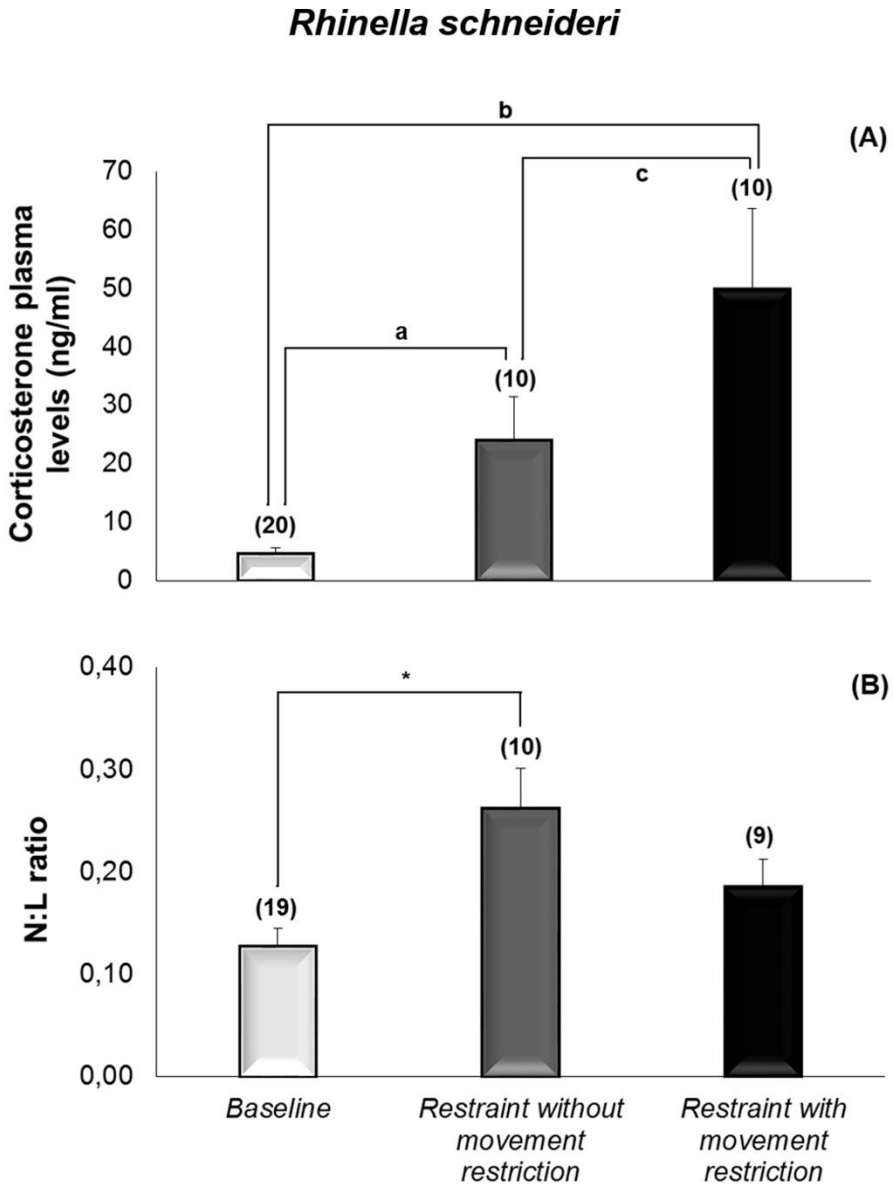

Figure 2. 5. Comparison on blood parameters at baseline and after 24h of the restraint challenge in *R. schneideri*. The bars represent the mean ± standard error with *N* in parentheses. **(A)** Corticosterone plasma levels: $^{a}P \leq 0.05$; $^{b}P \leq 0.0001$; $^{c}P = 0.04$ (1-tailed). **(B)** Neutrophil/ Lymphocyte ratio (N:L): $^{*}P \leq 0.01$.

Table 2. 3. Descriptive statistics of blood parameters, body mass and snout-vent-length for individuals of *R. schneideri* under field conditions and after restraint challenge with and without movement restriction.

		Parameter	N	Minimum	Maximum	Mean ± SD
FIELD		BKA (%)	19	54.00	93.00	74.74 ± 10.95
		N:L	19	0.02	0.25	0.13 ± 0.07
		CORT (ng/ml)	20	0.84	17.84	4.61 ± 4.50
		T (ng/ml)	19	0.26	7.98	2.52 ± 2.20
		Body Mass (g)	20	72.92	379.00	152.77 ± 80.56
		SVL (mm)	20	100.09	145.29	118.34 ± 14.67
AFTER 24H OF RESTRAIT CHALLENGE	**Without movement restriction**	BKA (%)	10	17.00	93.00	76.70 ± 21.48
		N:L	10	0.16	0.54	0.26 ± 0.12
		CORT (ng/ml)	10	1.36	57.91	23.92 ± 23.72
		T (ng/ml)	10	0.46	3.04	1.05 ± 0.93
		Body Mass (g)	10	93.35	289.03	164.34 ± 76.47
		SVL (mm)	10	103.97	143.49	120.76 ± 16.98
	With movement restriction	BKA (%)	10	34.00	93.00	66.80 ± 20.07
		N:L	10	0.07	0.33	0.19 ± 0.09
		CORT (ng/ml)	10	4.79	138.41	49.87 ± 43.53
		T (ng/ml)	10	0.43	1.12	0.73 ± 0.21
		Body Mass (g)	10	72.92	379.00	141.20 ± 86.93
		SVL (mm)	10	100.09	145.29	115.93 ± 12.38

BKA: Bacterial killing ability; **N:L**: Neutrophil/ Lymphocyte ratio; **CORT**: Corticosterone plasma levels; **T:** Testosterone plasma levels; **SVL:** Snout-vent-length.

When compared to the baseline values, the T levels were 2.5 times (t = 3.578, df = 8, P = 0.007) and 3.5 times (t = 2.062, df = 9, P = 0.035 [1-tailed]) lower in animals after 24h of restraint challenge without and with movement restriction, respectively (Figure 2.6; Table 2.3). There was no correlation between T and CORT in any moment (r = -0.275; $P \geq 0.255$).

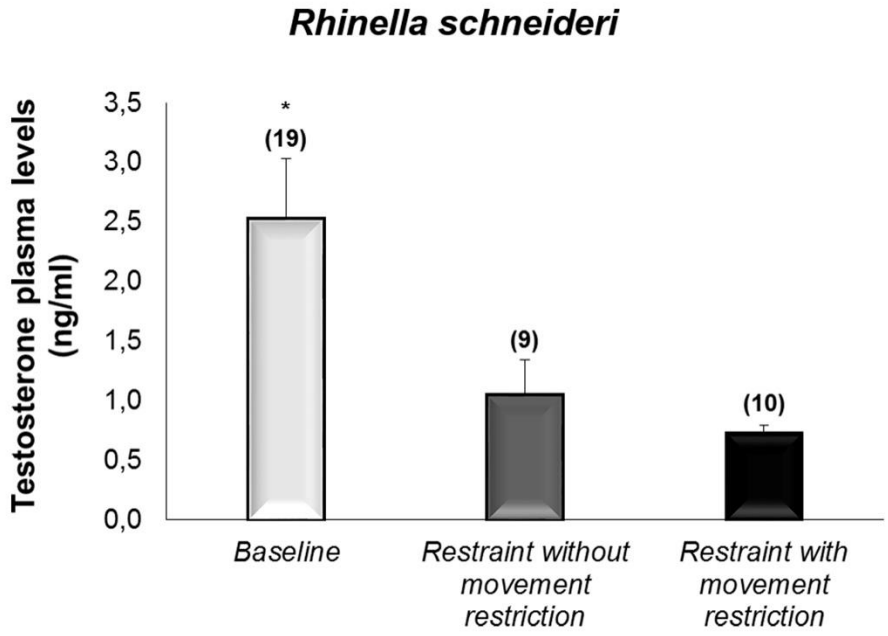

Figure 2. 6. Comparison on testosterone plasma levels at baseline and after 24h of the restraint challenge in *R. schneideri*. The bars represent the mean ± standard error with *N* in parentheses. $^{*}P \leq 0.05$.

d) Rhinella marina

When New Port Richey and Miami Springs populations were compared, there were no differences on baseline values (**CORT:** t = -0.482, df = 30, P = 0.633; **N:L ratio:** t = -1.603, df = 32, P = 0.119; **BKA:** t = 0.312, df = 32; P = 0.757).

After 24h of the restraint challenge without movement restriction, New Port Richey showed 25% lower BKA (t = -1.882, df = 14, P = 0.041 [1-tailed]) and 3 times lower NL ratio (t = -3.816, df = 14, P = 0.002) when compared to toads from Miami Springs. After 24h of the restraint challenge with movement restriction, Miami Springs population showed 40% higher CORT (t = -2.061, df = 16, P = 0.028 [1-tailed]) and 4.5 times higher NL ratio (t = -3.301, df = 16, P = 0.005). For this reason, the analyses for *R. marina* were run separated by population.

- <u>*New Port Richey population (NPR)*</u>

CORT levels in the restrained animals without movement restriction were 5.5 times higher (t = -4.022, df = 6, P = 0.007) in relation to baseline values (Figure 2.7; Table 2.4). In addition, when the restrained animals with movement restriction were compared to baseline values (t = -1.616, df = 8, P= 0.145; Figure 2.7); or when the two restrained groups were compared (t = 1.121, df = 14, P = 0.281; Figure 2.7), there were no differences.

There were no differences in N:L ratio and BKA from both stressed groups when compared to baseline values (t = -1.766, df = 6, $P \geq 0.128$; Table 2.4). Additionally, there were no differences between the two restrained groups (t = -1.337, df = 14, $P \geq 0.202$; Table 2.4). There was no correlation between BKA and CORT in any circumstance (r = -0.513; $P \geq 0.158$).

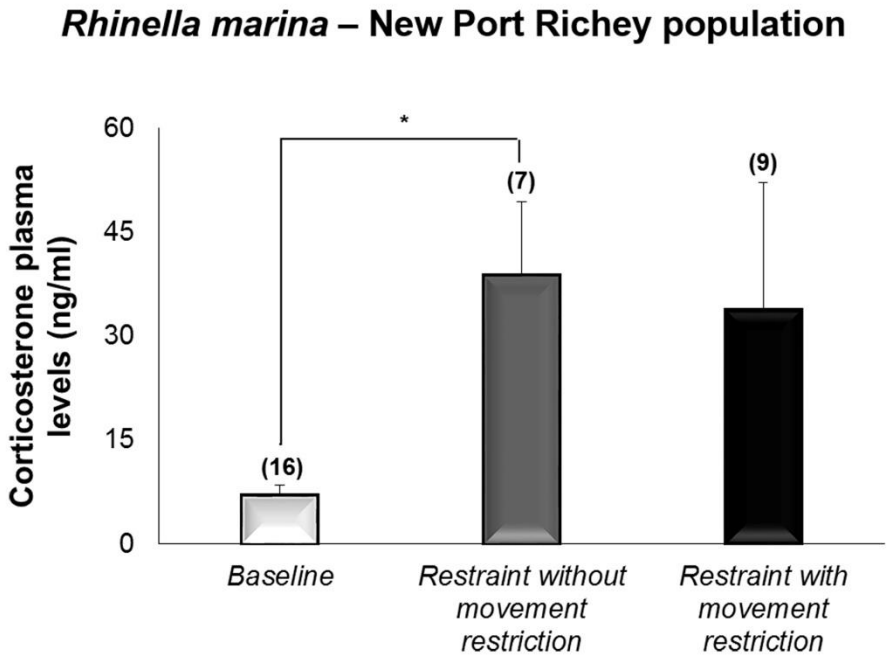

Figure 2. 7. Comparison on corticosterone plasma levels at baseline and after 24h of the restraint challenge in *R. marina* from New Port Richey population. The bars represent the mean ± standard error with *N* in parentheses. *$P \leq 0.01$.

Table 2. 4. Descriptive statistics of blood parameters, body mass and snout-vent-length for individuals of *R. marina* from New Port Richey population under field conditions and after restraint challenge with and without movement restriction.

		Parameter	N	Minimum	Maximum	Mean ± SD
FIELD		BKA (%)	16	0.00	100.00	62.31 ± 31.79
		N:L	16	0.04	0.68	0.26 ± 0.20
		CORT (ng/ml)	16	2.62	24.07	6.98 ± 5.81
		Body Mass (g)	16	65.97	165.45	119.88 ± 32.07
		SVL (mm)	16	89.50	128.00	107.59 ± 10.32
AFTER 24H OF RESTRAINT CHALLENGE	**Without movement restriction**	BKA (%)	7	9.00	85.00	57.57 ± 25.53
		N:L	7	0.06	1.14	0.43 ± 0.36
		CORT (ng/ml)	7	7.01	73.40	38.73 ± 28.33
		Body Mass (g)	7	65.97	137.62	93.43 ± 26.76
		SVL (mm)	7	89.50	110.00	100.07 ± 6.85
	With movement restriction	BKA (%)	9	26.00	99.00	72.00 ± 21.51
		N:L	9	0.21	0.68	0.46 ± 0.17
		CORT (ng/ml)	9	3.26	170.71	33.82 ± 55.08
		Body Mass (g)	9	116.65	165.45	140.45 ± 17.41
		SVL (mm)	9	103.00	128.00	113.44 ± 8.75

BKA: Bacterial killing ability; **N:L**: Neutrophil/ Lymphocyte ratio; **CORT**: Corticosterone plasma levels; **SVL:** Snout-vent-length.

- *Miami Springs population (MS)*

After 24h of restraint challenge, CORT levels increased 8 times (t = ,-2.521 df = 7, P = 0.040) and 5 times (t = -4.186, df = 7, P = 0.004) relative to the baseline values, respectively without and with movement restriction (Table 2.5; Figure 2.8A). There were no differences between the two restrained groups (t = -0.514 df = 16, P = 0.614; Table 2.5).

When compared to the baseline values, the N:L ratio was 3.5 times (t = -7.531, df = 8, $P \leq 0.0001$) and 5 times higher (t = -4.868, df = 8, P = 0.001) in animals after 24h of restraint challenge without and with movement restriction, respectively (Figure 2.8B; Table 2.5). Moreover, BKA was 25% higher (t = -2.483, df = 8, $P \leq 0.038$) in both stressed groups when compared to baseline values (Figure 2.8C; Table 2.5). Additionally there were no differences between the two restrained groups (t = 0.629, df = 16, $P \geq 0.487$; Table 2.5). There was no correlation between BKA and CORT in any circumstance (r = 0.319; $P \geq 0.228$).

Table 2. 5. Descriptive statistics of blood parameters, body mass and snout-vent-length for individuals of *R. marina* from Miami Springs population under field conditions and after restraint challenge with and without movement restriction.

		Parameter	N	Minimum	Maximum	Mean ± SD
FIELD		BKA (%)	18	10.00	98.00	61.06 ± 24.89
		N:L	18	0.03	0.92	0.40 ± 0.29
		CORT (ng/ml)	16	1.34	35.19	9.50 ± 9.61
		Body Mass (g)	18	42.70	201.32	117.75 ± 46.55
		SVL (mm)	18	72.00	122.00	102.28 ± 13.15
AFTER 24H OF RESTRAINT CHALLENGE	**Without movement restriction**	BKA (%)	9	36.00	98.00	77.78 ± 24.97
		N:L	9	0.36	2.68	1.41 ± 0.67
		CORT (ng/ml)	9	2.05	230.09	53.45 ± 68.11
		Body Mass (g)	9	58.99	189.90	122.29 ± 51.92
		SVL (mm)	9	84.00	122.00	102.78 ± 12.97
	With movement restriction	BKA (%)	9	59.00	99.00	74.89 ± 14.82
		N:L	9	0.38	6.88	2.13 ± 2.06
		CORT (ng/ml)	9	13.19	85.51	47.78 ± 26.55
		Body Mass (g)	9	42.70	201.32	113.21 ± 43.16
		SVL (mm)	9	72.00	120.00	101.78 ± 14.10

BKA: Bacterial killing ability; **N:L**: Neutrophil/ Lymphocyte ratio; **CORT**: Corticosterone plasma levels; **SVL:** Snout-vent-length.

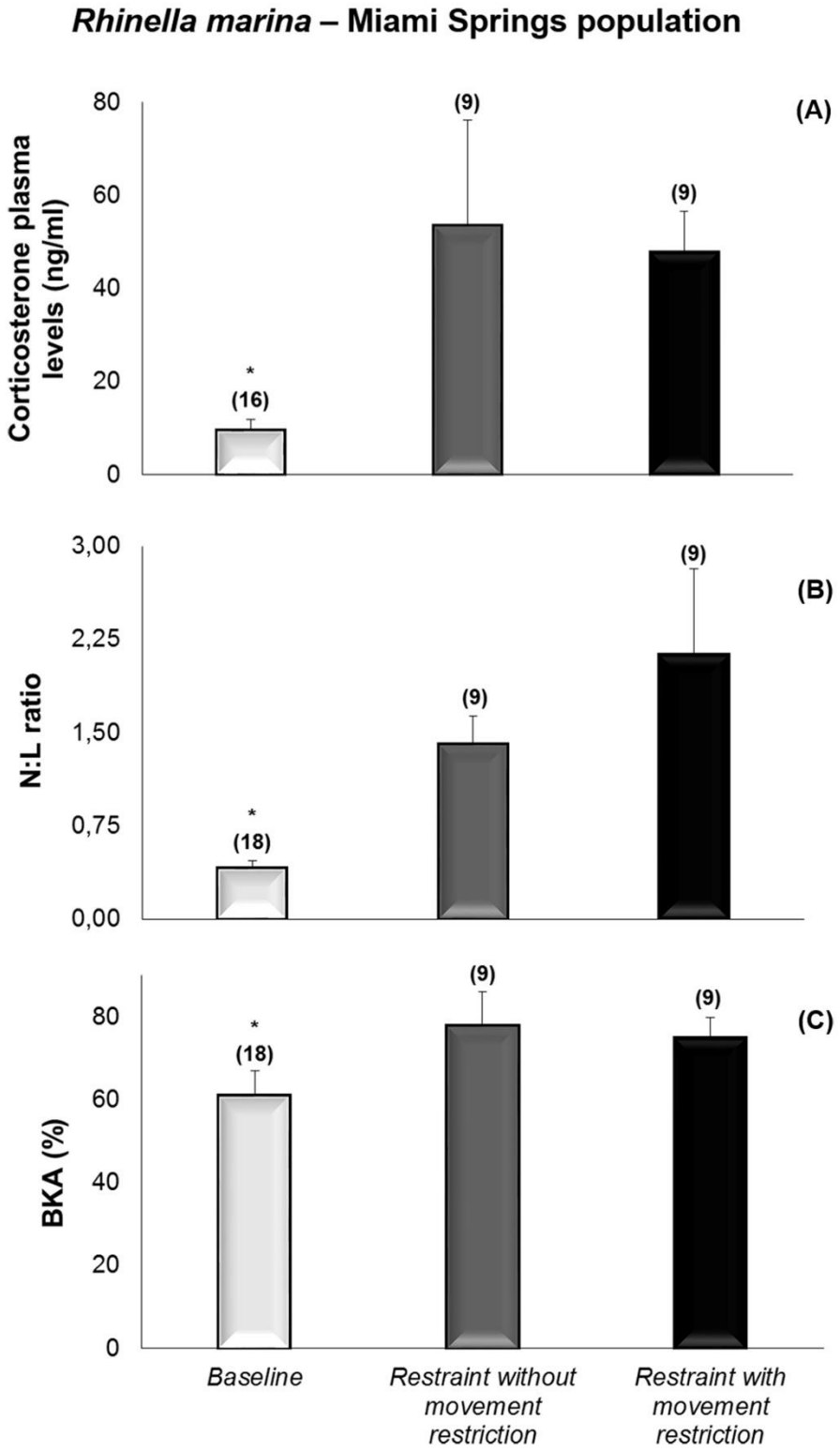

Figure 2. 8. Comparison on blood parameters at baseline and after 24h of the restraint challenge in *R. marina* from Miami Springs population. The bars represent the mean ± standard error with *N* in parentheses. **(A)** Corticosterone plasma levels: $^{*}P \leq 0.05$. **(B)** Neutrophil/ Lymphocyte ratio (N:L): $^{*}P \leq 0.001$. **(C)** Bacterial killing ability of plasma (BKA): $^{*}P \leq 0.05$.

2.4.2. Interspecific comparison

a) Baseline values

CORT differs between species ($F_{4,79}$ = 12.952; P < 0.0001) and is not affected by body mass ($F_{1,79}$ = 2.090; P = 0.152), with *R. ornata* showing CORT levels approximately 15 times higher than all the others species ($P \leq 0.001$; Figure 2.9A).

