Universidade de São Paulo
Instituto de Biociências
Departamento de Fisiologia
Programa de Pós-Graduação em Ciências Biológicas - Fisiologia Geral

RELAÇÃO SAZONAL ENTRE REPRODUÇÃO, ENERGÉTICA E IMUNOCOMPETÊNCIA EM SAPOS DA CAATINGA

CARLA BONETTI MADELAIRE

SÃO PAULO - SP

2017

Madelaire, Carla Bonetti
RELAÇÃO SAZONAL ENTRE REPRODUÇÃO, ENERGÉTICA E IMUNOCOMPETÊNCIA EM SAPOS DA CAATINGA / Carla Bonetti Madelaire; orientador Fernando Ribeiro Gomes. -- São Paulo, 2017.

157 f.

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

1. anura. 2. testosterona. 3. corticosterona. 4. metabolismo. 5. reguladores metabólicos. I. Gomes, Fernando Ribeiro, orient. II. Título.

Catalogação da Publicação
Serviço de Biblioteca do Instituto de Biociências

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RELAÇÃO SAZONAL ENTRE REPRODUÇÃO, ENERGÉTICA E IMUNOCOMPETÊNCIA EM SAPOS DA CAATINGA

SEASONAL RELATIONSHIP BETWEEN REPRODUCTION, ENERGETICS AND IMMUNOCOMPETENCE IN FROGS FROM THE BRAZILIAN SEMI-ARID, CAATINGA

CARLA BONETTI MADELAIRE

Tese apresentada ao Instituto de Biociências, Universidade de São Paulo - USP - para obtenção do título de doutora em Ciências Biológicas - Fisiologia geral

Orientador: Prof. Dr. Fernando Ribeiro Gomes

SÃO PAULO - SP

2017

Comissão julgadora:

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Prof. Dr. Fernando Ribeiro Gomes

Orientador

AGRADECIMENTOS

Ao Prof. Dr. Fernando Ribeiro Gomes, meu orientador e amigo. Por toda paciência e fé que você teve na minha capacidade. Você sabiamente me guiou, orientou e inspirou a cientista que eu me tornei. Eu serei eternamente grata!

À Profa. Dra. Inna Sokolova, por gentilmente ter me recebido no seu laboratório, e por ter me guiado e orientado na arte das ciências moleculares.

À Bruna de Oliveira Cassettari, pela ajuda preciosa no campo e no laboratório.

Ao Francisco de Assis (Seu Assis), Neci de Assis (Dona Neci) e toda a Fazenda São Miguel, pelo apoio e por fazer eu me sentir como parte da família enquanto eu coletava todos aqueles sapos.

Ao Prof. Dr. Carlos Arturo Navas, pelos conselhos e ensinamentos, e por inspirar a ciência que eu faço.

Aos professores do Departamento de Fisiologia - IB - USP, pelos momentos que contribuíram para minha formação.

Aos meus colegas de laboratório no Brasil, por todos os ensinamentos, discussões científicas, amizade e apoio, e por fomentar a competição que me incentivou a sempre dar o meu melhor.

Aos meus colegas de laboratório nos USA, por todos os ensinamentos e apoio.

Aos meus colegas de departamento, pelo apoio e discussões científicas.

Aos familiares que me apoiaram e incentivaram durante esta jornada.

Às minhas colegas de casa no Brasil e EUA, pela paciência, amizade e apoio.

A todos os meus amigos no Brasil e no resto do mundo (EUA, Europa e China). Vocês sempre me apoiaram e estiverem comigo durante os piores e os melhores momentos. Eu serei eternamente grata. S2

À Fundação do Amparo à Pesquisa do Estado de São Paulo, pelas bolsas no Brasil e no exterior e pelos auxílios financeiros (2013/24206-4 e 2015/02484-0, auxílio regular à pesquisa 2013/00900-1 e SPRINT 2014/50643-8).

