Stefanny Christie Monteiro Titon

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Immune response, plasma corticosterone and testosterone levels, and body condition variation of Brazilian toads in response to captivity

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

Orientador: Fernando Ribeiro Gomes
Co-orientador: Pedro A.C.M. Fernandes

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RESUMO GERAL

O aumento dos níveis plasmáticos de glicocorticoides com concomitante diminuição de níveis plasmáticos de andrógenos em resposta a estressores é comumente observado em diversos vertebrados. Adicionalmente, os glicocorticoides, bem como os andrógenos, exercem importantes efeitos imunomodulatórios. Populações naturais de anfíbios respondem a agentes estressores de maneiras variadas, incluindo aumento dos níveis plasmáticos de corticosterona (principal glicocorticoide em anfíbios - CORT), alterações na resposta imune e a diminuição dos níveis plasmáticos de testosterona (T). Utilizando o cativeiro como um agente estressor para populações naturais de anfíbios, investigamos os efeitos da manutenção em cativeiro sobre os níveis plasmáticos de CORT e T, resposta imune (capacidade bactericida do plasma - CBP e fagocitose de células peritoneais) e condição corpórea de indivíduos machos de *Rhinella schneideri* e *R. icterica,* a partir de indivíduos coletados em populações naturais e mantidos em cativeiro por 60 e 90 dias, respectivamente. Machos adultos foram amostrados em campo, para coleta de dados basais, transportados e mantidos em cativeiro, onde amostras foram coletadas após 7, 30, 60 e 90 dias. Inicialmente, foi realizada a padronização da metodologia do ensaio de fagocitose das células peritoneais em sapos. Nossos resultados demonstraram que a o ensaio de fagocitose e a análise por citometria de fluxo por imagem resultam em um método confiável e preciso para a análise de atividade fagocítica de células peritoneais de sapos. Em resposta ao cativeiro, ambas as espécies (*R. schneideri* e *R. icterica*) apresentaram aumento dos níveis plasmáticos de CORT, diminuição dos níveis plasmáticos de T e resposta imune (CBP e fagocitose), indicando uma condição de estresse crônico. Indivíduos da espécie *R. schneideri* apresentaram uma acentuada perda da condição corpórea em resposta ao cativeiro, enquanto os indivíduos da espécie *R. icterica* mantiveram a condição corpórea ao longo do tempo em cativeiro. A CBP esteve positivamente correlacionada com a CORT plasmática quando não houve variação da condição corpórea (*R. icterica*), enquanto que em *R. schneideri*, os

indivíduos que perderam massa ao longo do tempo apresentaram níveis elevados de CORT plasmática e menores valores de CBP. Adicionalmente, os níveis plasmáticos de T estavam positivamente correlacionados com a fagocitose em ambas as espécies estudadas. Estes resultados indicam que a manutenção em cativeiro pode ser considerada um estressor crônico para sapos, uma vez que promove o aumento de CORT e diminuição de T e resposta imune nestes animais. De maneira associada, a variação da resposta imune em resposta à manutenção em cativeiro depende do parâmetro avaliado e tempo de resposta (dias em cativeiro). Nossos resultados ainda sugerem que a imunomodulação nas condições de cativeiro em sapos pode estar associada às variações de CORT e T plasmática, dependendo de sinalização da condição corpórea do animal.

Palavras-chave: Estresse, corticosterona, testosterona, resposta imune, condição corpórea.

GENERAL ABSTRACT

Increased plasma glucocorticoid levels with concomitant decrease in plasma androgen levels in response to stressors are commonly observed in several vertebrates. In addition, glucocorticoids, as well as androgens, plays important immunomodulatory effects. Natural populations of amphibians respond to stressors in different ways, including increased corticosterone (main glucocorticoid in amphibians – CORT) plasma levels, changes in immune responses, and decreased plasma testosterone (T) levels. Given that captivity is a stressor for amphibian natural populations, we investigated the effects of captivity maintenance on plasma CORT and T levels, immune response (plasma bacterial killing ability – BKA and phagocytosis of peritoneal cells) and body condition of male individuals of *Rhinella schneideri* and *R. icterica*, from individuals collected in natural populations and kept in captivity for 60 and 90 days, respectively. Adult males were sampled in the field for baseline measurements, transported and kept in captivity, where samples were collected after 7, 30, 60 and 90 days. Initially, we standardized the phagocytosis of peritoneal cells assay for toads. Our results demonstrated that the phagocytosis assay and the imaging flow cytometry analysis result in a reliable and accurate method for the analysis of phagocytic activity of peritoneal cells of toads. In response to captivity, both species (*R. schneideri* and *R. icterica*) showed increased plasma CORT, decreased plasma T levels and immune response (BKA and phagocytosis), indicating a chronic stress condition. Individuals of *R. schneideri* showed accentuated body condition loss in response to captivity, while individuals of *R. icterica* displayed sustained body condition over time in captivity. BKA was positively correlated with plasma CORT when there was no change in body condition (*R. icterica*), whereas in *R. schneideri*, individuals showing the highest body mass loss over time, also presented the highest CORT levels and the lowest BKA values. Additionally, plasma T levels were positively correlated with phagocytosis in both studied species. These results indicate that captivity maintenance can be considered a chronic

stressor for toads, promoting increased CORT and decreased T and immune response in these animals. Moreover, the variation in immunity in response to captivity maintenance depends on the evaluated parameter and time (days in captivity). Our results further suggest that immunomodulation in captivity conditions in toads may be associated with changes in plasma CORT and T, depending on the individual's body condition.

Keywords: Stress, corticosterone, testosterone, immune response, body condition.

INTRODUÇÃO GERAL

Relações entre estresse, resposta imune e andrógenos

Em resposta a um estímulo estressor, ocorre ativação do sistema nervoso central e concomitante ativação do eixo hipotálamo-hipófise-adrenais/interrenais (HPA/I) (Sapolsky et al., 2000; Sapolsky, 2002). Esta ativação simultânea do sistema nervoso e endócrino resulta na liberação de catecolaminas e glicocorticoides (GC), os quais promovem alterações fisiológicas como: aumento do tônus muscular, aumento da disponibilização de substratos energéticos e imunomodulação (Sapolsky, 2000). Embora a resposta de estresse apresente efeitos benéficos, que promovem a sobrevivência do animal a uma determinada condição estressante, a ativação frequente do sistema nervoso e do eixo HPA/I em resposta a estressores pode resultar em efeitos deletérios para o animal, como a inibição da reprodução e supressão da resposta imune (Sapolsky et al., 2000; Sapolsky, 2002; Marketon e Glaser, 2008).

Diversos estudos mostram que a inibição de diferentes pontos do eixo hipotálamo-hipófise-gônadas em resposta a ativação do eixo HPA/I tem sido demonstrada em vertebrados, incluindo anfíbios (Sapolsky, 2002; Deviche et al, 2010; Tokarz e Summers, 2011; Barsotti et al., 2017). Como resultado, frequentemente observamos a inibição do comportamento reprodutivo, assim como a diminuição dos níveis plasmáticos de andrógenos em resposta a diferentes estressores em machos (Paolucci et al., 1990; Moore et al., 1991; Sapolsky et al., 2000; Jones e Bell, 2004; Deviche et al., 2010; Narayan et al., 2012; Assis, 2015). Os andrógenos, por sua vez, além de exercerem um importante papel na reprodução, também apresentam ações imunomoduladoras (Nava-Castro et al., 2012). As células imunes, uma vez que apresentam receptores para andrógenos, podem ser moduladas diretamente pelos andrógenos (Nava-Castro et al., 2012). É importante ressaltar que ações indiretas dos andrógenos também podem ser observadas, uma vez que a testosterona (T - principal andrógeno nos machos) pode competir pelas globulinas de ligação de GCs, alterando a fração livre destes

hormônios (Bradley et al., 1980; McDonald et al., 1981; Bradley, 1987 McEwen et al., 1997). Portanto, uma vez que as células imunes também apresentam receptores para GCs, estando sujeitas a modulação direta dos GCs (Wiegers e Reul, 1998), é possível que os andrógenos, associados aos GCs, exerçam um papel fundamental na regulação da resposta imune, particularmente durante a resposta de estresse.

Em relação ao sistema imune, a resposta de estresse promove uma imunomodulação direta, mediada pela ativação do sistema nervoso central (inervação direta dos órgãos linfoides), e também, via ação hormonal, através, principalmente, da atuação das catecolaminas e dos GCs sobre as células imunes (Marketon e Glaser, 2008). Em resposta ao estresse agudo, o sistema imune apresenta alterações como redistribuição de leucócitos (linfócitos T e B, células natural-killer, e monócitos) do sangue para tecidos periféricos e linfonodos, resultando em uma diminuição do número de células imune na corrente sanguínea (Dhabhar, 2009; Dhabhar, 2014). De maneira associada, durante uma resposta de estresse agudo, ocorre um aumento da resposta inflamatória, assim como de diversos componentes da resposta inata e adquirida (Dhabhar, 1999; Barriga et al., 2001; Dhabhar, 2009; Graham et al., 2012). Por outro lado, o estresse crônico apresenta, em sua maioria, efeitos imunossupressores. De fato, diminuições da resposta inflamatória e reduções na atividade de células fagocitárias e de componentes da resposta humoral e mediada por células são comumente observadas em resposta ao estresse crônico (Hoffman-Goetz et al, 1986; Yin et al, 1995; Dhabhar, 2009; Dhabhar, 2014; Assis et al., 2015; Neuman,-Lee et al., 2015). Deste modo, apesar da imunomodulação associada à resposta de estresse ser muito mais refinada do que o exposto (Dhabhar, 2014), observamos que os efeitos estimulatórios ou supressores da resposta imune apresentam uma forte relação com a duração da resposta de estresse.

Variações na amplitude e duração da elevação dos níveis plasmáticos de corticosterona (CORT – principal GC em anfíbios), assim como nas alterações no perfil da resposta imune,

têm sido reportadas em diversos anfíbios expostos a diferentes agentes estressores. Algumas espécies de anuros Brasileiros tem demonstrado um aumento da CORT e diminuição da capacidade bactericida plasmática em resposta ao estresse de contenção (Gomes et al., 2012) e cativeiro (Assis et al., 2015). Entretanto, apesar de Graham et al. (2012) terem demonstrado um efeito imunossupressor sobre a capacidade de lise do plasma, também foi observado um efeito estimulador sobre o burst oxidativo de leucócitos sanguíneos em resposta ao estresse de restrição em sapos Australianos. Por outro lado, Assis (2015) demonstrou que algumas espécies de anuros não apresentam variação da capacidade bactericida plasmática mesmo quando submetidos ao estresse de contenção de movimentos por 24h. Estes estudos demonstram que diferentes segmentos do sistema imune podem ser modulados de forma distinta em resposta a estressores em diversos anfíbios. De maneira associada, uma diminuição nos níveis de T tem sido frequentemente observada em resposta a estressores em anuros (Jones e Bell, 2004; Narayan et al., 2012; Assis, 2015). Entretanto, estudos que incorporem medidas de CORT, T e reposta imune com anuros são escassos (Assis, 2015), principalmente no contexto de estresse crônico.

Imunomodulação mediada por glicocorticóides

Os GCs são hormônios esteroides que promovem alterações fisiológicas que garantem a sobrevivência do animal durante a resposta de estresse (Sapolsky et al., 2000). Estudos realizados com diversos vertebrados demonstraram que elevações agudas de GCs promovem um aumento em diversos aspectos da resposta imune: resposta mediada por células (Dhabhar e McEwen, 1999); fagocitose de leucócitos do sangue (Graham et al., 2012; Assis et al., 2017); aglutinação mediada por anticorpos (McCormick e Langkilde, 2014); os quais poderiam estar associados a uma maior resposta imune em um período de recuperação pós estresse (Dhabhar, 2014). De fato, GCs liberados de forma aguda na corrente sanguínea promovem a liberação de

moléculas constituintes da resposta imune humoral que podem, por sua vez, ativar os mediadores celulares da resposta imune e direcionar os neutrófilos e linfócitos circulantes para tecidos específicos (Wiegers & Reul, 1998; Riccardi, et al, 2002; Cavalcanti et. al., 2006). Adicionalmente, estudos *in vitro* demonstraram que níveis fisiológicos de GCs aumentam a fagocitose e a quimiotaxia em macrófagos peritoneais de ratos (Barriga et al., 2002; Zhong et al., 2013) e a proliferação celular em ovelhas (Ciliberti et al., 2016), evidenciando o efeito imuno estimulador do aumento agudo dos GCs.

Por outro lado, níveis cronicamente elevados dos GCs apresentam efeitos majoritariamente imunossupressores (Dhabhar, 2014). Estudos abordando elevações crônicas experimentais de GCs demonstraram que estes hormônios podem retardar o processo de cicatrização, diminuir a resposta imune mediada por células, bloquear e inibir a maturação e desenvolvimento dos linfócitos, além de atuarem na diminuição da massa e função dos órgãos linfoides (Ahmed et. al., 1985; Wiegers & Reul, 1998; Dhabhar e McEwen, 1999; Riccardi, et al, 2002; Cavalcanti et. al., 2006; Thomas e Woodley, 2015), caracterizando a função imunossupressora destes esteroides. Tratamentos *in vitro*, demonstraram que o uso crônico de GCs, assim como altas doses destes hormônios também têm apresentam efeitos supressores sobre a resposta imune celular, atuando na diminuição da proliferação de células do sangue e da atividade (quimiotaxia e fagocitose) de macrófagos (Wiegers et al., 1993; Lim et al., 2007; Ciliberti et al., 2016). Adicionalmente, estudos realizados com camundongos demonstraram que baixas doses de CORT aumentam a produção de citocinas (TNF e IL-6), enquanto que altas doses do mesmo hormônio apresentam um efeito oposto (Chantong et al., 2012), especialmente quando combinada com uma injeção de lipopolissacarídeos (Liao et al., 1995). Por outro lado, é interessante ressaltar que a mesma dose de GCs pode apresentar tanto efeito supressor quanto estimulador sobre a resposta imune, variando de acordo com o tempo de exposição das células a estes hormônios (Wiegers et al., 1995; Ciliberti et al., 2016). Deste modo, os efeitos bimodais

dos GCs parecem estar associados a variação na duração e intensidade da resposta de estresse, além de também estar relacionados a ação dos distintos receptores de GCs (Wiegers et al., 1993; Wiegers & Reul, 1998; Salpolsky et al., 2000; Chantong et al., 2012).

Embora os efeitos imunomodulatórios diretos dos GCs estejam bem estabelecidos, sabe-se que a condição corpórea do indivíduo parece ser fundamental durante a modulação da resposta imune mediada pelo estresse. Em condições de vida livre, iguanas com maiores níveis de CORT apresentaram uma menor condição corpórea e resposta inflamatória (Berger et al., 2005). De maneira semelhante, trabalhos com mamíferos e aves demonstraram que, em resposta ao estresse agudo, o aumento da resposta imune concomitante ao aumento dos níveis circulantes de GCs, normalmente ocorre quando o indivíduo apresenta uma boa condição corpórea (Dhabhar, 2009; Harms et al., 2010). Por outro lado, Bourgeon et al. (2007) demonstraram que patos submetidos a um desafio com LPS apresentaram uma correlação positiva entre a condição corpórea e a resposta imune (níveis plasmáticos de oxido nítrico), entretanto, os animais com maior resposta imune e condição corpórea foram os que apresentaram os menores níveis de CORT. Estes estudos demonstram que os GCs associados com a sinalização de condição corpórea parecem exercer um papel fundamental sobre a resposta imune em vertebrados.

Imunomodulação mediada por andrógenos

Apesar de exercerem um papel intimamente relacionado à reprodução, os hormônios sexuais, incluindo a testosterona (T - principal andrógeno em vertebrados), são importantes reguladores de diversos aspectos da resposta imune inata e adquirida nos vertebrados (Ahmed et al., 2010). Os efeitos imunossupressores da T são bastante conhecidos, e incluem a redução de tecidos linfoides, assim como a diminuição da resposta imune celular e humoral (Grossman, 1985; Casto et al, 2001; Ahmed et al., 2010; Nava-Castro et al, 2012). Estudos *in vivo* e *in vitro* demonstraram que o tratamento com a T diminui a proliferação de linfócitos, produção de

anticorpos, assim como a atividade celular de macrófagos, representada pela fagocitose, produção de nitrito e interleucina-1 em diversos táxons (Savita e Rai, 1998; Casto et al., 2001; Mondal e Rai, 2002; Tripathi e Singh, 2014). Adicionalmente, aplicações agudas e sustentadas de T podem reduzir a atividade de células natural-killer, assim como diminuir as respostas humoral e mediada por células (Inman, 1978; Grossman, 1984; Ahmed et al., 1985; Hou e Zheng, 1988). Entretanto, embora uma imunossupressão associada a T em animais seja relatada para diversas espécies de vertebrados, uma meta-análise incluindo mamíferos, aves e répteis demonstrou que o efeito supressor da T varia de acordo com o tipo de resposta imune estudado, bem como dentre os grupos filogenéticos (Roberts et al., 2004).

Efeitos estimuladores da T são observados, principalmente, relacionados à resposta inflamatória (McCrohon et al., 1999; Greenman et al., 2005; Martin et al., 2008; Gilliver, 2010). Entretanto, há estudos demonstrando que a T também influencia positivamente a atividade celular de macrófagos (aumento da produção de óxido nítrico) e a produção de anticorpos em alguns animais (Chao et al., 1994; Evans et al., 2000; Peters, 2000). Adicionalmente, estudos recentes têm demonstrado que os efeitos estimuladores da T são observados, principalmente, associados a uma boa condição corpórea do animal. Desprat et al. (2015) demonstraram que pererecas tratadas com T, que apresentaram maiores níveis plasmáticos de T e com uma melhor condição corpórea, também apresentaram uma maior resposta inflamatória quando comparados com indivíduos com menor condição corpórea e com o grupo placebo (sem aplicação de T). Por outro lado, Roberts et al. (2007) demonstraram que aves tratadas com implantes de T, demonstrando menor massa corpórea, mas apresentando maiores níveis de T concomitantemente com maiores níveis de CORT, exibiram uma maior produção de anticorpos em resposta a um dado antígeno, sugerindo uma possível interação entre estes dois esteroides na regulação do sistema imune (Roberts et al., 2007). Consequentemente, é possível que a T possa participar de maneira fundamental da regulação da resposta imune durante a resposta de

estresse; e que a interação entre a CORT, T e condição corpórea sejam determinantes no controle do sistema imune em resposta a estressores.

Justificativa

Diversos estudos abordam diferentes respostas fisiológicas associadas à resposta de estresse do animal (Sapolsky et al., 2000), no entanto, o enfoque se dá em poucos aspectos fisiológicos em conjunto (ex. estresse e susceptibilidade a doenças – Kindermann et al., 2012; Peterson et al., 2013; GCs, condição corpórea e fragmentação – Janin et al., 2011; Tennessen et al, 2014; sistema imune e GCs – Buehler et al., 2008; Dhabhar, 2014). Uma vez que o organismo coordena múltiplos sistemas fisiológicos para lidar com as demandas ambientais, incluindo a resposta a estressores, um estudo mais completo, integrando diversos aspectos fisiológicos se faz necessário para uma compreensão mais integrada destes sistemas. Os anfíbios são particularmente sensíveis a alterações ambientais, representando desta forma um grupo interessante como modelo de estudo para compreender as mudanças integradas de diversos aspectos fisiológicos, como GCs, hormônios reprodutivos, condição corpórea e sistema imune associados a resposta de estresse.

Dentro do presente contexto, desenvolver ferramentas apropriadas para a avaliação da resposta imune em animais não modelo, como os anuros, se faz necessário. Incialmente, a nossa pesquisa foi motivada pela necessidade de avaliar diferentes aspectos da resposta imune onde a aplicação possa ser amplamente utilizada em diversas espécies. Para tal, adaptamos protocolos de fagocitose a partir de macrófagos peritoneais, provenientes do uso em mamíferos, para o uso com sapos. A partir de metodologias para avaliação da resposta imune (capacidade bactericida do plasma e fagocitose de células peritoneais - desenvolvida durante este estudo), de dosagens hormonais no plasma e medidas de condição corpórea (amplamente utilizada na literatura), nós avaliamos o efeito da manutenção em cativeiro (comumente utilizado como agente estressor

em vertebrados, incluindo anfíbios) sobre variáveis associadas à resposta de estresse e imunidade em sapos brasileiros.

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CAPÍTULO 1 – PHAGOCYTIC ACTIVITY OF PERITONEAL LEUKOCYTES FROM ANURANS AND MAMMALS BY CONVENTIONAL AND IMAGING FLOW CYTOMETRY

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1.1. Abstract

Leukocytes phagocytosis has been used to evaluate immune responses in different contexts in many vertebrate groups. The phagocytic activity can be measured by flow cytometry or confocal microscopy. However, both methodologies show limited performance. Recently, imaging flow cytometry has emerged as a powerful tool to study cellular functions, bringing new approaches to analyze phagocytosis in immune cells. The present study compared the performance of conventional and imaging flow cytometry to describe the phagocytic activity of peritoneal leukocytes from toads (*Rhinella schneideri*) and Wistar rats. Our results showed fast and accurate measurements regarding cell aggregation and phagocytic activity by using flow cytometry analyses in both rats and toads. Based on the cell morphology, we demonstrated that imaging flow provides a more comprehensive and robust procedure for identifying immune cells populations and allow the successful characterization of phagocytic activity using multi-parameter analyses (number of internalized particles and phagocytic score). Since phagocytosis is a non-specific aspect of immunity, the study of the phagocytic activity allows important investigations regarding to cellular activity in several natural and laboratory contexts, as well as, in comparative studies.

Keywords: Immunity, Macrophage, Neutrophil, Amphibia, Wistar rat.

1.2. Introduction

Phagocytosis, a conserved process in invertebrate and vertebrate groups (Bayne, 1990; Desjardins et al., 2005; Underhill and Ozinsky, 2002), allows an active internalization of particulate substrates, cell debris and engulfing and destruction of foreign organisms (e.g. bacteria, fungus spores), and infected cells (Aderem and Underhill, 1999; Jutras and Desjardins, 2005). In mammals, professional phagocytes, such as neutrophils and macrophages responsible for clearing cell debris and pathogens, may play relevant roles in immune function and, in specific conditions, dysfunctions (Jutras and Desjardins, 2005). There are several studies evaluating the physiological, pathophysiological and pathological relevance of this process in mammals (Barriga et al., 2002; Krishnamoorthy et al., 2010; Morceli et al., 2013; Henning et al., 2015; Mcfarlin et al., 2013; Kobayashi et al., 2016). Nevertheless, this highly relevant process is poorly investigated in other vertebrate groups, such as amphibians. Indeed, the only non-mammalian organism well studied is *Xenopus laevis*, which is highly used as a model for investigation of pathophysiological processes (Robert and Ohta, 2009; Haynes-Gilmore, et al., 2014; Andino et al., 2016). Therefore, only few and limited tools are currently available for assessing immunity in amphibians under natural, laboratory or ecoimmunological contexts (Clulow et al., 2015; Rollins-Smith and Woodhams, 2012).

Assessment of the immune response in amphibians has been commonly carried out by phytohemagglutinin challenge, plasma bacterial killing ability and lymphocyte proliferation (Morales and Robert, 2008; Assis et al., 2013; Titon et al., 2016). However, the aforementioned techniques do not provide information regarding the nature of immune response, such as which specific cell type and/or protein are involved or which specific cellular activities defend against a foreign antigen. Although phagocytic activity has been investigated in a few studies with amphibians, it has often been measured by chemiluminescence (Marnila et al., 1995; Gilbertson et al., 2003; Albert et al., 2007; Graham et al., 2012). The whole-blood lucigenin-dependent

chemiluminescence assay measures the production of reactive oxygen species (ROS) by circulating neutrophils (Lilius and Marnila, 1992). However, this technique is an indirect measurement of phagocytosis, since ROS production may be related to cellular functions other than phagocytic activity (Lilius and Marnila, 1992; Hancock et al., 2001). In this way, ROS production may not provide accurate measures regarding the phagocytosis itself (Lilius and Marnila, 1992; Hancock et al., 2001; Robinson, 2009).