The N:L ratio also differs between species ($F_{4,79}$ = 7.623; P < 0.0001) and is not affected by body mass ($F_{1,79}$ = 1.491; P = 0.226), with *R. ornata* showing 2.5 higher values than *R. icterica*, *R. schneideri* and *R. marina* from New Port Richey ($P \leq 0.01$; Figure 2.9B). *Rhinella marina* from Miami Springs also shows 3 times higher N:L values than *R. schneideri* ($P \leq 0.001$; Figure 2.9B).

BKA differs between species ($F_{4,79}$ = 13.989; P < 0.0001) and is not affected by body mass ($F_{1,79}$ = 1.714; $P \geq 0.194$), with *R. icterica* showing 20% higher BKA than *R. schneideri* and 30% higher BKA than *R. marina* from the both populations ($P \leq 0.001$; Figure 2.9C). Additionally, *R. ornata* shows 10% higher values than *R. marina* from the both populations ($P \leq 0.018$; Figure 2.9C).

T levels differed between species (t = 3.147, df = 36, P = 0.003), with *Rhinella icterica* showing 3.5 times higher values than *R. schneideri* (Figure 2.10).

There is a negative interspecific correlation between CORT and body mass (r = -0.996; $P \leq 0.001$).

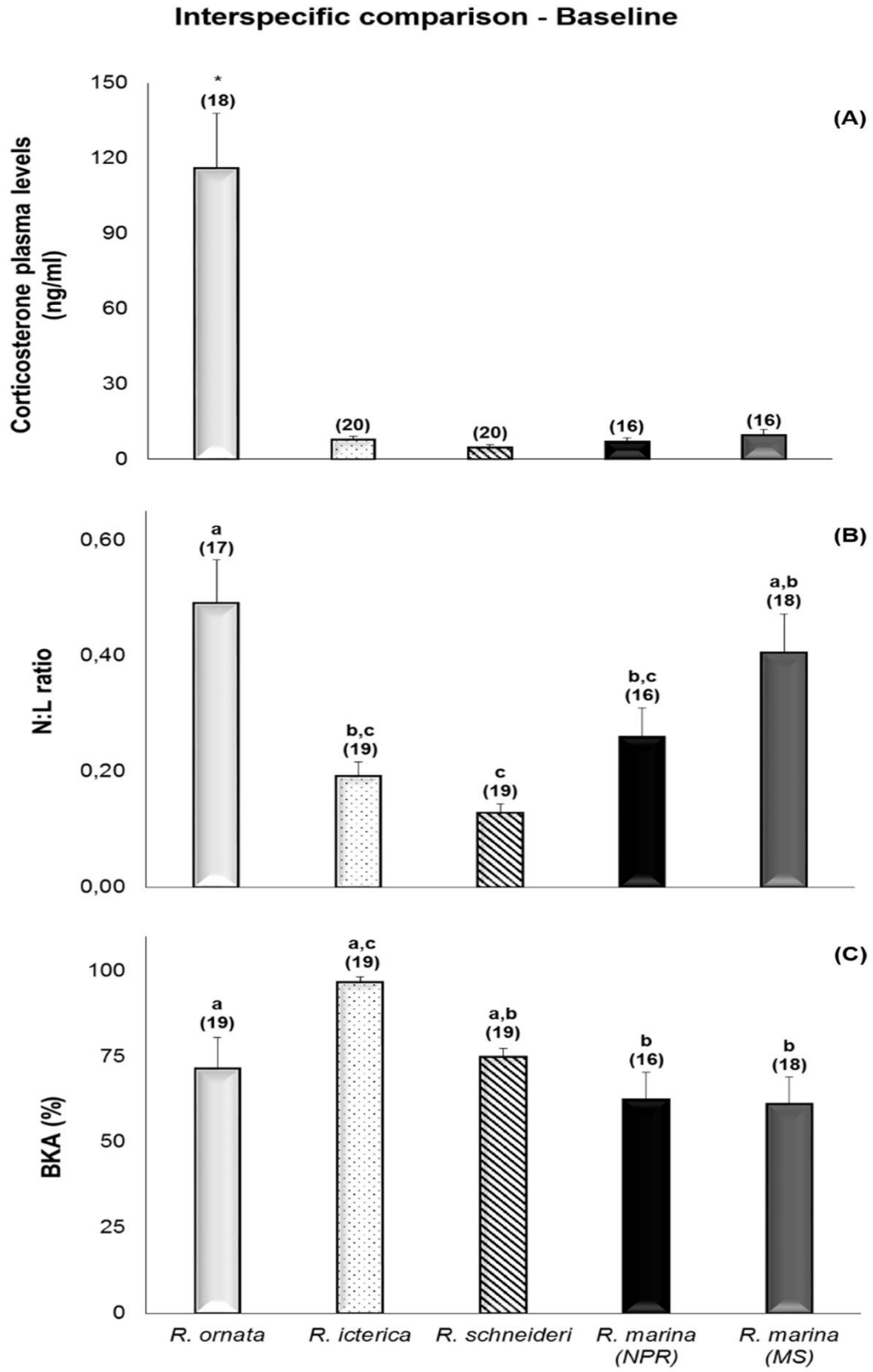

Figure 2. 9. Interspecific baseline comparison on blood parameters. The bars represent the mean ± standard error with *N* in parentheses. **(A)** Corticosterone plasma levels: $^{*}P \leq 0.001$. **(B)** Neutrophil/ Lymphocyte ratio (N:L): $^{a}P \leq 0.01$; $^{b}P \leq 0.001$; $^{c}P \geq 0.217$. **(C)** Bacterial killing ability of plasma (BKA): $^{a}P \leq 0.018$; $^{b}P \leq 0.001$; $^{c}P \geq 0.133$. **NPR:** New Port Richey population; **MS:** Miami Springs population.

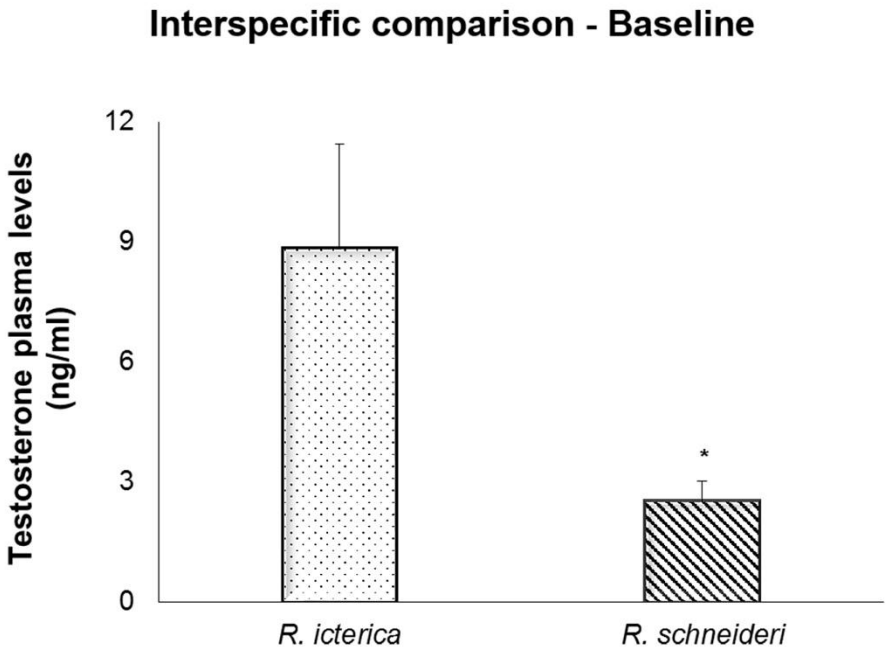

Figure 2. 10. Interspecific baseline comparison on testosterone plasma levels between *R. icterica* and *R. schneideri*. The bars represent the mean ± standard error, the *N* is the same for both species (*N* = 19). $^{*}P \leq 0.01$.

b) After the restraint challenge

- *Without movement restriction*

CORT did not differ between species ($F_{4,39} = 0.439$; $P = 0.439$) and is affected by body mass ($F_{1,39} = 5.254$; $P = 0.027$).

The N:L ratio differs between species ($F_{4,39} = 13.050$; $P < 0.0001$) and is not affected by body mass ($F_{1,39} = 1.505$; $P = 0.227$), with *Rhinella marina* from Miami Springs showing 5 times higher N:L values than *R. schneideri* ($P \leq 0.001$; Figure 2.11A); 4 times higher N:L values than *R. icterica* ($P \leq 0.001$; Figure 2.11A); and 3 times higher N:L values than *R. marina* from New Port Richey ($P \leq 0.001$; Figure 2.11A). *Rhinella ornata* shows 3 times higher values than *R. icterica* and *R. schneideri* ($P \leq 0.01$; Figure 2.11A).

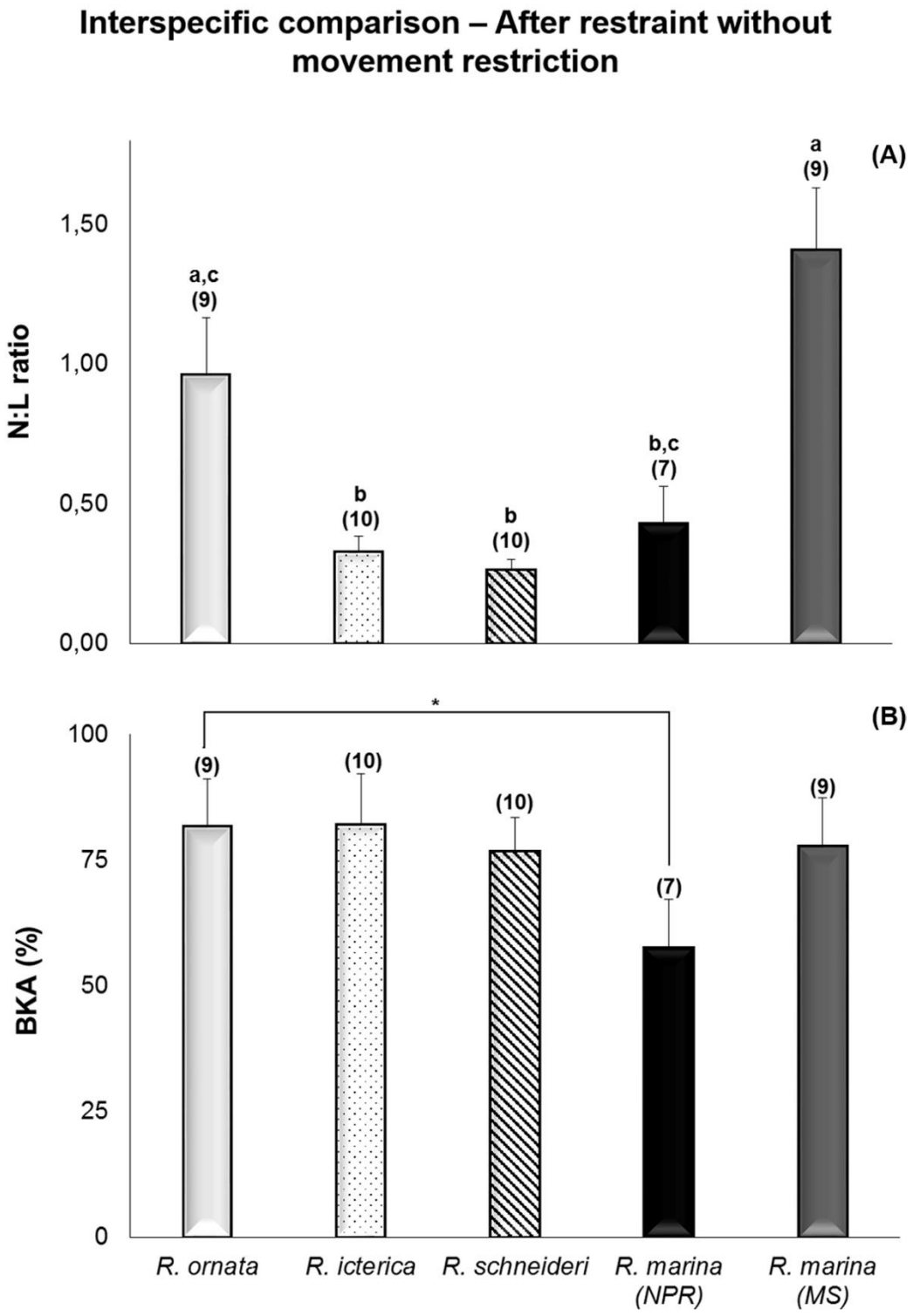

Figure 2. 11. Interspecific comparison on blood parameters after 24h of the restraint challenge without movement restriction. The bars represent the mean ± standard error with *N* in parentheses. **(A)** Neutrophil/ Lymphocyte ratio (N:L): $^{a}P \leq 0.01$; $^{b}P \leq 0.001$; $^{c}P \geq 0.05$. **(B)** Bacterial killing ability of plasma (BKA): $^{*}P \leq 0.017$. **NPR:** New Port Richey population; **MS:** Miami Springs population.

BKA differs between species ($F_{4,39}$ = 3.493; P = 0.016) and is affected by body mass ($F_{1,39}$ = 6.173; P = 0.017), with *R. ornata* showing 24% higher BKA than *R. marina* from New Port Richey ($P \leq 0.017$; Figure 2.11B).

T levels not differed between species (t = 1.225, df = 16, P = 0.238).

There is a negative interspecific correlation between CORT and body mass (r = -0.939; P = 0.018).

- <u>*With movement restriction*</u>

CORT did not differ between species ($F_{4,41}$ = 2.006; P = 0.112) and is not affected by body mass ($F_{1,41}$ = 1.301; P = 0.261).

The N:L ratio differs between species ($F_{4,41}$ = 7.144; $P < 0.0001$) and is not affected by body mass ($F_{1,41}$ = 0.008; P = 0.931), with *Rhinella marina* from Miami Springs showing 11 times higher N:L values than *R. schneideri* ($P \leq 0.001$; Figure 2.12); and 4.5 times higher N:L values than *R. marina* from New Port Richey ($P \leq 0.01$; Figure 2.12). *Rhinella ornata* shows 10 times higher values than *R. schneideri* ($P \leq 0.01$; Figure 2.12).

BKA do not differs between species ($F_{4,41}$ = 1.244; P = 0.308) and is not affected by body mass ($F_{1,41}$ = 0.009; P = 0.924).

T levels differ between species (t = 2.000, df = 17, P = 0.031; 1-tailed), with *Rhinella icterica* showing 2 times higher values than *R. schneideri* (Figure 2.13).

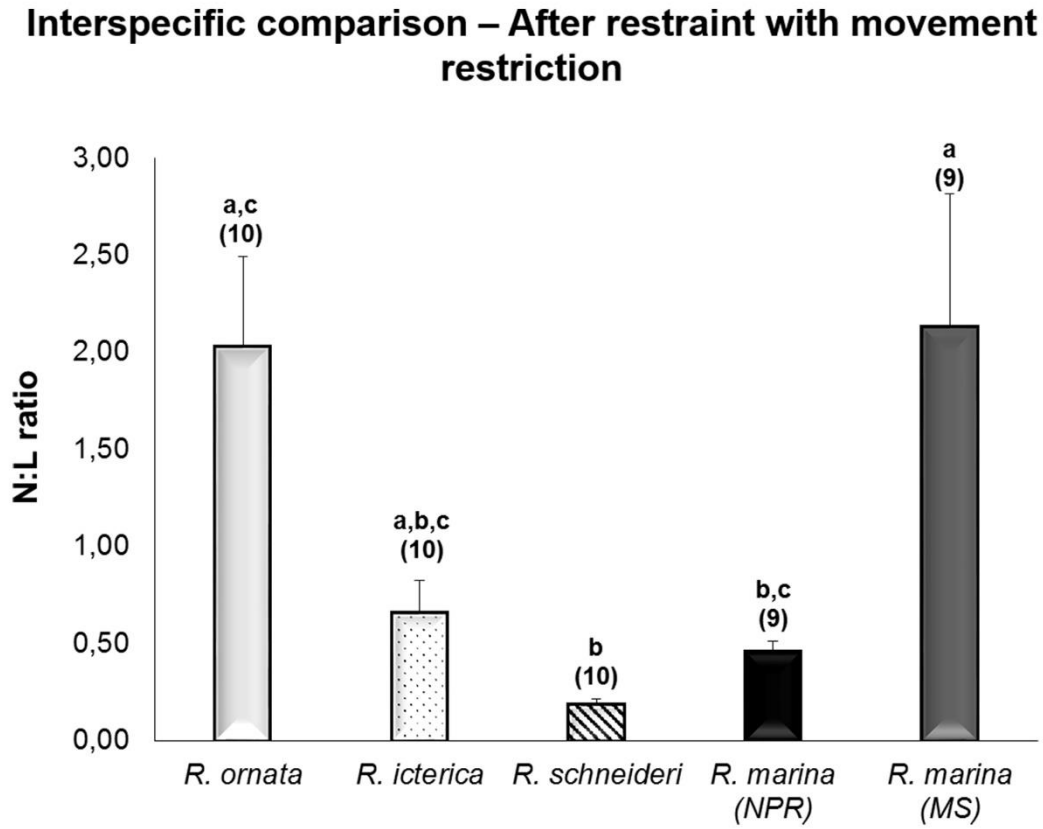

Figure 2. 12. Interspecific comparison on Neutrophil/ Lymphocyte ratio after 24h of the restraint challenge with movement restriction. The bars represent the mean ± standard error with *N* in parentheses. * $^{a}P \leq 0.01$; $^{b}P \leq 0.01$; $^{c}P \geq 0.07$. **NPR:** New Port Richey population; **MS:** Miami Springs population.

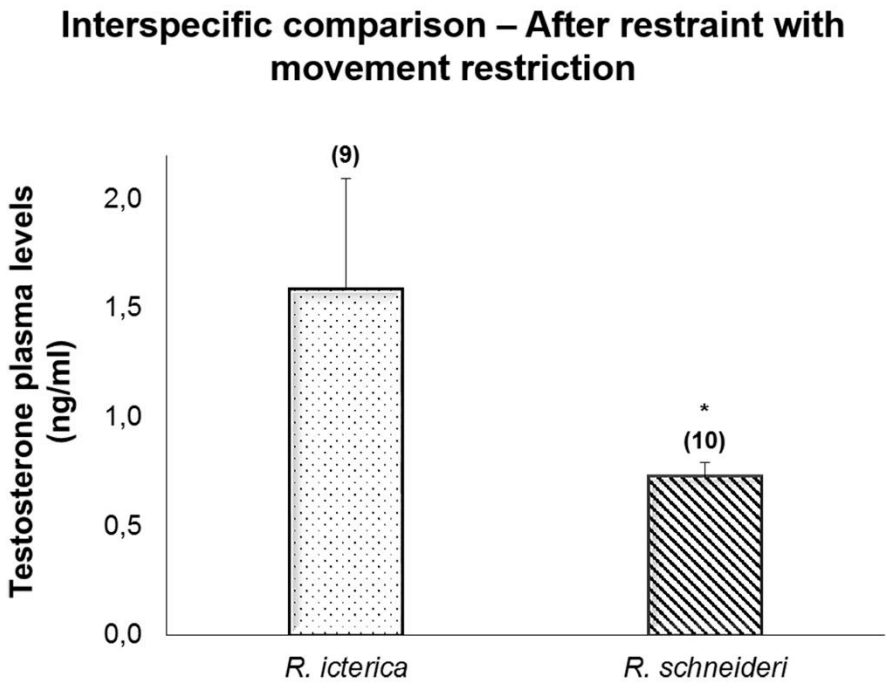

Figure 2. 13. Interspecific comparison on testosterone plasma levels after 24h of the restraint challenge with movement restriction between *R. icterica* and *R. schneideri*. The bars represent the mean ± standard error with *N* in parentheses. $^{*}P = 0.031$ (1-tailed).

2.5. Discussion

2.5.1. Intraspecific effects

In a general way, all the species were affected by the restraint challenge, with and/or without movement restriction, and the general patterns of responses included an increase in the CORT levels and N:L ratio, and a decrease in T levels. Restraint challenge decreased and increased BKA in *Rhinella icterica* and one population of *R. marina*, respectively.

Rhinella ornata was the only species not showing an increase in CORT levels after the restraint challenge. However, it was the species showing the highest baseline CORT levels, probably because males were collected during calling activity, despite all the other species were collected during the breeding season. CORT are high during reproductive season in different vertebrates, including anurans (Moore and Jessop, 2003; Assis et al., 2012), and it is probably

associated to facilitation of reproductive behavior through mobilization of energy reserves (Moore and Jessop, 2003; Landys et al., 2006; Carr, 2011). In the Energetics-Hormone Vocalization model, proposed by Emerson (2001), the CORT and T are positively correlated with vocal effort in anurans when males start calling. Over consecutive nights of vocal activity, CORT increases until a threshold evoking a stress response, reducing T and inhibiting the expression of vocal behavior (Emerson, 2001). In this way, it is possible that males of *R. ornata* did not increase CORT after restraint challenge because they were already experiencing a short-term stress response, related to vocal activity. All the other four species showed higher CORT levels after restraint challenge, corroborating previous studies with anurans (Narayan et al., 2011a; Narayan et al., 2012; Gomes et al., 2012; Grahan et al., 2012).