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico, pela bolsa no meu primeiro mês de doutorado (140162/2013-5).

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Resumo

Nesta tese de doutorado foram investigados parâmetros imunológicos de 3 espécies de anuros ao longo de diferentes estágios de história de vida, bem como a relação dos níveis plasmáticos de hormônios esteroides com as variáveis imunológicas (Capítulo 1). O capítulo 2 aborda os ajustes sazonais de reguladores metabólicos em diferentes músculos relacionados a reprodução e locomoção no período reprodutivo e de seca em três espécies de anuros. O capítulo 3 aborda a relação causal entre o aumento dos níveis plasmáticos de hormônios esteroides e a imunomodulação da resposta inflamatória e da resposta mediada por proteínas do sistema complemento na espécie *R. jimi*. Adicionalmente, foram investigados possíveis ajustes anuais do custo energético da resposta inflamatória, da taxa metabólica padrão, e a relação dessas variáveis com os níveis plasmáticos de hormônios esteroides na espécie *R. jimi* (Capítulo 4). O conjunto de dados dos capítulos 1 e 4 apontam na direção contrária da hipótese de imunossupressão durante a temporada reprodutiva, altos níveis de hormônios esteroides (testosterona e corticosterona) aumentam os paramêtros imunitários, bem como a resposta imune. Os resultados apresentados no capítulo 3 corroboram os efeitos imunomodulatórios do tratamento agudo de testosterona e corticosterona em anfíbios anuros. Por fim, o capítulo 2 mostra a variação sazonal de expressão de proteínas que agem como reguladores metabólicos. Essas reguladores, mediam a manutenção de células e tecidos durante a seca, fazendo com que os músculos associados a reprodução e locomoção não degradem durante este período. Adicionamente, a espécie estivadora ativa vias que diminuem o consumo de ATP, fazendo que com que haja economia de reservas energéticas durante a estivação.

Introdução Geral

Ambientes com drástica variação sazonal são caracterizados por mudanças nas condições bióticas e abióticas, como disponibilidade alimentar e temperatura (Chesson et al. 2004). Para sobreviverem a essas variações e completarem seus ciclos de vida, os animais necessitam apresentar ajustes fisiológicos e comportamentais sazonais (Dayton and Fitzgerald, 2005; Storey e Storey, 2005; Navas e Carvalho, 2010). Dentre os ajustes fisiológicos, a variação sazonal da secreção de hormônios esteroides (e.g. testosterona e corticosterona) e, consequentemente, o desenvolvimento e manutenção de características sexuais secundárias (Moore e Jessop, 2003; Madelaire e Gomes, 2016) são de importância fundamental, pois garantem que a atividade reprodutiva ocorra em períodos favoráveis. Em machos, a testosterona e a corticosterona, além do papel desempenhado na reprodução (Moore e Jessop, 2003), também são importantes na regulação de parâmetros imunitários, como número de leucócitos circulantes na corrente sanguínea, a resposta inflamatória e a resposta mediadas por proteínas do sistema complemento (Roberts et al. 2004; Dhabhar 2009). Assim, a variação sazonal desses hormônios também poderia resultar em uma variação na resposta imunológica em espécies de ambientes sazonais. Adicionalmente, ajustes fisiológicos sazonais em nível molecular também são importantes para garantir a integridade das células, tecidos e órgãos, bem como regular o balanço energético metabólico durante períodos críticos, como durante a estação seca em ambientes áridos e semi-áridos (Storey e Storey, 2005; Storey e Storey, 2010).