The studies of phagocytosis must combine methods that need specific antibodies to segregate cellular types by flow cytometry (Brown and Wittwer, 2000; Lehmann et al., 2000), confocal microscopy (Barriga et al., 2002; Pires-Lapa, et al 2013), and chemiluminescence (used as indicative of phagocytosis) (Plytycz and Bayne, 1987; Gilbertson et al., 2003; Marnila et al., 1995). These methods are reliable only if associated to cell morphology identification, which demands manual and time-consuming sample account. Imaging flow cytometry, on the other hand, brings together traditional flow cytometry, fluorescence microscopy and advanced data-processing algorithms (Wabnitz et al., 2015). The concomitant acquisition of morphological and multicolor fluorescence data allows the high-throughput evaluation of features such as percentage of phagocytosis and cell markers expression, providing more accurate (since it photographs all events) and fast measurements (one equipment and program allows all the necessary analysis) (Erdbrügger et al., 2014). Besides that, image-based analyses allow a more complete interpretation of structural alterations in phagocytic cells and the identification of immunophenotypes (Hanning et al 2015; McFarlin et al., 2013), improving the knowledge of phagocytosis itself and other cellular processes and analysis (Havixbeck et al., 2015).

The use of more precise tools for assessing immune function might result in an important evolution in the quality of investigations about immune response in several vertebrate groups. Even though a widespread technique, flow cytometry has been only occasionally used to

evaluate phagocytosis in amphibians (Froese et al., 2005; Christin et al., 2013; Assis et al., 2017). In this sense, the more detailed the study of phagocytosis, the more comprehended this process and its regulation not only in amphibians, but also in a wide range of organisms. In that regard, the present study compares the performance of conventional flow cytometry and imaging flow cytometry in the investigation of phagocytic activity of peritoneal cells in toads. We also described improved analyses from imaging technique in Wistar rats and provided detailed morphological and functional aspects of peritoneal phagocytic cells of a Brazilian toad (*Rhinella schneideri*).

1.3. Material and methods

1.3.1. Study site and toad collection

Adult males of toads (*Rhinella schneideri*; N =7) were collected in February 2015 in Luiz Antônio (21° 32′ 58″ S, 47° 42′ 24″ W), São Paulo/Brazil. Individuals were located by visual inspection and manually captured. Thereafter, animals were kept in individual plastic containers for three days, until they were taken to the laboratory. The containers had holes to allow air circulation, and were placed in a room exposed to the natural light cycle and temperatures compatible with natural thermal regime. Animal capture and procedures for the collection and use of biological samples were performed under license from Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio, process number 8132-1) and the approval of the Ethic Committee of the Institute of Bioscience of the University of São Paulo (protocol: 054/2013).

1.3.2. Toads and Wistar rast's laboratory maintenance

Toads were individually placed in plastic containers (43.0 x 28.5 x 26.5 cm), which lids had holes to allow air circulation. Animals had free access to water and were fed with

cockroaches once per week. The room was exposed to a 13/11 LD cycle (lights on at 6:40 am and off at 7:40 pm) and temperature of 21 ± 2°C. All individuals were kept in captivity during 120 days.

Male Wistar rats (N = 4; 75 days old), from the Department of Physiology Animal Facility at the Biosciences Institute of the University of São Paulo, were exposed to a 12/12 LD cycle (lights on at 6 am and lights off at 6 pm) and kept at 22 ± 2°C with free access to water and food ad libitum. Wistar rat's procedures were performed in accordance with the Ethic Committee of the Biosciences Institute of the University of São Paulo (protocol: 207/2014).

1.3.3. Retrieval of peritoneal cells

Toads were euthanized by immersion in a lethal solution of benzocaine (0.2%) and Wistar rats were killed by decapitation without anesthesia. After euthanasia, the abdomen of each animal was cleansed with ethanol 70% and a small incision was made into the peritoneal cavity with sterile surgical material. Ten milliliters of sterile PBS were injected into the peritoneum with a sterile Pasteur pipette. Then, the abdominal region was carefully massaged, and lavage fluid (resulting solution of PBS + cells) was removed with a sterile Pasteur pipette and placed in a 15ml conical tube. The lavage fluid was centrifuged (1200 rpm, at 4°C for 9 min), supernatant was discarded, and cells were resuspended in 1ml of PBS to perform phagocytosis assay.

1.3.4. Phagocytosis assay

Phagocytosis assay from peritoneal cells of toads and rats was performed according to Titon et al. (2017). Lavage fluid was diluted in a 0.4% stock solution of Trypan Blue (dilution factor: 1:1) to perform count and viability of the cells. The cells were counted in a glass hemocytometer. The number of phagocytic cells (macrophages and neutrophils – for toads;

macrophages – for rats) was adjusted to 2 x 10^5 cells/200 µl for toads and 1 x 10^6 cells/200 µl for Wistar rats. Zymosan A-CFSE-labeled work solution (1 x 10^7 particles/ml PBS) was prepared from a stock solution (Zymosan A – from Saccharomyces cerevisiae, Sigma A-Aldrich Z4250 - of 2 mg/ml labeled previously with CFSE at 5µM). Thereafter, 200 µl of the lavage fluid were diluted in 800 µl of PBS, and then 100 µl of zymosan work solution was added to the samples (in a proportion of 5-zymosan particles per cell). Negative controls were obtained by incubating the cells diluted in PBS in the same proportion without zymosan. Samples were covered with aluminum foil, to protect from light, and incubated under agitation, for 65 min. For toads, the assay was performed at 25°C, since this is the mean thermal preference for R. schneideri (Bicego et al., 2002), and for Wistar rats, the assay was performed at 37°C (Zhang et al., 2010). After incubation, the reactions were stopped, by adding 2 ml of cold (4°C) EDTA (6 mM) followed by a subsequent centrifugation (1200 rpm, at 4°C for 7 min). Thereafter, the supernatant was discarded and 200 µl of cold (4°C) paraformaldehyde (PFA 1%) was added. Samples were then kept during 1 hour at 4°C for cell fixation. After this period, 500 µl of PBS solution were added and samples were then centrifuged (1200 rpm, at 4°C for 7 min). The supernatant was discarded and 100 µl of PBS solution were added for flow cytometry. For Wistar rats, macrophages were marked with CD11b/c+ (Abcam - AB112239), and the cells were kept at 4°C until flow cytometry analysis (24h). Direct measurements of mean fluorescence in the green channel were recorded as phagocytosis.

1.3.5. Conventional flow cytometry

A flow cytometer (FACS Calibur, Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) interfaced with a Macintosh G4 computer was used to validate the measurements of phagocytic activity from peritoneal phagocytic cells of toads by flow cytometry. Data from 20,000 events were acquired in a list mode and analyzed in Cell Quest

(Becton Dickinson Immunocytometry Systems). Discrete cell populations were identified based on their properties on forward scatter *vs.* side scatter plots. Data from neutrophils and macrophages were collected applying gates, which sorted out lymphocytes and erythrocytes clusters. Fluorescence data was collected in log scale. Green fluorescence from zymosan-CFSE was measured at 530 ± 30 nm (FL1 detector). Dot plot cytograms were chosen for peritoneal neutrophils and macrophages frequency analysis, which was performed using FlowJo software (tree Star Inc., San Carlos, California, USA) version 7.6.4 for Windows.

1.3.6. Imaging flow cytometry and analyses

Phagocytosis assay samples from rat's cells were acquired on a AMNIS Flowsight imaging flow cytometer (Merck-Millipore, Darmstadt, Germany) interfaced with a DELL computer. To recognize, describe and analyze phagocytosis events in toad's peritoneal cells by this new technology, additional 2 individual samples (2-blank and 2-zymosan) were analyzed on this imaging cytometer. Data from 20,000 events were acquired, utilizing the 488nm laser, at a 20x magnification, using the INSPIRE software. Single cells were first identified based on a scatter plot of bright field area vs. the aspect ratio. A gate was drawn around each population containing putative single cells, based on the criteria of the area being large enough to exclude debris, and the aspect ratio being greater than 0.6, which eliminates debris and clusters. Neutrophils and/or macrophages were identified through gate images in focused-single cells plotted on the SSC *vs.* brightfield area plot for Wistar rats, and SSC *vs.* bright detail intensity - brightfield plot for toads. Acquired data were analyzed using IDEAS analysis software (EMD Millipore) version 6.1 for windows.

The number of internalized particles is calculated using an algorithm that determines whether a pixel is connected to a particular spot or to the background. The algorithm first obtains bright regions from an image, regardless of the intensity differences from one spot to

another. Next, using the spot to cell background ratio, it identifies the number of intensity areas that have local maxima (brightness). The spot to cell background ratio is calculated dividing the spot pixel value by the background intensity in the bright detail image. The Phagocytic Score, on the other hand, is a measurement of internalization efficiency defined as the ratio of intensity inside the cell to the intensity of the entire cell. The thickness of the membrane (in pixels) determines which pixels are used to define the boundary and the membrane portions of the cell. The higher the score, the greater the concentration of intensity inside the cell. Internalized cells typically have positive scores while cells with little internalization have negative scores. Cells with scores around zero have a mix of internalization and membrane intensity. All pixels are background-subtracted and the feature is invariant to cell size and can accommodate concentrated bright regions and small dim spots. The parameters analyzed were calculated using the "Internalization" and "Spot Count" wizards of the IDEAS analysis software (EMD Millipore) version 6.1 for windows.

1.4. Results

Toad's peritoneal lavage fluid was first analyzed through conventional flow cytometry, which showed a typical forward scatter *vs.* side scatter cytograms for *R. schneideri*, displaying different cell populations (Fig. 1.1A). Most peritoneal lavage fluid cells were macrophages (38%; Fig 1.1A). Neutrophils were the second most abundant cells (27%), followed by lymphocytes (20%; Fig. 1.1A). Although there were lymphocytes in the peritoneal lavage fluid, only macrophages and neutrophils showed phagocytic activity, observed and confirmed by the conventional (indicated by median fluorescence intensity - MFI) (Fig. 1.2A, B and C) and imaging flow cytometry (Fig. 1.2D-I).

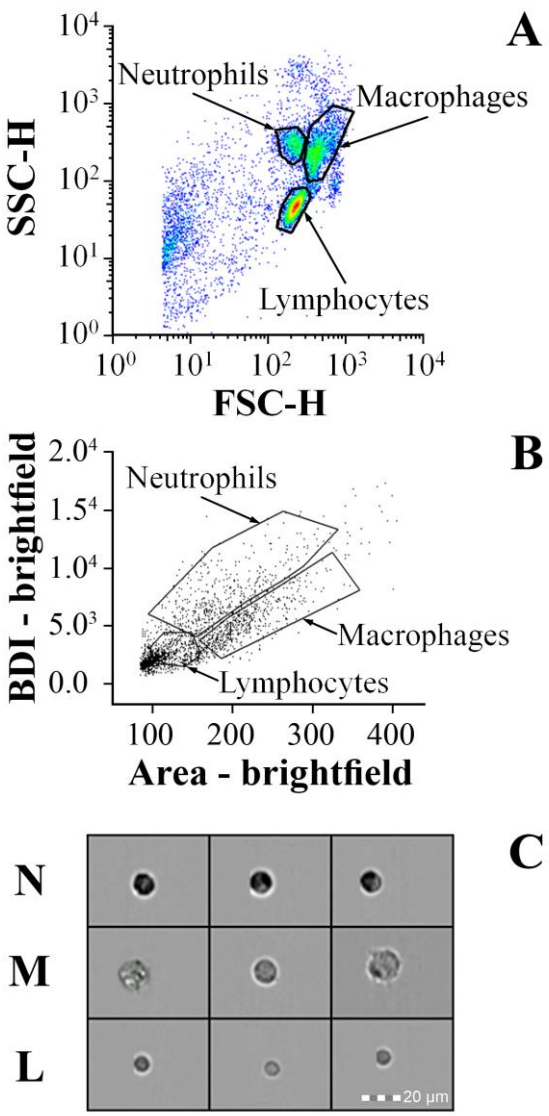

Figure 1.1. Representative conventional and imaging flow cytometry analysis of *R. schneideri* peritoneal lavage fluid. (A) FACS Calibur Plot: side scatter (SSC) *vs.* forward scatter (FSC) logarithmic scale dot plot, with cell populations gated. (B) AMNIS Flowsight Plot: bright detailed intensity - brightfield *vs.* SSC dot plot, with cell populations gated. (C) AMNIS Flowsight images from peritoneal cell populations in brightfield channel. Abbreviations: **SSC:** side scatter; **FSC:** forward scatter; **BDI:** Bright detail intensity; **N:** Neutrophils; **M:** Macrophages; **L:** Lymphocytes.

Imaging flow cytometry also showed a typical side scatter *vs.* bright detail intensity - brightfield cytograms for *R. schneideri* (Fig. 1.1B), displaying three different gates: macrophages (31%), neutrophils (24%) and lymphocytes (18%) (Fig. 1.1B and C). Brightfield images allowed us to further inspect the morphology of cells in each gate, providing more power and precision in the distinction of immune cells population. Toads imaging flow cytometry also showed neutrophils and macrophages engulfing a different number of zymosan particles, which allows a detailed analysis of phagocytosis (related to the number of zymosan particles engulfed by cells) in anurans (Fig. 1.2D, E, G and H). Imaging flow cytometry histogram and images also confirmed no lymphocytes performing phagocytosis (Fig. 1.2F and I).

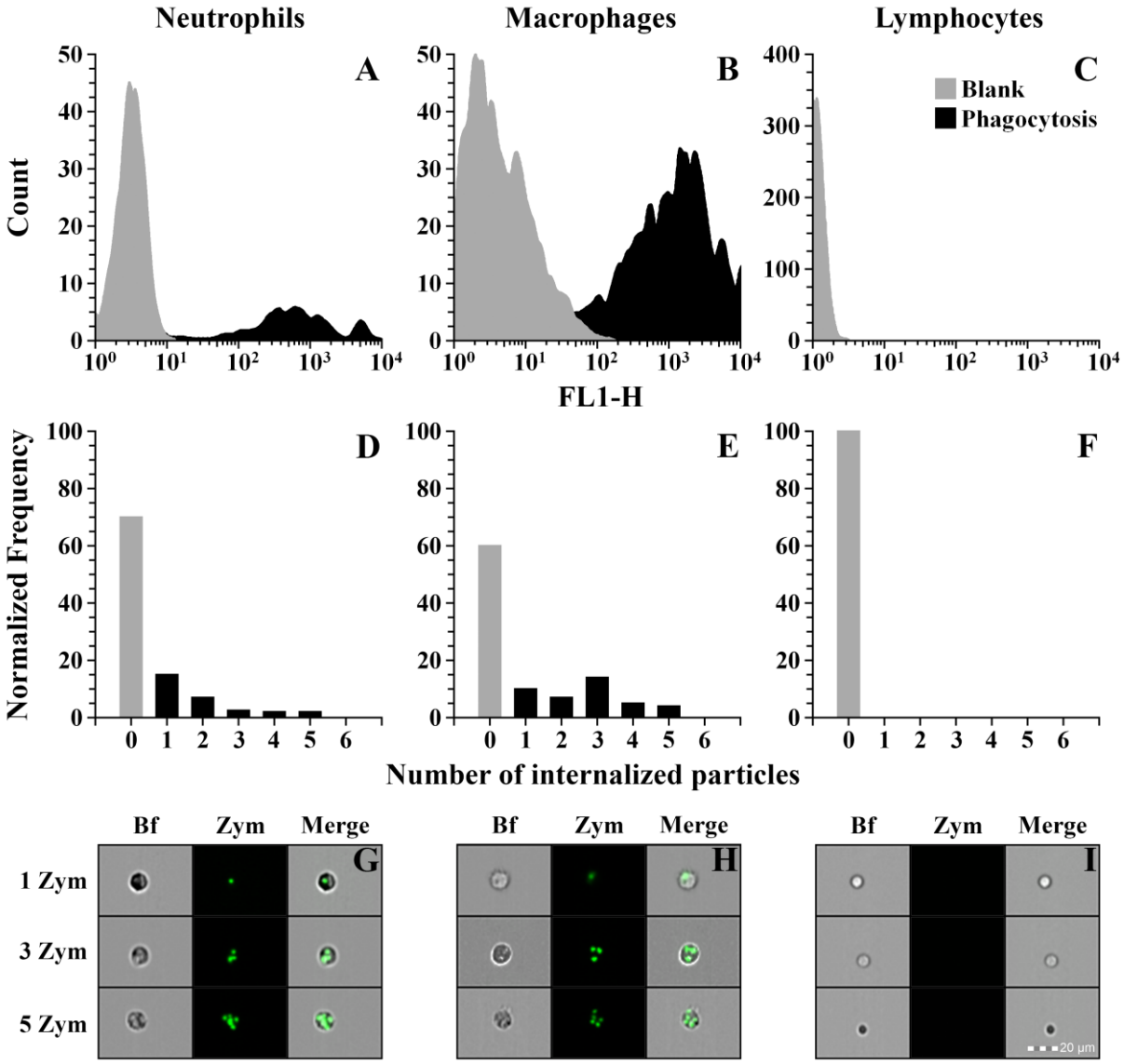

Figure 1.2. Representative histograms of peritoneal cells phagocytic activity of *R. schneideri*. Representative FACS Calibur green fluorescence (FL1 channel) histograms from population gates with 65 minutes of interaction (cells + zymosan): (A) neutrophils, (B) macrophages and (C) lymphocytes, respectively. AMNIS Flowsight green fluorescence (Ch 2) spot count histograms (D to I) from cell populations at 65 minutes of interaction: (D) neutrophils, (E) macrophages, and (F) lymphocytes. AMNIS Flowsight images: (G) neutrophils and (H) macrophages engulfing 1, 3, or 5 zymosan particles, and (I) lymphocytes with no zymosan particle. Legend: **Blank:** fluorescence of cells without zymosan; **Phagocytosis:** fluorescence of internalized zymosan particles (by cells). Abbreviations: **Bf:** Brightfield; **Zym:** Zymosan; **Merge:** Brightfield + Zymosan; **1 Zym:** One zymosan particle engulfed by the cell; **3 Zym**: Three zymosan particles engulfed by the cell; **5 Zym:** Five zymosan particles engulfed by the cell.

In order to confirm the relevance of imaging flow cytometry in multicolor fluorescence analyses, we evaluated the phagocytic activity of peritoneal macrophages obtained from Wistar rats. A CD11b/c+ membrane cell marker, which identify microglia and macrophage cells in rats, confirmed the macrophage population and phagocytosis activity by the cells (Fig. 1.3). The detailed spot count results, based on the number of particles internalized by the cell, are depicted in Figure 1.3A and B. When cell membranes are labeled (i.e. when cells are stained with plasma membrane markers antibodies, such as anti-CD11b/c) the IDEAS internalization algorithm was used to calculate phagocytic scores, a measure of internalization efficiency (Fig. 1.3C and D).

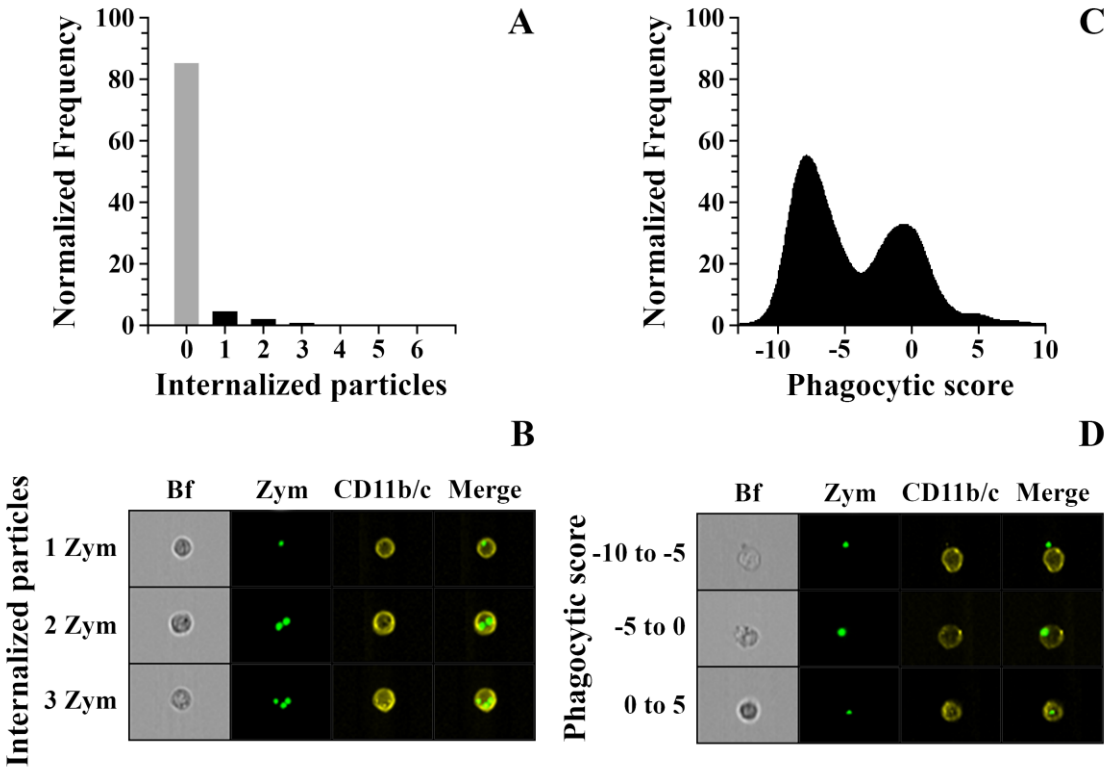

Figure 1.3. Phagocytic activity of Wistar rat's peritoneal cells. (A) AMNIS Flowsight green fluorescence (Ch 2) spot count histograms (A) from cell macrophages at 65 minutes of interaction. (B) Macrophages that engulfed 1, 2 or 3 zymosan particles. (C) Phagocytic score at 65 minutes of interaction. (D) Degree of zymosan internalization according to the phagocytic score. Points and bars represent mean and standard error, respectively (N = 4). Abbreviations: **Bf:** Brightfield; **Zym:** Zymosan; **Merge:** Zymosan + CD11b/c; **1 Zym:** One zymosan particle engulfed by the cell; **2 Zym**: Two zymosan particles engulfed by the cell; **3 Zym:** Three zymosan particles engulfed by the cell.

1.5. Discussion

Toad's phagocytosis measurements from peritoneal cells were made using a conventional and an imaging flow cytometer. Our results showed typical forward scatter *vs.* side scatter plot by conventional cytometry, with macrophages, neutrophils and lymphocytes grouped in different populations (Perticarari et al., 1994; Massoco and Palermo-Neto, 2003). A similar graph depicting the same three populations of cells was confirmed by imaging flow cytometry, demonstrating that both techniques were useful in grouping the peritoneal cells of these animals.

Though a widespread technique, conventional flow cytometry has been used to evaluate phagocytosis in a few studies with anurans, showing a quantitative analysis of phagocytosis, measured as percentage of cells positive for the zymosan-associated fluorochrome (Froese et al., 2005; Christin et al., 2013; Assis et al., 2017). This analysis of phagocytosis is based on the MFI (Brekke et al., 2007; Khanfer et al., 2012; Assis et al., 2017), which indicates the total the amount of zymosan-associated fluorescence present in the cells. As previously described by our group for *R. icterica* toads (Assis et al., 2017), we demonstrated MFI-based analysis using conventional cytometry for describing phagocytosis of peritoneal cells in *R. schneideri* toads. Based on the MFI we observed that macrophages showed a great percentage of cells engulfing a higher amount of zymosan particles (higher fluorescence), while neutrophils engulfed a smaller number of zymosan particles (low fluorescence), but showing great percentage of cells performing phagocytosis. In mammals, macrophages are the most important phagocytic cells, with distinct macrophages presenting differences in phagocytic activity varying according to the cell phenotype (Gordon and Taylor, 2005). In the meantime, neutrophils are important microbicidal polymorphonuclear cells, sharing some similar characteristics with macrophages (Kantari et al., 2008). Therefore, the differences between toads' macrophage and neutrophil phagocytic activity may reflect differences in these cells characteristics (e.g. type/ number of

specific receptor) and physiology (e.g. signaling molecules) that may contribute to efficient particle internalization (Kantari et al., 2008).