Relative to baseline values, CORT tended to increase more in response to restraint with movement restriction than to restraint without movement restriction. This difference was statistically significant for *Rhinella icterica* and *R. schneideri*. Even without showing significant differences, the same pattern was observed for *R. ornata*. These results suggest that the animals are able to respond differently to the intensity of the stressor. *Rhinella marina* from both populations did not show the same pattern of increase in CORT with the intensity of the stressor applied, displaying very similar levels for both groups. Maybe it is because the both types of restraint, with and without movement restriction, were much more alike than the stimuli we applied in Brazil. The boxes where we kept the animals in USA were much smaller than the ones used in Brazil, and rather than been kept in white cloth bags (what means it had opacity), the animals in USA were kept in clear plastic bags. To be able to identify the source of this variation, we would need to collect *R. marina* in Brazil, and run for the native and invasive populations the same restraint protocol we applied for the other Brazilian species.

The N:L ratio was higher for all the species after the restraint challenge. This is in accordance with the pattern reported by other studies, where an increase in N:L ratio was

observed after a restraint stress in *macaques* (Morrow-Tesch et al., 1993), captivity stress in *salamanders* (Davis and Maerz, 2008) and *lizards* (Seddon and Klukowski, 2012); and after exogenous administration of corticosterone in *salamanders* (Davis and Maerz, 2010). The leukocyte profile is altered by stress, and can be directly related to the plasma levels of stress hormones. Specifically, the changes caused by stress or by treatment with glucocorticoids include an increase in the number of circulating neutrophils and a decrease in the number of circulating lymphocytes (Davis et al, 2008). Although the general pattern exhibited by the species in this study have been an increase in CORT and N:L ratio after application of the stressor, supporting the interpretation given by other authors, in general, there was no correlation between these two parameters. Interestingly *Rhinella ornata* responded to restraint stress increasing N:L ratio, while not having increased CORT. In general, the 24h of restraint challenge stressed all species. The restraint without movement restriction promoted an increase in CORT, while the restraint with movement restriction promoted a higher increase in CORT accompained by an increase in N:L ratio. It is possible that the individuals of *Rhinella ornata* had already presented high CORT levels at field (baseline) and, when restricted,, they showed the common response to more intense stressor for other species (increase in N:L ratio).

The glucocorticoid plasma levels have been used as an index to monitor stress levels in natural populations (Dunlap and Wingfield, 1995; Hopkins et al., 1997; Norris et al., 1997; Janin et. al., 2011). Additionally, some authors assume that changes in CORT directly causes a proportional change in N:L ratio, and the N:L ratio could be used as an indication of changes in CORT over time (Davis et al., 2008). However, a positive correlation between these two variables were found only in two of our toad species, and only after the restraint challenge with movement restriction. In this way, we recommend caution to consider the N:L ratio alone as a truthful representative of the stress condition and a good indicative of elevated CORT, because apparently these two measures cannot be used interchangeably as indicators of the same levels

of stress response. Similar results were found in studies with free-living birds (Vleck et al., 2000; Muller et al., 2011), that also found no correlation between N:L ratio and CORT. We agree with Muller et al. (2011) that N:L ratio and CORT can be used together, to provide a comprehensive picture about the stress condition of animals.

The T levels were measured in only two species: *Rhinella icterica* and *R. schneideri*, and both species showed a reduction on T after the restraint challenge. Additionally, *R. icterica* the species with higher T levels, showed a higher decrease on T. This same pattern of variation was found when comparing individuals in birds (Deviche et al., 2012; Deviche et al., 2014). The authors suggested that stressed birds defend against decreasing T below a minimum level that is needed for maintenance of T-dependent behavioral and morphological sexual characteristics, and/or physiological functions, particularly the negative feedback on gonadotropin secretion (Deviche et al., 2014). In our study and both studies with birds (Deviche et al., 2012; Deviche et al., 2014), the reduction on T was not correlated to increase on CORT, suggesting that the reduction on T was not simply a function of increased CORT secretion (Deviche et al., 2012).

Studies in a diversity of vertebrate species have shown that stressors typically suppress T, sometimes quite rapidly (Greenberg and Wingfield, 1987; Deviche et al., 2010, Deviche et al., 2012; Deviche et al., 2014). Reduction on T levels has also been reported after induced acute stress in reptiles (reviewed in Tokarz and Summers, 2011), including the responses to confinement (Jones and Bell, 2004) and handling stress (Moore et al., 1991), and after a restraint challenge and toe-clipping in toads (Narayan et al., 2011b; Narayan et al., 2012). Multiple possible physiological mechanisms can explain the transition between positive to negative relationships between CORT and T. These may include a rapid direct inhibition of testicular function by glucocorticoids (Dong et al., 2004; Hardy et al., 2005; Hu et al., 2008; Martin and Tremblay, 2008); and an acceleration of T clearance through interactions of CORT with plasma corticosterone-binding globulin (Deviche et al., 2001). More studies are necessary in anurans

to determine which mechanisms are involved in how stress, and even CORT, influences changes in T.

Rhinella icterica and one of the populations of *R. marina* showed opposite responses of BKA to the restraint challenge. Studies using a restraint stress protocol in birds showed a reduction in BKA (Matson et al., 2006) and a negative correlation with CORT (Millet et al., 2007). In anuran amphibians, studies using a restraint stress protocol (Graham et al., 2012) and long-term captivity (Assis et al., 2015) reported declines in BKA, but there were no significant correlations between CORT and BKA in any of them. In the present study, there were no correlation between BKA and CORT (except for a positive correlation in *R. ornata*, after the restraint with movement restriction), and the decrease in BKA on *R. icterica* was expected and supported by aforementioned studies. Interestingly, a positive relationship between the stress response by restraint and BKA was found in a salamander (*Cryptobranchus alleganiensis*), showing that the BKA may increase after an acute stress (Hopkins and DuRant, 2011). We know that BKA differ between species (Assis et al., 2013), and different patterns of response to the same stress protocol are completely plausible. Moreover, *R. icterica* individuals were colleted in their original distribution area, while the individuals of *R. marina* came from invasive populations. There is a prediction that invasive species should present weaker systemic inflammatory responses, but stronger humoral responses, given that systemic inflammatory response is the cause of some of the most familiar symptoms of illness, including fever, fatigue, and loss of appetite (Lee and Klasing, 2004). To better evaluate and understand the variation in immune response and its modulation by stress and CORT, we need to analyze different segments of the immune response, such as humoral and natural antibodies (e.g. BKA, hemagglutination), cellular mediated processes (phagocytosis, lymphocyte proliferation), and inflammatory response (PHA or LPS challenge), given that the investment in each of this segments can be different within and between species.

2.5.2. Interspecific comparisons

Rhinella ornata, the species with the highest degree of dependence on forested habitats, showed the highest baseline CORT, corroborating our predictions and also previous observations (Gomes et al., 2012). Species of anurans may differ in sensitivity to environmental changes, and variation in baseline CORT levels may have important implications for exploring the impacts of environmental change. Some observations suggest that the broadness of geographical distribution and the sensitivity to environmental changes are inversely related in anurans. Species more dependent on forested environments, for example, show higher genetic variance than more generalist ones when populations from fragments of Atlantic Forest are compared (Carnaval, 2002). Interspecific differences in glucocorticoids sensitivity may be useful to predict which taxa are most likely to be challenged by environmental changes. If increased glucocorticoids sensitivity is associated with reduced tolerance for change, then species with more responsive glucocorticoids physiologies may be particularly vulnerable to anthropogenic or other sources of environmental modification (Jessop et al., 2013; Wikelski and Cooke, 2006; Wingfield, 2013; Hammond et al., 2015). Our data are in agreement with a possible physiological explanation proposed by Gomes et al. (2012) that more generalist species, which normally can occupy and persist in naturally open and/or disturbed areas, would adjust more efficiently to these stressful conditions by maintaining lower CORT levels. When compared with four more generalist species, *R. ornata* is showing higher baseline and after restraint CORT levels, even considering that, after the restraint, the interspecific differences are not significant.

Moreover, it is known that anuran males show correlated variation in calling performance and CORT (Moore et al., 2005; Assis et al., 2012), and even phylogenetically close species can vary dramatically in calling performance (Bevier et al., 2008). The interspecific variation in baseline CORT during the breeding season might reflect, at least in part, differences in calling

behavior. Moreover, *R. ornata* was the only species calling during the nights of data collection, despite all the species were collected within the breeding season. In accordance with this, behavioral differences might be at least partially responsible for the interspecific variation in baseline CORT observed in the present study.

Interestingly, baseline CORT values from *Rhinella ornata* in this study (~ 120ng/ml) were 3.5 times higher than baseline values (~ 35ng/ml) and equivalent to values after a restraint challenge (~120ng/ml) found by Gomes et al. (2012) for this same population. This difference between years, between animals coming from the same population and same period (breeding season), could be driven by the intensity of calling activity, as explained on intra-specific discussion, and following the prediction of the Energetics-Hormone Vocalization model proposed by Emerson (2001). Furthermore, *R. ornata* is the species with more explosive reproductive behavior, following heavy rains, which does not occur in other species. This also should be associated with comparatively higher calling rates and higher CORT levels at the field.

In addition, the difference in baseline CORT of *R. ornata* from this study and the study conducted by Gomes et al. (2012) could be driven by a stress condition associated to changes in climatic conditions. In a comparative study with *Rhinella ornata*, *R. icterica* and *R. schneideri*, Titon Jr. et al. (2010) showed that *R. ornata* displays higher sensitivity of locomotor performance to dehydration in different temperatures, which suggests that the activity patterns of this species are highly sensitive to rainfall. During the reproductive period of *R. ornata* in the region where the individuals were sampled (August to October), rainfall was much lower in 2014 (0.97 ± 1.92mm – Mean ± Standard Deviation) than in 2008 (48.45 ± 75.27mm – Mean ± Standard Deviation; INPE – Jau/SP – ID: 31978). In this way, the drastic reduction in the amount of rain in 2014 could be a stressor for this species, promoting an elevation on baseline CORT levels. Reduced rainfall has been described as a possible cause of anuran population

declines observed during long-term studies at the Savannah River Site (SRS) in South Carolina, USA (Daszak et al., 2005; Navas and Otani, 2007).

After the restraint challenge, the proportional increase in CORT was higher in the species that showed lower CORT values at baseline. Animals under chronic stress usually show higher baseline levels (Assis et al., 2015) and a lower increase in CORT when submitted to acute stress (Gadek-Michalska and Bugajski, 2003; Love et al., 2003). This attenuation of the stress response with chronic stress has been alternativelty interpreted as habituation (the perception or cognitive interpretation of the stressors), exhaustion (the state in which the animal can no longer compensate for sustained stress and effects become life-threatening), or downregulation (the mechanism that alters CORT release could be a function of the adrenal tissue itself, through downregulation of ACTH receptors or a decreased ability to manufacture the steroids) (Rich and Romero, 2005). Unlike these studies, we are observing this pattern when comparing different species, what means that mechanisms may not be the same, and more studies are required to understand the functional causes and evolutionary implications of this pattern. Additionally, there is a negative correlation between CORT and body mass. In birds, variation in baseline CORT among species was inversely related to body mass and length of the breeding season (Hau et al., 2010). Individuals of smaller species have higher mass-specific metabolic rates (Calder, 1996), and given that some studies have found that glucocorticoids can increase metabolic rates (DuRant et al., 2008; Preest and Cree, 2008; Wack et al., 2012), it is possible that there are energetic implications associated to this allometric function of CORT.

Rhinella ornata and *R. marina* from Miami Springs are the species showing higher N:L ratio at baseline and after restraint challenge. As previously discussed on intra-specific discussion section, changes in CORT have been functionally associated to altered relative numbers of neutrophils and lymphocytes in the circulation (Davis et al., 2008; Seddon and Klukowski, 2012). Even though there was no significant correlation between N:L ratio and

CORT, there is a strong tendency for species with higher CORT after the restraint challenge also show higher N:L ratio under the same conditions, reinforcing the pattern previously observed by other authors at intraspecific level (Vleck et al., 2000; Muller et al., 2011).

Rhinella ornata and *R. icterica* showed the highest baseline BKA values, while BKA differed only between *R. ornata* and *R. marina* from New Port Richey after the restraint challenge without movement restriction. These results were not expected; given that previous comparative results showed the highest baseline BKA values for *R. schneideri*, when compared to other species of *Rhinella* (Gomes et al., 2012) and the highest baseline BKA values for *R. icterica* when compared to anurans from other phylogenetic groups (Assis et al., 2013). Additionally, we expected that *R. marina* could also have high BKA, since this species is more phylogenetically related to *R. schneideri*, and both species are more generalists in terms of environmental occupancy (Maciel et al., 2010). Glucocorticoid levels and the immune response vary greatly even at intraspecific level, depending on environmental conditions and time of life cycle (Norris & Evans, 2000; Lee & Klasing, 2004; Verhulst *et al.*, 2005; Lee, 2006; Romero, 2002; Latin and Romero, 2013; Wingfield and Romero, 2001). This huge intra-specific variation complicates the inter-specific comparison. The finer sampling in temporal terms for all species could help to better discern the sources of variation at intra and interspecific levels.

Rhinella icterica showed higher T than *R. schneideri* at baseline and after the restraint challenge with movement restriction. This difference might be related to the time of breeding season when samples were collected. We know that the T levels can show huge variation even within the breeding season (Zerani et al., 1991; Houck and Woodley, 1995; Canosa and Ceballos, 2002, Assis et al., 2012). To test this hypothesis, we need to collect animals from both species throughout their reproductive season (Assis et. al., 2012).

2.6. Conclusion

Twenty-four hours of the restraint challenge promoted a stress response in different species of toads (*Rhinella*), including increased CORT and N:L, and decreased T. *Rhinella ornata* was the only species not showing an increase in CORT in response to the restraint challenge, possibly because they were already experiencing a short-term stress response related to vocal activity. Moreover, CORT and N:L tended to increase more in response to restraint with movement restriction than to restraint without movement restriction, relative to baseline values, suggesting that these toads are able to respond differently to the intensity of the stressor applied. The only exception to this trend was *Rhinella marina*, which showed similar CORT levels for both restrained groups. However, this altered pattern of response could be due to methodological issues associated to the fact that this species was subjected to different restraint stimuli. *Rhinella icterica* showed a reduction in BKA, while*R. marina* showed increased values after the restraint challenge, suggesting that the impacts of stress response on immune response can be variable in toads.

Species of *Rhinella* differ in steroid levels (CORT and T), N:L and BKA at field conditions and after restraint challenge. *Rhinella ornata* showed higher baseline CORT levels when compared to other species. Explanations for this pattern might be associated to interspecific differences in sensitivity to environmental changes and intensity of calling behavior. *Rhinella ornata* and *R. marina* from Miami Springs are the species showing higher N:L ratio at baseline and after restraint challenge, no correlation with CORT levels. We expected, from previous comparative results, that more generalist species in terms of environmental occupancy (*R. schneideri* and *R. marina)*, would be those characterized by higher baseline BKA, but *R. ornata* and *R. icterica* showed the highest baseline values. Interspecific comparisons in glucocorticoid plasma levels and the immune response are difficult to stablish, since these physiological variables vary greatly even at intraspecific level,

depending on environmental conditions and time of life cycle. *Rhinella icterica* showed higher T levels than *R. schneideri*, and this difference might be related to the time of breeding season when samples were collected for both species.

Based on our results, we consider that a carefull evaluation is necessary in order to understand the interrelationship between immune system and its modulation by stress and CORT levels, at both intra and interspecific levels. The inclusion of different segments of the immune response (e.g. humoral and natural antibodies, cellular mediated processes and inflammatory response) may be very informative, since the investment in each segment of immune response can differ within or between species. Additionally, it is necessary to standardize data collection for all the species under the same period (e.g. inside or outside of breeding season) and same activity (e.g. calling or foraging), given that vocal activity is a source of great variability in hormonal levels and probably on immune response.

2.7. Acknowledgments

We would like to thank Beny Spira (ICB/USP), Cristina O. M. S. Gomes (FMVZ/USP) and Eliana R. Matushima (FMVZ/USP) for methodological discussion and assistance. We would like to thank Braz Titon Jr. (IB/USP), Adriana M. G. Barsotti (IB/USP) and Jasmine Dagg (BS/AU) for helping us during the fieldwork. This research was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) through Regular grant for FRG (2013/00900-1); PhD scholarship (2011/10560-8) and BEPE scholarship (2014/05806-6) for VRA (http://www.fapesp.br). By Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPq) through a PhD scholarship (140272/2011-9 GD) for VRA (http://www.cnpq.br) and by Auburn University Undergraduate Research Fellowhip for C.S.M.

2.8. References

Angelier, F. & Wingfield, J.C. 2013. Importance of the glucocorticoid stress response in a changing world: theory, hypotheses and perspectives. *Gen. Comp. Endocrinol.* 190, 118-128.

Assis, V.R., Navas, C.A., Mendonça, M.T. & Gomes, F.R. 2012. Vocal and territorial behavior in the Smith frog (*Hypsiboas faber*): Relationships with plasma levels of corticosterone and testosterone. *Comp. Biochem. Physiol.* 163, 265–271.

Assis, V.R., Titon, S.C.M., Barsotti, A.M.G., Spira, B. & Gomes, F.R. 2013. Antimicrobial Capacity of Plasma from Anurans of the Atlantic Forest. *South Amer. J. Herpetol*. *8*, 155–160.

Assis, V.R., Titon, S.C.M., Barsotti, A.M.G., Titon Jr., B. & Gomes, F.R. 2015. Effects of acute restraint stress, prolonged captivity stress and transdermal corticosterone application on immunocompetence and plasma levels of corticosterone on the Cururu toad (*Rhinella icterica*). *PLOS ONE*, 10, 1–21.

Baldissera Jr., F.A., Caramaschi, U. & Haddad, C.F.B. 2004. Review of the Bufo crucifer species group, with descriptions of two new related species (Amphibia, Anura, Bufonidae). Arquivos do Museu Nacional. 62, 255–282.

Bernardo, J., Ossola, R.J., Spotila, J. & Crandall, K.A. 2007. Interspecies physiological variation as a tool for cross-species assessments of global warming-induced endangerment: validation of an intrinsic determinant of macroecological and phylogeographic structure. *Biol. Letters*. 3, 695-699.

Bevier, C.R., Gomes, F.R. & Navas, C.A. 2008. Variation in call structure and calling behavior in treefrogs of the genus Scinax. *South Amer. J. Herpetol.* 3, 196-206.

Brown, G.P., Kelehear, C. & Shine, R. 2011a. Effects of seasonal aridity on the ecology and behaviour of invasive cane toads (*Rhinella marina*) in the Australian wet-dry tropics. *Funct. Ecol.* 25, 1339–1347.

Brown, G.P., Phillips, B.L., Dubey, S. & Shine, R. 2014. Invader immunology: invasion history alters immune system function in cane toads (*Rhinella marina*) in tropical Australia. *Ecol. Letters.* 18, 57–65.

Brown, G.P., Shilton, C.M. & Shine, R. 2011b. Measuring amphibian immunocompetence: validation of the phytohemagglutinin skin-swelling assay in the cane toad, *Rhinella marina. Meth. Ecol. Evol.* 2, 341–348

Calder III, W.A. 1996. Size, function, and life history, 2nd ed. Cambridge, MA: Harvard University Press.

Campbell, T.W. 2006. Hematologia de Anfíbios. *Hematologia e Bioquímica Clínica Veterinária*, eds. Trall, M.A., Backer, D.C., Campbell, T.C., DeNicola, D.B., Fettman, M.J., Lasse, E.D., Rebar, A. & Weiser, G. Ed. Roca. Pp. 291-300.

Canosa, L.F. & Ceballos, N.R. 2002. Seasonal changes in testicular steroidogenesis in the toad *Bufo arenarum* H. *Gen. Comp. Endocrinol.* 125, 426–434.

Carey, C., Cohen, N. & Rollins-Smith, L. 1999. Amphibian declines: an immunological perspective**.** *Develop. Comp. Immunol.* 23, 459-472.

Carnaval, A.C.O.Q. 2002. Phylogeography of four frog species in forest fragments of northeastern Brazil – a preliminary study. *Integr. Comp. Biol.* 42, 913-921.

Carr, J.A., 2011. Stress and reproduction in amphibians. In: Norris, D.O., Lopez, K.H. (Eds.), Hormones and reproduction of vertebrates. Amphibians, Volume 2. Academic Press, Burlington and San Diego, pp. 99–116.