Os anfíbios anuros do semi-árido Brasileiro, a Caatinga, enfrentam variações sazonais drásticas de disponibilidade alimentar e de água (Abe, 1995; Rodrigues, 2003; Carvalho et al., 2010). Esse ambiente apresenta uma temporada de chuvas (Janeiro a Maio), quando há maior umidade e disponibilidade alimentar comparada com o período de seca (Julho a Novembro). Mesmo assim, os anfíbios dependem de uma grande quantidade de chuvas (100mm) para se reproduzir, o que pode não ocorrer em alguns anos. Assim, diz-se que o evento reprodutivo nessa região é imprevisível (Carvalho et al. 2010; Madelaire e Gomes, 2016). O período de chuvas é considerado o período reprodutivo das espécies de anuros dessa região, quando os machos apresentam altos níveis de andrógenos no plasma. Já os níveis plasmáticos de corticosterona só aumentam durante os eventos de chuvas, quando os machos começam a vocalizar para

atrair as fêmeas (Madelaire e Gomes, 2016). Na seca, por sua vez, os níveis plasmáticos de ambos os esteroides estão baixos (Madelaire e Gomes, 2016). Outro aspecto relevante é que os anuros podem apresentar diferentes estratégias comportamentais para lidar com período de seca. Na região de Angicos (RN), por exemplo, os sapos *Rhinella jimi* e *R. granulosa* continuam ativos e se alimentando próximos a corpos de água permanentes. Por outro lado, *Pleurodema diplolister* se enterra na areia próxima aos lençóis freáticos e entra em depressão metabólica (comportamento de estivação), não se alimentando até o próximo evento reprodutivo (Carvalho et al. 2010; Madelaire e Gomes, 2016). Essa diversidade de estratégias também poderia resultar em diferenças de intensidade nos ajustes fisiológicos da espécie estivadora *versus* as espécies que se mantêm em atividade durante a seca.

Na minha tese de doutorado investigamos se esses três anuros demonstram ajustes em parâmetros imunológicos ao longo de diferentes estágios de história de vida [no período reprodutivo (quando os níveis de andrógenos estão elevados, porém os animais não apresentam atividade reprodutiva), durante a atividade reprodutiva (quando os machos estão vocalizando e ambos os esteroides, andrógenos e corticosterona, estão elevados) e período de seca (quando ambos os esteroides estão em níveis baixos no plasma], bem como a relação dos níveis plasmáticos de hormônios esteroides com as variáveis imunológicas (Capítulo 1. Seasonal patterns of variation in steroid plasma levels and immune parameters in anurans from Brazilian semiarid area). Investigamos também os ajustes de reguladores metabólicos em diferentes músculos relacionados a reprodução e locomoção no período reprodutivo e de seca (Capítulo 2: Muscle maintenance and energy status of anurans living in an extremely seasonal semi-arid environment, the Brazillian Caatinga). A partir dos resultados encontrados no artigo do capítulo 1, decidimos explorar a relação funcional causal entre o aumento dos níveis plasmáticos de hormônios esteroides e a imunomodulação da resposta inflamatória e da resposta mediada por proteínas do sistema complemento na espécie *R. jimi* (Capítulo 3: Immunomodulation by testosterone and corticosterone in toads: experimental evidences from transdermal application). Adicionalmente, investigamos possíveis ajustes anuais do custo energético da resposta inflamatória, da taxa metabólica padrão, e a relação dessas variáveis com os níveis plasmáticos de hormônios esteroides na espécie *R. jimi*

(Capítulo 4. Seasonality of steroid hormones, immune response and metabolic cost to inflammation in toads).

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1. Seasonal patterns of variation in steroid plasma levels and immune parameters in anurans from Brazilian semiarid area

RUNNING TITLE: **Anuran steroids and immune variation**

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Key words: androgens, corticosterone, phytohemagglutinin, leukocyte profile, parasite load

Summary statement

Anurans from a semi-arid habitat show higher number of circulating leukocytes and immune responsiveness during the reproductive period, when steroid plasma levels are higher.

Accepted 1/22/2017; Electronically Published 4/11/2017.