Meantime, the imaging flow cytometry allows the measurements of additional parameters of phagocytic function, such as the study of different phagocytosis stages, including distinguishing binding for those internalized particles, number of particle engulfed by the cells, among others (Erdbrügger et al., 2014; Havixbeck et al., 2015). Our results demonstrated that Wistar rats' CD11b/c macrophages presented different stages of zymosan internalization at 65 min (attached to the membrane, inside the cell, close to the central zone of the cell), allowing the study of the phagocytosis process. Phagocytosis involves two distinct steps, attachment and internalization, with the recognition and binding of targets to membrane receptors and the actin-dependent internalization being independent processes that involve different sets of molecules (Aderem and Underhill, 1999; Underhill and Goodrige, 2012). Therefore, the dynamics of phagocytosis can be modulated at the level of particle recognition and/or particle engulfment and internalization (Underhill and Ozinsky, 2002). Hence, the use of this membrane marker allowed the quantification of the efficiency of particle internalization, measured as phagocytic score. In addition, the development of membrane markers would be of great interest in studies related to non-mammalian vertebrates.

For *R. schneideri* toads, the imaging flow cytometry, which is based on the cell morphology, allowed detailed analyses regarding to phagocytic activity of peritoneal macrophages and neutrophils. The phagocytosis process initiates by the recognition of the antigen to be engulfed, which is performed by proper receptors, then, initiates the engulfing process (Janeway, 1992; Aderem and Underhill, 1999). The MFI-based analysis relies on positive fluorescence, including cells with the particle attached to the cell surface (not engulfed by the cell) as positive for phagocytosis (Nuutila and Lilius, 2005). The imaging flow cytometry analyses, in turn, is based on the cell morphology and automatically excludes attached zymosan

to the surface of the cell. Therefore, quantifying the percentage of cells by the spot count analysis, based on the numbers of internalized particles by the cell, reduced the incidence of false positives due to unspecific binding of zymosan to the cell membrane, representing a more accurate measurement of phagocytosis.

Regarding the phagocytic activity, the spot count analyses allowed a detailed investigation regarding to the number of zymosan particles engulfed by the peritoneal cells for both, toads and Wistar rats. Phagocytosis has been measured by the total number of cells performing phagocytosis; with some studies showing that phagocytosis percentage can be altered by some in vitro and in vivo treatments (Barriga et al., 2002; Dohm et al., 2005; Assis et al., 2017). Meantime, Titon et al. (2017) demonstrated that, although phagocytosis percentage was not altered, the number of cells engulfing three or more zymosan particles could be modulated by long-term stress conditions. The modulation of the relation between: cell and number of engulfed particles, is subjected to different treatments (Barriga et al., 2001; Barriga et al., 2002; Pires-Lapa et al., 2013; Titon et al., 2017). Therefore, the analyses of the percentage of cells engulfing different number of zymosan (antigen) particles, highlight the importance of the study of phagocytosis itself. Moreover, the spot count analyses can allow a more comprehensive investigation regarding to the dynamic and regulation of phagocytosis.

1.6. Conclusions

Flow cytometry is a useful tool in phagocytosis analysis of toad's peritoneal cells. Imaging flow cytometry provides a more detailed study, bringing new approaches for analyses of phagocytosis of peritoneal cells from anurans and mammals. Moreover, the detailed analysis of phagocytosis (number of internalized particles and in internalization efficiency) can bring a more comprehensive study about immune cells' function and regulation in a wide range of species at several *in vivo* and *in vitro* contexts.

1.7. Acknowledgments

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CAPÍTULO 2 – CAPTIVITY EFFECTS ON IMMUNE RESPONSE AND STEROID PLASMA LEVELS OF A BRAZILIAN TOAD (*Rhinella schneideri*)

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2.1. Abstract

Stressors can increase plasma glucocorticoid levels and decrease plasma androgen levels in different species of vertebrates. Glucocorticoids can have immune-enhancing or immunosuppressive effects, which are dependent upon stress duration and intensity. The worldwide decline in amphibian populations is strongly linked to an array of different stressors. The impacts of stress on glucocorticoids, androgens and the immune response are important to clarify, and should lead to the better development of conservation strategies. The present study in adult male toads of *Rhinella schneideri* investigated the effects of captivity (7, 30 and 60 days) on plasma corticosterone (CORT) and plasma testosterone (T), as well as innate immune responses, specifically humoral and cell mediated responses, as indicated by bacterial killing ability (BKA) and phagocytosis by peritoneal cells, respectively. Captivity increased CORT 3-fold and decreased T versus controls. CORT maintained a 3-fold elevation throughout the captivity period, while body mass and T gradually decreased with time in captivity. BKA was lower at day 30, versus days 7 and 60, whilst peritoneal cell phagocytic efficiency decreased after day 30, remaining low at day 60. Moreover, phagocytosis efficiency was positively associated with T and body condition, suggesting that the effects of chronic stress on reproductive potential and immune response might be associated with the state of energetic reserves.

Keywords: Stress, amphibian, corticosterone, testosterone, phagocytosis, bacterial killing ability.

2.2. Introduction

Glucocorticoids (GCs) are steroid hormones produced by the adrenal or interrenal glands (Sapolsky, 2002). Their production and release can be modulated by the activation of the hypothalamus-pituitary-adrenal/interrenal axis in response to different stressors in all vertebrates (Sapolsky, 2002). Acute stress-induced transient increases in plasma GCs may have beneficial effects, such as gluconeogenesis and the temporary suppression of reproduction, as well as immune modulation, including immune-enhancement (Sapolsky et al., 2000; Wingfield and Romero, 2001). However, chronic stress-induced increases in plasma GCs generally have deleterious effects, including discontinued reproduction in association with lowered androgen levels, and immune response suppression (Sapolsky et al., 2000; Landys et al., 2006). The immune-enhancing effects of acute stress-induced GCs include the release of humoral components, which can activate cell-mediated immunity, increase lymphocyte proliferation, and heighten the phagocytic activity of blood leukocytes (Wiegers et al., 1995; Wiegers and Reul, 1998; Graham et al., 2012; Dhabhar, 2014). However, chronically elevated GCs reduce the size and function of lymphoid organs and exhibit anti-inflammatory effects, including by inhibiting the development and action of immune cells (Ahmed et al., 1985; Wiegers and Reul, 1998; Sapolsky et al., 2000; Riccardi et al., 2002; Cavalcanti et al., 2006). As such, GCs have a bimodal effect on the immune response, driven by variations in stress duration and intensity (Wiegers and Reul, '98; Sapolsky et al., 2000, Dhabhar, 2014).

Amphibian populations have been declining around the world in recent decades (Ficetola, 2015). Climate change, habitat loss and degradation, landscape fragmentation, and emerging infectious diseases are currently recognized as major causes, sometimes synergistic, of the decline and extinction of many populations (Carey et al., 1999; Lips et al., 2005; Navas et al., 2012; Ficetola, 2015). As stress can modulate the immune response, stress may modulate the vulnerability to infections associated with amphibian population decline (Dobson and

Foufopoulos, 2001; Rollins-Smith, 2001; Acevedo-Whitehouse and Duffus 2009; Kiesecker, 2010; Gabor et al., 2015). Symptomatic signs of infection with Batrachochytrium dendrobatidis can increase the plasma levels of the main amphibian GC, corticosterone (CORT), when compared to infected but not diseased or non-infected animals (Kindermann et al., 2012; Peterson et al., 2013). Environmental stress due to landscape alterations is also associated with increased CORT, in both anurans and salamanders (Homan et al., 2003; Janin et al., 2011; Tennessen et al., 2014). Therefore, increases in symptomatic infection-regulated CORT may interact with the immune response, whilst increased CORT may be an integral part of the amphibian adaptive response to novel environmental challenges.

Captivity is considered a stressor for many vertebrate species, commonly resulting in higher plasma CORT levels and lower plasma T levels, as well as altered immune responses in birds, reptiles, and amphibians (Jones and Bell, 2004; Matson et al., 2006; Buehler et al., 2008; Fokidis et al., 2011; Assis et al., 2015). However, the effects of captivity on the levels of CORT, T, and immune response may change over time and vary across species (Dickens and Romero, 2013). In anurans, captive individuals commonly show an increase in plasma CORT levels and decrease in plasma T levels, versus natural field conditions. However, immune responses can vary, which is dependent upon a number of factors, including the immune parameters evaluated, time in captivity, and studied species (Hopkins and Durant, 2011; Gomes et al., 2012; Graham et al., 2012; Falso et al., 2015). Such data highlight how stress-associated captivity can modulate the interactions of plasma CORT and T levels, with immune responses in different species, including amphibians.

The present study investigated the effects of captivity, for 7, 30, and 60 days, on plasma CORT and T levels as well as the innate immune response, as indicated by humoral: bacterial killing ability (BKA) and cell mediated: peritoneal cell phagocytic capacity, in adult male toads of *R. schneideri*. Considering that captivity might be a stressor for anurans, we tested the

following hypotheses: 1) captivity increases plasma CORT and decreases plasma T levels as well as suppressing immune responses; 2) these effects are modulated by the length of time that toads are maintained in captivity; 3) plasma CORT levels and the immune responses are inversely correlated over the course of captivity.

2.3. Material and methods

2.3.1. Study site and animals

Rhinella schneideri is a large toad from the *Rhinella marina* group (Maciel et al. 2010). This species shows a wide geographic distribution, occupying open areas in the Brazilian Atlantic Rainforest and Cerrado (Maciel et al., 2010).

Adult males of *R. schneideri* (*N* = 30) were collected in October 2015 in Botucatu (22° 46′ 55″ S, 48° 28′ 29″ W), São Paulo/Brazil. The animals were collected under license from Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (ICMBio, process number 29896-1). The procedures for the collection and use of biological material were performed with the approval of the Comissão de Ética no Uso de Animais (CEUA) do Instituto de Biociências da Universidade de São Paulo (CEUA - n° 054/2013).

2.3.2. Field procedures

All animals were located by visual inspection and manually captured. Blood samples from the toads were collected in the field (~ 200 µl) by cardiac puncture using heparinized 1 ml syringes and 26 Gx1/2” needles for baseline measurements. Only samples collected within 3 min after animal disturbance were considered, in order to avoid any influence of handling on hormone levels (Romero and Reed, 2005). All blood samples were identified and kept on ice (< 5 h), thereafter being centrifuged in order to isolate the plasma (4 min at 3000 rpm). Plasma samples were stored in cryogenic tubes, and kept in liquid nitrogen until they could be

transferred to a -80°C freezer, for hormone assays and BKA analysis. Additionally, hour of capture was recorded and included in later analyses, given that hormone levels may vary throughout the night (Jessop et al, 2014; Titon et al., 2016). All individuals were weighed (0.01g) and the snout-vent length (SVL) was measured (0.01 mm) after capture. The animals were kept in individual plastic containers, from 2 to 4 days, until they were taken to the laboratory. The lids of the containers had holes to allow air circulation. The animals were exposed to the natural light cycle and at temperatures compatible with their natural thermal regime.

2.3.3. Captivity conditions

In the laboratory, the animals were individually maintained in plastic containers (43.0 cm x 28.5 cm x 26.5 cm). The lids of the containers had holes to allow air circulation. The animals had free access to water and were fed with cockroaches once per week, a protocol previously applied in our laboratory to the same species and other large toads from genus *Rhinella*, and allowed maintenance of body mass under long periods. All toads ate the cockroaches offered. The animals were exposed to a 13/11 LD cycle (lights on at 06 h 40 min and off at 19 h 40 min) and 21 ± 3°C. Individuals were weighed 2 days before the experimental procedures. Captivity conditions were the same for all individuals collected, with captivity duration varying among experimental groups, as described below.

2.3.4. Captivity duration and experimental design

Captive animals were divided into three groups, which were sampled at day 7, day 30 and day 60, allowing an analysis of time effects on plasma CORT and T as well as immune responses indicated by BKA and peritoneal cell phagocytic activity. Before euthanasia, a blood sample from each individual was collected and processed in accordance with the methods

described in section 2.3.2. Plasma samples were later used for hormone assays and BKA. After blood collection, animals were euthanized by immersion in a lethal solution of benzocaine (0.2%), with the retrieval of peritoneal cells occurring thereafter. Blood collection and retrieval of peritoneal cells were performed between 19:00 and 20:00 h.

2.3.5. Retrieval of peritoneal cells

Due to methodological limitations in field, this assay was performed only for individuals in captivity, at d7, d30, and d60. Following euthanasia, the abdomen was cleansed with ethanol 70%, and a small incision was made into the peritoneal cavity with a sterile surgical instrument. Using a sterile Pasteur pipette, 10 ml of sterile PBS was injected into the peritoneum, and the abdominal region of the toad was carefully massaged. Lavage fluid (resulting solution of PBS + cells) was then removed with a sterile Pasteur pipette, placed in a 15 ml conical tube, and kept on ice. The lavage fluid was then used to perform cell counts and viability. Ten microliters of the lavage fluid were diluted in 10 µl of a 0.4% stock solution of Trypan Blue (dilution factor: 1:1) to perform cell counting in a glass hemocytometer. Lavage fluid was then centrifuged (1200 rpm, at 4°C for 9 min), and the supernatant discarded. The cells were then re-suspended in 1ml of PBS to perform the phagocytosis assay.

2.3.6. Phagocytosis assay

The phagocytosis assay is a measure of cellular, primarily macrophage and neutrophil, ability to recognize and engulf foreign particles or infected dying cells (Aderen and Underhill, 1999). The phagocytosis assay from peritoneal cells of *R. schneideri* was performed according to Massoco and Palermo-Neto (2003), with modifications. The number of phagocytic cells was adjusted to 2 x 10^5 cells/200 µl. Zymosan A-CFSE work solution (1 x 10^7 particles/ml PBS)

was prepared from a zymosan (SIGMA Z-4250) stock solution which was kept frozen (-20°C) and protected from light. Thereafter, 200 µl of the lavage fluid with phagocytic cells (2 x 10^5 cells) were diluted in 800 µl of PBS, and 100 µl of zymosan work solution was added to the samples. Blank was made with the lavage fluid containing phagocytic cells (2 x 10^5 cells) diluted in PBS in the same proportion. Samples were covered with aluminum foil to protect from light, and incubated under agitation at 25°C, for 35 min. A temperature of 25°C was chosen to run the phagocytic assay, as this is the mean thermal preference for R. schneideri according to Bicego et al. (2002). Reactions were stopped by adding 2 ml of cold (4°C) EDTA solution (6 mM). After centrifugation (1200 rpm, at 4°C for 7 min) the supernatant was discarded and 200 µl of cold (4°C) paraformaldehyde (PAF 1%) were added. The samples were then kept for 1 hour at 4°C for cell fixation. After this period, 500 µl of PBS solution was added, and the samples were centrifuged (1200 rpm, at 4°C for 7 min). The supernatant was then discarded, and 100 µl of PBS solution was added for later readings on flow cytometer (details in section 2.7). Quantification of phagocytosis was estimated by mean zymosan-CFSE fluorescence cell. Direct measurements of mean fluorescence of green channel were recorded as phagocytosis.

2.3.7. Flow cytometry and analysis

An image flow cytometer (AMNIS Flowsight imaging flow cytometer – Merck-Millipore, German) interfaced with a DELL computer was used for phagocytic activity measurements of peritoneal macrophages by flow cytometry. Data from 20,000 events were acquired utilizing the 488 nm laser at a 40x magnification, using the INSPIRE software. Acquired data were analyzed using IDEAS analysis software (EMD Millipore) version 6.1 for windows. Single cells were first identified based on a scatter plot of bright field area *versus* the aspect ratio. A gate was drawn around each population containing putative single cells, based

on the criteria of the area being large enough to exclude debris, and the aspect ratio being greater than 0.6, which eliminates debris and clusters. Macrophages and neutrophils were identified through gate images in focused-single cells plotted on the bright field area *vs.* the side scatter (SSC) plot. Quantification of phagocytosis was estimated by mean zymosan-CFSE fluorescence cell. Direct measurements of mean fluorescence in the green channel were recorded as phagocytosis. The percentage of macrophages and neutrophils that ingested at least one zymosan particle (with green fluorescence divided by the total number of cells [multiplied by 100]) was expressed as the phagocytosis percentage (PP). Phagocytic efficiency (PE) was calculated by the percentage of cells (macrophages and neutrophils) that ingested three or more zymosan particles.

2.3.8. Bacterial killing ability (BKA)

The BKA is a humoral innate immune response based on the ability of soluble proteins, such as complement, natural antibodies and lysozymes, to recognize and promote cell lyses of a foreign microorganism (Matson et al., 2006; Millet et al., 2007). This assay was conducted according to Assis et al. (2013). Briefly, plasma samples diluted (1: 20) in amphibian Ringer's solution (10 µl plasma: 190 µl Ringer) were mixed with 10 µl of *Escherichia 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 60 min at 37°C (optimal temperature for bacterial growth). After the incubation period, 500 µl of tryptic soy broth (TSB) was added to each sample. The bacterial suspensions were thoroughly mixed and 300 µl of each was transferred (in duplicate) to a 96 wells microplate. The microplate was incubated at 37ºC for 2h, 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 BKA was evaluated at the beginning of the bacterial exponential growth phase.

2.3.9. Hormonal assays

Steroid hormones were initially extracted with ether according to Mendonça et al. (1996). Briefly, 3 ml of ethyl ether were added to the plasma samples, vortexed for 30 seconds, and centrifuged at 4°C for 9 min at 1800 rpm. The tubes were then kept at -80°C for 7 min. 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 (~ 24h). The samples were resuspended in EIA buffer and plasma CORT and T levels were assayed using EIA kits (CORT number 500655; T number 582701, Cayman Chemical), according to the manufacturer's instructions and previous studies conducted with toads in our laboratory (Assis et al., 2015; Madelaire and Gomes, 2016). Different dilutions were used to run hormone assays, ranging from 1:15 to 1:20 for CORT and 1:15 to 1:30 for T samples. Intra-assay variation, estimated from sample duplicates, were 5.60% for CORT and 6.46% for T. Inter-assay variation, estimated using the average of four intermediate values from the standard curve (as recommended by the kit instructions), were 4.06% for CORT and 2.70% for T. Sensitivity of the assays, calculated as 80% B/B0 of curve value, was 52 pg/ml for CORT and 15 pg/ml for T.

2.3.10. Statistical analysis

Descriptive statistics were performed for all variables, and data were then submitted to a Shapiro-Wilk normality test. With the exception of body mass, all variables showed an absence of normality and were transformed to log10 to fit the prerequisites of parametric tests. Pearson

correlation tests were used to investigate the correlations between variables in the field and after d7, d30, and d60, as appropriate. Paired t-tests were used to investigate differences between data collected in field and after each captivity period. Paired t-tests were also used to investigate differences between field and grouped captivity values for plasma CORT levels, which did not differ across days in captivity.

Plasma CORT levels increase in amphibians kept captive when compared to field conditions (Narayan et al, 2011; Assis et al, 2015). Moreover, CORT is a catabolic hormone, which may promote body mass loss when elevated for prolonged periods (Bentley, '98; Sapolsky et al., 2000). Hence, an index of body mass loss was calculated as the percentage of body mass change from initial to final body mass records (1 - body mass after captivity days/body mass in field) and included in subsequent analyses. Two sets of ANCOVAs for independent measures were used to investigate the effect of time in captivity. The first set used plasma CORT levels as the dependent variable, the index of body mass loss (BML) as a co-variate, and captivity duration as a factor. The second set used T plasma levels, BKA, PP, and PE as dependent variables, with BML and CORT as co-variate, and captivity duration as a factor. For the dependent variables not significantly affected by co-variates, a set of independent measures ANOVAs, using captivity duration as a factor, were then performed. All ANOVAs were followed by tests for mean multiple comparisons with Bonferroni adjustment.

In order to better understand the relations among the studied variables, we also performed structural equation modeling (SEM). SEM is a multivariate statistical analysis used to test hypothesized cause-effect relationships. Models are proposed and tested against empirical data, and the magnitude of causal relationships between variables can be described (Shipley, 2000; Hershberger, 2003). The variables were transformed according to Box & Cox (1964) to fit the prerequisites of SEM analysis, which include data with multivariate normality (Shipley, 2000). Six models were proposed based on Pearson correlation tests, with predictions based on the

available knowledge about the relations between the following physiological traits: high plasma CORT levels may promote BML when elevated for prolonged periods (Sapolsky et al., 2000); plasma T levels tend to decrease in response to stressors, being inversely associated with plasma CORT levels in some species (Paolucci et al, 1990; Deviche et al, 2010; Narayan et al., 2012); BML can promote an increase in plasma CORT levels (Romero and Wikelski, 2001; Sockman and Schwabl, 2001); while body condition and energetic constraints can influence plasma T levels (Ligon et al., 1990; Pérez-Rodriguez et al., 2006), and immune responses in some vertebrate groups (Eraud et al., 2005; Adams et al., 2009; Desprat et al, 2015); In addition, immune cells express glucocorticoid and androgen receptors, and therefore, can be directly modulated by these hormones (Wiegers and Reul, 1998; Nava-Castro et al, 2012). The six proposed models are available as supplementary material (Fig. 2S1). The overall model fit was assessed based on $\chi 2$ statistic, which is computed comparing the observed and expected elements of the covariance matrix (Shipley, 2000). A non-significant result on $\chi 2$ test ($p > 0.05$) indicates that data support the proposed model (Shipley, 2000). Akaike's information criterion (AIC) was used to identify the best model among those proposed. In this way, models that were supported by $\chi 2$ test and had smaller AIC (dAICc < 2.0 on model selection analysis) were selected for better explaining the relationships among the variables (Shipley, 2000; Mazerolle, 2006) (Table 2S1).

We performed descriptive statistics, correlations, ANCOVAs, and ANOVAs using IBM SPSS Statistics 22, and structural equation modeling in R 3.2.5 (R Development Core Team, 2016), using the packages lavaan (Yves et al., 2015), semPlot (Epskamp, 2015), bbmle (Bolker, 2015), MVN (Korkmaz et al., 2015) and car (Fox et al., 2015).

2.4. Results

Descriptive statistics of variables from males of *R. schneideri* in the field and after 7, 30, and 60 days of captivity are shown in Table 2.1.

Table 2.1. Descriptive statistics of physiological variables and body measures for individuals of *R. schneideri* in field and captivity.

Variable	CD (day)	N	Mean ± SE	Min	Max
Snout-vent length (mm)	Field	25	117.87 ± 1.70	103.88	134.91
	7	8	120.55 ± 3.41	104.86	129.61
	30	6	120.47 ± 4.36	108.28	134.91
	60	11	114.50 ± 1.65	103.88	121.53
Body mass (g)	Field	27	145.32 ± 6.06	90.63	195.35
	7	8	144.18 ± 11.20	100.01	189.54
	30	6	137.50 ± 17.63	94.05	213.50
	60	11	121.37 ± 7.07	70.22	152.33
Plasma corticosterone levels (ng/ml)	Field	27	1.48 ± 0.25	0.18	4.24
	7	8	4.61 ± 1.36	0.16	11.14
	30	6	4.10 ± 1.28	0.95	9.00
	60	11	3.76 ± 0.86	0.32	8.43
Plasma testosterone levels (ng/ml)	Field	26	5.59 ± 1.15	0.08	20.93
	7	8	1.94 ± 0.66	0.23	4.96
	30	6	0.84 ± 0.30	0.14	1.72
	60	11	0.19 ± 0.02	0.13	0.34
Bacterial killing ability (%)	Field	27	54.81 ± 5.09	11.00	100.00
	7	8	69.50 ± 7.92	36.00	100.00
	30	6	42.67 ± 7.53	9.00	62.00
	60	11	72.09 ± 4.22	49.00	98.00
Phagocytosis percentage (%)	7	8	9.67 ± 2.97	3.57	28.10
	30	6	6.98 ± 2.05	0.83	14.50
	60	9	9.31 ± 2.22	2.59	22.40
Phagocytic efficiency (%)	7	8	4.33 ± 1.13	0.00	9.43
	30	6	1.70 ± 0.56	0.00	3.92
	60	9	1.33 ± 0.21	0.55	2.43

Abbreviation as follow: **CD:** Captivity duration; **Mean ± SE:** Mean ± standard error. **Min:** Minimum value; **Max:** Maximum value.

Regarding field data, a negative correlation was found between plasma CORT levels and hour of capture (ρ = -0.458, P = 0.016). Captivity duration periods did not show any significant

differences for variables measured in field conditions ($P \geq 0.078$). An ANOVA table showing detailed results from these comparisons is available as supplementary material (Table 2S2).