Casto, J.M., Nolan, V.Jr. & Ketterson, E.D. 2001. Steroid hormones and immune function: Experimental studies in wild and captive dark-eyed juncos (*Junco hyemalis*). *Am. Nat.* 157, 408-420.

Daszak, P., Scott, D.E., Kilpatrick, A.M., Faggioni, C., Gibbons, J.W. & Porter, D. 2005. Amphibian population declines at savannah river site are linked to climate, not chytridiomycosis. *Ecol.* 86, 3232–3237.

Davis, A.K. & Maerz, J.C. 2008. Comparison of Hematological Stress Indicators in Recently Captured and Captive Paedomorphic Mole Salamanders, *Ambystoma talpoideum. Copeia*. 3, 613–617.

Davis, A.K. & Maerz, J.C. 2010. Effects of exogenous corticosterone on circulating leukocytes of a salamander (*Ambystoma talpoideum*) with unusually abundant eosinophils. *Int. J. Zool. 2010*, 1–9.

Davis, A.K., Maney, D.L. & Maerz, J.C. 2008. The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. *Funct. Ecol. 22*, 760–772.

Deviche, P., Beouche-Helias, B., Davies, S., Gao, S., Lane, S. & Valle, S. 2014. Regulation of plasma testosterone, corticosterone, and metabolites in response to stress, reproductive stage, and social challenges in a desert male songbird. *Gen. Comp. Endocrinol. 203*, 120–131.

Deviche, P., Breuner, C. & Orchinik, M. 2001. Testosterone, corticosterone, and photoperiod interact to regulate plasma levels of binding globulin and free steroid hormone in dark-eyed juncos, *Junco hyemalis. Gen. Comp. Endocrinol.* 122, 67–77.

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

Deviche, P., Hurley, L., Fokidis, H., Lerbour, B., Silverin, B., Silverin B, et al. 2010. Acute stress rapidly decreases plasma testosterone in a free- ranging male songbird: potential site of action and mechanism. *Gen. Comp. Endocrinol.* 169, 82–90.

Dhabhar FS & McEwen BS. 1999. Enhancing versus suppressive effects of stress hormones on skin immune function. *Proc. Natl. Acad. Sci.* 96, 1059–1064.

Dhabhar, F.S. 2009. Enhancing versus suppressive effects of stress on immune function: Implications for immunoprotection and immunopathology. *Neuroimmunomodulation*. 16, 300–317.

Dong, Q., Salva, A., Sottas, C.M., Niu, E., Holmes, M. & Hardy, M.P. 2004. Rapid glucocorticoid mediation of suppressed testosterone biosynthesis in male mice subjected to immobilization stress. *J. Androl.* 25, 973-981.

Duellman, W.E. 1999. Global distribution of amphibians: patterns, conservation, and future challenges, pp. 1-30. In: W.E. Duellman (Ed.), Patterns of Distribution of Amphibians: a Global Perspective. The John Hopkins University Press, Baltimore.

Dunlap, K.D. & Wingfield, J.C. 1995. External and internal influences on indices of physiological stress. I. Seasonal and population variation in adrenocortical secretion of free-living lizards, *Sceloporus occidentalis*. *J.Exp. Zool.* 271, 36-46.

DuRant, S.E., Romero, L.M., Talent, L.G. & Hopkins, W.A. 2008. Effect of exogenous corticosterone on respiration in a reptile. *Gen. Comp. Endocrinol.* 156, 126–133.

Emerson, S.B. 2001. Male advertisement calls: behavioral variation and physiological processes. In: Ryan, M.J. (Ed.), Anuran Communication. Smithsonian Institution Press, Washington and London, pp. 36–44.

Folstad, I. & Karter, A.J. 1992. Parasites, bright males, and the immunocompetence handicap. *Am. Nat.* 139, 603-622.

Gadek-Michalska, A., & Bugajski, J. 2003. Repeated handling, restraint, or chronic crowding impair the hypothalamic-pituitary-adrenocortical response to acute restraint stress. J. Physiol. Pharmacol. 54, 449-460.

Gomes, F.R., Oliveira, R.V., Assis, V.R., Titon Jr., B., Moretti, E.H. & Mendonça, M.T. 2012. Interspecific Variation in Innate Immune Defenses and Stress Response of Toads from Botucatu (São Paulo, Brazil). South Amer. J. Herpetol. 7, 1-8.

Graham, S.P, Kelehear, C., Brown, G.P. & Shine, R. 2012. Corticosterone-immune interactions during captive stress in invading Australian cane toads (*Rhinella marina*). *Horm. Behav*. 62, 146–53

Greenberg, N. & Wingfield, J.C. 1987. Stress and reproduction: reciprocal relationships. In: Norris DO, Jones RE, editors. Hormones and reproduction in fishes, amphibians and reptiles. New York: Plenum Press. Pp. 461–502.

Greenman, C. G., Martin, L. B. & Hau, M. 2005. Reproductive state, but not testosterone, reduces immune function in male house sparrows (*Passer domesticus*). *Physiol. Biochem. Zool.* 78, 60–68.

Hamilton, W.D. & Zuk, M. 1982. Heritable true fitness and bright birds: a role for parasites? *Science.* 218, 384-387.

Hammond, T.T., Palme, R. & Lacey, E.A. 2015. Contrasting stress responses of two co-occurring chipmunk species (*Tamias alpinus* and *T. speciosus*). *Gen. Comp. Endocrinol.* 211, 114–122.

Hardy, M.P., Gao, H.B., Dong, Q., Ge, R., Wang, Q., Chai, W.R. et al. 2005. Stress hormone and male reproductive function. *Cell Tissue Res.* 322, 147-153.

Hau, M., Ricklefs, R.E., Wikelski, M., Lee, K.A. & Brawn, J.D. 2010. Corticosterone, testosterone and life-history strategies of birds. *Proceedings of the Royal Society B: Biological Sciences.* 277, 3203-3212.

Hayes, T.B., Falso, P., Gallipeau, S. & Stice, M. 2010. The cause of global amphibian declines: a developmental endocrinologist's perspective. *J. Experim. Biol.* 213, 921-933.

Hopkins, W.A. & DuRant, S.E. 2011. Innate immunity and stress physiology of eastern hellbenders (*Cryptobranchus alleganiensis*) from two stream reaches with differing habitat quality. *Gen. Comp. Endocrinol.* 174, 107–115.

Hopkins, W.A., Mendonça, M.T. & Congdon, J.D. 1997. Increased circulating levels of testosterone and corticosterone in southern toads, *Bufo terrestris*, exposed to coal combustion wastes. *Comp. Biochem. Physiol*. 122C, 191-196.

Horrocks, N.P.C., Matson, K.D. & Tieleman, B.I. 2011. Pathogen pressure puts immune defense into perspective. *Integr. Comp. Biol.* 51, 563–576.

Houck, L.D. &Woodley, S.K. 1995. Field studies of steroid hormones and male reproductive behavior in amphibians. In: Heatwole, H. (Ed.), Amphibian biology. Social Behaviour, 2. Surrey Beatty & Sons Pty Ltd., Australia, pp. 677–703

Hu, G.X., Lian, Q.Q., Lin, H., Latif, S.A., Morris, D.J., Hardy, M.P. & Ge, R.S. 2008. Rapid mechanisms of glucocorticoid signaling in the Leydig cell. *Steroids*. 73, 1018-1024.

INPE (Instituto Nacional de Pesquisas Espaciais): http://sinda.crn2.inpe.br/PCD/SITE/novo/site/index.php

Janin, A., J.P. Léna, & Joly, P. 2011. Beyond occurrence: body condition and stress hormone as integrative indicators of habitat availability and fragmentation in the common toad. *Biol. Conserv.* 144, 1008-1016.

Jessop, T.S., Woodford, R. & Symonds, M.R. 2013. Macrostress: do large-scale ecological patterns exist in the glucocorticoid stress response of vertebrates? *Funct. Ecol.* 27, 120-130.

Jones, S. & Bell, K. 2004. Plasma corticosterone concentrations in males of the skink *Egernia whitii* during acute and chronic confinement, and over a diel period. *Comp. Biochem. Physiol*. 137,105–113.

Landys, M.M., Ramenofsky, M. & Wingfield, J.C., 2006. Actions of glucocorticoids at a seasonal baseline as compared to stress-related levels in the regulation of periodic life processes. *Gen. Comp. Endocrinol.* 148, 132–149

Lattin, C.R. & Romero, L.M. 2013. Seasonal variation in corticosterone receptor binding in brain, hippocampus, and gonads in House Sparrows (*Passer domesticus*). *The Auk*. 130, 591-598.

Lee, K.A. & Klasing, K.C. 2004. A role for immunology in invasion biology. *Trends Ecol. Evol.* 19, 523–529.
Lee, K.A. 2006. Linking immune defenses and life history at the levels of the individual and the species. *Integr. Comp. Biol.* 46, 1000-1015.
Llewellyn, D., Thompson, M.B., Brown, G.P., Phillips, B.L. & Shine, R. 2012. Reduced investment in immune function in invasion-front populations of the cane toad (*Rhinella marina*) in Australia. *Biol. Inv.* 14, 999–1008.
Love, O.P., Shutt, L.J., Silfies, J.S. & Bird, D.M. 2003. Repeated restraint and sampling results in reduced corticosterone levels in developing and adult captive American kestrels (*Falco sparverius*). Physiol. Biochem. Zool. 76, 753-761.
Maciel, N.M., Collevatti, R.G., Colli, G.R. & Schwartz, E.F. 2010. Late Miocene diversification and phylogenetic relationships of the huge toads in the *Rhinella marina* (Linnaeus, 1758) species group (Anura: Bufonidae). *Mol. Phylog. Evol. 57*, 787-797.
Martin, L.B., Hopkins, W.A., Mydlarz, L.D. & Rohr, J.R. 2010. The effects of anthropogenic global changes on immune functions and disease resistance. *Ann. NY Acad. Sci.* 1195, 129-148.
Martin, L.B., Weil, Z.M. & Nelson, R.J. 2008. Seasonal changes in vertebrate immune activity: mediation by physiological trade-offs. *Philos. Trans. R. Soc. London Ser. B.* 363, 321-339.
Martin, L.J. & Tremblay, J.J. 2008. Glucocorticoids antagonize cAMP-induced Star transcription in Leydig cells through the orphan nuclear receptor NR4A1. *J. Mol. Endocrinol.* 41, 165-175.
Matson, K.D., Tieleman, B.I. & Klassing, K.C. 2006. Capture stress and the bactericidal competence of blood and plasma in five species of tropical birds. *Physiol. Biochem. Zool.* 79, 556-564.
Mendonça, M.T., Chernetsby, S.D., Nester, K.E. & Gardner, G.L. 1996. Effects of sex steroids on sexual behavior in the big brown bat, *Eptesicus fuscus*. *Horm. Behav.* 30, 153-161.
Millet, S., Bennett, J., Lee, K.A., Hau, M. & Klassing, K.C. 2007. Quantifying and comparing constitutive immunity across avian species. *Develop. Comp. Immun.* 31, 188-201.
Mills, S.C., Grapputo, A., Jokinen, I., Koskela, E., Mappes, T. & Poikonen, T. 2010. Fitness trade-offs mediated by immunosuppression costs in a small mammal. *Evol.* 64, 166-179.
Mitchell, C.E. & Power, A.G. 2003. Release of invasive plants from fungal and viral pathogens. *Nature*. 421, 625–627.
Moore, F.L., Boyd, S.K. & Kelley, D.B. 2005. Historical perspective: Hormonal regulation of behaviors in amphibians. *Horm. Behav.* 48,373-383.
Moore, I.T. & Jessop, T.S. 2003. Stress, reproduction, and adrenocortical modulation in amphibians and reptiles. *Horm. Behav*. 43, 39–47.
Moore, M.C., Thompson, C.W. & Marler, C.A. 1991. Reciprocal changes in corticosterone and testosterone levels following acute and chronic handling stress in the tree lizard, *Urosaurus ornatus*. *Gen. Comp. Endocrinol.* 81, 217–226.
Morrow-Tesch, J.L., McGlone, J.J. & Norman, R.L. 1993. Consequences of restraint stress on natural killer cell activity, behavior, and hormone levels in rhesus macaques (*Macaca mulatta*). *Psychoneuroendocrinol. 18*, 383–395.
Muller, C., Jenni-Eiermann, S. & Jenni, L. 2011. Heterophils/lymphocytes-ratio and circulating corticosterone do not indicate the same stress imposed on Eurasian kestrel nestlings. *Funct. Ecol.* 25, 566–576.
Narayan E., Hero J.M. & Cockrem, J.F. 2012. Inverse urinary corticosterone and testosterone responses to different durations of restraint in the cane toad (*Rhinella marina*). *Gen. Comp. Endocrinol.* 179, 345–349.

Narayan, E.J., Cockrem, J.F. & Hero, J.M. 2011a. Urinary corticosterone metabolite responses to capture and captivity in the cane toad (*Rhinella marina*). *Gen. Comp. Endocrinol.* 173, 371–377

Narayan, E.J., Molinia, F.C., Kindermann, C., Cockrem, J.F. & Hero, J.M. 2011b. Urinary corticosterone responses to capture and toe-clipping in the cane toad (*Rhinella marina*) indicate that toe-clipping is a stressor for amphibians. *Gen. Comp. Endocrinol.* 174, 238-245.

Navas, C.A. & Otani, L. 2007. Physiology, environmental change, and anuran conservation. *Phyllomedusa*. 6, 83–103.

Norris, D.O., Felt, S.B., Woodling, J.D. & Dores, R.M. 1997. Immunocytochemical and histological differences in the interregnal axis of feral brown trout, *Salmo trutta*, in metal-contaminated waters. *Gen. Comp. Endocrinol.* 108, 343-351.

Norris, K. & Evans, M.R. 2000. Ecological immunology: life history trade-offs and immune defense in birds. *Behav. Ecol.* 11, 19-26.

Phillips, B.L., Brown, G.P., Travis, J.M. & Shine, R. 2008. Reid's paradox revisited: the evolution of dispersal kernels during range expansion. *Am. Nat.* 172, S34–S48

Preest, M.R. & Cree, A. 2008. Corticosterone treatment has subtle effects on thermoregulatory behavior and raises metabolic rate in the New Zealand common gecko, *Hoplodactylus maculatus*. *Physiol. Biochem. Zool.* 81, 641–650.

Rich, E.L. & Romero, L.M. 2005. Exposure to chronic stress down regulates corticosterone responses to acute stressors. *Amer. J. Physiol.* 288, R1628-R1636.

Roberts, M.L., Buchanan, K.L. & Evans, M.R. 2004. Testing the immunocompetence handicap hypothesis: a review of the evidence. *Anim. Behav.* 68, 227-239.

Romero, L.M. & Reed, J.M. 2005. Collecting baseline corticosterone samples in the field: is under 3 min good enough? *Comp. Biochem. Physiol.* 140, 73-79.

Romero, L.M. 2002. Seasonal changes in plasma glucocorticoid concentrations in free-living vertebrates. *Gen.Comp. Endocrinol.* 128, 1–24.

Sapolsky, R.M. 1992. Stress, the aging brain and the mechanisms of neuron death. Cambridge, MA: MIT Press.

Sapolsky, R.M., Romero, L.M. & Muck, A.U. 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocrinol Rev.* 21, 55–89.

Seddon, R. J., & Klukowski, M. 2012. Influence of Stressor Duration on Leukocyte and Hormonal Responses in Male Southeastern Five-Lined Skinks (Plestiodon inexpectatus). *J. Experim. Zool. 317*, 499–510.

The IUCN red list of threatened species: http://maps.iucnredlist.org/

Tingley, M.W., Monahan, W.B., Beissinger, S.R. &Moritz, C. 2009. Birds track their grinnellian niche through a century of climate change. *Proc. Natl. Acad. Sci.* 106, 19637–19643.

Titon Jr., B., Navas, C.A., Jim, J. & Gomes, F.R. 2010. Water balance and locomotor performance in three species of neotropical toads that differ in geographical distribution. *Comp. Biochem. Physiol.* 156, 129–135.

Tokarz, R.R. & Summers, C.H. 2011. Stress and reproduction in reptiles. In: Norris, D.O., Lopez, K.H. editors. Hormones and reproduction in vertebrates. Vol. 3: reptiles. New York: Academic Press. Pp. 169–213.

Tomanek, L. 2012. Environmental proteomics of the mussel Mytilus: implications for tolerance to stress and change in limits of biogeographic ranges in response to climate change. *Integr. Comp. Biol.* ics114.

Tompkins, D.M., White, A.R. & Boots, M. 2003. Ecological replacement of native red squirrels by invasive greys driven by disease. *Ecol. Lett.* 6, 189–196.

Torchin, M.E., Lafferty, K.D., Dobson, A.P., McKenzie, V.J. & Kuris, A.M. 2003. Introduced species and their missing parasites. *Nature.* 42, 628–630.

Verhulst, S., Riedstra, B. & Wiersma, P. 2005. Brood size and immunity costs in zebra finches *Taeniopygia guttata*. *J. Avian Biol.* 36, 22-30.

Vleck, C.M., Vertalino, N., Vleck, D. & Bucher, T.L. 2000. Stress, corticosterone, and heterophil to lymphocyte ratios in free-living adelie penguins. *The Condor*. 102, 392–400.

Wack, C.L., DuRant, S.E., Hopkins, W.A, Lovern, M.B., Feldhoff, R.C. & Woodley, S. K. 2012. Elevated plasma corticosterone increases metabolic rate in a terrestrial salamander. *Comp. Biochem. Physiol.* 161, 153–158.

Wiegers, G.J. & Reul, J.M.H.M. 1998. Induction of cytokine receptors by glucocorticoids: functional and pathological significance. *Trends Pharmacol. Sci.* 19, 317–321.

Wikelski, M. & Cooke, S.J. 2006. Conservation physiology. *Trends Ecol. Evol.* 21, 38–46.

Wilckens, T. & DeRijk, R. 1997. Glucocorticoids and immune function: unknown dimensions and new frontiers. *Immunol. Today.* 18, 418–424.

Wingfield, J.C. & Romero, L.M. 2001. Adrenocortical responses to stress and their modulation in free-living vertebrates," in Coping with the Environment: Neural and Endocrine Mechanisms, B. S. McEwen and H. M. Goodman, Eds., vol. 4 of Handbook of Physiology, Section 7: The Endocrine System,pp. 211-234, Oxford University Press, New York, NY, USA.

Wingfield, J.C. 2013. The comparative biology of environmental stress: Behavioural endocrinology and variation in ability to cope with novel, changing environments. *Anim. Behav.* 85, 1127–1133.

Wingfield, J.C., Hunt, K., Breuner, C., Dunlop, K., Fowler, G.S., Freed, L. & Lepson, J. 1997. Environmental stress, field endocrinology, and conservation biology. In Behavioral approaches to conservation in the wild: 95–131. Clemmons, J. R. & Buchholz, R. (Eds). Cambridge: Cambridge University Press.

Zerani, M., Vellano, C., Amabili, F., Carnevali, O., Andreoletti, G.E. & Polzonetti-Magni, A. 1991. Sex steroid profile and plasma vitellogenin during the annual reproductive cycle of the crested newt (*Triturus carnifex laur.*). *Gen. Comp. Endocrinol.* 82, 337–344.

CHAPTER 3 – EFFECTS OF ACUTE RESTRAINT STRESS, PROLONGED CAPTIVITY STRESS AND TRANSDERMAL CORTICOSTERONE APPLICATION ON IMMUNOCOMPETENCE AND PLASMA LEVELS OF CORTICOSTERONE ON THE CURURU TOAD (*Rhinella icterica*)

Vania Regina de Assis[1*], Stefanny Christie Monteiro Titon[1], Adriana Maria Giorgi Barsotti[1], Braz Titon Jr[1], Fernando Ribeiro Gomes[1]

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

* Corresponding author. Tel.: +55 11 3091 7522; fax: +55 11 3091 8095

E-mail address: v.regina.a@gmail.com

This chapter is a published paper: Assis, V.R., Titon, S.C.M., Barsotti, A.M.G., Titon Jr., B. & Gomes, F.R. 2015. Effects of Acute Restraint Stress, Prolonged Captivity Stress and Transdermal Corticosterone Application on Immunocompetence and Plasma Levels of Corticosterone on the Cururu Toad (*Rhinella icterica*). ***PLOS ONE,*** *10*(4), 1–21. doi:10.1371/journal.pone.0121005

3.1. Abstract

Glucocorticoid steroids modulate immunocompetence in complex ways with both immunoenhancing and immunosuppressive effects in vertebrates exposed to different stressors. Such bimodal effects have been associated with variation in duration and intensity of the stress response. Given that natural populations have been exposed to a multitude of stressors, a better understanding of the functional association between duration and intensity of the stress response, the resulting changes in glucocorticoid plasma levels and their impact on different aspects of immunocompetence emerges as a cornerstone for vertebrate conservation strategies. We investigated the effects of a restraint challenge (with and without movement restriction), long-term captivity, and transdermal corticosterone application on plasma levels of corticosterone (hereinafter referred to as CORT) and different parameters of innate immunocompetence in the male cururu toads (*Rhinella icterica*). We show that for *R. icterica* restraint for 24h proved to be a stressful condition, increasing CORT by 3-fold without consistent immunological changes. However, the application of a more intense stressor (restraint with movement restriction), for the same period, potentiated this response resulting in a 9-fold increase in CORT, associated with increase Neutrophil/Lymphocyte ratio (N:L) and a lower bacterial killing ability (BKA). Transdermal application of corticosterone efficiently mimics repeated acute stress response events, without changing the immune parameters even after 13 days of treatment. Interestingly, long-term captivity did not mitigate the stress response, since the toads maintained 3-fold increased CORT even after 3 months under these conditions. Moreover, long-term captivity in the same condition increased total leukocyte count (TLC) and generated an even greater decrease in BKA, suggesting that consequences of the stress response can be aggravated by time in captivity.