Physiological and Biochemical Zoology 90(4):415–433

1.1. Abstract

Elevated androgens and glucocorticoids displayed by males during the reproductive season have been proposed to mediate a possible trade-off between reproduction and immunocompetence. Anurans living in arid and semiarid environments display a strong seasonal reproduction, which could accentuate the variation in physiological, immunological, and behavioral parameters. We studied covariation between steroid plasma levels, morphometric variables associated with body condition and immunity, leukocyte profile, parasite load, and response to an immunological challenge across different phases of the annual life-history cycle of three anuran species from a Brazilian semiarid area. Our results showed a seasonal pattern of covariation among leukocyte parameters, kidney mass, and steroid plasma levels, with higher values measured during the reproductive season, particularly when males were sampled during calling activity. Moreover, these anurans showed a stronger response to an immunological challenge during the reproductive period. The immunosuppression during the dry period was particularly evident for the species that aestivate, indicating that the availability of energetic resources might be an important factor determining seasonal variation in inflammatory response. Intensity of the helminth infection was associated with eosinophil count but showed a more complex pattern with regard to androgens levels. These data emphasize that variations in the intensity of helminth infection might be more closely related to specific aspects of the immune response than to the general seasonal patterns of variation in steroid plasma levels, total circulating leukocytes, and inflammatory response.

1.2. Introduction

Many vertebrates display seasonal reproduction, during which males present increased androgen plasma levels (Norris and Lopez 2010). Elevated androgen plasma levels are necessary for development and maintenance of male primary and secondary sexual characteristics (Goldey and van Anders 2015). However, several studies point to potential immunosuppression during the reproductive period, which has been attributed to elevated androgen plasma levels (Folstad and Karter 1992; Cézilly et al. 2002; Opplinger et al. 2004; Cox and JohnAlder 2007) or to an energetic trade-off emerging from investment in reproduction and immunocompetence (Zuk and Stoehr 2002; Martin et al. 2008). The empirical support for the immunocompetence handicap hypothesis has been controversial in the literature (Roberts et al. 2004). Higher levels of testosterone increase parasite load (Nordling et al. 1998; Mills et al. 2010); reduce lymphoid organs and tissues (Bilbo et al. 2002; Nelson et al. 2002; Nelson 2004; Buehler 2008); and decrease bacterial-killing capacity of the blood (Pap et al. 2010), humoral immune response (Casto et al. 2001), and cell-mediated immune response (Peter et al. 2000; Belliure et al. 2004) in experimental males and in the wild. Alternatively, several studies demonstrate that increased levels of testosterone enhance humoral and cell-mediated responses when males are immunologically challenged (Evans et al. 2000; Peters 2000; Roberts et al. 2007; Pap et al. 2010), especially in males characterized by higher energetic conditions (Ruiz et al. 2010; Husak et al. 2016). Energy constraint is an important factor modulating possible tradeoffs between immunocompetence and reproductive investment (French et al. 2007 b). Additionally, previous studies demonstrated a modulatory effect on the inflammatory response in humans and rats by androgens and their receptors, which increase the release of proinflammatory cytokines including TNF-a, IL-6, IL-10, the numbers of inflammatory monocytes, chemotaxis of monocytes, and infiltration of macrophages into wound areas (Angele et al. 1999; Ashcroft and Mills 2002; Lai et al. 2009).

Glucocorticoids plasma levels are also elevated during reproductive season in several vertebrates (Crossin et al., 2015). Elevated glucocorticoid secretion during this period is probably associated with the mobilization of energy stores to cope with the energy costs of reproduction (Buchanan, 2000; Sapolsky et al., 2000). Changes in plasma glucocorticoids may exert a complex immunomodulatory effect (Dhabhar, 2009;