When data from captivity were grouped, and compared to field values, captive toads showed a 3-fold increase in the levels of plasma CORT (t_{20} = -3.206, P = 0.004). Toads maintained for 7 days in captivity showed decreased plasma T levels (t_7 = 3.097, P = 0.017), and increased plasma CORT levels (t_7 = -2.853, P = 0.025), whilst toads maintained captive for 60 days showed decreased plasma T levels (t_7 = 11.805, $P \leq 0.001$), and increased BML (t_7 = 5.146, $P \leq 0.001$), when compared to field data. Although there is a tendency to decrease at day 30, BKA from captive toads did not differ when compared to field values ($P \geq 0.07$).

Toads displayed increasing BML over time in captivity ($F_{2,18}$ = 4.658, P = 0.023; Fig 2.1 A), and BML was not correlated with SVL (P = 0,183). Plasma CORT levels did not differ between periods of captivity duration (Fig 2.1 B), but was higher in individuals characterized by greater BML (Table 2.2), with no correlation with SVL (P = 0,603). When periods of time in captivity were compared, plasma T levels were higher after 7 days, gradually decreasing over time (Fig 2.1 C; Table 2.3). PP did not differ between periods of captivity (Fig 2.2 A; Table 2.3). PE decreased from 7 to 30 days in captivity, remaining low after 60 days under these conditions (Fig 2.2 B; Table 2.3). Moreover, PE showed a negative correlation with BML (Fig 2.3 A). BKA was affected by time in captivity, with lower values when toads were maintained captive for 30 days, when compared to shorter and longer periods under these conditions (Fig 2.2 C; Table 2.3). Toads in captivity displaying higher BML also displayed higher plasma CORT levels and lower plasma T levels (Fig 2.3B, C). Individuals in captivity with lower plasma T levels also had lower PE (Fig 2.3 D). Detailed ANCOVAs for all variables tested are available as supplementary material (Table 2S3).

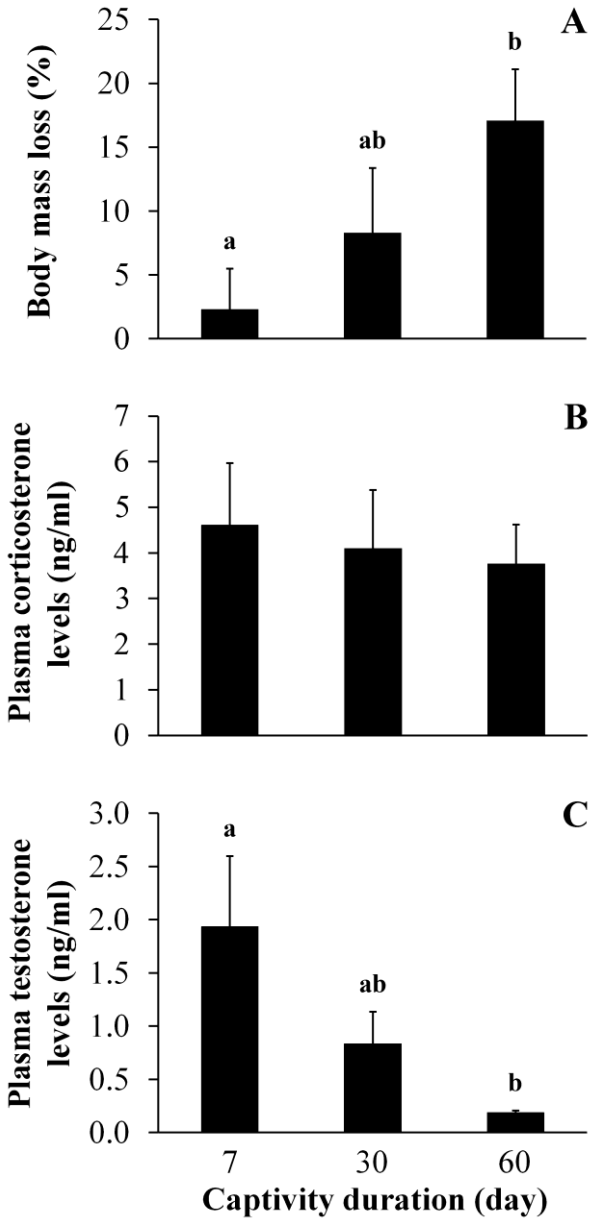

Figure 2.1. Effects of captivity on body mass and plasma hormone levels of toads (*Rhinella schneideri*). Differences in (A) body mass loss, (B) plasma corticosterone and (C) plasma testosterone levels of individuals during captivity (N = 8, 5 and 8, for 7, 30, and 60 days of captivity, respectively, for all variables). Letters above the bars represent statistical differences for ANOVA, with different letters representing statistical difference with $P \leq 0.05$. Bars represent mean ± standard error.

Table 2.2. Effect of captivity duration on plasma corticosterone levels, with plasma corticosterone levels as dependent variable, body mass loss percentage as co-variable and captivity duration (7, 30 and 60 days) as factor.

Dependent Variable	Source	Type III SS	DF	MS	*F*	*P*
CORT	**Model**	1.61	5	0.32	1.565	0.230
	Intercept	2.53	1	2.53	12.289	**0.003**
	CD	0.06	2	0.03	0.150	0.862
	BML	1.33	1	1.33	6.483	**0.022**
	CD*BML	0.29	2	0.14	0.696	0.514
	Error	3.08	15	0.21		
	Total	16.08	21			

Abbreviation as follow: **Type III SS:** Type III sum of squares; **DF:** Degrees of freedom; **MS:** Mean square; **CORT:** Plasma corticosterone levels; **CD:** Captivity duration; **BML:** Body mass loss percentage; Variables with *P* significant < 0.05 are highlighted in bold.

Table 2.3. Effect of captivity duration on plasma testosterone levels and immune response tested through a set of ANOVAs, with plasma testosterone levels, bacterial killing ability and phagocytic percentage and efficiency as dependent variables and captivity duration (7, 30 and 60 days) as factor.

Dependent Variable	Source	Type III SS	DF	MS	*F*	*P*
T	**Model**	3.03	2	1.51	10.518	**0.001**
	Intercept	2.36	1	2.36	16.408	**0.001**
	CD	3.03	2	1.51	10.518	**0.001**
	Error	3.17	22	0.14		
	Total	9.65	25			
BKA	**Model**	0.34	2	0.17	5.308	**0.013**
	Intercept	71.65	1	71.65	2219.051	**0.000**
	CD	0.34	2	0.17	5.308	**0.013**
	Error	0.71	22	0.03		
	Total	79.57	25			
PP	**Model**	0.15	2	0.08	0.573	0.573
	Intercept	14.64	1	14.64	111.788	**0.000**
	CD	0.15	2	0.08	0.573	0.573
	Error	2.62	20	0.13		
	Total	18.33	23			
PE	**Model**	0.58	2	0.29	11.355	**0.001**
	Intercept	5.25	1	5.25	204.301	**0.000**
	CD	0.58	2	0.29	11.355	**0.001**
	Error	0.46	18	0.03		
	Total	6.34	21			

Abbreviation as follow: **Type III SS:** Type III sum of squares; **DF:** Degrees of freedom; **MS:** Mean square; **T:** Plasma testosterone levels; **CD:** Captivity duration; **BKA:** Bacterial killing ability; **PP:** Phagocytosis percentage; PE: Phagocytosis efficiency. Variables with *P* significant < 0.05 are highlighted in bold.

The models that better explained the relations between plasma CORT levels, BML, plasma T levels and PE are presented in Figure 2.3 E, F and G, with model 2 showing the highest weight value (Table 2S3). All three models showed consistent results concerning the relations between the investigated variables, with higher plasma CORT levels positively correlating with BML over time in captivity. Moreover, increased BML promoted a decrease in plasma T levels, with plasma T levels positively associating with PE in peritoneal cells.

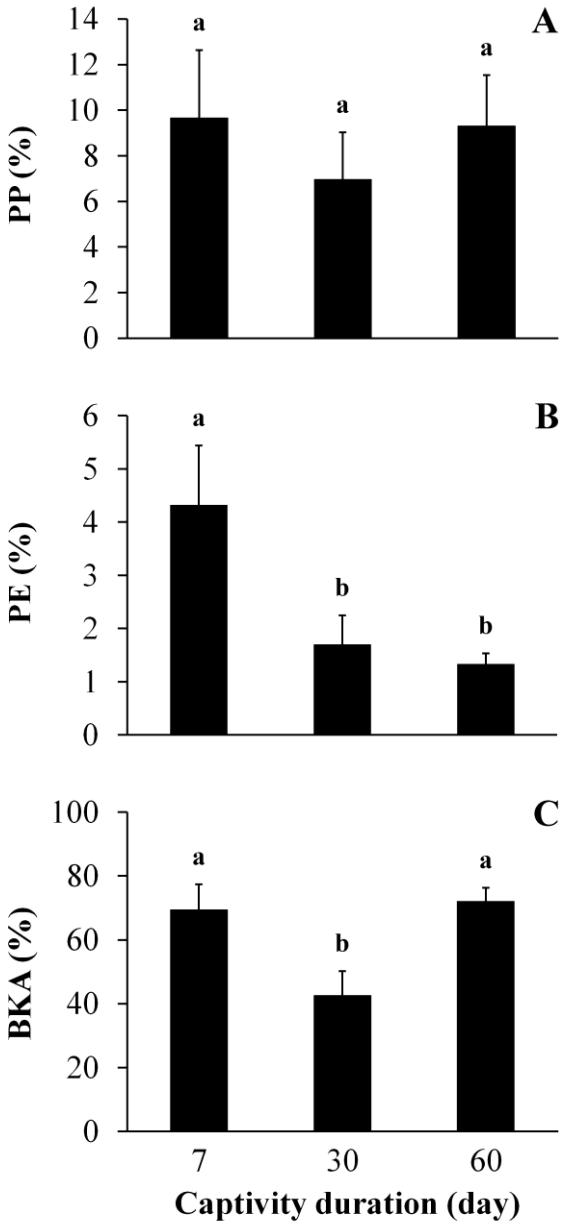

Figure 2.2. Effects of captivity on immune responses of toads (*Rhinella schneideri*). Differences in (A) Phagocytosis percentage, (B) Phagocytosis efficiency, and (C) Bacterial killing ability of individuals during captivity (N = 8, 5 and 8, for 7, 30, and 60 days of captivity duration, respectively, for all variables). Letters above the bars represent statistical differences, with different letters representing statistical difference with $P \leq 0.05$. Bars represent mean ± standard error. Abbreviations: **PP:** Phagocytosis percentage; **PE:** Phagocytic efficiency; **BKA:** Bacterial killing ability

2.5. Discussion

Individuals of *R. schneideri* collected earlier in the night showed the highest plasma CORT levels, a pattern previously described for other anuran species (Leary et al., 2015; Titon et al., 2016). A circadian GC peak anticipates the activity period in many species of vertebrates (Goldman, et al., 2004). For *R. schneideri*, field plasma CORT levels were measured between 18:30 h and 23:00 h, with higher values found in the first hours of observations, indicating a general pattern of higher CORT at the beginning of the activity period for this nocturnal species.

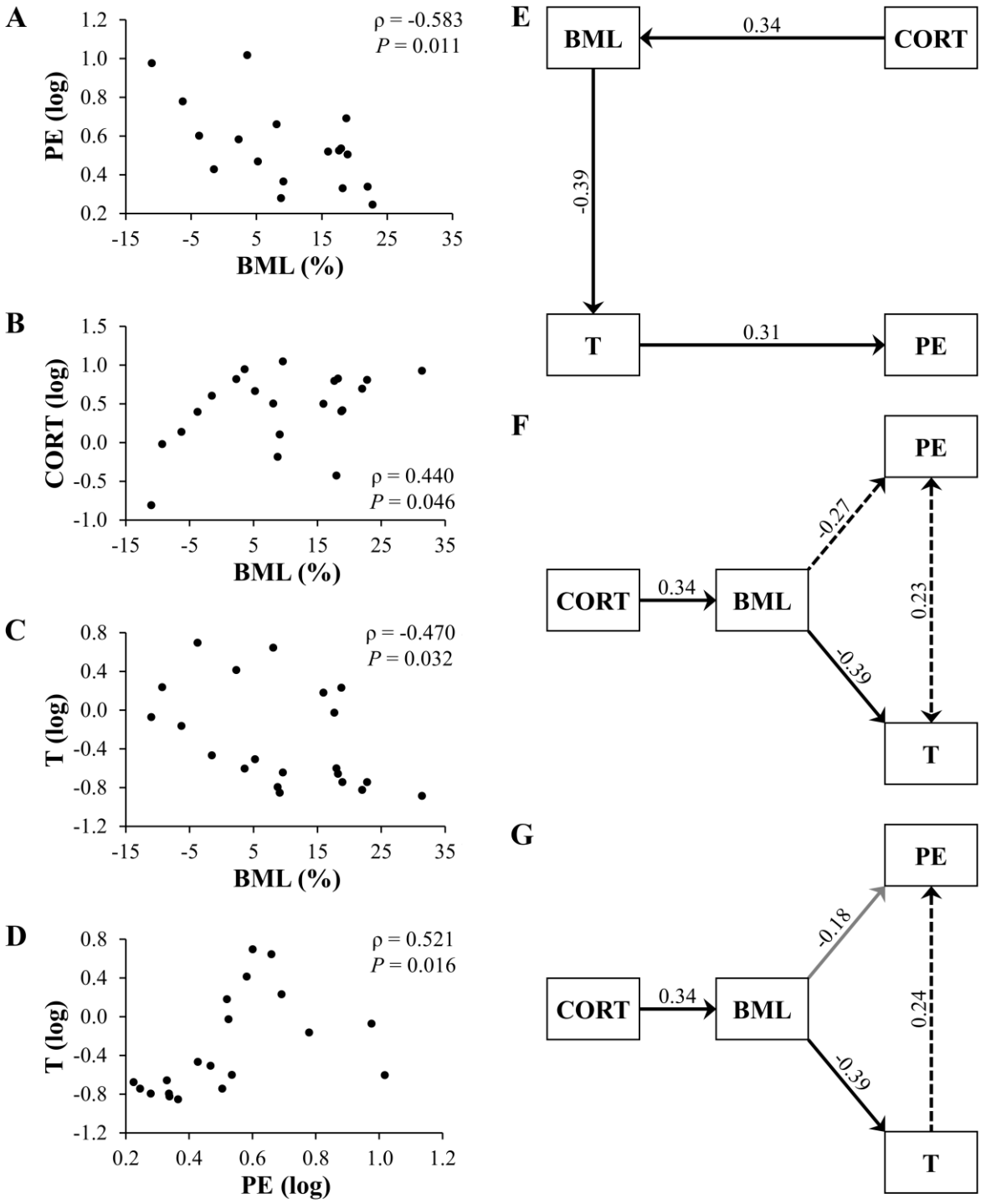

Figure 2.3. Correlations between physiological variables and selected structural equation modelling for these variables in toads (*Rhinella schneideri*) in captivity. Significant correlations between captivity data are represented by letters A to D. Letters E, F and G are path diagrams of the three causal selected models. Path coefficients shown are all standardized values in sequence with higher AIC and dAIC < 2.0: (E) Model 2 χ^2= 0.719, df=3, p= 0.869, AIC= -93.926; (F) Model 3 χ^2=0.125, df=2, p=0,939, AIC= -92.520; (G) model 4 χ^2=0,125, df=2, p= 0.939, AIC= -92.520. Abbreviations as follow: **CORT:** Plasma corticosterone levels; **BML:** Body mass loss; **T:** Plasma testosterone levels; **PE:** Phagocytic efficiency. (N = 20).

Captivity increased plasma CORT levels and decreased plasma T levels, versus field conditions, in *R. schneideri*. Many vertebrates show increased GCs when in captivity, with the amplitude of response varying in response to captivity conditions and duration (Moore et al., 1991; Cree et al., 2003; Markham et al., 2006; Adams et al., 2011; Gomes et al., 2012; Narayan et al., 2012; Assis et al., 2015). Capture and captivity increase plasma CORT levels in different vertebrates, with a longer captivity duration resulting in sustained increases in plasma CORT levels in some species (Moore et al., 1991; Sparkman et al., 2014; Dickens et al., 2009; Martin et al., 2012), and short-term attenuation of the acute increases in plasma CORT levels in other vertebrates (Jones and Bell, 2004; Adams et al., 2011). For amphibians, the relation between plasma CORT levels and captivity may vary with capture restraint intensity. Male toads (*R. icterica*) showed increased plasma CORT levels of 9-fold and 3-fold in response to restraint challenge for 24h with and without movement restriction, respectively, indicating that more intense stress conditions result in stronger activation of the hypothalamus-pituitary-interrenal axis (Assis et al., 2015). Additionally, interspecific differences were observed in anurans, for plasma CORT levels to the same stressor (Gomes et al., 2012). Interspecific differences also seem to exist in the attenuation of the stress response, as influenced by captivity duration. *R. marina* showed increased plasma CORT levels after the first day of captivity, with plasma CORT levels decreasing over time (Narayan et al., 2011; Narayan and Marc-Hero, 2014), while *R. icterica* showed a sustained 3-fold increase in plasma CORT levels after 3 months in captivity (Assis et al, 2015). In our study, captive toads showed a sustained 3-fold increase in plasma CORT levels, versus field conditions. Thus, long-term maintenance under these conditions does not attenuate the stress response in these toads, indicating that captivity can be considered a chronic stressor for *R. schneideri*.

A decrease in plasma T levels in response to stressors was previously described for vertebrate species (Greenberg and Wingfield, 1987; Moore et al., 1991; Deviche et al., 2010),

including anurans (Paolucci et al., 1990; Narayan et al., 2010; Narayan et al., 2012), with a few studies showing T plasma level alterations in response to long-term captivity (Moore et al., 1991; Jones and Bell, 2004). The acute stress response to capture decreases plasma T levels (Paolucci et al., 1990; Woodley and Lacy, 2010), which can continue to decrease over time in captivity, as evident in birds, reptiles, and amphibians (Jones and Bell, 2004; Deviche et al., 2012; Deviche et al., 2014; Assis, 2015). This pattern was also found for *R. schneideri* in the present study, corroborating previous observations. As found in other tetrapod groups, plasma CORT and T levels were not correlated in *R. schneideri*, suggesting that the plasma T level reduction occurs in response to stress, but not as a direct result of plasma CORT levels increase (Jones and Bell, 2004; Deviche et al., 2012; Narayan et al., 2012).

BML increased throughout the period of captivity, with the percentage of BML positively correlated with plasma CORT levels and negatively correlating with plasma T levels over time. Stressors, including captivity, can increase plasma CORT levels and decrease body condition in many tetrapods (Romero and Wikelski, 2001; Bliley and Woodley, 2012; Martin et al., 2012; Narayan and Hero, 2014; Thomas and Woodley, 2015). A negative correlation between body condition and plasma CORT levels has been found in amphibians living in forest fragments with different habitat quality (Homan et al., 2003; Janin et al., 2011), as well as in anuran males calling in natural choruses (Leary et al., 2015; Titon et al., 2016). Stressors and exogenous corticosterone promote elevations in plasma CORT levels and thyroid hormones, resulting in enhanced amphibian metabolic rates (Denver, 1997; Denver, 2009; Wack et al., 2012). Higher energetic demands, in turn, could promote increased catabolism of energy stores and consequently BML over time. Also of note, an inter-individual positive correlation between body mass and plasma T levels has been found in anurans, suggesting that individuals that maintain body mass may also have better reproductive performance (Leary and Harris, 2013; Leary et al., 2015; Madelaire and Gomes, 2016). In this way, although toads were fed according

to a protocol that has been previously shown efficient to allow maintenance of body conditions in similar-sized toads and were not anorexic, it is possible that high plasma CORT levels resulting from captivity might have promoted a catabolic state resulting in BML and, consequently, lower plasma T levels and reproductive condition. However, further studies measuring plasma CORT and T levels, testes histological analyses, measurement of metabolic rates and food consumption should provide clarification as to the effects of captivity, including on reproductive capacity.

Variables associated with the immune response showed different patterns of response to captivity in *R. schneideri*. While PP did not vary over time, PE decreased with days in captivity and BKA showed a decrease, followed by an increase, throughout the captivity period. Several cellular and humoral immunosuppressive effects in response to stressors have been described in many vertebrate groups (Sapolsky et al., 2000; Martin, 2009). Previous studies have shown that the phagocytic activity (chemiluminescence measures) of blood neutrophils and macrophages in anurans respond in contrasting ways to long-term stress conditions (Gilbertson et al., 2003; Dohm et al., 2005; Albert et al., 2007; Graham et al., 2012; Falso et al., 2015). Although plasma CORT levels increased in response to stressors, and macrophage phagocytosis may be directly modulated by glucocorticoids (Belayat et al., 1998; Barriga et al., 2002), we found no correlation between PP and PE of peritoneal cells (macrophages and neutrophils) with plasma CORT levels in *R. schneideri*. Interestingly, PE was higher in individuals with higher plasma T levels over time in captivity. Although T shows several immunosuppressive effects (Roberts et al., 2004; Martin et al., 2008), a positive correlation between plasma T levels and immune response, in an acute stress paradigm, have been reported in birds (Davies et al, 2016). Additionally, an immune enhancing effect of T-implants has been demonstrated in anuran males, characterized by increased body mass (Desprat et al., 2015). In our study, individuals with higher plasma T levels and PE also presented with lower BML percentage over time. Our

results are in accordance with the conclusions from Desprat et al. (2015), and indicate that individuals with better maintenance of body mass could have an immune response that is up regulated by higher plasma T levels, coupled to increased reproductive capacity. Immune defense can be costly, and individuals with less energy reserves could present with a reduced immune response (Reviewed in Martin et al., 2008). Additionally, diseased amphibians showed higher CORT plasma levels and lower body mass (Peterson et al., 2013), which could be indicative of an even less optimal immune response. These results indicate that stress response over a long-period can decrease body mass, immune response and plasma T levels in toads. However, the effects of stress response on hormone-immune regulations, and its association with body mass need a more detailed investigation in anurans.

BKA was affected by captivity duration, with the lowest values found in *R. schneideri* captive for 30 days when compared to field conditions, as well as 7, and 60 days in captivity. Captivity and restraint protocol decreases BKA in birds (Matson et al., 2006; Millet at al., 2007) and amphibians (Graham et al., 2012; Assis et al., 2015), indicating that stressors may decrease BKA response in some vertebrate groups. Additionally, long-term captivity can further decrease BKA in birds (Buehler et al., 2008) and anurans (Assis et al., 2015). In our study, long-term captivity (30 days) also decreased BKA in males of *R. schneideri*. However, contrary to our predictions, BKA increased in toads after 60 days in captivity, showing 18% and 20% increases in BKA, when compared to field condition and 30 captive days, respectively. A similar pattern of changes was observed in birds, with BKA showing a tendency to decrease in the first weeks of captivity, but recovering after 5 weeks in captivity (Martin et al., 2012). In amphibians, increased BKA in response to stressors was previously reported, but in a context of acute stress response (restraint) (Hopkins and DuRant, 2011). Although BKA was affected by captivity duration in *R. schneideri*, BKA showed no correlation with plasma CORT and/or T levels, as in previous studies conducted with toads (Gomes et al., 2012; Graham et al., 2012;

Assis et al., 2015). Therefore, the present study and previous findings show BKA to be affected by stressors, although not directly by CORT in anurans.

2.6. Conclusions

Captivity over a prolonged period increased CORT and BML, whilst decreasing T in *R. schneideri*. However, changes in immune response may vary over time in captivity, depending on the immune parameter studied. Individual trajectory of immune response, particularly the phagocytic percentage of macrophages and neutrophils, was positively associated with T and body mass, suggesting that T might present immune-stimulatory effects depending on body mass. Based on our results, long-term captivity can be considered a chronic stressor for *R. schneideri*, with stress response effects on body mass, plasma hormone levels, and immune responses.

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2.9. Supplementary file

Supplementary Materials for

Captivity effects on immune response and steroid plasma levels of a Brazilian toad (*Rhinella schneideri*)

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This file includes:

Tables 2S1 to 2S3

Figure 2S1

Table 2S1. SEM analysis result including all fitted models for plasma corticosterone and testosterone levels, body mass loss percentage and phagocytic efficiency.