Keywords: Amphibia; Corticosterone; Captivity; Toad; Immunocompetence; Restraint; Stress; Transdermal application

3.2. Introduction

Glucocorticoid hormones are produced by adrenal or interrenal glands, and their release is modulated by several stressors through the activation of the hypothalamic pituitary-interrenal axis (HPI) in ectotherms vertebrates [1]. In a short-term period, the activation of the HPI axis may have beneficial effects, such as temporary suppression of reproduction, increased foraging activity, gluconeogenesis, and regulation of the immune response [2,3]. However, when the HPI axis is activated for longer periods, negative functional consequences such as chronic suppression of growth, reproductive and immune function, and neuronal death have been reported [3–5]. Regarding the immune modulation, the immunosuppressive effects of glucocorticoids have been more commonly observed in contexts of intense and chronic activation of the HPI axis. These immunosuppressive effects include inhibition of the synthesis, release and efficiency of several cytokines and other mediators that promote the immune response and inflammatory reactions, and atrophy of lymphoid tissues, particularly the thymus [1,6]. Moreover, immune-enhancing effects have been very commonly observed in the context of low-intensity and short-term activation of the HPI axis, include an increased expression of receptors for different cytokines [6], and redistribution of immune cells within the body, with corresponding increased traffic of leukocytes and enhanced immune function in organs such as the skin [7,8]. Studies have also identified mechanisms involving dendritic cell, neutrophil, macrophage, and lymphocyte trafficking, maturation, and function through which acute stressors may enhance innate as well as adaptive immunity [7,8]. This bimodal effect of glucocorticoids on the immune response can be mediated by different concentrations of these hormones and possibly different receptors [6,9–11]. As an example, glucorticoid increase T-cell response at low concentration, an effect possibly mediated through mineralocorticoid receptors. Otherwise, these hormones decrease the T-cell response at high concentration, an effect possibly mediated through glucocorticoid receptors [9–11].

Experiments conducted in captivity, where conditions can be carefully controlled, are useful for examining complex biological phenomena, such as the inter-relationships between the levels of glucocorticoids and immune function in different stages of the life cycle [12,13]. However, few studies have examined how the captivity itself affects the activation of the HPI axis and immune function. Captive birds (*Calidris canutus*) show reduced antimicrobial killing ability *in vitro* and the number of circulating heterophils and eosinophils when compared to free-ranging individuals [14]. Moreover, the effects of captivity maintenance may change through time, and such changes are presently difficult to predict for different vertebrates [15,16]. Newly captured animals might show an initial strong release of glucocorticoids and a consequent immunosuppression [17]. However, they might habituate to captivity conditions through time, decreasing glucocorticoid plasma levels and improving immune response. Alternatively, the stress response could increase with time, resulting in an even stronger immunosuppression [18–24]. These responses might be species-specific, and might reflect ecological associations through evolutionary history and show important implications for strategies of conservation.

Amphibian populations are undergoing large declines, and the main causes of this decline include habitat loss and fragmentation, occurrence of infectious diseases, habitat pollution, and introduction of exotic species that may become predators/competitors to native species [25,26]. Several infectious agents have been associated with population declines of amphibians including: 1) Chytrid fungus parasite (Bd: *Batrachochytrium dendrobatidis*) [27,28]; 2) Pathogenic bacteria, such as *Aeromonas hydrophilla* [29,30]; and 3) Ranavirus [31]. An interesting fact is that in most cases of mortality events caused by these infectious agents, there were reports of other non-affected amphibian species occupying the same habitat [25]. Amphibians are a vertebrate group that is particularly sensitive to different environmental changes and, consequently, studies of the effects of stressors on immune and reproductive

functions are needed to define conservation strategies, including captive breeding [32] and immunization [33].

We investigated the effects of short-term restraint (with and without movement restriction), long-term captivity, and transdermal corticosterone application on plasma levels of corticosterone (hereinafter referred as CORT) and innate immunocompetence in male cururu toads (*Rhinella icterica*). We tested the following hypotheses: 1) Restraint challenge in newly captured toads (maintaining the toads for 24h of captivity) can be considered a stressor, promoting elevation of CORT and reduced immunocompetence relative to baseline values in natural environment; 2) Restraint challenge with movement restriction in newly captured toads (maintained in moistened cloth bags for 24h) can be considered a stronger stressor, promoting more intense effects of CORT elevation and immunossupression than captivity maintenance; 3) Keeping animals in prolonged captivity conditions (three months) could mitigate the effects of restraint challenge for 24h (with or wihout movement restriction); 4) Transdermal corticosterone application should cause an increase in CORT and a reduction in immunocompetence, mimicking the effects of a potent stressor, such as restraint with movement restriction.

3.3. Materials and methods

3.3.1. Animals and study site

Rhinella icterica is a large toad from the *R. marina* group [34]. This species shows geographic distribution associated with forested habitats (Atlantic rain forest), although these toads are common in anthropomorphized areas [34]. Males were collected in February 2012 (*N* = 23), in São Luiz do Paraitinga (23°13'23" S; 45°18'38" WO), São Paulo, Brazil. These individuals were used to test the effects of prolonged captivity stress and for the experimental manipulation of corticosterone levels (transdermal corticosterone application). Twenty

additional male toads were collected in January 2013, at the same location, and were used to test the effects of restraint stress with and without movement restriction. Although all these toads were kept during the reproductive season, they were not calling. The collections were performed under authorization from Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA, process 17895-1) and laboratory procedures were performed under the approval of the Comissão de Ética no Uso de Animais (CEUA) do Instituto de Biociências da Universidade de São Paulo (CEUA - n° 142/2011).

3.3.2. Collecting and processing of blood samples

Animals were located by visual inspection, captured and blood was collected in the field (about 200 µl) via cardiac puncture with 1 ml syringes and needles 26Gx1/2" previously heparinized. The blood samples were considered only if collection was performed within 3 min after animal capture, in order to avoid any influence of the stress of capture and manipulation on hormone levels [35].

All blood samples were identified and kept on ice until they were divided into two aliquots on the same night. One of these aliquots was used for total leukocyte count, blood smear (for further analysis of leukocyte profile), and analysis of hematocrit. The other aliquot was centrifuged to isolate the plasma (4 min at 3000 rpm). Plasma samples (a range 100-150 µl) were stored in cryogenic tubes, and kept in liquid nitrogen until they could be transferred to a -80°C freezer, for posterior hormone assays and analyses of bacterial killing ability.

3.3.3. Analysis of blood parameters

d) Total leukocyte count (TLC)

On the same night of blood sampling, 5 µl of blood were diluted in 120 µl of saline solution of toluidine blue (0.01%). The toluidine blue stains cells, facilitating differentiation of

leukocytes and erythrocytes. Ten microliters of this dilution was placed on a hemocytometer, and TLC were performed under a light microscope (40X objective - Nikon E200, 104c). The number of leukocytes was counted in one quadrant and multiplied by the dilution factor (25X).

e) Hematocrit (HEM)

HEM was calculated as the proportion of blood cells in relation to the total volume of blood after centrifugation of the blood contained in a microhematocrit tube (4 min at 3000 rpm).

f) Leukocyte profile

A drop of blood (about 2 µl) was used to perform each blood smear slide. Two slides were made for each animal and, subsequently, one of these slides was stained with Giemsa solution (10%) and observed under an optical microscope (100X objective, using oil immersion - Nikon E200, 104c). For differential leukocyte counts, 100 leukocytes were counted on each slide, and classified based on morphology as neutrophils, lymphocytes, eosinophils, basophils, and monocytes [36]. Based on the leukocyte profile, the ratio between neutrophils and lymphocytes (N:L) was calculated.

g) Bacterial killing ability (BKA)

This assay was conducted according to [37]. Briefly, plasma samples diluted (1: 20) in Ringer's solution (10 µl plasma: 190 µl Ringer) were mixed with 10 µl of *E. coli* working solution (~10^4 microorganisms). Positive controls consisted of 10 µl of *E. coli* working solution in 200 µl of Ringer's solution, and negative control contained 210 µl of Ringer's solution. All samples and controls were incubated for 30 min at 37ºC. After the incubation period, 500 µl of tryptic soy broth (TSB) were added to each sample. The bacterial suspensions were thoroughly mixed and 300 µl of each one were transferred (in duplicates) to a 96 wells microplate. The

microplate was incubated at 37ºC for 2 hours, and thereafter the optical density of the samples was measured hourly in a plate spectrophotometer (wavelength 600 nm), totaling 4 readings. The BKA was 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. The bacterial killing ability was evaluated at the beginning of the bacterial exponential growth phase.

h) Hormonal assay

- Plasma extraction

Plasma samples were extracted with ether according to [38]. Briefly, 3 ml of ether was added to 10 µl of each sample, and then vortexed for 30 seconds and centrifuged (4°C, 9 min, at 1800 rpm). Next, the samples were allowed to decant in -80ºC freezer for 7 min and the liquid phase was transferred to another tube. These tubes were kept in laminar flow hood at room temperature (20 ± 2°C), until all of the ether had evaporated (approximatelly 24h). The samples were resuspended in EIA buffer and CORT was assayed using EIA kits (number 500655, Cayman Chemical), according to the manufacturer's instructions.

- Validation of the enzyme-immunoassay

To validate the use of the Cayman kit for anurans, we tested if the kits were sensitive to detect alterations in CORT in response to: 1) 24h of captivity; and 2) Transdermal corticosterone application in *R. icterica.*

Based on previous studies, mean baseline CORT values measured by radioimmunoassay for *R. icterica* were 18.5 ng/ml [22]. Given that the standard curve of this Cayman kit is expressed in pg/ml, we knew that large sample dilutions would be necessary. In this way, all samples were run in duplicates, and at three different dilutions. For baseline values, we used

1:50, 1:75 and 1:150 dilutions and after aforementioned stress procedures, we used 1:100, 1:200 and 1:300.

Baseline CORT values for *R. icterica* (7.7 ± 6.1 ng/ml) were within the range expected based on [22]. The somewhat lower values measured with EIA reflect the fact that these toads were not calling. Maintenance of *R. icterica* in captivity for 24h promoted a 3-fold increase in CORT (20.3 ± 9.5 ng/ml), and transdermal corticosterone application increased CORT in 12-fold (156.9 ± 90.2 ng/ml).

By testing 15 duplicates on each plate, we estimated intra-assay variation to be 8.3% and inter-assay variation was estimated using the average of four intermediate values from the standard curve (as recommended by the kit instructions) and it was 14.8% for *R. icterica.* Sensitivity of the assay was 30 pg/ml.

3.3.4. Phytohemagglutinin (PHA) skin swelling assay

An immunological challenge with PHA was performed to assess the cell-mediated innate immunity, and this procedure was performed 24h after the end of treatment with transdermal corticosterone application.

The hind fleshy base of the right foot was injected with 10 µl of a 20 mg/ml solution of PHA (Sigma L8754) in sterile saline solution using a 10 µl glass syringes and 30Gx1/2" needles. As a control, the hind fleshy base of the left foot was injected with 10 µl of sterile saline solution (Figure 3.1A) [39-41]. The thickness of each injected hind fleshy base of the foot was measured prior to injection and 12h after injection using a thickness gauge (Digimess - 0.01mm precision; Figure 3.1B).

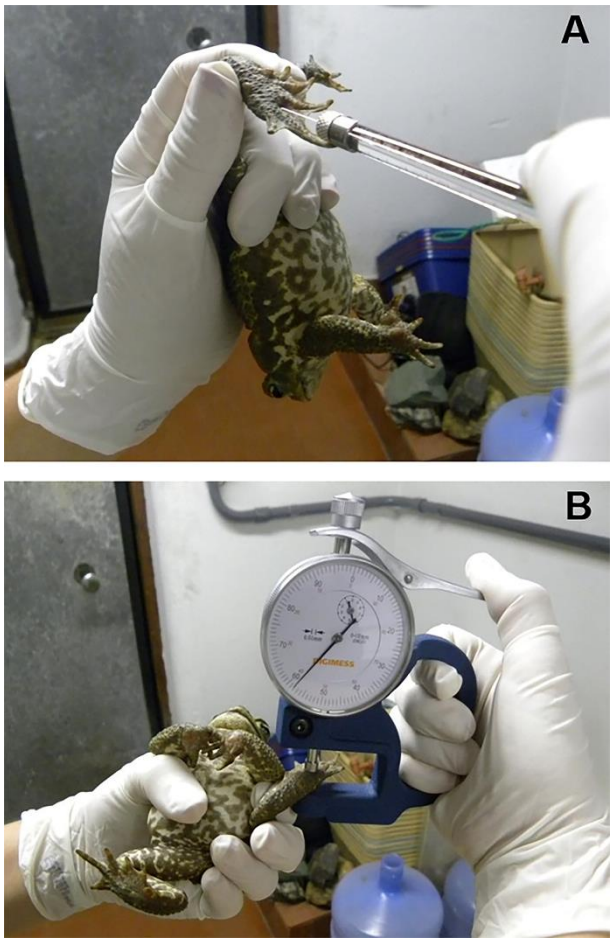

Figure 3. 1. Phytohemagglutinin (PHA) skin-swelling assay. The hind fleshy base of the foot was injected with 10 µl of PHA or sterile saline solution, using a glass syringe (A). The thickness of each hind fleshy base of the foot was measured prior and after 12h of the injection using a thickness gauge: Digimess - 0.01mm precision (B) (Photographs by Stefanny C. M. Titon).

The swelling in response to PHA was calculated from the proportional increase in hind fleshy base of the foot thickness before and postinjection. Thickness of the fleshy base of the foot was consecutively measured three times and a mean of these values was used for the calculations.

3.3.5. Experimental manipulation of hormone levels - transdermal corticosterone application

a) Captive maintenance conditions

Immediately after blood collection in the field, animals were placed into individual plastic containers [20L - 43.0 (L) x 28.5 (W) x 26.5 (H) cm] with free access to water. The lids of the containers had holes to allow air circulation. Animals were exposed to a natural light cycle and temperatures compatible with a natural thermal regime, until they were transferred to the laboratory. In the laboratory, the animals were kept individually for three months in these containers, with free access to water and objects to provide hiding. Lighting conditions and temperature were kept constant (LD 13:11h - 13h of light [light turned on 06h 30min] and 11h of dark [light turned off 19h 30min] and 22 ± 2°C). While in captivity, all animals had free access to water and containers were cleaned and toads fed with crickets and cockroaches once per week.

b) Corticosterone working solution

Based on post-restriction CORT for *R. icterica* [22] and on corticosterone concentrations and volumes used by [42] and [43], we have defined our working concentration as 3 µg corticosterone/ 1 µl sesame oil. We applied one 5 µl drop the solution, comprising 15 µg corticosterone per application. The working solution was prepared by diluting 7.5 mg of crystalline corticosterone (Sigma - 27840) in 750 µl absolute ethanol. This corticosterone dilution was added to 2.5 ml of sesame oil, and then this mixture was vortexed, and remained in an open vial overnight for ethanol evaporation. All animals received the same amount of hormone application, since no relationship was found between body mass and CORT in the field ($r = -0.042$; $P = 0.865$). The two aforementioned studies by [42] and [43] found no

relationship between hormone levels and body mass or body index and neither study corrected the volumes applied to body mass.

c) Transdermal application

One week before starting the application, a blood sample was collected from each individual (around 200 µl) between 18h and 20h, within 3 min, to assess the immunocompetence and CORT after long-term maintenance in captivity (3 months). Therefore, the animals were divided into three groups: Control (N = 7), Placebo (N = 8) and Experimental (N = 8). Animals from the control group did not receive anything on the skin, but the plastic container was opened and a micropipette with an empty tip was approached to their back (between the front legs), simulating an application. For placebo group, the container was opened and one drop of 5 µl sesame oil was administered on the back of the animals using a micropipette. The same procedure was used for individuals from the experimental group using one drop of 5 µl working solution (a mixture of sesame oil + hormone). The time chosen for the application was between 17h and 19h, with 5 min intervals between individuals, in order to temporally match the baseline samples carried out in the field. Additionally, given that these animals are nocturnal, sampling occurred at a time near to the expected peak of CORT, associated with the onset of the activity period [44].

Water was removed to ensure complete absorption of application for all groups before daily application of treatment and was returned 3h after treatment. The transdermal application treatment occurred for 13 consecutive days and, at the end of this period, a new blood sample was collected from each individual (around 200 µl) between 18h and 20h, 1h after the last application, to assess immune parameters and CORT.

One day after the end of the experiment of transdermal application, animals were tested for subcutaneous inflamation response to PHA. The swelling was measured 12h postinjection

of saline and PHA. At the end of this procedure, individuals were euthanatized with an intraperitoneal injection (75mg/kg) of sodium thiopental (Thiopenthax®) solution (25mg/ml).

3.3.6. Comparison between baseline values of CORT and immunocompetence with values after restraint challenge with and without movement restriction

Immediately after blood collection in the field, individuals from the second group of toads captured were randomly placed directly into the individual plastic containers (restraint) or within moistened cloth bags and then in the individual plastic containers (restraint with movement restriction), where they remained for exactly 24h. The lids of the containers had holes to allow air circulation. Animals were exposed to the natural light cycle and temperatures compatible with its natural thermal regime. At the end of 24h, the individuals were bled again to assess measures of immunocompetence and CORT. Upon termination of this experimental protocol, animals were measured (mm), weighed (0.01g) and returned to their collection point at night.

3.3.7. Statistical analysis

The data were initially analyzed with descriptive statistics and Shapiro-Wilk normality test. Some variables showed absence of normality and were transformed to fit the prerequisites of parametric tests: 1) BKA – arccosine; 2) N:L – ln; and 3) CORT – $\log_{10}$. To test for the effects of long-term captivity (three months) on blood parameters (BKA, HEM, TLC, N:L, CORT, neutrophils, lymphocytes, eosinophils, basophils and monocytes) and body mass, we compared these data with those obtained in the field by using paired samples t-test. To test for repeatability in these variables, parametric correlations (Pearson) between data in field and after long-term captivity were used. Correlation tests (Pearson) were also used to investigate possible relationships between variables in the field and after long-term captivity. Treatment effects of

transdermal corticosterone application on blood parameters, body mass and response on the PHA skin-swelling challenge were tested by one-way ANOVA with treatment group as a factor. To test for swelling differences between feet that received saline and PHA, we used paired samples t-tests. To compare the variables in the field and after 24h of restraint with and without movement restriction, we used t-tests for paired and independent samples. All analyzes were performed using SPSS version 17.

3.4. Results

3.4.1. Effects of long-term captivity and transdermal corticosterone application on CORT and immunocompetence

Descriptive statistics for males of *R. icterica* in field conditions, after long-term captivity (3 months), and at the end of the transdermal corticosterone application are shown in Table 3.1. Leukocyte profile for these males, in the field and after long-term captivity, is shown in Figure 3.2.

Long-term captivity resulted in a mean reduction of 41% in BKA and 45% in HEM, in addition to a mean increase of 3-fold in CORT and 2-fold in TLC, without changing the leukocyte profile, N:L and body mass (Figure 3.3, Table 3.2). Individuals with higher HEM also had higher BKA in the field ($r = 0.564$, $P = 0.008$) and in captivity ($r = 0.498$, $P = 0.016$), and individuals with higher TLC also showed higher HEM in field ($r = 0.539$, $P = 0.014$) and in captivity ($r = 0.534$, $P = 0.010$). Individuals showed consistent variation for BKA ($r = 0.448$, $P = 0.037$) and body mass ($r = 0.754$, $P \leq 0.001$), when data in the field and after long-term captivity were compared.

Transdermal corticosterone application for 13 days produced a 6-fold average increase in CORT compared to values found for the Placebo and Control groups ($F_{2,18} = 20.693$, $P \leq 0.001$,

Figure 3.4) 1h post-application, without changing any other blood parameter or body mass ($P \geq 0.115$).