Assis et al., 2015; Thomas and Woodley, 2015). While acutely elevated glucocorticoid plasma levels frequently enhance immunity, increasing cell-mediated immunity (Dhabhar et al. 1996, 1999) and traffi cking of leukocytes (Dhabhar et al. 1996; Bowers et al. 2008), chronically elevated levels usually result in immunosuppression (Dhabhar and McEwen 1999; Dhabhar 2000; Sapolsky et al. 2000; Dhabhar 2009). The dual effects of glucocorticoids on the immune response depend on the concentration of circulating hormones (Dhabhar 2009), the temporal pattern of glucocorticoid induction (French et al. 2010), and the type of receptors (mineralocorticoid/glucocorticoid) activated (Breuner and Orchinik 2001; Wada et al. 2006). Additionally, the corticosteroneinduced immunosuppression tends to be more pronounced in individuals in the poorest energetic conditions (French et al. 2007 a, 2007 c, 2010).

Vertebrates from arid and semiarid environments live under a drastic seasonal regime that may accentuate the variation of physiological and behavioral aspects throughout the year (Withers and Guppy 1996; Tieleman et al. 2002; Brown et al. 2011; Kordonowy et al. 2016). Like other species from arid environments, anurans from the Caatinga, a Brazilian semiarid zone, depend on unpredictable rain events occurring during the short rain season to reproduce (Abe 1995; Rodrigues 2003). Male anurans from the Caatinga show elevated plasma levels of androgens throughout the reproductive period (January–May), while corticosterone (CORT) plasma levels increase during rain events only when animals are calling (Leary et al. 2008; Madelaire and Gomes 2016). During the dry period (August–November), plasma levels of both androgens and CORT are low. Moreover, lipid reserves are lower during breeding events and inversely related to CORT plasma levels, indicating that interrenal steroids are involved in mobilization of energy reserves to sustain calling activity (Madelaire and Gomes 2016).

We studied the relationship between the seasonal changesin endocrine, bioenergetics, and immune parameters across different phases of the annual life-history cycle of three anuran species (*Rhinella jimi* [Stevaux, 2002], *Rhinella granulosa* [Spix, 1824], and *Pleurodema diplolister* [Peters, 1870]) from a drastically seasonal environment, the Brazilian semiarid Caatinga. Two of these species (*R. jimi* and *R. granulosa*) continue to forage during the drought in the studied locality, while *P. diplolister* aestivates near underground water sources. We expected to find a strong

seasonal variation of the studied endocrine, immunological, and energetic parameters, especially in the aestivating species. These anurans are explosive breeders and show increased plasma levels of testosterone and dihydrotestosterone (T-DHT) during the reproductive period (Madelaire and Gomes 2016); therefore, we expect that higher T-DHT plasma levels and body condition will be associated with activation of the immune system and stimulation of the immune response in the three studied species. During the breeding event, when animals are calling and CORT levels are higher, we anticipate that the immune system will be further stimulated, associated with the higher numbers of circulating immune cells compared to the reproductive period. To test these hypotheses, we assessed steroid plasma levels (CORT and T-DHT), morphometric variables associated with energetic body condition and immune system (mass of spleen, kidneys, fat bodies, stomach content, and body condition index), leukocyte profile (total and differential number of circulating leukocytes), parasite load (cavitary helminths), and response to an immunological challenge (edema response to phytohemaglutinin [PHA] injection). These data were collected in the three species from the same region during (A) the reproductive season, (B) the active breeding period when males are calling, and (C) the dry period.

1.3. Materials and Methods

1.3.1. Field Collection and Animal Maintenance

Fieldwork was conducted at Fazenda São Miguel, a private area located in the Municipality of Angicos, in the State of Rio Grande do Norte, Brazil (5730′43 0 0S, 36736′18 0 0W). This area is within the Brazilian semiarid Caatinga and is characterized by high temperatures and seasonally concentrated precipitation. The annual average temperature from 1950 to 2000 is 26.6°C (http://worldclim.org). January is the hottest month, with an average temperature of 27.47C (minimum: 22.8°C; maximum: 32.07C), and July is the coldest month, with an average temperature of 24.3°C (minimum: 20.3°C; maximum: 28.3°C; http://pt.climate-data.org/location/312354/). The rainy season occurs from January to May (96.4 mm of precipitation per month), while the dry season occurs from August to November (2.5 mm of precipitation per month). Anurans in this region reproduce during the rainy season, when heavy rain occurs (150 mm or higher precipitation; C.B. Madelaire, personal observation; Arzabe 1999). However, there are years when it does not rain in