	X^2	DF	*P*	AIC	dAIC	weight
Model 2*	0.719	3	0.869	-93.9	0.0	0.378
Model 3*	0.125	2	0.939	-92.5	1.4	0.187
Model 4*	0.125	2	0.939	-92.5	1.4	0.187
Model 1	1.119	2	0.571	-91.5	2.4	0.114
Model 6	3.605	3	0.307	-91.0	2.9	0.089
Model 5	3.011	2	0.222	-89.6	4.3	0.044

Abbreviation as follow: **DF:** degrees of freedom; **AIC:** akaike information criterion; **dAIC:** difference between akaike information criterion. (N = 20) * = Selected models (P > 0.05 and dAIC < 2.0).

Table 2S2. Physiological variables and body measures for individuals of *R. schneideri* in field analyzed by a set of ANOVAs, with snout-vent length, body mass, plasma corticosterone and testosterone levels, and bacterial killing ability as dependent variables and captivity duration groups (7, 30 and 60 days) as factor.

Dependent Variable	Source	Type III SS	DF	MS	*F*	*P*
SVL	**Model**	0.01	2	0.00	2.983	0.078
	Intercept	77.74	1	77.74	86425.560	**≤ 0.001**
	CD	0.01	2	0.00	2.983	0.078
	Error	0.02	17	0.00		
	Total	86.06	20			
Body mass	**Model**	1435677.00	2	717.84	0.738	0.493
	Intercept	421630.56	1	421630.56	433.766	**≤ 0.001**
	CD	1435.68	2	717.84	0.738	0.493
	Error	16524.40	17	972.02		
	Total	469803.97	20			
CORT	**Model**	0.08	2	0.04	0.255	0.778
	Intercept	1.23	1	1.23	8.351	**0.010**
	CD	0.08	2	0.04	0.255	0.778
	Error	2.50	17	0.15		
	Total	3.89	20			
T	**Model**	0.29	2	0.15	0.559	0.582
	Intercept	5.53	1	5.53	21.118	**≤ 0.001**
	CD	0.29	2	0.15	0.559	0.582
	Error	4.45	17	0.26		
	Total	11.67	20			
BKA	**Model**	0.10	2	0.05	0.610	0.555
	Intercept	50.68	1	50.68	595.848	**≤ 0.001**
	CD	0.10	2	0.05	0.610	0.555
	Error	1.45	17	0.09		
	Total	56.85	20			

Abbreviation as follow: **Type III SS:** Type III sum of squares; **DF:** Degrees of freedom; **MS:** Mean square; **SVL:** Snout-vent length; **CD:** Captivity duration groups; **CORT:** Plasma corticosterone levels; **T:** Plasma testosterone levels; **BKA:** Bacterial killing ability. Variables with *P* significant < 0.05 are highlighted in bold.

Table 2S3. Effects of captivity duration on plasma testosterone levels and immune response tested through a set of ANCOVAs, with plasma testosterone levels, bacterial killing ability and percentage and efficiency of peritoneal phagocytic cells as dependent variables, body mass loss percentage and plasma corticosterone levels as co-variables and captivity duration groups (7, 30 and 60 days) as factor.

Dependent Variable	Source	Type III SS	DF	MS	*F*	*P*
T	**Model**	2.26	8	0.28	1.120	0.420
	Intercept	0.11	1	0.11	0.436	0.523
	CD	0.49	2	0.24	0.964	0.411
	CORT	0.01	1	0.01	0.025	0.878
	BML	0.00	1	0.00	0.004	0.950
	CD*CORT	0.06	2	0.03	0.122	0.886
	CD*BML	0.07	2	0.03	0.131	0.879
	Error	2.78	11	0.25		
	Total	6.45	20			
BKA	**Model**	0.57	8	0.07	1.983	0.145
	Intercept	12.23	1	12.23	339.538	**≤ 0.001**
	CD	0.00	2	0.00	0.007	0.993
	CORT	0.08	1	0.08	2.132	0.172
	BML	0.00	1	0.00	0.027	0.872
	CD*CORT	0.15	2	0.08	2.149	0.163
	CD*BML	0.16	2	0.08	2.174	0.160
	Error	0.40	11	0.04		
	Total	62.29	20			
PP	**Model**	1.13	8	0.14	1.249	0.357
	Intercept	2.39	1	2.39	21.095	**0.001**
	CD	0.23	2	0.12	1.024	0.391
	CORT	0.05	1	0.05	0.477	0.504
	BML	0.20	1	0.20	1.806	0.206
	CD*CORT	0.03	2	0.02	0.144	0.868
	CD*BML	0.34	2	0.17	1.494	0.267
	Error	1.25	11	0.11		
	Total	16.77	20			
PE	**Model**	0.56	8	0.07	2.501	0.097
	Intercept	0.47	1	0.47	17.021	**0.003**
	CD	0.11	2	0.05	1.927	0.201
	CORT	0.00	1	0.00	0.003	0.956
	BML	0.00	1	0.00	0.021	0.888
	CD*CORT	0.01	2	0.00	0.174	0.843
	CD*BML	0.06	2	0.03	1.116	0.369
	Error	0.25	9	0.03		
	Total	6.20	18			

Abbreviation as follow: **Type III SS:** Type III sum of squares; **DF:** Degrees of freedom; **MS:** Mean square; **T:** Plasma testosterone levels; **CD:** Captivity duration; **CORT:** Plasma corticosterone levels; **BML:** Body mass loss percentage; **BKA:** Bacterial killing ability; **PP:** Phagocytosis percentage; **PE:** Phagocytic efficiency. Variables with *P* significant < 0.05 are highlighted in bold.

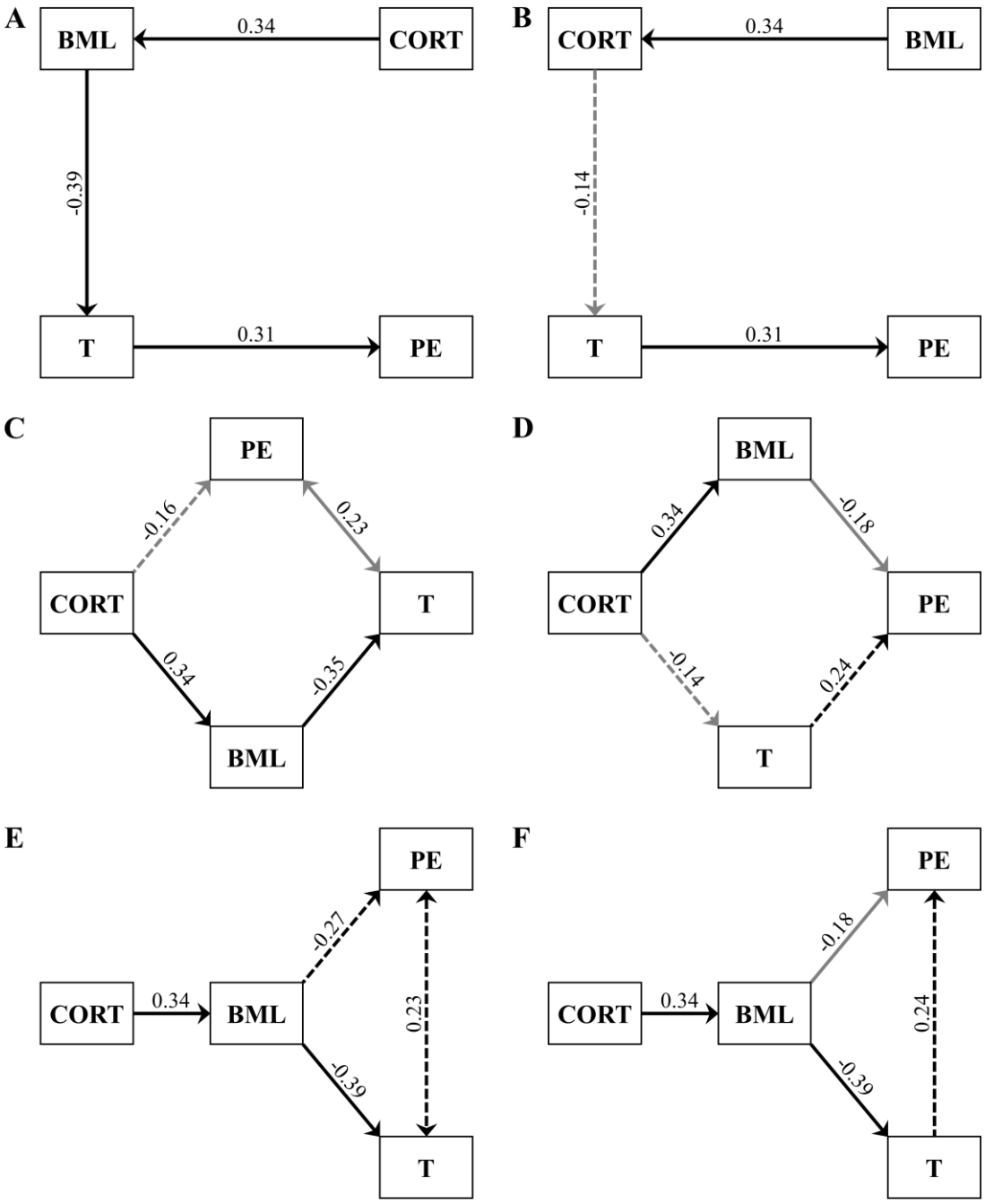

Figure 2S1. Proposed models for *R. schneideri*. Path diagrams of all six causal models tested. Path coefficients shown are all standardized values. (A) Model 2 χ^2= 0.719, df=3, p= 0.869, AIC= -93.926; (B) Model 6 χ^2= 3.605 df=3, p= 0.307, AIC= -91.040; (C) 1.119, df=2, p= 0.571, AIC= -91.525; (D) Model 5 χ^2= 3.011, df=2, p=0.222, AIC= -89.634; (E) Model 3 χ^2=0.125, df=2, p=0,939, AIC= -92.520; (F) Model 4 χ^2=0.125, df=2, p= 0.939, AIC= -92.520. Abbreviations as follow: **CORT:** Plasma corticosterone levels; **BML:** Body mass loss; **T:** Plasma testosterone levels; **PE:** Phagocytic efficiency. (N = 20).

CAPÍTULO 3 – INTERPLAY AMONG STEROIDS, BODY CONDITION AND IMMUNITY IN RESPONSE TO LONG-TERM CAPTIVITY STRESS IN TOADS

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3.1. Abstract

Glucocorticoid (GC) hormones are released in response to stressors in many vertebrate species. During chronic stress, individuals can show a decrease in androgen plasma levels, body condition and immune response, which, in turn, could be negatively correlated with increased GCs plasma levels. The relations between body condition and stress modulated physiological traits have been poorly studied in toads, particularly regarding immune parameters. In this study, we investigated the effects of captivity-associated stress (7, 30, 60, and 90 days) on plasma corticosterone (CORT) and testosterone (T) levels, body index (BI), and innate immune responses: bacterial killing ability (BKA) and phagocytosis from peritoneal cells, in adult males of *Rhinella icterica* toads. Captivity maintenance resulted in elevated CORT levels, whilst decreased T, BKA and phagocytosis, showing no effect on body condition. CORT and BKA were positively correlated, and, T was positively correlated with phagocytosis efficiency of peritoneal cells throughout captivity, suggesting immunomodulatory effects of CORT and T under the course of this stress condition in *R. icterica*. Additionally, given that *R. icterica* individuals showed no effect of captivity on BI and *R. schneideri*, a related species, displayed body mass loss over time in captivity (under the same conditions), we also investigated the relations among CORT, T, BI, and immunity (BKA and phagocytosis) in both species of Brazilian toads. While T and phagocytosis showed consistent and similar positive relations throughout period of captive maintenance in both species, the relations between CORT and BKA changed in association with BI alterations. CORT and BKA are positively associated in toads that kept BI throughout the period under captivity. Otherwise, CORT is negatively associated with BI, which is positively associated with BKA, in those animals characterized by decreasing BI in response to captivity maintenance. These results suggest that variations in toad´s immunity in response to long-term captivity stress are associated with changes in CORT and T, and these relations might be affected by BI.

Keywords: Captivity, Amphibian, Corticosterone, Testosterone, Phagocytosis, Bacterial killing ability.

3.2. Introduction

Stress events and intensity may be assessed by measuring glucocorticoid (GC) hormone levels in plasma and other fluids in most vertebrates (Sapolsky, 2002). While short-term stress response can be adaptive, promoting survival during fight-or-flight response, chronically elevated GCs may decrease fitness through several effects such as reproductive inhibition and depression of the immune response (McEwen et al., 1997, Sapolsky et al., 2000; Dhabhar, 2014). During stress response, elevated GCs levels are associated with increased energy mobilization for immediate and future needs (Myers et al., 2014). Therefore, GCs can directly modulate individuals' body condition, which may be negatively associated with increased GCs during long-term stress response (Moore et al., 2000; Bliley and Woodley, 2012; Titon et al., 2017). In the meantime, the reproductive axis can also be influenced by stressors (Wingfield et al., 2001). Studies on different vertebrates indicate that androgen plasma levels can decrease in response to capture and confinement stress (Lance and Elsey, 1986; Narayan et al., 2012; Deviche et al., 2010; 2016). Moreover, stress-induced downregulation of testosterone plasma levels (T) is more accentuated by chronic stress (Jones and Bell, 2004; Narayan et al., 2011a; Titon et al., 2017). Chronic stress still suppresses or dysregulates immunity by decreasing proinflammatory cytokines production, as well as, suppressing trafficking and function of immune cells (Dhabhar and McEwen, 1997; Dhabhar, 2014).

The immunomodulatory role of GCs is well explored and described, especially for mammals, which bimodal effects depend on intensity and duration of stressors (Dhabhar, 2014). In this context, immunostimulatory effects (e.g. increased cellular function and inflammatory

responses) are frequently observed in response to acutely increased GCs levels, while suppressive actions (e.g. decreased cell proliferation and proinflammatory cytokines production) are more commonly observed in chronically elevated GCs conditions (Dhabhar and McEwen, 1997; Dhabhar, 2014). A wide array of hormones can additionally modulate vertebrate's immune functions, including androgens and leptin (a hormone that signalizes individual´s body condition) (Demas and Sakaria, 2005; Ahmed et al., 2010). Testosterone-induced immune suppression includes a reduction of lymphoid tissues, as well as a decrease in humoral and cellular immune responses (Grossman, 1985; Casto et al, 2001; Ahmed et al., 2010; Nava-Castro et al, 2012), while stimulatory effects are indicated by increased inflammatory responses (Greenman et al., 2005; Martin et al., 2008; Desprat et al., 2015). Furthermore, mounting an immune response requires a substantial energetic investment (Demas, 2004; Demas et al., 2012). Accordingly, a poorer body condition (which indicates reduced endogenous energy availability) can suppress immune function (Carlton et al., 2012; Ashley and Demas, 2017). Therefore, androgens and body condition in association with GCs can play an important integrative role in the regulation of immune responses (Ahmed et al., 2010).

Experiments conducted in captivity have examined the relations among plasma levels of GCs and sex steroids, body condition and immune function (Casto et al., 2001; Fokidis et al., 2011; Narayan et al., 2011b). However, few studies have examined how captivity itself affects stress physiology and immune response, particularly in amphibians (Narayan and Hero, 2011; Assis et al., 2015: Titon et al., 2017). In this context, the first aim of the present study was investigating the effects of captivity maintenance (7, 30, 60, and 90 days) on plasma corticosterone (CORT) and testosterone (T) levels, BI, and innate immune responses (measured as bacterial killing ability - BKA and phagocytosis from peritoneal cells) in male toads of *R. icterica*. Given that previous studies suggested that long-term captivity (three months) is a

chronic stressor for this species (Assis et al., 2015), the following hypotheses were tested: 1) Captivity maintenance increases CORT and decrease T, BI and immune response; 2) These effects are accentuated throughout the time toads are kept in captivity; 3) CORT and immune response are negatively correlated over time in captivity; 4) T, BI and immune response are positively correlated over time in captivity.

Secondly, the maintenance and activation of immune system is costly, with a great availability of energy resources being critical for individuals' survival (French et al., 2011; Neumann-Lee and French, 2017). We have observed that toads tend to respond differently to the same conditions of captive maintenance regarding BI variation. While in Titon et al. (2017) adult male toads (*R. schneideri*) displayed a marked body loss over time in captivity, toads (*R. icterica*) did not show variation in body mass throughout days in captivity in this study. Comparing the different patterns of BI in response to captivity conditions might additionally contribute to understand the associations between steroids, body condition and immunity in toads. In this way, we investigated the relations between CORT, T, BI, and immunity (BKA and phagocytosis) in these Brazilian toads (*R. icterica* and *R. schneideri*) kept under the same captivity conditions. We tested the following additional hypothesis: 5) When BI decreases in response to long-term captivity, immune responses are directly associated with BI variation and indirectly related with CORT and T; and 6) Once BI does not vary throughout time in captivity, immune responses are directly associated with plasma CORT and T levels.

3.3. Material and methods

3.3.1. Animals and study site

Rhinella icterica and *R. schneideri* are both species belonging to *Rhinella marina* group, characterized by large body size and broad geographical distribution (Stevaux, 2002). *Rhinella schneideri* occurs predominantly in the Cerrado, but is also present in the Atlantic Forest,

Amazon Forest and Caatinga (Stevaux, 2002); and *R. icterica* geographical distribution is more associated with Atlantic Forest, but also occurring in some areas of Cerrado (Stevaux, 2002).

Thirty-eight adult males of *R. icterica* were collected in Botucatu (22° 46′ 55″ S, 48° 28′ 29″ W) - São Paulo/Brazil in July 2015. Animals were collected under license from Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio, process number 8132-1). Procedures and use of biological material were performed with approval of the Comissão de Ética no Uso de Animais (CEUA) do Instituto de Biociências da Universidade de São Paulo (CEUA - n ° 054/2013). Data related to field collection and captivity maintenance for R. schneideri were obtained from Titon et al. (2017).

3.3.2. Capture and blood sample collection

Rhinella icterica toads (N = 10) were located by visual inspection and manually captured. Blood samples (~ 200 µl) from those males were collected in the field by cardiac puncture using heparinized 1 ml syringes and 26 Gx1/2" needles. Only samples collected within 3 min after animal disturbance were considered, in order to avoid influence of handling on hormone levels (Romero and Reed, 2005). After blood collection, these animals were weighed (0.01 g) and the snout-vent length (SVL) was measured (0.01 mm). These ten toads were kept isolated in order to avoid resampling and released in field at the end of the field work. Blood samples were identified and kept on ice (< 4 hours) until they were centrifuged to isolate the plasma (4 min at 604 g). Plasma samples were stored in cryogenic tubes, and kept in liquid nitrogen until they could be transferred to a -80°C freezer, for hormone assays and bacterial killing ability (BKA). Other twenty-eight males were captured (not bled in field), transported to the laboratory and kept in captivity (see 3.3.3 and 3.3.4 for additional information). The captive individuals (N = 28) were weighed and kept in individual plastic containers, which lids had holes to allow air

circulation, for 3 days, until they were taken to the laboratory. The animals were exposed to the natural local climate and photoperiod conditions.

Additionally, all *R. icterica* toads were found in a chorus at the moment of capture. Given that anuran calling behavior is associated with changes in CORT and androgens (Leary et al., 2004; Assis et al., 2012), as well as to variation in innate immune response (Titon et al., 2016; Madelaire et al., 2017), the presence or absence of calling behavior (calling [N = 21] or non-calling [N = 12]) at the moment of capture was registered for each individual and included in further analysis.

3.3.3. Captivity conditions

At the laboratory, toads were kept in captivity under the same conditions of *R. schneideri* in Titon et al. (2017). The animals were individually housed in plastic containers (43.0cm x 28.5cm x 26.5cm). The lids of the containers had holes to allow air circulation. Toads were exposed to an 11/13 LD cycle (lights on at 7:40 am and off at 6:40 pm) and temperature of 21 ± 2°C. The animals had free access to water, and fed cockroaches once per week. Toads were weighed two days before the experimental procedure. Captivity conditions were the same for all individuals, with captivity duration varying among experimental groups, as described below.

3.3.4. Captivity duration and experimental design

Toads were divided in four groups to be sampled after 7, 30, 60, and 90 days under captivity, in order to evaluate the effects of captivity duration on CORT, T, and immune response (BKA and phagocytosis of peritoneal cells). A blood sample from each individual was collected and processed in accordance to the methods described in section 3.3.2. Plasma samples were used for BKA and hormone assays. After blood collection, animals were euthanized by immersion in a lethal solution of benzocaine (0.2%), the SVL was measured, and

then the retrieval of peritoneal cells was performed. Blood collection and retrieval of peritoneal cells were performed between 7 to 8 pm.

3.3.5. Phagocytosis

a) Retrieval of peritoneal cells

Once the animals were euthanized, the lavage fluid (cells + PBS) of the peritoneal cavity was collected with sterile surgical material according to Titon et al. (2017). Lavage fluid was centrifuged (259 g, at 4°C for 9 min), the supernatant was discarded, and cells were resuspended in 1ml of PBS to perform phagocytosis assay. Due to methodological limitations in field, this assay was carried out only for individuals in captivity (7, 30, 60, and 90 days).

b) Phagocytosis assay

The zymosan phagocytosis assay from peritoneal cells of *R. icterica* was carried out following a previously described protocol (Titon et al., 2017). Briefly, aliquots of 200 µl of the lavage fluid (PBS with macrophages and neutrophils adjusted to 2 x 10^5 cells) were diluted in 800 µl of PBS. Subsequently, 100 µl of zymosan (SIGMA Z-4250 A-CFSE at a concentration of 1 x 10^7 particles/ml PBS) were added to the samples, followed by 35 min of incubation at 25°C. A negative control was made with the lavage fluid diluted in PBS in the same proportion. Reactions were stopped by adding 2 ml of EDTA solution (6 mM). After centrifugation (259 g, at 4°C for 7 min), the supernatant was discarded and 200 µl of paraformaldehyde (1%) were added. The samples were then kept at 4°C during 1 hour for cell fixation. Thereafter, 500 µl of PBS were added and the samples were centrifuged (259 g, at 4°C for 7 min). Supernatant was then discarded and 100 µl of PBS were added for flow cytometry.

c) Imaging flow cytometry

Samples were analyzed on a Flowsight imaging flow cytometer (Merck-Millipore, German) interfaced with a DELL computer. Data from 20,000 events were acquired utilizing the 488nm laser at a 20x magnification, through INSPIRE software. Macrophages and neutrophils were identified through gate images in focused-single cells plotted on the bright field area *vs.* the side scatter (SSC) plot. Quantification of phagocytosis was estimated by mean zymosan-CFSE fluorescence cell. The percentage of cells that engulfed at least one zymosan particle (with green fluorescence divided by the total number of cells [multiplied by 100]) was expressed as the phagocytosis percentage (PP). The percentage of cells that ingested three or more zymosan particles was expressed as phagocytosis efficiency (PE). Acquired data were analyzed using IDEAS analysis software (EMD Millipore) version 6.1 for windows.

3.3.6. Bacterial killing ability (BKA)

The plasma BKA of *R. icterica* was accessed by the assay conducted according Assis et al. (2013). Briefly, 10 µl of plasma was combined with 10 µl of bacteria (*Escherichia coli* diluted to 10^6 microorganisms per ml) and 190 µl of Ringer's solution. Positive controls consisted of 10 µl of bacteria in 200 µl of Ringer's solution, and negative control contained 210 µl of Ringer's solution. All samples and controls were incubated by 60 min at 37°C (optimal temperature for bacterial growth). Tryptic soy broth (500 µl) was then added to each sample. The bacterial suspensions were thoroughly mixed and 300 µl of each one was transferred (in duplicates) to a 96 wells microplate. The microplate was incubated at 37ºC for 2h, and thereafter the optical density of the samples was measured hourly in a plate spectrophotometer (wavelength 600 nm). The BKA was evaluated at the beginning of the bacterial exponential growth phase, and calculated according to the formula: 1 - (optical density of sample / optical

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

3.3.7. Hormonal assays

A single ether extraction was performed on 10 µL of plasma (Titon et al., 2017), and CORT and T concentrations were then determined by duplicates in standard ELISA kits (CORT number 501320; T number 582701, Cayman Chemical), according to the manufacturer's instructions and previous studies conducted with this same species (Assis et al., 2015; 2017). Intra-assay variation, were 3.52% for CORT and 4.00% for T. Inter-assay variation were 3.00% for CORT and 4.06% for T. Sensitivity of the assays was 94 pg/ml and 9 pg/ml for CORT and T, respectively.