PHA injection caused an increase in hind fleshy base of foot thickness after 12hours ($t = -3.201$, $P = 0.004$), whereas saline did not ($t = 0.747$, $P = 0.464$). Transdermal corticosterone application did not alter the response to injections of PHA ($F_{2,18} = 0.197$, $P = 0.823$) and saline ($F_{2,18} = 0.632$, $P = 0.543$).

3.4.2. Effects of restraint challenge with and without movement restriction on CORT and immunocompetence

Descriptive statistics for males of *R. icterica* in the field, after 24h of restraint with or without movement restriction are shown in Table 3.3.

Individuals kept in captivity within plastic containers for 24h (restraint without movement restriction) showed a mean decrease of 38% in TLC and 3-fold increase in CORT (Figure 3.5), without changes in other measured parameters (Table 3.4). Otherwise, individuals maintained within moistened cloth bags for 24h (restraint with movement restriction), showed a mean decrease of 12% in BKA, a 4-fold increase in N:L ratio, and a 9-fold increase in CORT (Figure 3.5), without changes in other measured parameters (Table 3.4).

When the groups maintained in captivity for 24h in different conditions (restraint with x without movement restriction) were compared, we found 2.4-fold higher CORT and a 2-fold higher N:L ratio for the toads restrained with movement restriction, without changing any other blood parameters (Table 3.5).

Table 3. 1. Descriptive statistics of blood parameters and body mass for individuals of *R. icterica* under field conditions, after long-term captivity (three months), and at the end of the experiment of transdermal corticosterone application for 13 days.

			Parameter	N	Minimum	Maximum	Mean ± SD
FIELD			BKA (%)	22	0.00	100.00	67.45 ± 40.65
			HEM (%)	22	7.00	45.00	29.32 ± 11.46
			TLC (cells/µl)	21	450	2900	1285 ± 715
			N:L	23	0.00	0.36	0.13 ± 0.09
			CORT (ng/ml)	19	5.56	42.64	12.85 ± 8.38
		Leukocyte Profile (%)	Neutrophil	23	0.00	20.00	8.09 ± 4.69
			Lymphocyte	23	49.00	97.00	71.60 ± 11.68
			Eosinophil	23	0.00	37.00	11.96 ± 8.16
			Basophil	23	0.00	4.00	1.04 ± 1.22
			Monocyte	23	1.00	20.00	7.74 ± 5.15
			Body mass	23	70.99	195.20	127.95 ± 34.74
LONG-TERM CAPTIVITY			BKA (%)	23	0.00	84.00	40.17 ± 28.30
			HEM (%)	23	6.00	25.00	16.02 ± 4.80
			TLC (cells/µl)	23	675	5075	2510 ± 1260
			N:L	23	0.01	0.41	0.12 ± 0.10
			CORT (ng/ml)	23	7.48	93.84	39.90 ± 23.89
		Leukocyte Profile (%)	Neutrophil	23	1.00	22.00	8.00 ± 5.20
			Lymphocyte	23	54.00	88.00	72.09 ± 10.84
			Eosinophil	23	1.00	25.00	10.70 ± 7.26
			Basophil	23	0.00	10.00	1.70 ± 2.63
			Monocyte	23	0.00	17.00	7.52 ± 4.64
			Body mass	23	78.10	175.52	122.69 ± 27.84
TRANSDERMAL CORTICOSTERONE APPLICATION	***Control***		BKA (%)	7	84.00	97.00	90.57 ± 4.65
			HEM (%)	7	13.00	21.00	18.00 ± 2,88
			TLC (cells/µl)	7	1850	3900	2782 ± 777
			N:L	7	0.02	0.39	0.16 ± 0.14
			CORT (ng/ml)	7	14.34	39.69	29.67 ± 10.32
		Leukocyte Profile (%)	Neutrophil	7	2.00	19.00	9.86 ± 7.99
			Lymphocyte	7	49.00	84.00	69.29 ± 12.05
			Eosinophil	7	2.00	14.00	8.86 ± 4.49
			Basophil	7	0.00	16.00	5.14 ± 5.79
			Monocyte	7	2.00	14.00	6.86 ± 4.41
			Body mass	7	89.82	179.98	130.48 ± 33.59
	Placebo		BKA (%)	7	0.00	95.00	56.43 ± 40.57
			HEM (%)	7	7.00	27.00	17.57 ± 6.27
			TLC (cells/µl)	7	600	3925	2439 ± 1313
			N:L	7	0.06	0.66	0.22 ± 0.20
			CORT (ng/ml)	7	7.88	54.43	26.92 ± 16.22
		Leukocyte Profile (%)	Neutrophil	7	5.00	31.00	13.14 ± 8.55
			Lymphocyte	7	47.00	87.00	70.43 ± 12.58
			Eosinophil	7	4.00	21.00	9.57 ± 5.86
			Basophil	7	0.00	7.00	2.71 ± 2.43
			Monocyte	7	1.00	11.00	4.14 ± 3.58
			Body mass	7	76.27	144.95	112.72 ± 21.78
	Experimental		BKA (%)	7	0.00	98.00	74.14 ± 33.68
			HEM (%)	7	10.00	27.00	16.43 ± 6.78
			TLC (cells/µl)	7	750	4375	3064 ± 1283
			N:L	7	0.13	0.64	0.25 ± 0.18
			CORT (ng/ml)	7	56.21	324.82	156.96 ± 90.20
		Leukocyte Profile (%)	Neutrophil	7	10.00	25.00	15.43 ± 4.86
			Lymphocyte	7	39.00	80.00	69.43 ± 14.72
			Eosinophil	7	2.00	10.00	6.57 ± 2.99
			Basophil	7	0.00	18.00	3.57 ± 6.60
			Monocyte	7	2.00	10.00	5.00 ± 3.06
			Body mass	7	82.51	173.87	126.40 ± 32.57

BKA: Bacterial killing ability; **HEM**: Hematocrit; **N:L**: Neutrophil/ Lymphocyte ratio; **TLC**: Total Leukocytes Count; **CORT**: corticosterone plasma levels.

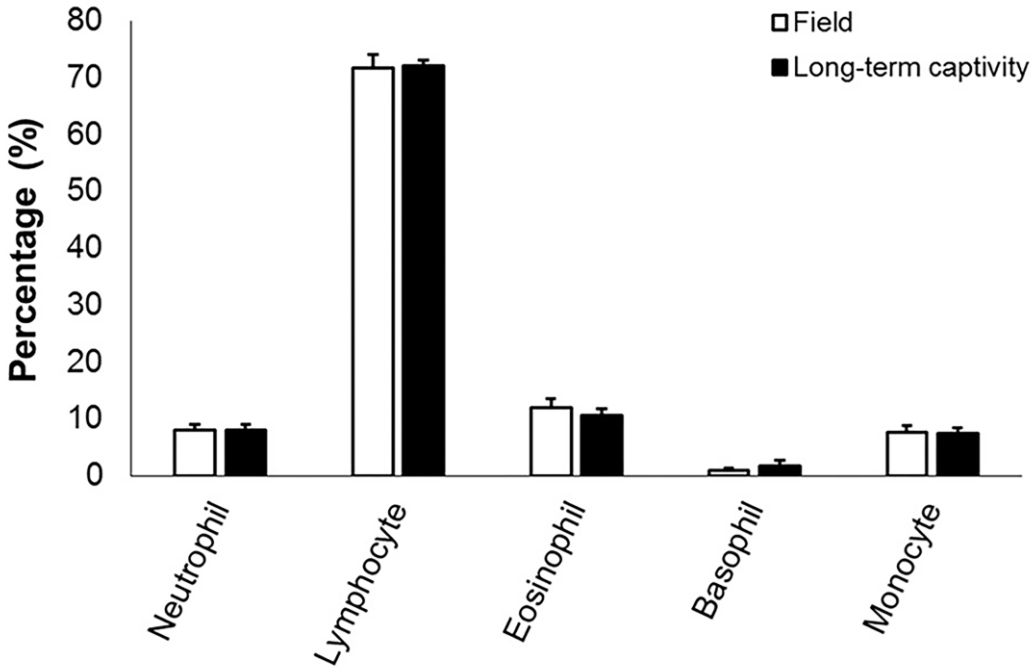

Figure 3. 2. Leukocyte profile of *Rhinella icterica*. Leukocyte profile of adult males of *Rhinella icterica* under field conditions and after long-term captivity (three months). Bars represent mean ± standard error. *N* is the same for all variables (*N* = 23).

Table 3. 2. Comparison between blood parameters and body mass for individuals of *R. icterica* under field conditions and after long-term captivity (three months)

	Parameter	***t-value***	**DF**	***P***
	BKA (%)	4.220	21	**≤ 0.001**
	HEM (%)	5.230	21	**≤ 0.001**
	TLC (cels/µl)	-4.965	20	**≤ 0.001**
	N:L	0.204	22	0.840
	CORT (ng/ml)	-5.659	18	**≤ 0.001**
Leukocyte Profile (%)	Neutrophil	0.056	22	0.956
	Lymphocyte	-0.139	22	0.891
	Eosinophil	0.695	22	0.494
	Basophil	-1.066	22	0.298
	Monocyte	0.179	22	0.860
	Body mass	1.103	22	0.282

Paired samples T-Test. Tests significant at 0.05 are in bold. **BKA**: Bacterial killing ability; **HEM**: Hematocrit; **N:L**: Neutrophil/ Lymphocyte ratio; **TLC**: Total Leukocytes Count; **CORT**: corticosterone plasma levels.

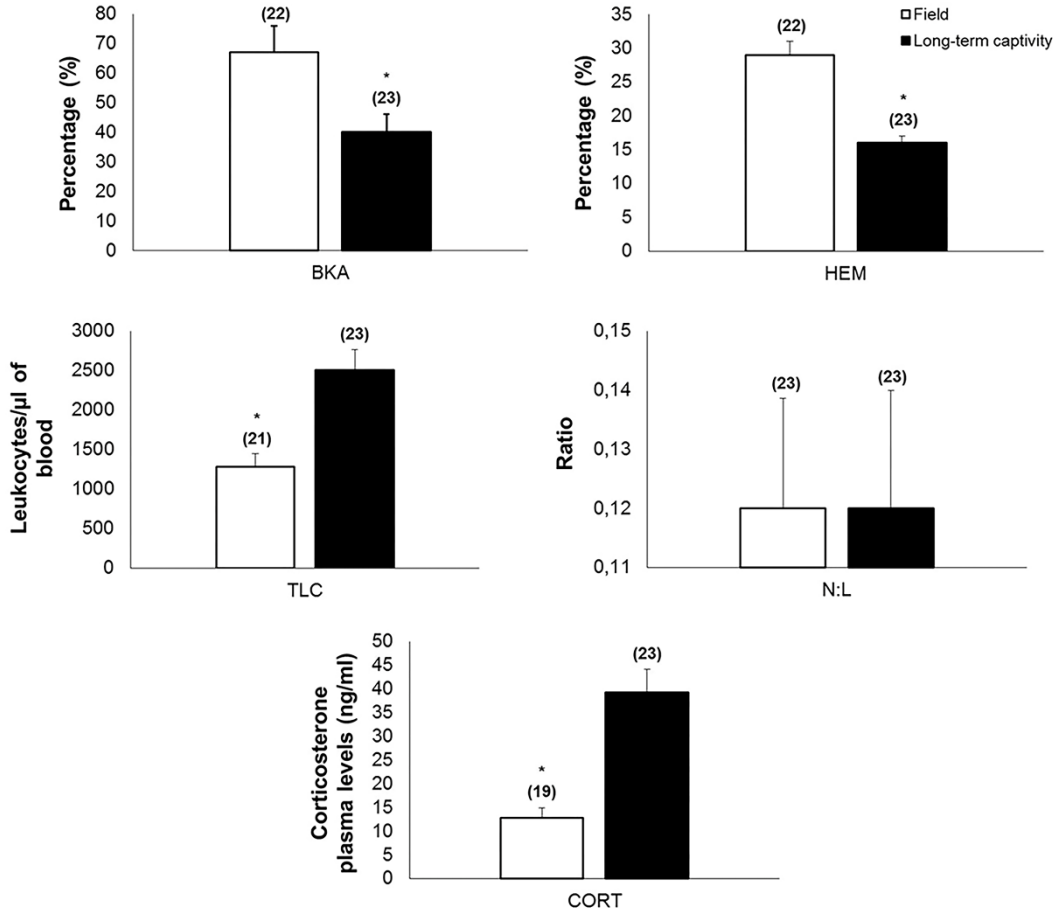

Figure 3. 3. Blood paramenters - comparison between baseline and after long-term captivity. Comparison of blood parameters of toads (*Rhinella icterica*) in the field and after long-term captivity (three months). Bars represent mean ± standard error with *N* in parentheses. **BKA:** Bacterial killing ability; **HEM**: Hematocrit; **N:L**: Neutrophil/ Lymphocyte ratio; **TLC**: Total Leukocytes Count; **CORT**: Corticosterone plasma levels. *$P \leq 0.001$.

Table 3. 3. Descriptive statistics of blood parameters and body mass for individuals of *R. icterica* under field conditions and after restraint challenge with and without movement restriction.

			Parameter	N	Minimum	Maximum	Mean ± SD
FIELD			BKA (%)	20	52.00	100.00	94.00 ± 12.00
			HEM (%)	20	11.00	55.00	33.00 ± 10.00
			TLC (cells/µl)	20	750	5550	2998 ± 1322
			N:L	20	0.08	0.63	0.21 ± 0.15
			CORT (ng/ml)	20	0.76	23.80	7.71 ± 6.12
		Leukocyte Profile (%)	Neutrophil	20	6.00	31.00	13.30 ± 6.34
			Lymphocyte	20	35.00	81.00	69.10 ± 11.10
			Eosinophil	20	4.00	33.00	12.40 ± 7.16
			Basophil	20	0.00	10.00	1.35 ± 2.28
			Monocyte	20	0.00	11.00	3.90 ± 3.00
AFTER 24H OF MAINTENANCE IN CAPTIVITY	***Restraint without movement restriction***		BKA (%)	10	0.00	100.00	82.00 ± 32.00
			HEM (%)	10	11.00	45.00	29.00 ± 12.00
			TLC (cells/µl)	10	700	3400	1903 ± 893
			N:L	10	0.12	0.65	0.33 ± 0.18
			CORT (ng/ml)	9	4.59	36.50	20.32 ± 9.48
		Leukocyte Profile (%)	Neutrophil	10	9.00	32.00	18.70 ± 8.03
			Lymphocyte	10	44.00	78.00	62.10 ± 11.06
			Eosinophil	10	4.00	32.00	12.90 ± 7.58
			Basophil	10	0.00	3.00	0.70 ± 1.16
			Monocyte	10	0.00	15.00	5.60 ± 5.17
	Restraint with movement restriction		BKA (%)	10	45.00	100.00	87.00 ± 19.00
			HEM (%)	10	9.00	38.00	30.00 ± 8.00
			TLC (cells/µl)	10	1300	3575	2323 ± 665
			N:L	10	0.12	1.81	0.66 ± 0.53
			CORT (ng/ml)	10	7.39	165.22	66.40 ± 48.85
		Leukocyte Profile (%)	Neutrophil	10	10.00	56.00	28.80 ± 14.62
			Lymphocyte	10	31.00	82.00	55.10 ± 17.48
			Eosinophil	10	1.00	24.00	9.80 ± 7.67
			Basophil	10	0.00	5.00	1.50 ± 1.90
			Monocyte	10	1.00	8.00	4.80 ± 2.44
			Body mass	20	44.20	188.60	119.88 ± 38.66

BKA: Bacterial killing ability; **HEM**: Hematocrit; **N:L**: Neutrophil/ Lymphocyte ratio; **TLC**: Total Leukocytes Count; **CORT**: corticosterone plasma levels.

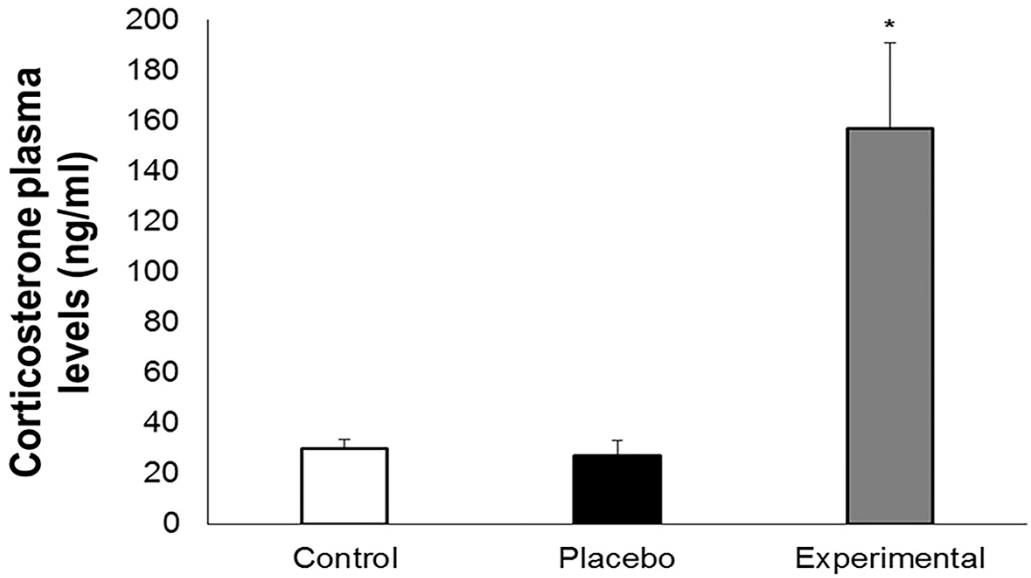

Figure 3. 4. Corticosterone plasma levels after transdermal application. Corticosterone plasma levels at the end of the experiment of transdermal application in *Rhinella icterica*. Bars represent mean ± standard error. *N* is the same for all groups (*N* = 7). **P* ≤ 0.010.

Table 3. 4. Comparison between blood parameters for individuals of *R. icterica* under field conditions and after restraint challenge with and without movement restriction.

			Parameter	*t-value*	DF	*P*
AFTER 24H OF MAINTENANCE IN CAPTIVITY	***Restraint without mevement restriction***		BKA (%)	0.916	9	0.383
			HEM (%)	0.417	9	0.687
			TLC (cells/µl)	2.352	9	**0.043**
			CORT (ng/ml)	-3.352	9	**0.012**
			N:L	-0.753	9	0.471
		Leukocyte Profile (%)	Neutrophil	-0.883	9	0.400
			Lymphocyte	0.931	9	0.376
			Eosinophil	-0.589	9	0.570
			Basophil	0.228	9	0.825
			Monocyte	-0.686	9	0.510
	Resraint with movement restriction		BKA (%)	2.059	9	**0.035***
			HEM (%)	1.310	9	0.223
			TLC (cells/µl)	1.679	9	0.127
			CORT (ng/ml)	-5.875	9	≤ **0.001**
			N:L	-3.074	9	**0.013**
		Leukocyte Profile (%)	Neutrophil	-4.281	9	**0.002**
			Lymphocyte	3.396	9	**0.008**
			Eosinophil	1.667	9	0.130
			Basophil	0.415	9	0.688
			Monocyte	-1.385	9	0.200

Paired samples T-Test. Tests significant at 0.05 are in bold. *Value corresponding to one-tailed test. **BKA**: Bacterial killing ability; **HEM**: Hematocrit; **N:L**: Neutrophil/ Lymphocyte ratio; **TLC**: Total Leukocytes Count; **CORT**: corticosterone plasma levels.