the Caatinga (Arzabe 1999), and anurans do not show reproductive activity in these years (C.B. Madelaire, personal observation). Anurans from this locality have adopted different behavioral strategies during the dry season. While *Pleurodema diplolister* aestivates burrowed in the sandy soil above the beds of temporary rivers (Carvalho et al. 2010), *Rhinella granulosa* and *Rhinella jimi* remain active, foraging close to artificial water sources and humid areas (Madelaire and Gomes 2016). We monitored plasma levels of androgens and CORT in R. jimi, *R. granulosa*, and *P. diplolister* during the reproductive and dry periods annually from 2011 to 2015. Despite some interannual variability, the plasma levels of androgens and CORT were consistently low throughout the dry period and consistently elevated throughout the reproductive period in all studied species.

The animals were collected during three different periods in 2011: (A) during the reproductive season but when there was little or no precipitation and no breeding activity (January 11–21), (B) during a breeding event when it was raining and animals were calling (January 22–24), and (C) during the dry season (October 6–17). Males of *R. granulosa* (N = 59) and R. jimi (N = 42) were collected during the three periods described above. Males of *P. diplolister* (N = 38) were collected only during period B and C. All individuals collected for this study also generated data for published studies on reproductive endocrinology (Madelaire and Gomes 2016) and parasite ecology and taxonomy (Madelaire et al., forthcoming). Blood samples were collected by cardiac puncture using heparinized 1-mL syringes with 26-gauge, 1/2-inch needles for *R. granulosa* and *R. jimi*. Given their small body size, individuals of *P. diplolister* were decapitated, and heparinized microtubes were used to collect blood samples. Blood samples were collected within 3 min of capture to avoid interference of manipulation stress with steroid plasma levels (Romero and Reed 2005). Blood samples were maintained on ice for up to 2h and then divided in three aliquots. Two aliquots were used for total and differential leukocyte count and one for quantification of the steroid plasma levels. To obtain plasma, blood was centrifuged for 5 min at 2,000 g (minicentrifuge for six tubes; MiniStar). Plasma samples were frozen in liquid nitrogen, transferred to the laboratory in the University of São Paulo, and stored at -20°C. Males of *R. jimi* and *R. granulosa* were individually maintained in plastic containers with access to water. On the morning after capture, they were weighed and euthanized with

sodium thiopental solution (25 mg/mL; Thiopenthax). After decapitation, the head and body of *P. diplolister* were individually maintained in aluminum foil and refrigerated at 47°C until the next morning when the bodies were weighed, measured, and submitted to dissection procedures. All animals were weighed to the nearest 0.001 g, and the snout-vent length was measured to the nearest 0.01 mm. The animals were dissected, and stomach content, fat bodies, kidneys, and spleen were weighed to the nearest 0.001 g. All abdominal organs were examined for the presence of endoparasites under stereomicroscope (LM300B; SKU), and the parasites were fixed in warm ethanol, formol, acetic acid, counted, and stored in individual tubes with 70% ethanol for taxonomic analysis (Madelaire et al., forthcoming). Additionally, males from the three studied species were submitted to a PHA immunological challenge, as described below ("PHA Immunological Challenge"). These individuals were collected during the reproductive period from March 5 to 10, 2015 (*R. jimi*, N = 9; *R. granulosa*, N = 10; *P. diplolister*, N = 10), and during the dry period from August 10 to 14, 2015 (*R. jimi*, N = 5; *R. granulosa*, N = 4; *P. diplolister*, N = 2). All the experiments and fieldwork were conducted under approved permission of Comissão de Ética no Uso de Animais do IB (protocol 140/2011) and Ministério do Meio Ambiente, ICMBio, SISBio (license 3747-1).