3.3.8. Statistical analyses

Descriptive statistics were performed for all variables of *R. icterica* toads, and data were then submitted to Shapiro-Wilk normality test. With the exception of SVL and PP, all variables showed absence of normality and were transformed to fit the prerequisites of parametric tests, as follow: body mass to log10; CORT and T to square root; BKA and PE to arccosine. A measure of body condition (body index - BI) was calculated as the residuals from the regression of body mass as a function of SVL and included in the analysis. Additionally, presence or absence of calling behavior at the moment of capture was also included as a factor in the analysis. Pearson correlation tests were used to investigate correlations between variables in the field and throughout captivity. Since three individuals, which were not calling in field, died of unknown causes in captivity, they were not included in the analysis.

A set of ANCOVA for independent measures was used to investigate the effect of time in captivity on studied variables. CORT, T, BKA, PP and PE were used as dependent variables,

body mass as a co-variate, and captivity duration as a factor. For the dependent variables not significantly affected by body mass, two sets of independent measures ANOVAs were then performed. The first included calling behavior (calling and non-calling) and captivity duration as factors. For variables not affected by calling behavior, ANOVAs were performed using only captivity duration as a factor. All ANOVAs were followed by tests for mean multiple comparisons with Bonferroni adjustment.

In order to investigate the relations among studied variables we performed structural equation modeling (SEM). Eight models were proposed based on Pearson correlation tests, with predictions based on the available knowledge about the relations between the studied physiological traits. Glucocorticoids show catabolic effects, promoting lipid oxidation and muscle proteolysis in stress conditions (Malisch et al., 2007). Moreover, CORT and body condition may be negatively related, with higher CORT associated with a lower BI (Moore et al., 2000; Janin et al., 2011). T decreases in response to stressors, being inversely associated with CORT in some studies (Paolucci et al., 1990; Narayan et al., 2012). In fact, the activation of the hypothalamus-pituitary-adrenal/interrenal axis can result in a decrease in T as a result of the inhibition of the hypothalamus-pituitary-gonad axis (Sapolsky 2002; Barsotti et al., 2016). Body condition seems to influence CORT, with individuals displaying a better body condition showing better ability to modulate release and clearance of corticosterone (Health & Dufty Jr., 1997; Raja-ajo et al., 2010). A better body condition may be positively associated with T (Ligon et al., 1990; Lind & Beaupre, 2015; Madelaire and Gomes, 2016), with testosterone exogenous administration decreasing body condition in some situations (Sockman and Schwabl, 2001; Roberts et al., 2007). As well as, body condition and energetic resources seem to be directly and indirectly associated with immune response (Demas & Sakaria, 2005; Smith et al., 2007; Ruiz et al., 2010; Desprat et al., 2015). Additionally, immune cells are subjected to direct modulation by CORT and T, since they express glucocorticoid and androgen receptors (Nava-

Castro et al., 2012; Segner et al., 2017). All proposed models are available as supplementary material (Fig. 3S1 and 3S2). The overall model fit was assessed based on $\chi 2$ statistic (Shipley, 2000). A nonsignificant $\chi 2$ result on test ($P > 0.05$) indicates that data support the proposed model (Shipley, 2000). Akaike's information criterion (AIC) was used to identify the best model among those proposed. In this way, models that were supported by $\chi 2$ test and had smaller AIC (dAICc < 2.0 on model selection analysis) were selected for better explaining the relationships among the variables (Shipley, 2000; Mazerolle, 2006) (Table 3S1).

We additionally investigated the relations among steroids, body condition and immunity in response to captivity maintenance in *R. schneideri*, by comparing the relations between the same studied variables for *R. icterica* in the SEM. The SEM for *R. schneideri* was performed by using CORT, T, PE and BKA obtained from Titon et al. (2017), and including and additional variable - BI, according to the models described for *R. icterica*. The eight proposed models for R. schneideri are available as supplementary material (Fig. 3S3 and 3S4), with the models selected for better explaining the relationships among the variables showed in Table 3S1.

We performed descriptive statistics, correlations, ANCOVAs and ANOVAs using IBM SPSS Statistics 22. Structural equation modeling was performed in R 3.2.5 (R Development Core Team, 2016), according Titon et al. (2017).

3.4. Results

3.4.1. Captivity effects on body condition and physiological traits in R. icterica

Descriptive statistics of variables from males of *R. icterica* in field and after captivity (7, 30, 60, and 90 days) are shown in Supplementary materials (Table 3S2 and 3S3). Body mass did not affect the studied physiological variables either in field or in captivity ($P \geq 0.247$; Table 3S4 and 3S5).

Regarding field data, CORT and T were higher in calling than non-calling toads ($P \leq 0.001$; Fig 3.1A and B). Captivity maintenance affected CORT of calling and non-calling males in different ways (Fig. 3.1A, Table 1). Calling individuals showed sustained high CORT after 7 and 30 days under captivity, while non-calling animals displayed gradual increasing in CORT within this period, with no differences between callers and non-callers after 30 days (Fig. 3.1A, Table 3.1). Thereafter, callers showed an abrupt decrease in CORT after 60 and 90 days under captivity, while non-callers showed a gradual decrease in CORT in the same period. In this way, callers showed lower CORT than non-callers after 60 days in captivity, while both groups showed equivalently low CORT after 90 days in captivity (Fig. 3.1A, Table 3.1). Although T were higher for calling toads in the field, T dropped sharply for callers and non-callers after 7 days in captivity, and T was equally low for both groups throughout the period of captivity maintenance (Fig. 3.1B, Table 3.1).

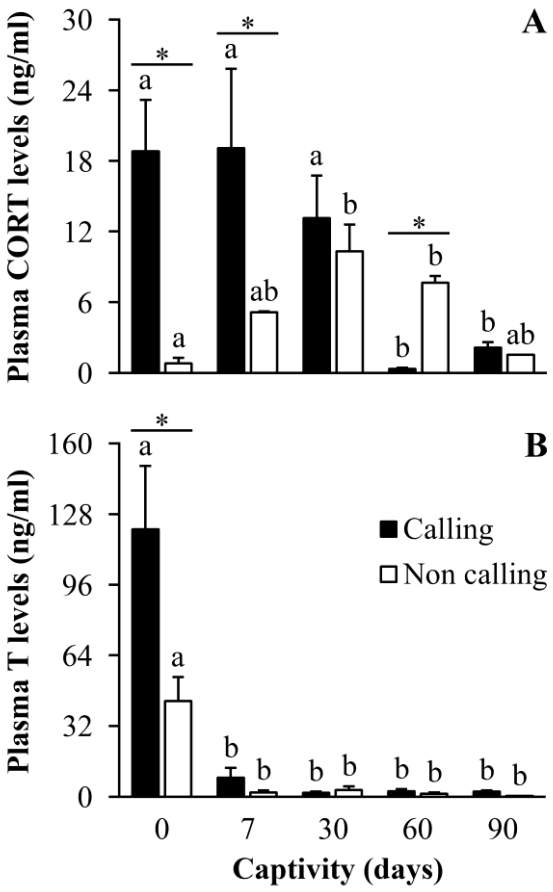

Figure 3.1. Field and captivity variation of plasma hormone levels of *Rhinella icterica* toads. Differences in (A) plasma corticosterone and (B) plasma testosterone levels of calling and non-calling individuals in the field and under captivity (N = 6 and 4 (field), 4 and 2 (d7), 3 and 2 (d30), 3 and 3 (d60), 5 and 1 (d90) for calling and non-calling individuals, respectively, for both variables). Letters above the bars represent statistical differences for ANOVA, with different letters representing statistical difference within groups with $P \leq 0.05$. Asterisks represent statistical differences between groups (calling or non-calling) at each specific time with $P \leq 0.05$. Bars represent mean ± standard error. Abbreviations: **CORT:** Corticosterone; **T:** Testosterone.

Table 3.1. Effect of captivity maintenance and calling behavior on plasma steroid levels in *R. icterica* tested through a set of ANOVAs, with plasma corticosterone and testosterone levels as dependent variables and captivity maintenance (0, 7, 30, 60, and 90 days) and calling behavior (calling and non-calling) as factors.

Dependent Variable	Source	Type III SS	DF	MS	*F*	*P*
Plasma corticosterone levels	**Corrected Model**	128.846	9	14.316	9.766	**0.000**
	Intercept	301.366	1	301.366	205.578	**0.000**
	Calling behavior	7.296	1	7.296	4.977	**0.036**
	CM	30.468	4	7.617	5.196	**0.004**
	Calling behavior * CM	64.245	4	16.061	10.956	**0.000**
	Error	33.717	23	1.466		
	Total	598.728	33			
	Corrected Total	162.563	32			
Plasma testosterone levels	**Corrected Model**	380.993	9	42.333	18.496	**0.000**
	Intercept	212.222	1	212.222	92.724	**0.000**
	Calling behavior	10.313	1	10.313	4.506	**0.045**
	CM	276.132	4	69.033	30.162	**0.000**
	Calling behavior * CM	20.830	4	5.208	2.275	0.094
	Error	50.353	22	2.289		
	Total	811.290	32			
	Corrected Total	431.345	31			

Abbreviation as follow: **Type III SS:** Type III sum of squares; **DF:** Degrees of freedom; **MS:** Mean square; **CM:** Captivity maintenance. Variables with *P* significant < 0.05 are highlighted in bold.

BI did not differ in in the field for callers and non-callers, and was maintained throughout captivity maintenance (Table 3.2). Also, there were no differences in immune response between callers and non-callers in the field or throughout the period of captivity ($P \geq 0.145$; Table 3S6). BKA decreased after 60 days in captivity and remained low in day 90th, when compared to values from the field and after 7 and 30 days under captivity (Fig. 3.2A, Table 3.2). PP showed a transient reduction in response to captivity maintenance, displaying lower values at the 30th day in captivity (Fig. 3.2B, Table 3.2). PE in response to captivity showed the same temporal trend of PP in response to captivity maintenance (Fig. 3.2C, Table 3.2). Moreover, CORT showed a positive correlation with BKA (Fig. 3.3A), and T was positively correlated with PP and PE (Fig. 3.3B and C) over time in captivity.

Table 3.2. Effect of captivity maintenance on body condition and immune response in *R. icterica* tested through a set of ANOVAs, with body index, bacterial killing ability, phagocytosis percentage and phagocytosis efficiency as dependent variables and captivity maintenance (0, 7, 30, 60, and 90 days) as factor.

Dependent Variable	Source	Type III SS	DF	MS	*F*	*P*
Body Index	**Corrected Model**	99.430	3	33.143	0.200	0.894
	Intercept	1.953	1	1.953	0.012	0.915
	CM	99.430	3	33.143	0.200	0.894
	Error	2479.816	15	165.321		
	Total	2579.245	19			
	Corrected Total	2579.245	18			
Bacterial Killing Ability	**Corrected Model**	5879.014	4	1469.754	15.721	**0.000**
	Intercept	166969.202	1	166969.202	1785.963	**0.000**
	CM	5879.014	4	1469.754	15.721	**0.000**
	Error	2617.712	28	93.490		
	Total	182957.828	33			
	Corrected Total	8496.727	32			
Phagocytosis	**Corrected Model**	780.847	3	260.282	3.499	**0.035**
	Intercept	7510.466	1	7510.466	100.973	**0.000**
	CM	780.847	3	260.282	3.499	**0.035**
	Error	1487.625	20	74.381		
	Total	9778.938	24			
	Corrected Total	2268.471	23			
Phagocytosis Efficiency	**Corrected Model**	109.301	3	36.434	1.552	0.232
	Intercept	1594.140	1	1594.140	67.915	**0.000**
	CM	109.301	3	36.434	1.552	0.232
	Error	469.454	20	23.473		
	Total	2172.895	24			
	Corrected Total	578.755	23			

Abbreviation as follow: **Type III SS:** Type III sum of squares; **DF:** Degrees of freedom; **MS:** Mean square; **CM:** Captivity maintenance. Variables with *P* significant < 0.05 are highlighted in bold.

3.4.2. Relations among steroids, body condition and immunity in response to chronic stress in R. icterica and R. schneideri

The two models that better explained the relations between CORT, T, BI, BKA, and PE for *R. icterica* are depicted in Figure 4A and B (Table 3S1). Both selected models showed consistent results concerning the relations among the investigated variables, with a sharply positive influence of CORT on BKA, and T on PE (Fig. 3.4A and B). A positive influence of

BI on T was also observed for *R. icterica* during captivity maintenance in the selected models (Fig. 3.4A and B).

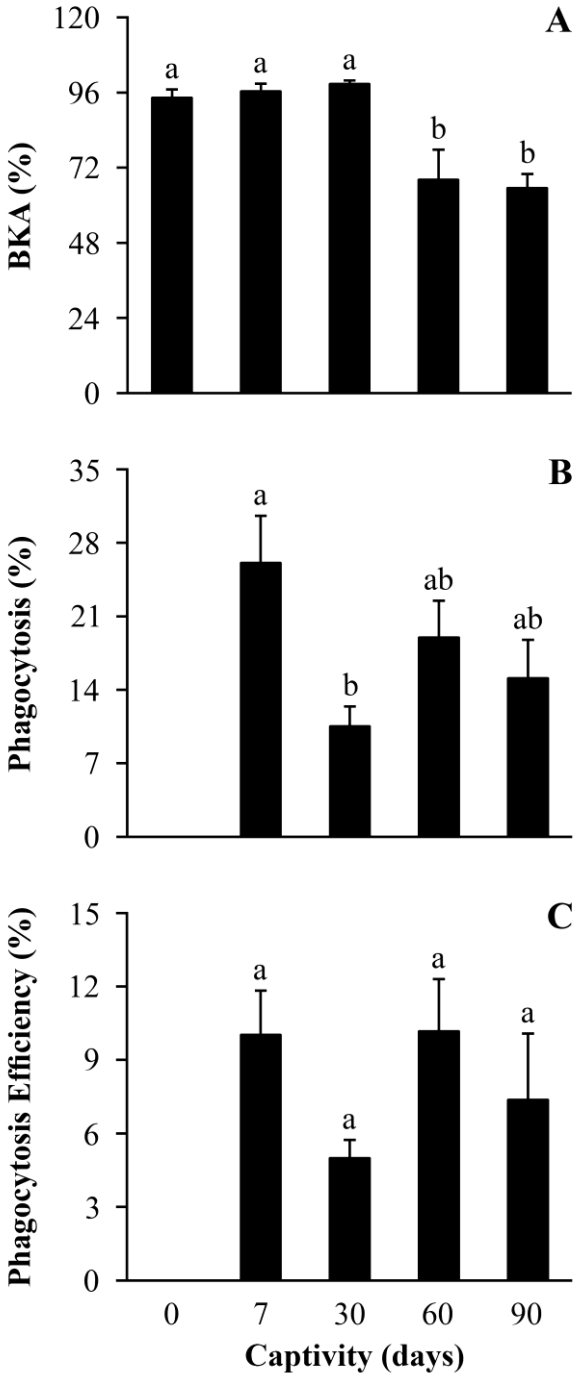

Figure 3.2. Field and captivity variation of immune response of *Rhinella icterica* toads. Differences in (A) Bacterial killing ability, (B) Phagocytosis (%) and (C) Phagocytosis efficiency (%) for individuals in field and captivity (N = 10 (field), 6 (d7), 6 (d30), 6 (d60), 6 (d90) for all variables). Letters above the bars represent statistical differences for ANOVA, with different letters representing statistical difference with $P \leq 0.05$. Bars represent mean ± standard error. Abbreviations: **BKA:** Bacterial killing ability.

Three models were selected as the ones which better explained the relations among the studied variables for *R. schneideri* (Fig. 3.4C, D and E; Table 3S1). All selected models showed consistent results regarding the relations among CORT, BI, T, BKA, and PE (Fig. 3.4C, D and

E). Higher CORT negatively influenced BI, which influenced positively T and BKA. T also influenced positively PE (Fig. 3.4C, D and E).

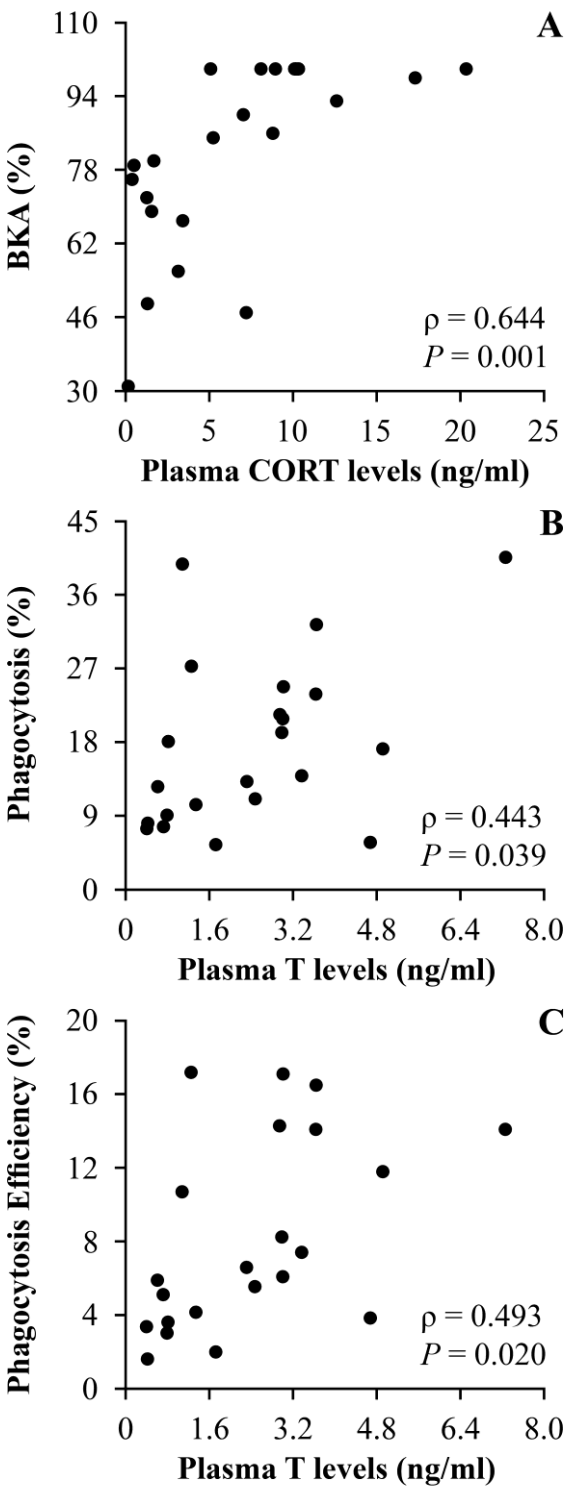

Figure 3.3. Correlations between plasma hormone levels and immune response in *Rhinella icterica* toads in captivity. (A) Correlation between CORT and BKA; (B and C) Correlations between T and phagocytic response. Abbreviations as follow: **CORT:** Corticosterone; **BKA:** Bacterial killing ability; **T:** Testosterone. (*N* = 22).

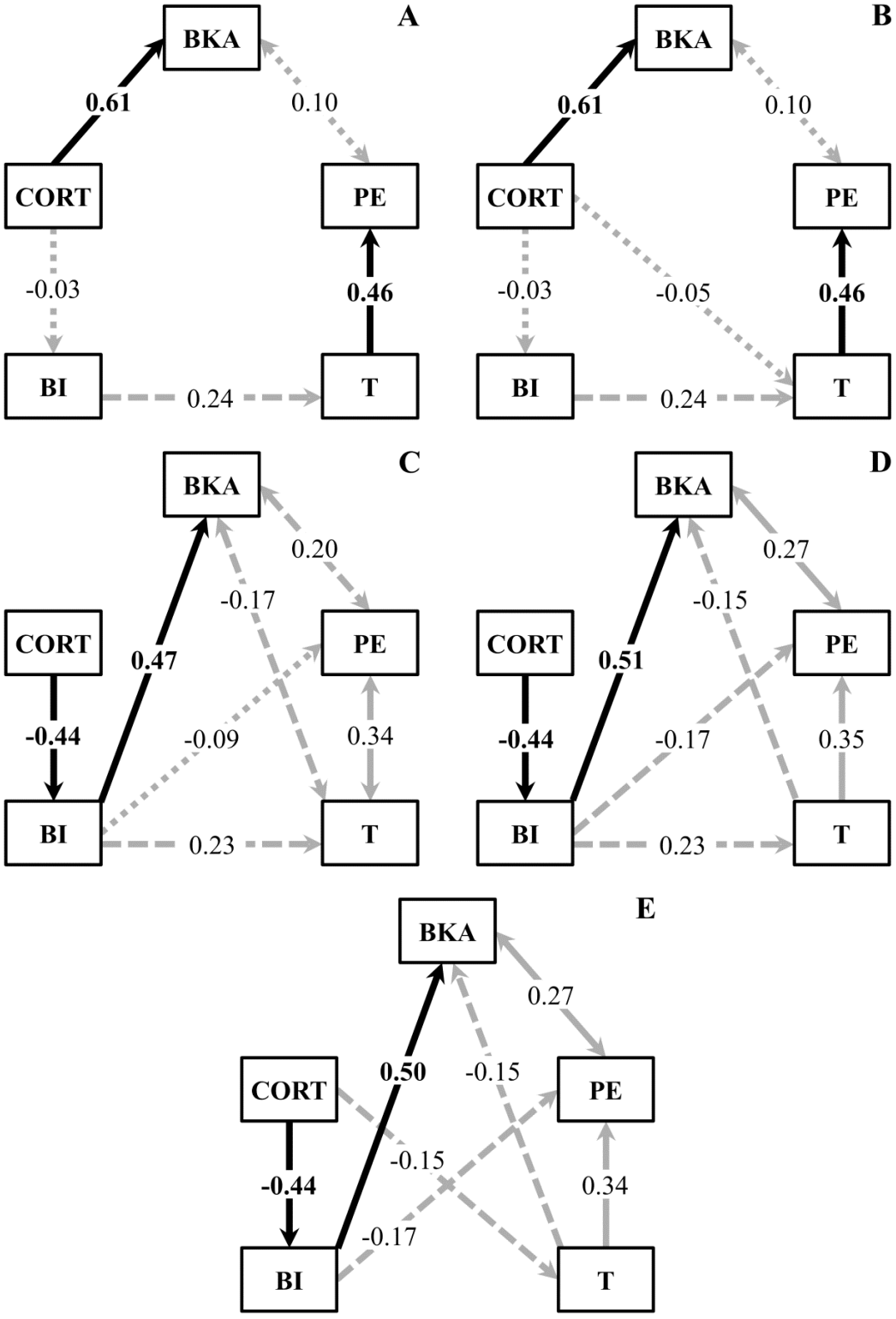

Figure 3.4. Selected structural equation modelling for BI, steroids and immune variables in *R. icterica* and *R. schneideri* in captivity. (A and B) Path diagrams of the two causal selected models for *R. icterica*. (C, D and E) Path diagrams of the three causal selected models for *R. schneideri*. Abbreviations as follow: **CORT:** Plasma corticosterone levels; **BI:** Body index; **T:** Plasma testosterone levels; **BKA:** Bacterial killing ability; **PE:** Phagocytic efficiency. (*N* = 19 and 20, for *R. icterica* and *R. schneideri*, respectively).

3.5. Discussion

3.5.1. Physiological traits and body condition in R. icterica in the field and following long-term captivity

Our study demonstrated that, in addition to promoting complex time-dependent adjustments in CORT, captivity maintenance decreased T and innate immune response in *R. icterica*. CORT trajectories for calling animals demonstrated that, at the moment of capture, calling toads presented higher CORT than non-calling. Calling individuals also maintained CORT similar to those in the field after 7 and 30 in captivity. Non-calling toads, in turn, increased CORT in response to captivity, with individuals at 30 days displayed similar CORT values to calling ones. These findings were in accordance to our predictions, since calling behavior is directly associated to high CORT values (Leary et al., 2004; Assis et al., 2012), and captivity increases CORT in anurans (Narayan and Hero, 2011; Narayan et al., 2011b; Assis et al., 2015; Titon et al., 2017). Interestingly, captive toads from both groups, calling and non-calling, displayed CORT similar to the calling animals at the moment of the capture, showing that captivity for 30 days increases CORT at physiological levels of calling behavior in *R. icterica*. However, while transient increases in CORT, in order to mobilize energy for calling activity, are beneficial (Moore and Jessop, 2003; Carr, 2011), increased CORT for a long-term are indicative of chronic stress (Sapolsky, 2002; Dhabhar, 2014). Therefore, *R. icterica* toads seem to be under chronic stress conditions in long-term captivity.