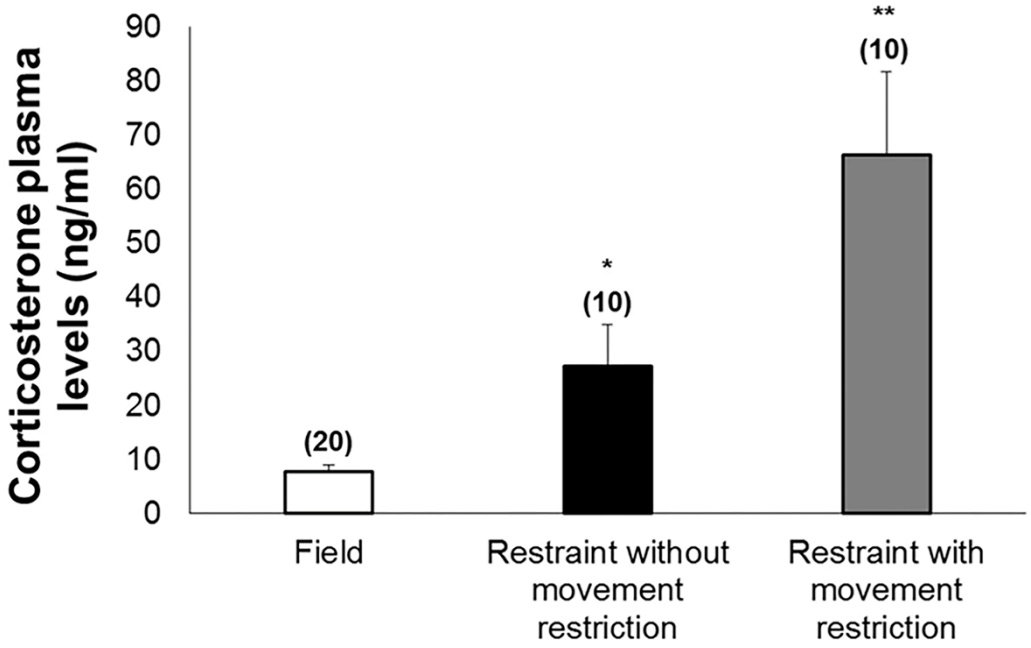

Figure 3. 5. Baseline and after restraint corticosterone plasma levels. Corticosterone plasma levels of toads (*Rhinella icterica*) under field conditions and after 24h of restraint challenge with and without movement restriction. Bars represent mean ± standard error with *N* in parentheses. *$P \leq 0.012$; **$P \leq 0.001$.

Table 3. 5. Comparison between blood parameters and body mass for individuals of *R. icterica* after 24h of restraint challenge with and without movement restriction.

	Parameter	***t-value***	**DF**	***P***
	BKA (%)	-0.266	18	0.793
	HEM (%)	-0.332	18	0.743
	TLC (cels/µl)	-1.193	18	0.248
	CORT (ng/ml)	-2.093	18	**0.051**
	N:L	-1.879	18	**0.038***
Leukocyte Profile (%)	Neutrophil	-1.637	18	0.119
	Lymphocyte	1.070	18	0.299
	Eosinophil	1.321	18	0.203
	Basophil	-0.994	18	0.333
	Monocyte	0.443	18	0.663
	Body mass	0.497	18	0.625

Paired samples T-Test. Tests significant at 0.05 are in bold. *Value corresponding to one-tailed test. BKA: Bacterial killing ability; **HEM**: Hematocrit; **N:L**: Neutrophil/ Lymphocyte ratio; **TLC**: Total Leukocytes Count; **CORT**: corticosterone plasma levels.

3.5. Discussion

3.5.1. Effects restraint challenge and long-term captivity on CORT and immunocompetence

As previously observed for other tetrapods, including anurans kept under captivity for hours to days [21–23,45–50], captivity maintenance for 24h increased CORT in *R. icterica* when compared to values found for free individuals in the field. These data support the interpretation that captivity is a stressor for these animals. Moreover, such increase in CORT levels associated with captivity is dependent on the restriction level imposed. While restraining toads within large plastic containers promoted a 3-fold increase in CORT, restraining toads within moistened bags, for the same period, increased CORT by nine times the free-range values. Previously similar results for *Rhinella* were shown in [22,23] , but in both studies, blood samples after the movement restriction have been collected during the day, while the basal samples were collected during the night. This difference in time of blood sampling employed by previous studies restritcted their conclusions about the scope of stress-response. Interestingly, the group of toads maintained for three months in captivity also showed a 3-fold increase in CORT when compared to the free-range values, suggesting that captivity remains a stressor for a long time, without reduction of the stress response.

Regarding the immune parameters, we found an increase in N:L in toads restrained with movement restriction when compared to both field conditions and restraint without movement restriction. It is known that the increase in CORT promotes changes in transmigration patterns of different leukocyte types between blood and other tissues. This modulation commonly results in a reduction in circulating lymphocyte levels and an increase in production and influx of neutrophils into the blood stream, consequently generating an increase in N:L ratio [51–54] . Although movement restriction increased CORT and N:L in *R. icterica*, as previously observed for other tetrapods [54–58] , a direct correlation between these variable has not been found.

Given that the toads exposed to restraint without movement restriction showed increased CORT without changes in N:L, our results suggest that only stronger stressors, associated with higher CORT, are associated with increased N:L.

Additionally, we have observed somewhat contradictory changes in TLC associated with short-term and long-term captivity stress. While the restraint without movement restriction of toads by 24h decreased TLC by 38%, three months in captivity increased TLC by 2-fold. The initial decrease in TLC might be associated to the short time interval between blood samples collected. However, the same sampling interval was applied to the restraint with movement restriction group, and such effect was not observed for these toads. Increased TLC has been used as a measure of stress (for example, [59]) and injections of steroid hormones in horses promoted doubling TLC within 2h [60]. In this way, the increased CORT along with higher TLC support the interpretation that long-term captivity represents a stressor for these toads.

Restraint with movement restriction and long-term captivity promoted 12% and 41% reduction respectively in the ability to eliminate *E. coli* in *R. icterica*, when compared to field conditions. Reduced BKA had been previously observed in response to restraint challenge in *R.marina* [23], and due to the captivity maintenance in birds [14]. Thus, our data demonstrate that the stress associated with restraint and long-term captivity results in immunosuppression, at least in the humoral innate response [12–14,23,61]. Moreover, these results also corroborate the hypothesis that the stronger acute stressor, associated with higher CORT, promote more intense immunosuppression. Long-term captivity maintenance is associated with an even higher reduction in BKA in *R. icterica*, reinforcing the point that the stress response of these toads does not reduce the stress level even after three months.

Along with the previously described effects of long-term captivity, toads showed reduced HEM in this condition. Due to their high tolerance of dehydration and low skin resistance to water loss, toads are subject to high rates of water loss by evaporation in the terrestrial

environment [62,63]. In this way, the reduction of HEM and BKA after three months in captivity could reflect a higher degree of hydration associated with free access to water under these conditions. However, the lack of long-term captivity effect on body mass and the 2-fold increased TLC do not support the possibility of a pronounced effect of hydration level on these results. Although treatment with hydrocortisone stimulated the production of erythrocytes in frogs [64], the relationship between erythropoiesis and glucocorticoids remains uncertain even in mammals, with evidence of stimulation and inhibition on erythropoiesis depending on dose and time of glucocorticoids application [65,66].

The positive correlations observed between BKA, TLC and HEM in the field and after long-term captivity, along with the evidence of increased stress response and reduced immunocompetence, indicate that these three variables might be used as indexes of allostatic state (definition according to [67]), with possible implications for detection of stressors in natural populations of this species and applications in conservation strategies [68,69]. Moreover, BKA showed repeatability when data from the field and after the long-term captivity were compared. Although long-term captivity reduced mean BKA, individuals characterized by higher BKA in the field continued to show higher values after three months in captivity. Previous studies also demonstrated the maintenance of the mean BKA in males from the same population of *R. ornata* collected during vocal activity in two different breeding seasons, and patterns of interspecific variation in BKA consistent with the existence of phylogenetic signal for this variable in anurans [22,37]. Given that the repeatability is a prerequisite for the detection of inter-individual variation, and inter-individual variation represents the substrate for the action of natural selection, these data reinforce the possibility of adaptive interpretations for interspecific variation of BKA in anurans [22,37]. A next step in this direction would be, however, the detection of heritability for this trait [70–72].

3.5.2. Transdermal corticosterone application

Transdermal corticosterone application resulted in a 6-fold increase in CORT on animals in the experimental group compared to the values from control and placebo groups, and 12-fold when compared to baseline values (obtained in the field). The volume and concentration applied of the hormone was sufficient to increase CORT in these animals, as previously observed for lizards [42] and salamanders [43]. Additionally, the lack of differences in CORT between our control and placebo groups shows the lack of effect of the manipulation performed on hormone levels. Despite the considerable increase in CORT on animals in experimental groups, no other blood parameter showed significant differences between groups at the end of the experiment.

In the study carried out by [43] male salamanders (*Desmognathus ocoee*) were treated for 9 consecutive days, with corticosterone through an application of a dermal patch that lasted 30 min. The authors found significantly elevated CORT one hour after removal of the dermal patch, but no differences were found between treated and non-treated animals 8h after removing the patch. Additionally, this treatment resulted in repeated acute elevations CORT over the 9 days, without changes in baseline values [43]. In our study, given that the samples were always collected 1h post-application, we were not able to explain the temporal dynamics of CORT changes due to treatment. However, in another experiment with males from this same species, collecting blood after 1h, 6h and 12h of application, we confirmed that differences in CORT between experimental and placebo groups occurred only 1h post-application, both for the first and for the last day of a 30 days period of daily application (Assis et al., unpublished results). In this way, these results suggest that the transdermal corticosterone application in *R. icterica* resulted in daily transient peaks of CORT, instead of promoting sustained hormonal peaks that would mimic a chronic stress condition [8,43,73]. The absence of changes in the immune parameters, unlike the observed effects of long-term captivity, also corroborates this interpretation.

Another possible explanation for the absence of changes in immune parameters following the transdermal corticosterone application would be that the experiment was conducted for an insufficient time. However, 30 days of treatment also did not change the immune parameters (Assis et al., unpublished results). An experimental protocol involving subcutaneous corticosterone implants would be interesting to tell apart the influence of acute and chronic exposition to corticosterone on immune parameters in these toads. An additional possibility is that the contrast of the effects of long-term captivity and transdermal corticosterone application on the immune variables in this study is the expression of a bimodal effect of this hormone [1,6,9–11,74]. In rats, for example, it is known that corticosterone may potentiate or inhibit the production of melatonin by pineal gland and leukocytes, indirectly modulating the inflammatory process, and this bimodal effect depends on corticosterone concentration [75,76]. Application of different concentrations of corticosterone, followed by melatonin and immunocompetence measures, would be needed to test this hypothesis of melatonin mediated bimodal action of corticosterone in anurans.

Despite the increased thickness of hind fleshy base of foot injected with PHA when compared to the saline control, the treatment of transdermal corticosterone application did not affect the response to PHA. Although a previous study with *R. marina* has shown a progression of swelling with the time postinjection of PHA, reaching maximum values after 24h, the cell infiltration associated with innate response occurred 12h postinjection [41]. Moreover, previous results obtained in our laboratory for a tree-frog (*Hypsiboas albopunctatus*), showed maximum swelling-response to PHA at 12h postinjection, and hind fleshy base of feet returned to basal conditions at 24h postinjection (Titon et al., unpublished data). These observations guided our initial choice to standardize the swelling measurements to 12h postinjection. However, we cannot rule out the possibility that an effect of corticosterone treatment would be more pronounced at 24h postinjection for this species. The measures of hind fleshy base of foot

thickness at later postinjection intervals in further studies will provide information needed to clarify this issue.

In summary, we showed that for *R. icterica*, restraint for 24h was a stressful condition, increasing CORT in 3-fold without consistent immunological changes. However, the application of a more invasive stress protocol (restraint with movement restriction) for the same period potentiated this response, resulting in 9-fold increase in CORT, associated with increased N:L ratio and lower BKA. Transdermal application of corticosterone efficiently mimicked repeated acute stress response events, without changing immune parameters even after 13 days of treatment. Interestingly, long-term captivity did not mitigate the stress response, since these toads maintained 3-fold increased CORT even after 3 months under these conditions. Moreover, long-term captivity increased TLC and generated an even stronger decrease in BKA, suggesting that consequences of the stress response can be aggravated by time in captivity. Such strong immune consequences of response to chronic stress in toads, if generalized to other stressors such as environmental pollution and habitat loss fragmentation, might show important impact on fitness of natural populations. Additionally, other physiological functions crucial to fitness, such as reproduction, might also be disrupted by response to chronic stress. The effects of biologically relevant chronic stressors on immune response and other physiological functions, such as growth and reproduction, represent important avenues of investigation.

3.6. Acknowledgments

We would like to thank Beny Spira (ICB/USP), Cristina O. M. S. Gomes (FMVZ/USP) and Eliana R. Matushima (FMVZ/USP) for methodological discussion and assistance, and Jeffrey M. Goessling (BS/AU) and Judy Heenan (EFLT/AU) for helping us review the English grammar. The study design, data collection and analysis, decision to publish, or preparation of the manuscriptstudy was supported by the Fundação de Amparo à Pesquisa do Estado de São

Paulo (FAPESP) through Young Researcher support (2006/54699-1) conducted by FRG, Regular grant for FRG (2013/00900-1) and PhD scholarship (2011/10560-8) for VRA (http://www.fapesp.br), and by Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPq) through a PhD scholarship (140272/2011-9 GD) for VRA (http://www.cnpq.br).

3.7. References

1. Sapolsky RM, Romero LM, Muck AU (2000) How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocrinol Rev 21: 55–89.
2. Wingfield JC, Hunt K, Breuner C, Dunlop K, Fowler GS (1997) Environmental stress, field endocrinology, and conservation biology. In Behavioral approaches to conservation in the wild: 95–131. Clemmons, J. R. & Buchholz, R. (Eds). Cambridge: Cambridge University Press.
3. Wingfield JC, Romero LM (2001) Adrenocortical responses to stress and their modulation in free-living vertebrates," in Coping with the Environment: Neural and Endocrine Mechanisms, B. S. McEwen and H. M. Goodman, Eds., vol. 4 of Handbook of Physiology, Section 7: The Endocrine System, pp. 211–234, Oxford University Press, New York, NY, USA.
4. Sapolsky RM (1992) Stress, the aging brain and the mechanisms of neuron death. Cambridge, MA: MIT Press.
5. Narayan E, Hero, J-M (2014) Repeated thermal stressor causes chronic elevation of baseline corticosterone and suppresses the physiological endocrine sensitivity to acute stressor in the cane toad (Rhinella marina). J Thermal Biol 41: 72–76.
6. Wiegers GJ, Reul, JMHM (1998) Induction of cytokine receptors by glucocorticoids: functional and pathological significance. Trends Pharmacol Sci 19: 317–321.
7. Dhabhar FS (2009) Enhancing versus suppressive effects of stress on immune function: Implications for immunoprotection and immunopathology. Neuroimmunomodul 16: 300–317.
8. McCormick GL, Langkilde T (2014) Immune responses of eastern fence lizards (*Sceloporus undulatus*) to repeated acute elevation of Corticosterone. Gen Comp Endocrinol 204: 135–140.
9. Wiegers GJ, Croiset G, Reul JMHM, Holsboer F, De Kloet ER (1993) Differential effects of corticosteroids on rat peripheral blood T-lymphocyte mitogenesis in vivo and in vitro. Am J Physiol 265: E825–E830.
10. Wiegers GJ, Reul JMHM, Holsboer F, De Kloet, ER. (1994) Enhancement of rat splenic lymphocyte mitogenesis after short term pre-exposure to corticosteroids in vitro. Endocrinol. 135, 2351–2357.
11. Wiegers GJ, Labeur MS, Stec IEM, Klinkert WEF, Holsboer F, et al. (1995) Glucocorticoids accelerate anti-T cell receptor-induced T cell growth. J Immunol 155: 1893–1902.

12. Casto JM, Nolan JV, Ketterson ED (2001) Steroid hormones and immune function: experimental studies in wild and captive dark-eyed juncos (*Junco hyemalis*). Am Nat 157: 408–420.
13. Mendes L, Piersma T, Hasselquist D, Matson KD, Ricklefs RE (2006) Variation in the innate and acquired arms of the immune system among five shorebird species. J Exp Biol 209: 284–291.
14. Buehler DM, Piersma T, Tieleman BI (2008) Captive and free-living red knots *Calidris canutus* exhibit diferences in non-induced immunity that suggest different immune strategies in different environments. J Avian Biol 39: 560–566.
15. Patchev VK, Patchev AV (2006) Experimental models of stress. Dialogues Clin Neurosci 8: 417–432.
16. Dickens MJ, Romero LM (2013) A consensus endocrine profile for chronically stressed wild animals does not exist. Gen Comp Endocrinol 191: 177–189.
17. Narayan E, Hero, J-M (2011) Urinary corticosterone responses and haematological stress indices in endangered Fijian ground frog (*Platymantis vitiana*) during transportation and captivity. Australian J Zool 59: 79–85.
18. Rich EL, Romero LM (2005) Exposure to chronic stress downregulates corticosterone responses to acute stressors. Am J Physiol Regul Integr Comp Physiol 288: R1628–R1636.
19. Berzins LL, Tilman-Schindel E, Burnes (2008) Sex-specific effects of handling time on an index of immune activity in zebra finches. Physiol Bioch Zool 81: 383–387.
20. Martin LB (2009) Stress and immunity in wild vertebrates: Timing is everything. Gen Comp Endocrinol 163: 70–76.
21. Narayan EJ, Cockrem JF, Hero JM (2011) Urinary corticosterone metabolite responses to capture and captivity in the cane toad (*Rhinella marina*). Gen Comp Endocrinol 173: 371–377.
22. Gomes FR, Oliveira RV, Assis VR, Titon Jr B, Moretti EH, et al. (2012) Interspecific Variation in Innate Immune Defenses and Stress Response of Toads from Botucatu (São Paulo, Brazil). South Amer J Herpetol 7: 1–8.
23. Graham SP, Kelehear C, Brown GP, Shine R (2012) Corticosterone-immune interactions during captive stress in invading Australian cane toads (*Rhinella marina*). Horm Behav 62: 146–53.
24. Brown GP, Shine R (2014) Immune response varies with rate of dispersal in invasive Cane Toads (*Rhinella marina***)**. PLoS ONE 9(6): e99734.
25. Carey C, Cohen N, Rollins-Smith L (1999) Amphibian declines: an immunological perspective. Develop Comp Immunol 23: 459–472.
26. Hopkins WA, DuRant SE (2011) Innate immunity and stress physiology of eastern hellbenders (*Cryptobranchus alleganiensis*) from two stream reaches with differing habitat quality. Gen Comp Endocrinol 174: 107–115.
27. Berger L, Speare R, Daszak P, Green DE, Cunningham AA, et al. (1998) Chytridiomycosis causes amphibian mortality associated with population declines in the rainforests of Australia and Central America. Proc Natl Acad Sci 95: 9031–9036.
28. Kindermann C, Narayan EJ, Hero JM (2012) Urinary corticosterone metabolites and chytridiomycosis disease prevalence in a free-living population of male Stony Creek frogs (*Litoria wilcoxii*). Comp Biochem Physiol 162: 171–176.
29. Kinney KS, Austin CE, Morton DS, Sonnenfield G (1999) Catecholamine enhancement of Aeromonas hydrophila growth. Microb Pathog 26: 85–91.
30. Taylor SK, Williams,ES, Thorne ET, Mills KW, Withers DI (1999) Causes of mortality of the Wyoming toad (*Bufo baxteri*). J Wildlife Diseases 35: 49–57.

31. Cunningham AA, Langton TES, Bennett PM, Lewin JF, Drury SEN, et al. (1996) Pathological and microbiological findings from incidents of unusual mortality of the common frog (*Rana temporaria*). Phil Trans R Soc Lond B 351: 1539–1557.
32. Narayan EJ, Christi K, Morley C (2009) Captive propagation of the endangered native Fijian frog *Platymantis Vitiana*: Implications for ex situ conservation and management. Pac Conserv Biol 15: 47–55.
33. McMahon TA, Sears BF, Venesky MD, Bessler SM, Brown JM, et al. (2014) Amphibians acquire resistance to live and dead fungus overcoming fungal immunosuppression. Nature 511: 224–227.
34. Maciel NM, Collevatti RG, Colli GR, Schwartz EF (2010) Late Miocene diversification and phylogenetic relationships of the huge toads in the *Rhinella marina* (Linnaeus, 1758) species group (Anura: Bufonidae). Mol Phylogenet Evol 57: 787–797.
35. Romero LM, Reed JM (2005) Collecting baseline corticosterone samples in the field: is under 3 min good enough? Comp Biochem Physiol A 140: 73–79.
36. Campbell TW (2006) Hematologia de Anfíbios. Hematologia e Bioquímica Clínica Veterinária, eds. Trall MA, Backer DC, Campbell TC, DeNicola DB, Fettman MJ, et al. Ed. Roca. pp. 291–300.
37. Assis VR, Monteiro SCG, Barsotti AMG, Spira B, Gomes FR (2013) Antimicrobial capacity of plasma from anurans of the Atlantic Forest. South Amer J Herpetol 8: 155–60.
38. Mendonça MT, Chernetsby, SD, Nester, KE, Gardner GL (1996) Effects of sex steroids on sexual behavior in the big brown bat, *Eptesicus fuscus*. Horm Behav 30: 153–161.
39. Gilbertson MK, Haffner GD, Drouillard KG, Albert A, Dixon B (2003) Immunosuppression in the northern leopard frog (*Rana pipiens*) induced by pesticide exposure. Environ. Toxicol Chem 22: 101–110.
40. Gervasi SS, Foufopoulos J (2008) Costs of plasticity: responses to desiccation decrease post-metamorphic immune function in a pond-breeding amphibian. Func Ecol 22: 100–108.
41. Brown, GP, Shilton CM, Shine R (2011) Measuring amphibian immunocompetence: validation of the phytohemagglutinin skin-swelling assay in the cane toad, *Rhinella marina*. Meth Ecol Evol 2: 341–348.
42. Meylan S, Dufty AM, Clobert J (2003) The effect of transdermal corticosterone application on plasma corticosterone levels in pregnant *Lacerta vivipara*. Comp Biochem Physiol 134: 497–503.
43. Wack CL, Lovern MB, Woodley SK (2010) Transdermal delivery of corticosterone in terrestrial amphibians. Gen Comp Endocrinol 169: 269–275.
44. Zerani M, Gobbetti A (1993) Corticosterone during the annual reproductive cicle and in sexual behavior in the crested newt, *Triturus carnifex*. Horm Behav 27: 29–39.
45. Cree A, Tyrrell CL, Preest MR, Thorburn D, Guillette LJ (2003) Protecting embryos from stress: corticosterone effects and the corticosterone response to capture and confinement during pregnancy in a livebearing lizard (*Hoplodactylus maculatus*). Gen Comp Endocrinol 134: 316–329.
46. Muller C, Jenni-Eiermann S, Blondel J, Perret P, Caro SP, et al. (2006) Effect of human presence and handling on circulating corticosterone levels in breeding blue tits (*Parus caeruleus*). Gen Comp Endocrinol 148: 163–171.
47. Markham JA, Beckel-Mitchener AC, Estrada CM, Greenough WT (2006) Corticosterone response to acute stress in a mouse model of Fragile X syndrome. Psychoneuroendocrinol 31: 781–785.