1.3.2. Hormone Plasma Level Determination

The circulating plasma levels of androgens (T-DHT) and CORT are also published in Madelaire and Gomes (2016) and were quantified using enzyme immunoassay kits (testosterone ELISA Kit 582701, corticosterone ELISA Kit 500655) from Cayman Chemical (Ann Arbor, MI). Assays were carried out in accordance with the manufacturer's instructions. Before the assay, hormones were extracted from plasma samples (range 3– 20 µL) by adding 3 mL of diethyl ether (C4H10O), agitating for 30 s, and centrifuging for 9 min at 47C (1,800 rpm). Samples were then allowed to decant in a 2807C freezer for 7 min, and the liquid phase was poured in a new assay tube (Assis et al. 2015). Samples were run in duplicate, and 18 samples were run in each assay plate. We estimated intra-assay variation to be 5.2% for androgens and 3.7% for CORT. As recommended by the manufacturer's instructions, inter assay variation was estimated using the average of four intermediate values from the standard

curve—8.4% for androgens and 6.1% for CORT. Sensitivity of the assay was 6 pg/mL for testosterone and 30 pg/mL for CORT.

1.3.3. Total and Differential Leukocyte Count

On the morning after the blood collection, 10 mL of blood from each individual was diluted in 190 mL of toluidine blue saline solution (0.01%) and placed in a Neubauer chamber to count the total number of leukocytes. Toluidine blue stains cells, facilitating differentiation of leukocytes and erythrocytes (Campbell 2015). The number of total leukocytes was counted under a light microscope (LM2100BL, Lumen; #40 objective). To obtain a leukocyte profile (differential count), we performed a blood smear on a glass slide for each individual. The glass slides were fixed using 100% methanol and then stained with Giemsa solution (10%). We counted and classified 100 leukocytes per smear under an optical microscope (#100 objective; oil immersion; Nikon E200, 104c). The classification of leukocytes (basophils, eosinophils, monocytes, neutrophils, and lymphocytes) was based on the anuran leukocyte morphology (Campbell 2015). The neutrophil/lymphocyte ratio (N/L ratio) was calculated for each blood smear.

1.3.4. PHA Immunological Challenge

To assess cell-mediated innate immunity, we individually maintained animals in plastic containers with access to water for 24 h. Hind limbs were measured using a thickness gauge (Digimess; 0.01 mm precision), and the hind fleshy base of the right foot was then injected with 10 mL of a 20 mg/mL solution of PHA (Sigma L8754) in saline using 10-mL glass syringes and 30-gauge, 1/2-inch needles. As a control, the hind fleshy base of the left foot of the same individual was injected with 10 mL of saline. The thickness of both feet was measured 12 and 24 h after the injections. Each measurement was repeated at least three times at each measurement event, and the mean of these values was used for the calculations. The proportional swelling in response to PHA was calculated by dividing the maximum swelling valueafter injection by the first measure minus 1 ((Sfinal/Sinitial) - 1).

1.3.5. Statistical Analysis

Descriptive statistics were conducted for all variables, and data were then log10 transformed to meet the assumptions of data normality. The residuals of a standard least

squares linear regression using snout-vent length as the independent variable and body mass as the dependent variable were used to calculate the body condition index. The morphological variables spleen, kidneys, and fat bodies were corrected for body mass using a standard least-square linear regression. Body mass was used as the independent variable and morphological features as the dependent variable. The residuals of these regressions were used in later analysis.