Captivity increases CORT in many vertebrates (Jones and Bell, 2004; Buehler et al., 2008; Fokidis et al., 2011), including amphibians (Coddington and Cree, 1995; Narayan and Hero, 2011; Assis et al., 2015). Nevertheless, whilst some anurans show sustained high CORT (Assis et al., 2015; Titon et al., 2017), others exhibit an attenuation regarding CORT over time in captivity (Narayan and Hero, 2011; Narayan et al., 2011b; Narayan and Marc-Hero, 2014). In our study, we observed that *R. icterica* toads exhibited sustained high CORT for 30 days, but

contrary to our predictions, we observed a decreasing pattern in CORT at day 60 and 90 when compared to 30-day values. Our results indicate that *R. icterica* showed attenuation in the stress response, however, it was only observed at the 90th day in captivity. These results suggest that these animals can adjust to captivity conditions, but only after a long period of captivity maintenance. In the meantime, sustained increased CORT after 3 months compared to baseline values was previously described for *R. icterica* (Assis et al., 2015). Given that they were maintained at the same conditions, differences between our results and the presented in Assis et al. (2015) may be due to the fact these animals were captured from different populations (distant from each other by 500km) (Martin II et al., 2005; Hammond et al., 2015), at different months (July in this study and February in Assis et al., 2015) (Pancak and Taylor, 1982; Madelaire and Gomes, 2016) as well as different years (Buck et al., 2007).

Plasma T levels were higher in calling than non-calling toads in the field. Calling activity is often positively correlated with T in anurans (Moore et al., 2005; Assis et al., 2012). While studies have shown that high T are necessary to initiate and maintain calling activity in anurans (Arch and Narins, 2009; Carr, 2011), some authors have also emphasized that exposition to chorus stimuli can also increase T (Brzoska and Obert, 1980; Chu and Wilczynski, 2001). In response to captivity maintenance, T showed an accentuated drop after 7 days in captivity for both calling and non-calling individuals. This result is also in accordance with general pattern described for many vertebrates, including amphibians (Paollucci et al., 1990; Woodley and Lacy, 2010; Deviche et al., 2010; Davies et al., 2016; Assis et al., 2017). Decreased T in response to stressors includes inhibition of gonadotropin-releasing hormone secretion and impairment of the testicular function (Tsigos and Chrousos, 2002; Hardy et al., 2005; Deviche et al., 2010). In this context, the drop in T in response to long-term captivity might be explained by the inhibition of the hypothalamic-pituitary-gonadal axis through the constant activation of the hypothalamic-pituitary-interrenal axis (Sapolsky, 2002; Barsotti et al., 2017).

Moreover, we demonstrated that after the accentuated decrease in T following the first days in captivity, T was sustained at similar low levels throughout captivity. In addition, as previously found for anurans (Paolucci et al., 1990; Narayan et al., 2012; Assis et al., 2017; Titon et al., 2017), there is no correlation between CORT and T in *R. icterica*, suggesting that the decrease of T in response to captivity may not be directly mediated by changes in CORT. Stress-induced T decrease can occur at multiple levels (e.g. inhibition of reproductive axis, acceleration of T clearance) and they may not be obligatory associated to changes in CORT levels (Sapolsky, 2002; Carr, 2011; Deviche et al., 2001; 2014). Further studies, focused on which T controlling mechanisms are influenced by stress response and GCs levels, are necessary in order to better understand the stress T-induced changes in amphibians.

Despite the differences in baseline CORT and T, and in CORT values under captivity, immune responses showed no differences between calling and non-calling toads in the field or in response to captivity. Variations in the immune response associated to calling behavior are expected since CORT and T are both modulators of the immune system (Nava-Castro et al., 2012; Segner et al., 2017). However, differences in immune responses along reproductive season may be time-dependent (Apanius, 1991; Gonzalez et al., 1999) and, therefore, might not be detected at the moment R. icterica individuals were captured (beginning of reproductive season). A more complete study, including CORT, T and immune samples throughout the reproductive season may shed light to these implications. In addition, *R. icterica* showed no differences in BI (a common a physiological condition associated to the immune response) between calling and non-calling toads in the field or during long-term captivity. Therefore, since calling and non-calling toads started at similar conditions and were subjected to the same captivity protocol, differences in immune responses between groups were no longer expected during captivity maintenance.

As we predicted, grouped values for calling and non-calling animals showed that immune responses decrease following captivity in males of *R. icterica*, with the dynamics of suppressive effects varying according to the studied immune variables. Compared to field conditions, plasma BKA decreased after 60 days in captivity, remaining low in the 90th day, whilst PP and PE were reduced after 30 days followed by an increase in the 60th in captivity. Decreased BKA after 3 months in response to captivity for *R. icterica* male toads was previously reported by Assis et al. (2015). Indeed, decreases in BKA associated with stress, including long-term captivity, have been reported in birds and anurans (Millet et al., 2007; Buehler et al., 2008; Graham et al., 2012; Titon et al., 2017). Long-term stress conditions frequently suppress or dysregulates immune responses by decreasing several cellular functions, including: specific cytokine and antibody production, cell proliferation, as well as, by inhibiting inflammatory processes, among others (reviewed in Dhabhar, 2014). Therefore, although the specific mechanisms remain to be investigated, the decreased BKA following captivity maintenance in *R. icterica* could be a result of reduced protein concentration, such as those from complement system.

Contrary to our prediction, BKA was positively correlated with CORT throughout time in captivity in *R. icterica*. Although CORT and immune responses are often negatively associated under chronic stress conditions, and after chronic treatment with exogenous corticosterone (French et al., 2006; Dhabhar, 2014; Ciliberti et al., 2017), a positive association between CORT and immune responses was previously described in amphibians in response to restraint stress and corticosterone treatment (Graham et al., 2012; Falso et al., 2015). Therefore, our results, in accordance with previous findings, suggest that CORT may positively influence toads' immunity under stress conditions. Interestingly, some studies showed decreased BKA in response to stressors (short and long-term stress), but not correlated with increased CORT levels in anurans (Gomes et al., 2012; Graham et al., 2012; Titon et al., 2017), including *R. icterica*

toads (Assis et al., 2015). Therefore, although stress conditions often decrease BKA response, its immunosuppressive mechanisms may not rely on CORT effects in all circumstances.

Regarding phagocytic activity, a transient decrease in PP and PE was observed in response to captivity maintenance in *R. icterica*. This transient pattern in immune response is in accordance with previous studies in birds and anurans (Martin et al., 2012; Titon et al., 2017). Although stressors can decrease many aspects of immunity, interspecific variation in immune response to stress is commonly observed, as well as intraspecific variation depending upon the immune parameter studied (Buehler et al., 2008; Hopkins and DuRant, 211; Graham et al., 2012; Love et al., 2017; Titon et al., 2017). As we expected, PP and PE were positively correlated with T over time in captivity in *R. icterica*. Mostly known for its immunosuppressive role (Nava-Castro et al., 2012), T have been positively correlated with immune response under stress conditions in birds and anurans (Davies et al., 2016; Titon et al., 2017). These findings suggest that high T may enhance the activity of immune cells, though, more studies, associating testosterone manipulation and immune response, in vivo, as well as in vitro, are necessary to clarify testosterone effects on the immune system of toads.

3.5.2. Relations among steroids body condition and immunity in toads

Our results suggest that plasma T and CORT may positively influence innate immunity (measured as PE and BKA) when toads display better body condition. Interestingly, plasma T levels showed stimulatory effect associated with cellular aspects of immunity (phagocytic activity) for both conditions (with or without BI variation in response to long-term captivity stress). Besides its generally described immunosuppressive effects, a meta-analysis showed that testosterone might enhance cell-mediated immune responses (Foo et al., 2017). Studies of the testosterone effects on immune cells showed that neutrophils activity and cytokines production by CD4+ lymphocytes are enhanced by testosterone treatment (Liva and Voskuhl, 2001;

Chuang et al., 2009). Moreover, T effects may interact with the effects of body condition for mounting and maintenance of immune responses. In this way, individuals in better body condition concomitantly with higher T can afford a better immune response (Ruiz et al., 2010; Desprat et al., 2015). Our results are in accordance with those aforementioned, since toads with higher T showed a tendency to display a better BI and the highest values for PE throughout time in captivity. Nevertheless, more studies with dietary controlling conditions are important to highlight the energetic state role in modulating the interactions between T and immunity in toads.

For those toads not showing body condition variation in response to long-term captivity, our results showed that CORT is positively associated with BKA. Plasma BKA reflects the activity of soluble proteins, such as complement proteins, natural antibodies, and lysozymes in response to foreign microorganisms (Matson et al., 2006). Accordingly, during long-term stress condition, chronic stress response leads to a decrease in natural antibodies and complement proteins levels and, in turn, reducing plasma innate immunity (reviewed in Dhabhar, 2014). Our results are in accordance with the well-documented long-term stress-induced suppressive effects on immune responses. However, we found a positive correlation between CORT and BKA in animals presenting no BI variation in response to chronic stress, suggesting that BKA can be positively modulated by CORT in toads capable to maintain a good body condition over long-term stress conditions. Increased humoral immunity and a trend to increase BKA in response to repeated elevation of corticosterone (transdermal application) has being previously described in lizards by McCormick and Langkilde (2014). Additionally, there are some studies showing that treatment with GCs (at baseline and stress-induced concentrations) can promote overexpression of cytokines (TNF, for example) and also immune-related transcriptional factors (nuclear factor-kB, for example), in mammal immune cells (Liao et al., 1995; Smyth et al., 2004), pointing to a GC-induced immunoenhancing role. Particularly in anurans, CORT

transdermal application increased blood phagocytic ability but showed no effects on BKA (Assis et al., 2017). Further research on CORT treatment is necessary to assess the contexts and how CORT can directly influence toad's immune system.

In toads presenting decreased BI throughout time in captivity, individuals showing higher CORT were characterized by lower BI and, consequently, by lower BKA. These results indicate that body condition is positively associated with this aspect of immunity in toads. Increased CORT stimulates glycogenolysis, lipolysis and facilitate the breakdown of stored triglycerides (Sapolsky et al., 2000; Landys et al., 2006). Therefore, high CORT can negatively influence body condition by decreasing energetic reserves under chronic stress conditions (Landys et al., 2006). In accordance, decreased BI associated with reduced BKA might be a result of the sustained high CORT levels leading to accentuated body mass loss over time in captivity in these toads (Titon et al., 2017). Innate immune system is the first line of defense against pathogens for most vertebrates, and its maintenance at baseline levels is necessary for constant surveillance (Alberts et al., 2006). Given that immune responses may be energetically expensive (Demas et al., 2012), individuals in a better body condition may display better immunity. Moreover, reduced total body fat, an indicative of body condition, correlates with impaired immunity in a wide range of species (reviewed in Demas, 2004). In mammals, surgical removal of adipose tissue impairs antibody production, with immune function being restored after compensatory regrowth of fat pads (Demas et al., 2003). Moreover, body condition can be signalized to immune cells through plasma leptin levels (Demas and Sakaria, 2005; French et al., 2011). In a study conducted by Demas and Sakaria (2005), lipectomy decreases circulating leptin and humoral immunity, whereas restoring leptin via treatment with exogenous leptin restores lipectomy-induced immune suppression. Therefore, it is possible that individuals in a poor body condition displayed a reduced immunity in response to low levels of leptin

throughout captivity. However, more research is needed to understand how energetic status is transduced in signals that regulate immunity in amphibians.

The present results suggest that CORT, BI, T and immune responses can be directly associated in anurans, however, it is important to point out that SEM analyses suggest causal relations that need to be experimentally tested. In the meantime, the BI variation in response to captivity maintenance differed between the two-studied species, and by comparing two species there are inferences about the adaptive significance of differences between species (Garland and Adolph, 1994). Therefore, studies with controlled dietary associated with steroid exogenous application are necessary in order to investigate the causal relations suggested by the SEM analyses for toads.

3.6. Conclusions

Captivity maintenance resulted in high CORT values over a prolonged period, whilst decreased T in *R. icterica*. In the meantime, immune response may vary over time in captivity, depending on the immune parameter studied. While PE showed a transient decrease, BKA displayed a consistent decrease in response to long-term captivity. Also, BKA is positively correlated with CORT and PE is positively associated with T throughout captivity. These results suggest that captivity maintenance can be considered a stressor for *R. icterica*, associated with multiple hormone-immune interactions.

Additionally, the analyses of endocrine-immune responses of toads (*R. icterica* and *R. schneideri*) to the same captive conditions also reveal common patterns of covariance and functional implications. While T and phagocytosis showed consistent and similar positive relations throughout period of captive maintenance, the relations between CORT and BKA changes in response to toads' BI. CORT and BKA are positively associated in toads that maintain BI throughout the period under captivity. Otherwise, CORT is negatively associated

with BI, which is positively associated with BKA, in those animals characterized by lowering BI in response to captivity maintenance. Furthermore, a better BI tends to be associated with higher T. Collectively, this study indicates coordinated changes in steroid plasma levels (CORT and T) and different immune parameters under time in captivity. Moreover, body condition may play a critical role in modulating the interactions among CORT, T and immune responses in toads. Since resources in nature may vary according to the environmental conditions, resulting in possible energetic trade-offs, our results suggest that toads in a long-term stress condition may reduce immunity, with individuals in a poorer body condition being more susceptible to impairment of the immune response.

3.7. Acknowledgments

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3.9. Suppementary file

Supplementary Materials for

Interplay among steroids, body condition and immunity in Brazilian toads

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This file includes:

Tables 3S1 to 3S6

Figure 3S1 to 3S4

Table 3S1. SEM analysis result including all fitted models for body index, plasma corticosterone and testosterone levels, bacterial killing ability and phagocytosis efficiency for *R. icterica* and *R. schneideri*.

Species	Model	X^2	DF	*P*	AIC	dAIC	weight
R. icterica	**Model 1***	2.841	5	0.724	610.9	0.0	0.593
	Model 7*	2.782	4	0.595	612.9	1.9	0.225
	Model 6	2.506	3	0.474	614.6	3.7	0.095
	Model 8	7.074	5	0.215	615.2	4.2	0.071
	Model 2	11.086	5	**0.050**	619.2	8.2	0.010
	Model 4	9.803	3	**0.020**	621.9	11.0	0.003
	Model 3	9.803	3	**0.020**	621.9	11.0	0.003
	Model 5	10.907	3	**0.012**	623.0	12.1	0.001
R. schneideri	**Model 3***	1.618	3	0.655	-97.1	0.0	0.306
	Model 4*	1.618	3	0.655	-97.1	0.0	0.306
	Model 5*	2.243	3	0.524	-96.4	0.6	0.224
	Model 1	8.657	5	0.124	-94.0	3.0	0.067
	Model 2	8.941	5	0.111	-93.7	3.3	0.058
	Model 7	8.588	4	0.072	-92.1	5.0	0.026
	Model 6	8.873	3	**0.031**	-89.8	7.3	0.008
	Model 8	14.065	5	**0.015**	-88.6	8.4	0.005

Abbreviation as follow: **DF:** Degrees of freedom; **AIC:** Akaike information criterion; **dAIC:** difference between Akaike information criterion. (*N* = 19 and 20 for *R. icterica* and *R. schneideri*, respectively). * = Selected models (P > 0.05 and dAIC <2.0). Variables with *P* significant < 0.05 are highlighted in bold.

Table 3S2. Descriptive statistics of body measures and plasma steroid levels for *R. icterica* male toads in field and captivity.

Variable	Group	CM	N	Mean ± SE	Min	Max
Snout-vent lenght (mm)	Calling	Field	6	111.57 ± 3.98	101.34	127.80
		7	4	121.67 ± 9.25	106.26	147.79
		30	4	104.97 ± 5.80	90.38	112.64
		60	4	109.36 ± 2.04	103.71	113.18
		90	5	103.36 ± 2.27	99.48	111.90
	Non-Calling	Field	4	114.76 ± 9.91	94.44	137.90
		7	2	97.88 ± 6.76	91.13	104.64
		30	2	127.70 ± 12.45	115.25	140.15
		60	3	107.56 ± 9.90	93.91	126.82
		90	1	91.71 ± 0.00		
Body mass (g)	Calling	Field	6	105.18 ± 15.44	70.10	173.70
		7	4	157.88 ± 49.78	90.01	302.26
		30	4	87.08 ± 9.99	65.96	104.89
		60	4	100.47 ± 5.96	86.01	114.59
		90	5	87.23 ± 4.39	74.71	98.57
	Non-Calling	Field	4	129.52 ± 28.60	65.80	197.40
		7	2	72.06 ± 19.66	52.40	91.72
		30	2	154.90 ± 60.83	94.08	215.73
		60	3	93.66 ± 20.90	63.86	133.95
		90	1	71.76 ± 0.00		
Plasma corticosterone levels (ng/ml)	Calling	Field	6	18.84 ± 4.36	9.16	34.96
		7	4	19.10 ± 6.74	10.10	38.71
		30	3	13.15 ± 3.61	8.95	20.34
		60	3	0.33 ± 0.10	0.15	0.48
		90	5	2.15 ± 0.46	1.25	3.40
	Non-Calling	Field	4	0.80 ± 0.46	0.03	1.92
		7	2	5.14 ± 0.07	5.07	5.22
		30	2	10.34 ± 2.26	8.08	12.60
		60	3	7.66 ± 0.57	7.01	8.79
		90	1	1.54 ± 0.00		
Plasma testosterone levels (ng/ml)	Calling	Field	5	121.16 ± 28.82	57.04	201.23
		7	4	8.64 ± 4.41	1.08	21.31
		30	4	1.74 ± 0.58	0.72	2.98
		60	3	2.54 ± 0.96	0.63	3.64
		90	5	2.36 ± 0.58	0.42	3.63
	Non-Calling	Field	3	43.41 ± 10.85	23.75	61.18
		7	2	1.87 ± 1.07	0.81	2.94
		30	2	3.00 ± 1.67	1.34	4.67
		60	3	1.39 ± 0.50	0.61	2.31
		90	1	0.40 ± 0.00		

Abbreviation as follow: **CM:** Captivity maintenance; **SE:** standard error; **Min:** minimum value; **Max:** maximum value.

Table 3S3. Descriptive statistics of immune response for *R. icterica* male toads in field and captivity.

Variable	Group	CM	N	Mean ± SE	Min	Max
Bacterial killing ability (%)	Calling	Field	6	93.00 ± 3.89	74	100
		7	4	98.50 ± 0.96	96	100
		30	4	100.00 ± 0.00	100	100
		60	3	62.00 ± 15.52	31	79
		90	5	64.80 ± 5.54	49	80
	Non-Calling	Field	3	97.33 ± 1.76	94	100
		7	2	92.50 ± 7.50	85	100
		30	2	96.50 ± 3.50	93	100
		60	3	74.33 ± 13.72	47	90
		90	1	69.00 ± 0.00		
Phagocytosis (%)	Calling	7	4	29.27 ± 6.33	17.20	40.60
		30	4	11.77 ± 2.57	7.69	19.20
		60	3	20.30 ± 6.05	13.90	32.40
		90	5	16.65 ± 4.08	5.51	24.80
	Non-calling	7	2	19.75 ± 1.65	18.10	21.40
		30	2	8.08 ± 2.32	5.77	10.40
		60	3	17.70 ± 4.80	12.60	27.30
		90	1	7.46 ± 0.00		
Phagocytosis Efficiency (%)	Calling	7	4	10.57 ± 1.77	5.69	14.10
		30	4	5.49 ± 1.07	3.03	8.25
		60	3	10.47 ± 3.02	7.42	16.50
		90	5	8.19 ± 3.16	1.63	17.10
	Non-calling	7	2	8.95 ± 5.34	3.62	14.29
		30	2	4.01 ± 0.16	3.85	4.17
		60	3	9.90 ± 3.65	5.91	17.20
		90	1	3.38 ± 0.00		

Abbreviation as follow: **CM:** Captivity maintenance; **SE:** standard error; **Min:** minimum value; **Max:** maximum value.

Table 3S4. Effect of captivity maintenance on plasma steroid levels of *R. icterica* tested through a set of ANCOVAs, with plasma corticosterone and testosterone levels as dependent variables, body mass as co-variable and captivity maintenance (0, 7, 30, 60, and 90 days) and calling behavior (calling and non-calling) as factors.

Dependent Variable	Source	Type III SS	DF	MS	*F*	*P*
Plasma corticosterone levels	**Corrected Model**	137.388	18	7.633	3.994	**0.007**
	Intercept	0.483	1	0.483	0.253	0.623
	Calling behavior	0.004	1	0.004	0.002	0.963
	CM	6.200	4	1.550	0.811	0.540
	BM	0.002	1	0.002	0.001	0.978
	Calling behavior * CM	3.136	3	1.045	0.547	0.659
	Calling behavior * BM	0.022	1	0.022	0.011	0.917
	CM * BM	6.354	4	1.589	0.831	0.529
	Calling behavior * CM * BM	2.571	3	0.857	0.448	0.723
	Error	24.843	13	1.911		
	Total	577.659	32			
	Corrected Total	162.231	31			
Plasma testosterone levels	**Corrected Model**	387.857	18	21.548	6.441	**0.001**
	Intercept	0.000	1	0.000	0.000	0.994
	Calling behavior	0.829	1	0.829	0.248	0.627
	CM	1.894	4	0.473	0.142	0.964
	BM	0.392	1	0.392	0.117	0.738
	Calling behavior * CM	4.861	3	1.620	0.484	0.699
	Calling behavior * BM	0.523	1	0.523	0.156	0.699
	CM * BM	0.938	4	0.235	0.070	0.990
	Calling behavior * CM * BM	3.988	3	1.329	0.397	0.757
	Error	43.488	13	3.345		
	Total	811.290	32			
	Corrected Total	431.345	31			

Abbreviation as follow: **Type III SS:** Type III sum of squares; **DF:** Degrees of freedom; **MS:** Mean square; **CM:** Captivity maintenance; **BM:** Body mass. Variables with *P* significant < 0.05 are highlighted in bold.

Table 3S5. Effect of captivity maintenance on immune response of *R. icterica* tested through a set of ANCOVAs, with bacterial killing ability, phagocytosis percentage and phagocytosis efficiency as dependent variables, body mass as co-variable and captivity maintenance (0, 7, 30, 60, and 90 days) and calling behavior (calling and non-calling) as factors.

Dependent Variable	Source	Type III SS	DF	MS	*F*	*P*
Bacterial Killing Ability	**Corrected Model**	11416.548	18	634.253	4.302	**0.003**
	Intercept	2.121	1	2.121	0.014	0.906
	Calling behavior	19.985	1	19.985	0.136	0.718
	CM	1579.001	4	394.750	2.678	0.072
	BM	213.569	1	213.569	1.449	0.247
	Calling behavior * CM	872.073	3	290.691	1.972	0.162
	Calling behavior * BM	15.329	1	15.329	0.104	0.752
	CM * BM	1386.165	4	346.541	2.351	0.101
	Calling behavior * CM * BM	770.137	3	256.712	1.741	0.202
	Error	2211.388	15	147.426		
	Total	182957.828	34			
	Corrected Total	13627.936	33			
Phagocytosis	**Corrected Model**	1479.298	14	105.664	1.205	0.399
	Intercept	19.758	1	19.758	0.225	0.646
	Calling behavior	125.724	1	125.724	1.434	0.262
	CM	167.607	3	55.869	0.637	0.610
	BM	56.648	1	56.648	0.646	0.442
	Calling behavior * CM	207.279	2	103.639	1.182	0.350
	Calling behavior * BM	131.844	1	131.844	1.504	0.251
	CM * BM	184.649	3	61.550	0.702	0.574
	Calling behavior * CM * BM	214.854	2	107.427	1.225	0.338
	Error	789.174	9	87.686		
	Total	9778.938	24			
	Corrected Total	2268.471	23			
Phagocytosis Efficiency	**Corrected Model**	408.895	14	29.207	1.548	0.258
	Intercept	19.154	1	19.154	1.015	0.340
	Calling behavior	62.944	1	62.944	3.335	0.101
	CM	142.230	3	47.410	2.512	0.124
	BM	32.942	1	32.942	1.745	0.219
	Calling behavior * CM	77.868	2	38.934	2.063	0.183
	Calling behavior * BM	67.039	1	67.039	3.552	0.092
	CM * BM	145.006	3	48.335	2.561	0.120
	Calling behavior * CM * BM	81.793	2	40.897	2.167	0.171
	Error	169.860	9	18.873		
	Total	2172.895	24			
	Corrected Total	578.755	23			

Abbreviation as follow: **Type III SS:** Type III sum of squares; **DF:** Degrees of freedom; **MS:** Mean square; **CM:** Captivity maintenance; **BM:** Body mass; Variables with *P* significant < 0.05 are highlighted in bold.