48. Zamudio SR, Quevedo-Corona L, Garcés L, De La Cruz F. 2009. The effects of acute stress and acute corticosterone administration on the immobility response in rats. Brain. Res. Bull. 80: 331–336.
49. Klukowski M (2011) Effects of breeding season, testosterone and ACTH on the corticosterone response of free-ranging male fence lizards (*Sceloporus undulatus*). Gen Comp Endocrinol 173: 295–302.
50. Narayan EJ (2013) Non-invasive reproductive and stress endocrinology in amphibian conservation physiology. Conserv Physiol 1: 1–16.
51. Bishop CR, Athens JW, Boggs DR, Warner HR, Cartwrig GE, et al. (1968) Leukokinetic Studies 13. A non-steady-state kinetic evaluation of mechanism of cortisone-induced granulocytosis. J Clin Investig 47: 249–260.
52. Cohen JJ (1972) Thymus-derived lymphocytes sequestered in bone marrow of hydrocortisone-treated mice. J Immunol 108: 841–844.
53. Fauci AS (1975) Mechanisms of corticosteroid action on lymphocyte subpopulations 1. Redistribution of circulating T-lymphocytes and B- lymphocytes to bone marrow. Immunol 28: 669–680.
54. Dhabhar FS (2002) Stress-induced augmentation of immune function - the role of stress hormones, leukocyte trafficking, and cytokines. Brain Behav Immun 16: 785–798.
55. Dhabhar FS, Miller AH, McEwen BS, Spencer RL (1996) Stress-induced changes in blood leukocyte distribution – role of adrenal steroid hormones. J Immunol 157: 1638–1644.
56. Davis AK, Maerz JC (2008) Comparison of hematological stress indi- cators in recently captured and captive paedomorphic mole salamanders, *Ambystoma talpoideum*. Copeia 2008: 613–617.
57. Davis AK, Maney DL, Maerz JC (2008) The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. Funct Ecol 22: 760–772.
58. Seddon RJ, Klukowski M (2012) Influence of stressor duration on leukocyte and hormonal responses in male southeastern five-lined skinks (*Plestiodon inexpectatus*). J Exp Zool 9999A:1–12.
59. Otis I, Murumagi A, Horak P (1998) Haematological health state indices of reproducing Great Tits: methodology and sources of natural variation. Func Ecol 12: 700–707.
60. Jain NC (1986) Schalm's Veterinary Hematology. Lea and Febiger, Philadelphia, PA.
61. Brown, GP, Shilton C, Phillips BL, Shine R (2007) Invasion, stress, and spinal arthritis in cane toads. Proc Natl Acad Sci USA 104: 17698–17700.
62. Reynolds S, Christian KA (2009) Environmental moisture availability and body fluid osmolality in introduced toads, *Rhinella marina*, in Monssonal Northern Australia. J Herpetol 43: 326–331.
63. Titon Jr B, Navas CA, Jim J, Gomes FR (2010) Water balance and locomotor performance in three species of neotropical toads that differ in geographical distribution. Comp Biochem Physiol 156: 129–35.
64. Meints RH, Carver, FJ (1973) Triiodothyronine and hydrocortisone effects on Rana pipiens erythropoiesis. Gen Comp Endocrinol 21: 9–15.
65. Fisher JW (1958) Increase in circulating red cell volume of normal rats after treatment with hydrocortisone or corticosterone. Proc Soc Exp Biol Med 97: 502–505.
66. Fisher, JW (1998) A quest for erythropoietin over nine decades. Annu Rev Pharmacol Toxicol 38: 1–20
67. McEwen S, Wingfield JC (2003) The concept of allostasis in biology and biomedicine. Horm Behav 43: 2–15.

68. Janin A, Léna JP, Joly P (2011) Beyond occurrence: Body condition and stress hormone as integrative indicators of habitat availability and fragmentation in the common toad. Biol Conserv 144: 1008–1016.
69. Janin A, Léna JP, Deblois S, Joly P (2012) Use of stress-hormone levels and habitat selection to assess functional connectivity of a landscape for an amphibian. Conserv Biol 26: 923–931.
70. Huey RB, Dunham AE (1987) Repeatability of locomotor performance in natural populations of the lizard, *Sceloporus merriami*. Evol 41: 1116–1120.
71. Bennett AF (1987) Interindividual variability. New directions in ecological physiology. (Eds M. E. Feder, A. F. Bennett, W. W. Burggren & R. B. Huey), pp. 147–169. Cambridge University Press, New York.
72. Dohm MR (2002). Repeatability estimates do not always set an upper limit to heritability. Funct Ecol 16: 273–280.
73. Wack CL, DuRant SE, Hopkins WA, Lovern MB, Feldhoff RC, et al. (2012) Elevated plasma corticosterone increases metabolic rate in a terrestrial salamander. Comp Biochem Physiol 161: 153–158.
74. Barriga C, Martín MI, Ortega E, Rodriguez AB (2002) Physiological concentrations of melatonin and corticosterone in stress and their relationship with phagocytic activity. J Neuroendocrinol 14: 691–695.
75. Ferreira ZS, Fernandes PACM, Duma D, Assreuy J, Avellar MCW, et al. (2005) Corticosterone modulates noradrenaline-induced melatonin synthesis through inhibition of nuclear factor kappa. J Pineal Res 38: 182–188.
76. Fernandes, PACM (2009) Regulação da produção hormonal da glândula pineal de ratos por moduladores do processo inflamatório. Tese de Doutorado. Instituto de Biociências, Universidade de São Paulo, São Paulo, SP, Brasil.

CONCLUSÕES GERAIS

Em relação a capacidade bactericida plasmática (CBP) medida por espectrofotometria, podemos afirmar que este é um método confiável e preciso para estimar este aspecto da imunocompetência inata de anfíbios anuros, e pode ser usado em estudos comparativos e ecofisiológicos. Testes adicionais, incluindo outras cepas de microrganismos, o uso de amostras de sangue, bem como peptídeos antimicrobianos sintetizados pelas glândulas granulares na derme podem fornecer uma avaliação mais completa da atividade antimicrobiana desses animais.

Quando quatro diferentes espécies de Bufonídeos foram submetidos às 24 horas do desafio de contenção sem e/ou com restrição de movimento, o padrão geral de resposta incluiu um aumento dos níveis plasmáticos de corticosterona (CORT) e da relação neutrófilo/linfócito (N:L) e uma diminuição nos níveis plasmáticos de testosterona (T). Com relação à CBP, a resposta à contenção foi bem mais variável, com *Rhinella icterica* mostrando uma diminuição e *R. marina* mostrando um aumento nos valores em relação aos níveis basais. Adicionalmente, na comparação intraespecífica, os níveis de CORT tenderam a aumentar mais em resposta à cotenção com restrição de movimentos do que em relação à contenção sem restrição de movimentos, quando comparados com os valores basais, sugerindo que os animais são capazes de responder de forma diferente, de acordo com a intensidade do estressor aplicado. Além disso, as mudanças na N:L, nos níveis de T e em CBP, não foram correlacionadas com o aumento em CORT.

Rhinella ornata, apresentou maiores níveis basais de CORT quando comparada com outras espécies. Esta diferença interespecífica pode estar assoicada à variação no comportamento vocal ou a uma resposta de estresse a condições climáticas prevalentes no momento da coleta. Com relação à CBP, *R. ornata* e *R. icterica* mostraram os valores basais mais altos de CBP, contrariando as nossas predições. Esperávamos que *R. schneideri* e *R.*

marina, as espécies mais generalistas em termos de ocupação, exibiriam maior CBP. Entretanto, os níveis plasmáticos de glicocorticóides e a resposta imune variam grandemente mesmo em nível intra-específico, dependendo das condições ambientais e da fase ciclo de vida em que os animais se encontram, e estas fontes de variação podem se sobrepor às diferenças interespecíficas nas mesmas condições.

Para tentar entender melhor a resposta imune e a resposta ao estresse, submetemos machos adultos de *R. icterica* a três tratamentos distintos: desafio de contenção, manutenção em cativeiro a longo prazo e aplicação transdérmica de corticosterona. O desafio de contenção sem restrição de movimento foi um estressor, aumentando CORT sem alterações imunológicas consistentes. Entretanto, a aplicação de um protocolo de estresse mais invasivo (contenção com restrição de movimentos) pelo mesmo período potencializou essa resposta, resultando no aumento de CORT e N:L e diminuição da CBP. A aplicação transdérmica de corticosterona eficientemente simulou eventos repetidos de resposta ao estresse agudo, sem alterar os parâmetros imunológicos, mesmo após treze dias de tratamento. Curiosamente, o cativeiro a longo prazo não atenuou a resposta ao estresse, uma vez que estes sapos mantiveram um aumento de três vezes em CORT mesmo depois de três meses sob estas mesmas condições. Mais do que isso, a manutenção em cativeiro a longo prazo gerou uma diminuição ainda maior na CBP, sugerindo que as consequências da resposta ao estresse podem ser agravadas pelo tempo em cativeiro. Tais fortes consequências na resposta imune promovidas pela resposta ao estresse crônico em sapos, se generalizada para outros estressores, tais como a poluição ambiental e a perda de hábitat por fragmentação, podem representar um impacto importante na aptidão de populações naturais.

Com base em nossos resultados, consideramos que, para melhor avaliar e entender as inter-relações entre o sistema imunológico e sua modulação pelo estresse e pelos níveis de CORT, se faz necessário analisar diferentes segmentos da resposta imune (em geral, anticorpos

naturais e componentes humorais; repostas mediadas por células e respostas inflamatórias), pois o investimento em cada segmento da resposta imune pode ser diferente dentro e/ou entre espécies. Devemos também priorizar a coleta de dados de todas as espécies sob o mesmo período de atividade (em geral, dentro ou fora da período reprodutivo) e exercendo o mesmo tipo de atividade (em geral, vocalizando ou forrageamento), pois a atividade vocal é uma fonte de grande variabilidade nos níveis hormonais e, provavelmente, na resposta imune.

ATTACHED FILE 1 – PARALLELISM TEST

To validate the use of the Corticosterone assay kit from Cayman Chemicals (number 500655) for toads, we conducted a parallelism test including plasma samples of *Rhinella schneideri* under two different situations: baseline and post-restraint challenge of 24h.

Pooled plasma samples were initially extracted with ether. Briefly, 5mL of ethyl ether were added to the plasma samples, vortexed for 30 seconds and centrifuged at 4°C for 9 minutes at 1800rpm. The tubes were then kept at -80°C for 7 minutes. The liquid phase was transferred to new tubes and kept in laminar flow hood at room temperature (20 ± 2°C), until all of the ether had evaporated (approximately 24h). The samples were resuspended and diluted in EIA buffer.

The top standard of the coricosterone kit was used for a serial dilution (neat, 1:2, 1:4; 1:8, 1:16, 1:32, 1:64 and 1:128). The pooled plasma samples were serially diluted in the same dilutions factors, and assayed against the diluted standards on the same plate. The standard and sample curves were plotted on the same XY axes, and the 50% binding point was considered the indicative of the best dilution factor to run the samples in the future assays. The curves were parallel, not crossing each other (Figure 1), corroborating the functionality of the assay for toads.

The best dilution factor for pooled plasma samples from *R. schneideri* are those that resulted in 50 % binding on the parallelism curve, corresponding to 1:8 for baseline (field condition) and 1:32 after the restraint challenge (Figure 1).

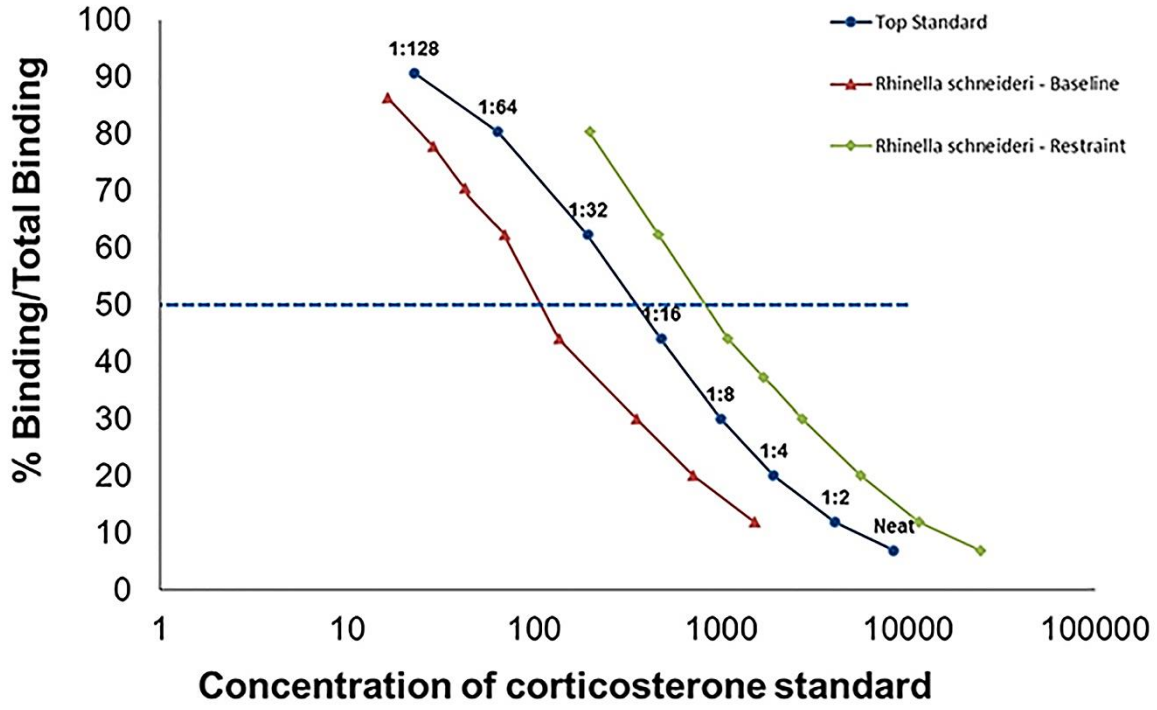

Figure 1: Binding displacement curves of serially diluted *Rhinella schneideri* pooled plasma at baseline conditions and after the restraint challenge, against the corticosterone standard used in the corticosterone enzyme-immunoassay. The y-axis shows the % Hormone Bound/Total Binding measured at 412 nm. The 50 % binding point is denoted using a dashed line, which determined dilution factors for the extracted plasma samples.

By testing 15 duplicates on each plate, we estimated an intra-assay variation of 6.31%. The inter-assay variation (12.07%) was estimated using the average of four intermediate values from the standard curve (as recommended by the kit instructions). Sensitivity of the assay was 30 pg/ml.

We would like to thank **Edward Narayan** (Charles Sturt University – Australia) for methodological discussion and assistance.

ATTACHED FILE 2 – APPROVAL OF THE COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA)

Instituto de Biociências

UNIVERSIDADE DE SÃO PAULO
INSTITUTO DE BIOCIÊNCIAS

OF.CEUA/IB/051/2011
Ref. 2011.1.1021.41.1

São Paulo, 26 de setembro de 2011.

Prezado Senhor

Dirijo-me a V. Sa. para informar que a Comissão de Ética em Uso de Animais do IB (CEUA), em reunião realizada nesta data, **APROVOU** o Projeto "Níveis plasmáticos de corticosterona, testosterona e imunocompetência em Bufonídeos" - **Protocolo 142/2011,** de sua responsabilidade (Colaboradores: Vânia Regina de Assis e Carlos Arturo Navas Iannini).

Lembramos que, para utilização de animais silvestres, o pesquisador responsável deve, ainda, ter/portar as autorizações de coleta, transporte e manutenção emitidas pelos órgãos ambienteis competentes.

Atenciosamente.

Profa. Dra. Mariz Vainzof
Coordenadora da Comissão de Ética no
Uso de Animais do IB (CEUA)

Ilmo. Sr.
Prof. Dr. FERNANDO RIBEIRO GOMES
Departamento de Fisiologia do IBUSP.

Rua do Matão - Travessa 14 nº 321 - CEP 05508-090 - Cidade Universitária
São Paulo - Brasil - http://www.ib.usp.br

ATTACHED FILE 3 – APPROVAL OF IACUC FOR FLORIDA CANE TOAD WORK

APPROVED

ANIMAL SUBJECTS REVIEW FORM

2013-2331

PRINCIPAL INVESTIGATOR: Mary T. Mendonca
RANK/TITLE: Professor
DEPARTMENT: Biological Sciences
COLLEGE/SCHOOL: College of Science and Math
CAMPUS ADDRESS: 331 Funchess — CAMPUS PHONE #: 334) 844 - 4856
E-MAIL: mendonca@auburn.edu — FAX #: 334) 844 - 9234

[X] Check if PI will serve as faculty advisor to the Lead Graduate Student or Resident associated with this activity.

LEAD GRADUATE STUDENT/RESIDENT: Jasmine N Dagg
RANK/TITLE: Graduate Student
DEPARTMENT: Biological Sciences — CAMPUS PHONE #: 334)844 - 4856
EMAIL: Jnd0008@auburn.edu — FAX #: 334) 844 - 9234

CO-INVESTIGATOR:
RANK/TITLE:
DEPARTMENT: — CAMPUS PHONE #:
EMAIL: — FAX #:

[ ] Check box if this protocol has more than one co-investigator. Additional co-investigators should be listed on page 2.

PROJECT TITLE: Measuring temperature constraints, energetic costs, stress response, and immune response associated with Anuran invasive spread in Florida.

STARTING DATE: ~~6/27/2013~~ 8/13/2013 (*Must not be prior to IACUC approval*) **EXPIRATION DATE:** ~~6/26/2016~~ 8/12/2016 (*Must not exceed three years*)

Is any part of the funding from a U.S. PUBLIC HEALTH SERVICE AGENCY: YES [ ] NO [X]

REQUIRED SIGNATURES

The information contained on this form provides an accurate description of the animal care and use protocol which will be followed. I agree to abide by governmental regulations and university policies concerning the use of animals. I will allow veterinary oversight to be provided to animals showing evidence of pain or illness. If the information provided for this project concerning animal use should be revised, or procedures changed, I will so notify the committee of those changes in writing, and no proposed changes will be implemented until full IACUC approval has been granted.

X Mary T. Mendonca 5/29/13
Principal Investigator **Date**

Medical care for animals will be available and provided as indicated by a qualified veterinarian. By accepting this responsibility, the veterinarian is providing assurance that any personal interest he/she might have in the project will not conflict with his/her responsibility for the provision of adequate veterinary care for the animals. Furthermore, the veterinarian provides assurance of review and consultation on the proper use of anesthetics and pain relieving medications for any painful procedures.

Emmett Blankenship
Project Veterinarian Name (print or type)

X [signature] 5/22/13
Project Veterinarian Signature **Date**

Unit Veterinarian Name (print or type)

X
Unit Veterinarian Signature **Date**

Jack Fontinella

X [signature] 5/29/13
Departmental Chairperson Name (print or type) **Departmental Chairperson Signature** **Date**

X Jasmine Dagg
Lead Graduate Student/Resident signature **Date**

X James J. Bannon 8/13/2013
***IACUC Chair Signature** **Date**
*IACUC Chair signs the protocol after IACUC approval has been granted

Received
JUN 05 2013
Research Compliance

Revised
AUG 12 2013

ASRF 04/2012
Page 1