Table 3S6. Effect of captivity maintenance on immune response of *R. icterica* male toads tested through a set of ANOVAs, with bacterial killing ability, phagocytosis percentage and phagocytosis efficiency as dependent variables and captivity maintenance (0, 7, 30, 60, and 90 days) and calling behavior (calling and non-calling) as factors.

Dependent Variable	Source	Type III SS	DF	MS	*F*	*P*
Bacterial Killing Ability	**Corrected Model**	6182.378	9	686.931	6.827	**0.000**
	Intercept	133810.573	1	133810.573	1329.809	**0.000**
	Calling behavior	0.984	1	0.984	0.010	0.922
	CM	4394.400	4	1098.600	10.918	**0.000**
	Calling behavior * CM	297.579	4	74.395	0.739	0.575
	Error	2314.349	23	100.624		
	Total	182957.828	33			
	Corrected Total	8496.727	32			
Phagocytosis	**Corrected Model**	1000.434	7	142.919	1.803	0.156
	Intercept	5096.587	1	5096.587	64.308	**0.000**
	Calling behavior	672.997	3	224.332	2.831	0.072
	CM	185.651	1	185.651	2.343	0.145
	Calling behavior * CM	49.370	3	16.457	0.208	0.890
	Error	1268.038	16	79.252		
	Total	9778.938	24			
	Corrected Total	2268.471	23			
Phagocytosis Efficiency	**Corrected Model**	135.434	7	19.348	0.698	0.673
	Intercept	1103.925	1	1103.925	39.842	**0.000**
	Calling behavior	117.710	3	39.237	1.416	0.275
	CM	21.293	1	21.293	0.769	0.394
	Calling behavior * CM	10.005	3	3.335	0.120	0.947
	Error	443.321	16	27.708		
	Total	2172.895	24			
	Corrected Total	578.755	23			

Abbreviation as follow: **Type III SS:** Type III sum of squares; **DF:** Degrees of freedom; **MS:** Mean square; **CM:** Captivity maintenance. Variables with *P* significant < 0.05 are highlighted in bold.

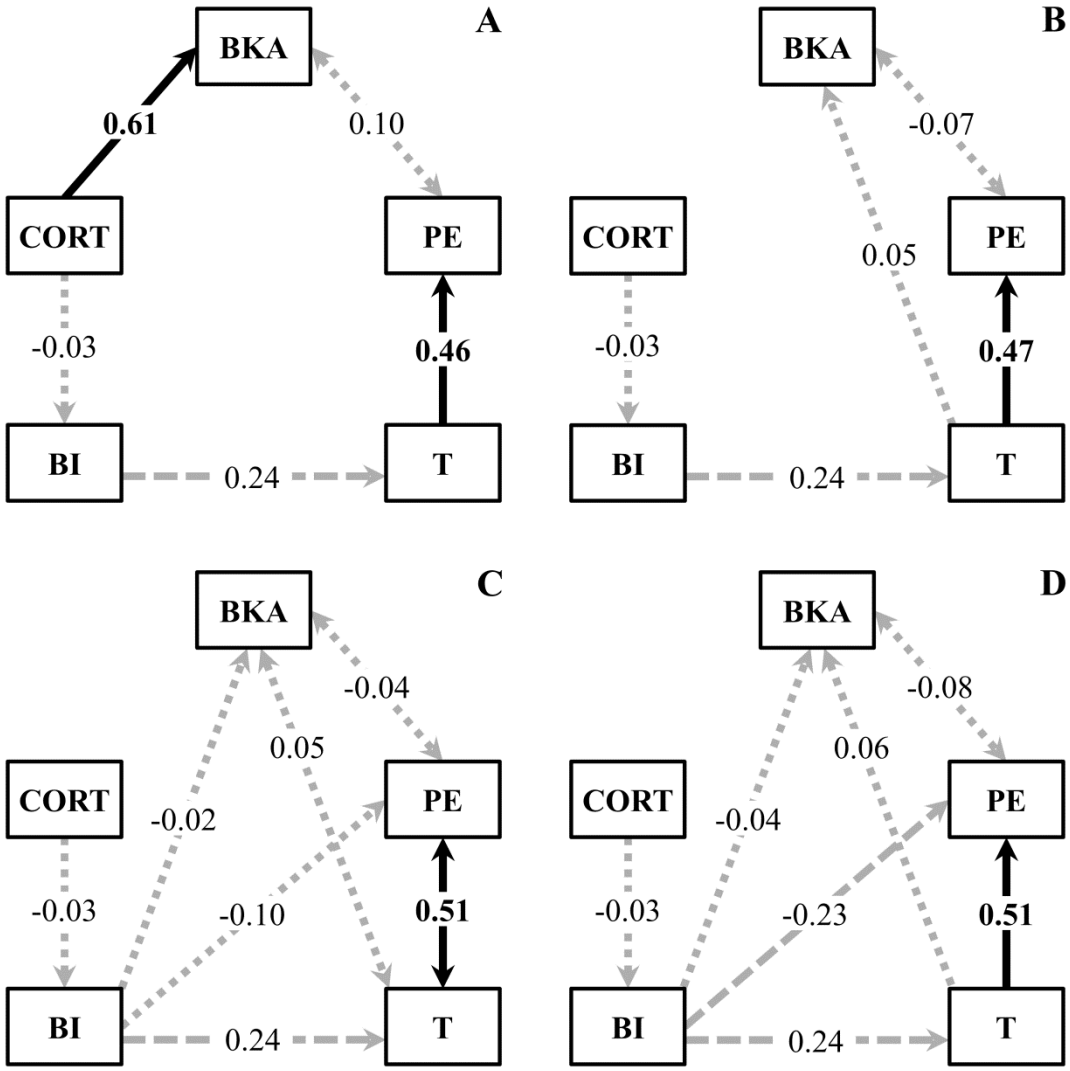

Figure 3S1. Proposed models (1-4) for *R. icterica*. Path diagrams of four causal models tested for *R. icterica*. Path coefficients shown are all standardized values (A) Model 1; (B) Model 2; (C) Model 3; (D) Model 4. Abbreviation as follow: **BKA:** Bacterial killing ability; **CORT:** Plasma corticosterone levels; **BI:** Body index; **PE:** Phagocytosis percentage; **T:** Plasma testosterone levels. (N = 19).

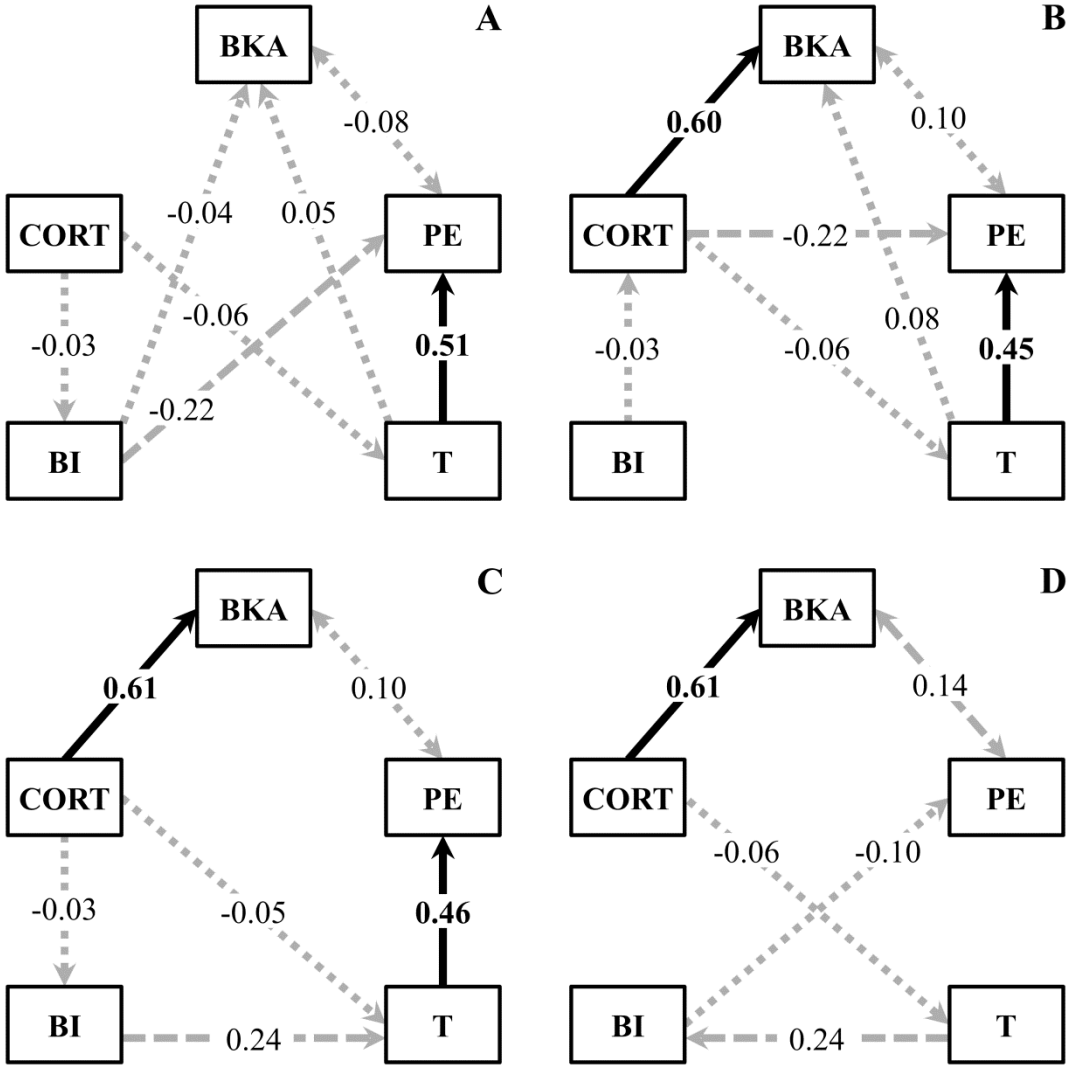

Figure 3S2. Proposed models (5-8) for *R. icterica*. Path diagrams of four causal models tested for *R. icterica*. Path coefficients shown are all standardized values (A) Model 5; (B) Model 6; (C) Model 7; (D) Model 8. Abbreviation as follow: **BKA:** Bacterial killing ability; **CORT:** Plasma corticosterone levels; **BI:** Body index; **PE:** Phagocytosis percentage; **T:** Plasma testosterone levels. (N = 19).

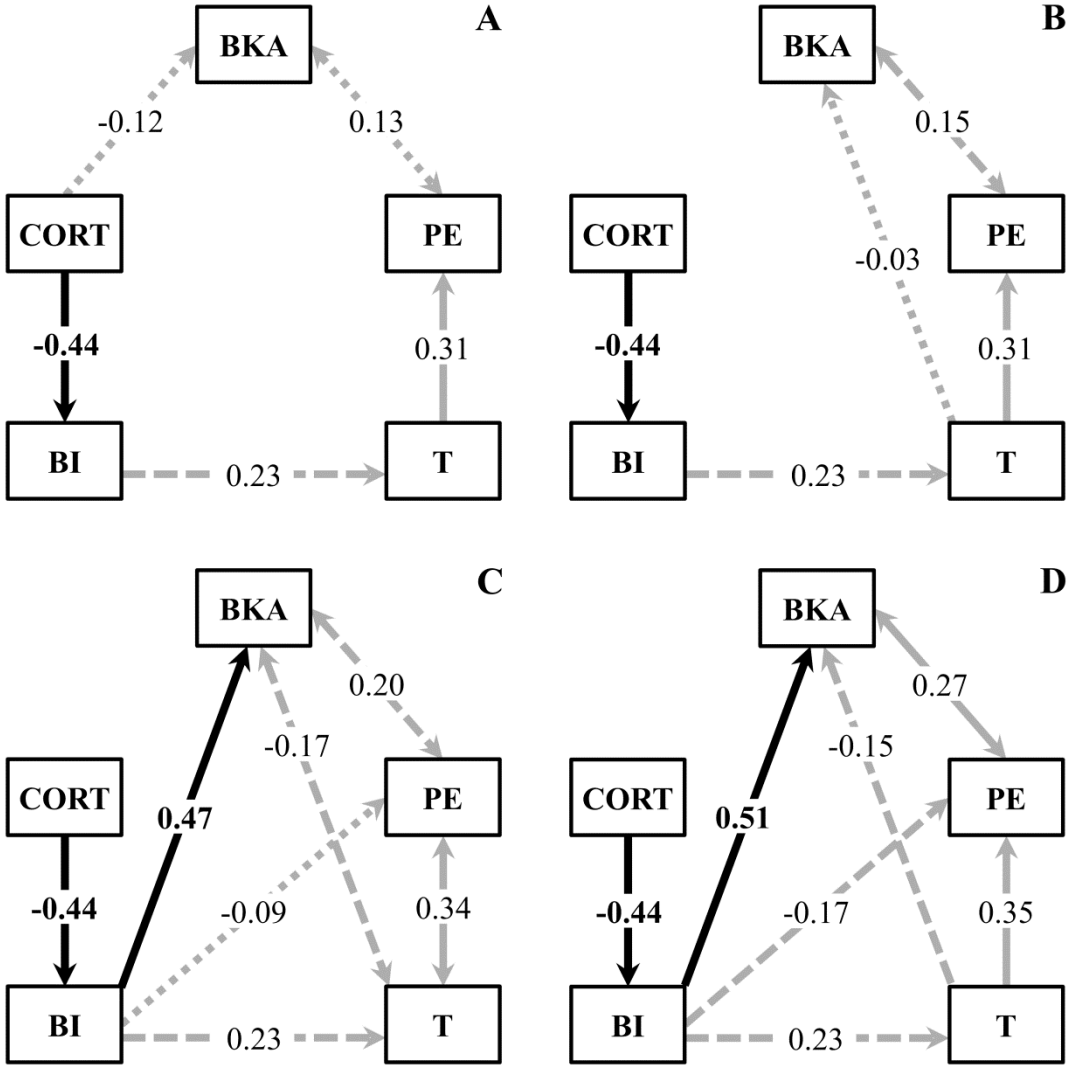

Figure 3S3. Proposed models (1-4) for *R. schneideri*. Path diagrams of four causal models tested for *R. schneideri*. Path coefficients shown are all standardized values (A) Model 1; (B) Model 2; (C) Model 3; (D) Model 4. Abbreviation as follow: **BKA:** Bacterial killing ability; **CORT:** Plasma corticosterone levels; **BI:** Body index; **PE:** Phagocytosis percentage; **T:** Plasma testosterone levels. (N = 20).

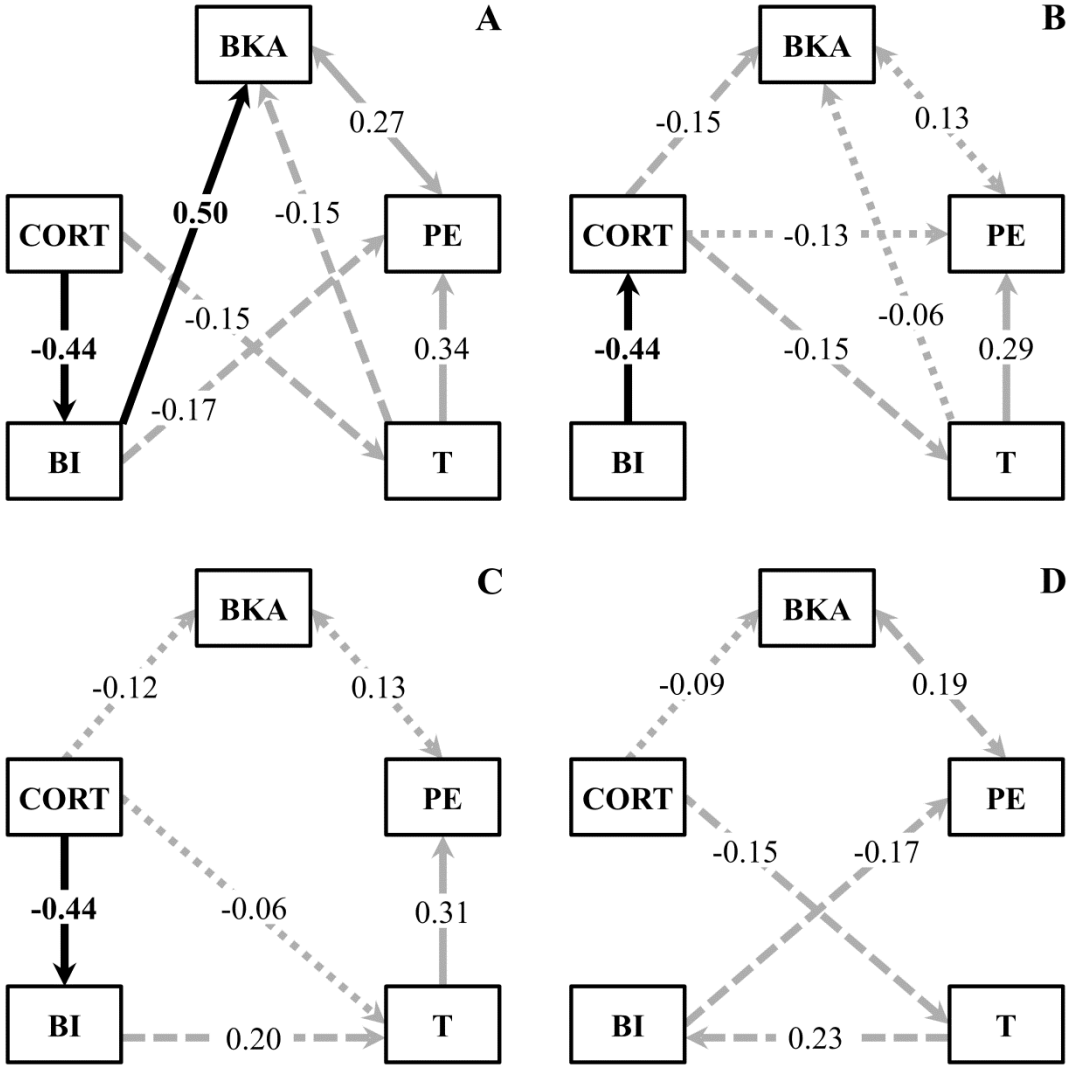

Figure 3S4. Proposed models (5-8) for *R. schneideri*. Path diagrams of four causal models tested for *R. schneideri*. Path coefficients shown are all standardized values (A) Model 5; (B) Model 6; (C) Model 7; (D) Model 8. Abbreviation as follow: **BKA:** Bacterial killing ability; **CORT:** Plasma corticosterone levels; **BI:** Body index; **PE:** Phagocytosis percentage; **T:** Plasma testosterone levels. (N = 20).

CONCLUSÕES GERAIS

Em relação à utilização da citometria de fluxo como ferramenta para analises de fagocitose, podemos afirmar que a citometria convencional é um método confiável para estimar medidas de fagocitose. No entanto, a citometria de fluxo por imagem é um método mais preciso de análise no estudo da fagocitose, dispensando o uso de outras ferramentas associadas para as análises (sorting e imagem por microscopia convencional). Além disso, permite análises de parâmetros adicionais que não poderiam ser realizados pela citometria convencional, sendo estes também mais precisos (qualitativamente e quantitativamente) quando comparados à citometria convencional associada à a contagem manual (microscopia convencional).

O cativeiro por tempo prolongado pode ser considerado um agente estressor para *Rhinella schneideri* e *R. icterica*, uma vez que a manutenção prolongada dos sapos nestas condições (*R. schneideri* – 60 dias; *R. icterica* – 90 dias) resultou no aumento dos níveis plasmáticos de corticosterona, diminuição dos níveis plasmáticos de testosterona e da resposta imune em ambas as espécies.

A fagocitose de células peritoneais, tanto quando avaliada como porcentagem de fagocitose, quanto como eficiência de fagocitose, esteve positivamente correlacionada com os níveis plasmáticos de testosterona ao longo do tempo em cativeiro em ambas as espécies estudadas. Uma vez que esta relação se manteve independente da variação da condição corpórea, é possível que haja um papel imunomodulatório direto entre dos níveis plasmáticos de testosterona sobre a resposta imune celular em sapos.

Os níveis plasmáticos de corticosterona estiveram positivamente correlacionados com a capacidade bactericida plasmática quando não houve perda de condição corpórea em resposta ao cativeiro, indicando que a corticosterona pode exercer efeitos estimulatórios sobre a resposta imune quando o animal apresenta uma boa condição corpórea. Havendo variação da condição corpórea em condições de estresse crônico, a corticosterona plasmática pareceu influenciar

indiretamente a capacidade bactericida plasmática através da influência sobre a condição corpórea do indivíduo, onde indivíduos com maior corticosterona plasmática apresentaram menor condição corpórea e menor capacidade bactericida plasmática ao longo do tempo em cativeiro. Nossos resultados sugerem que, a resposta imune inata parece estar associada às variações dos níveis plasmáticos de corticosterona e testosterona, assim como com à condição corpórea de anuros sob condições de estresse de manutenção a longo prazo em cativeiro.

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

Instituto de biociências

UNIVERSIDADE DE SÃO PAULO

OF.CEUA/IB/054/2013
Ref. 2013.1.1443.41.5

São Paulo, 12 de dezembro de 2013

Prezado Senhor,

Dirijo-me a V.Sa. para informar que a Comissão de Ética no Uso de Animais do IB (CEUA), em reunião realizada no dia 10 de dezembro de 2013, **APROVOU** o Projeto "Efeito da temperatura sobre diversos parâmetros da imunocompetência e níveis plasmáticos de corticosterona de três populações de anfíbios, *Rhinella schneideri* (Spix, 1824)" - **Protocolo 194/2013**, de sua responsabilidade (Colaboradora Stefanny Christie Monteiro Titon).

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

O pesquisador responsável deverá comunicar a esta Comissão qualquer alteração e/ou intercorrência que houver durante a realização da pesquisa.

Lembramos que deverão ser apresentados os relatórios anuais ou final, conforme modelo disponível no link desta Comissão.

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 - IBUSP

Rua do Matão - Travessa 14 nº 321 - CEP 05508-900 - Cidade Universitária
São Paulo - Brasil - http://www.ib.usp.br

ANEXO 2 – CERTIFICADO DE APROVAÇÃO DE ALTERAÇÃO DO PROJETO PELO CEUA IB/USP

Universidade de São Paulo
Instituto de Biociências

www.ib.usp.br

CERTIFICADO

Certificamos que a proposta intitulada **"Efeitos do cativeiro de curto e longo prazo sobre níveis plasmáticos de corticosterona e testosterona e resposta imunitária em sapos do gênero *Rhinella*"**, registrada com o nº 194/2013 (Proc. 13.1.1443.41.5), sob a responsabilidade do Prof. Dr. Fernando Ribeiro Gomes e com a participação da colaboradora Stefanny Christie Monteiro Titon (IB/USP), que envolve a utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica encontra-se de acordo com os preceitos da Lei nº 11.794, de 08 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009 e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovada pela Comissão de Ética no Uso de Animais – CEUA do Instituto de Biociências da Universidade de São Paulo, em reunião de 14 de junho de 2016.

Aprovada a alteração do título do projeto acima mencionado.

Vigência da autorização: 14/06/2016 a 13/06/2018

Finalidade: Pesquisa Científica

Nº da solicitação ou autorização SISBIO: 1904018

Atividade: Captura

Espécies/Grupos Taxonômicos: Anfíbios/*Rhinella icterica* e *Rhinella ornata* (aprovada à inclusão de duas novas espécies)

Locais de realização das atividades: Caatinga, Cerrado e Mata Atlântica

OBS.: Qualquer alteração e/ou intercorrência deverá ser comunicada a CEUA-IB.

Profa. Dra. Mariz Vainzof

Coordenadora da Comissão de Ética no Uso de Animais

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