To integrate the large number of studied variables and increase the power of model explanation, we performed two Varimax normalized principal component analyses (PCA) for each species. The first PCA was performed for morphological variables including body condition index; stomach content mass; and residuals of fat bodies, kidneys, and spleen masses. Since the spleens from *P. diplolister* were too small for our scale, these data were not included in the morphological PCA for this species. The second PCA was performed for leukocyte variables: total number of circulating leukocytes; number of circulating basophils, eosinophils, and monocytes; and the N/L ratio. The principal components with eigenvalues > 1.00 were considered for interpretation. Subsequently, we calculated results from each component extracted by regression and saved these as compound variables. Using general linear models, we tested whether morphological and leukocyte variables could be explained by period (reproductive season [a], breeding event [b], and dry season [c]), CORT and androgen plasma levels (T-DHT; data from Madelaire and Gomes 2016), or parasite load (data from Madelaire et al., forthcoming), or the additive effects of these explanatory variables. We also tested for an interaction between hormone plasma levels and parasite load (Table 1). The general linear models for T-DHT and CORT were run separately since adding an additional variable to the models decreases the valid N and substantially increases the number of tested models. Since these models have different numbers of parameters, we calculated the second order Akaike information criterion (AICc; Akaike 1974), which penalizes the likelihood of a given model as a function of the number of parameters and corrects for low sample sizes. The AICc value (dAICc < 2.0) and the Akaike weight were used to determine which models had the most support. Akaike weight describes the relative strength of the evidence in support of a particular model. The best model corresponds to the one with the lowest AICc value, providing a good fit to the data with the fewest parameters (Burnham and Anderson 2002). We also

considered the Akaike weight in the power of explanation between competing models with dAICc < 2.0. Additionally, when only null models were selected, they were not included in the table results. To verify whether the PHA immunological challenge worked, we performed a t-test comparing the proportional edema from the foot injected with PHA to the proportional edema from the foot injected with saline. Additionally, a t-test was used to investigate differences in foot thickness in response to PHA and saline injections during reproductive and dry periods for each species. PCA and t-tests were carried out using SPSS 5.0, and the general linear models were run in R, version 2.10.0 (R Development Core Team).

1.4. Results

Descriptive analyses of all variables are presented by season and species (Table A1).

1.4.1. Principal Component Analyses

The PCA for morphological variables of *Rhinella jimi* resulted in three components with eigenvalues > 1 (Table A2). The first component explained 28.8% of the variance and was related to the variation of stomach content mass and body condition index in one direction and spleen mass in the opposite direction. The second component explained 25.5% of the variance and was related to kidney mass. The third component explained 20.6% of the variance and was related to the mass of the fat bodies. The PCA for the morphological variables of *Rhinella granulosa* resulted in two components with eigenvalues > 1 (Table A3). The first component explained 31.3% of the variance and was related to the variation in the mass of the fat bodies and spleen in the same direction. The second component explained 26.4% of the variance and consisted of stomach content, kidney mass, and body condition index variation in the same direction. The PCA for the morphological variables from *Pleurodema diplolister* resulted in three components with eigenvalues > 1 (Table A2). The first component explained 34.8% of the variance and evidenced a negative relation between the mass of the fat bodies and stomach content mass. The second component explained 27.2% of the variance and consisted of body condition index. The third component explained 25.6% of the variance and was related to kidney mass.

The PCA for leukocyte variables of *R. jimi* resulted in three components with eigenvalues > 1 (Table A3). The first component explained 26.8% of the variance and consisted of a positive relationship between the numbers of basophils and monocytes. The second component explained 22.8% of the variance and was related to the variation of the N/L ratio and total leukocyte number in the same direction. The third component explained 21.1% of the variance and was related to the number of eosinophils. PCA for leukocyte variables of *R. granulosa* resulted in two components with eigenvalues > 1 (Table 3). Component 1 explained 36.3% of the variance and it was related to the variation of total leukocyte number, monocyte number and N/L ratio in the same direction. Component 2 explained 23.0 % of the variance, and consisted of basophil numbers being negatively correlated to eosinophil numbers. The PCA for leukocyte variables of *P. diplolister* resulted in two components with eigenvalues > 1 (Table 3). Component 1 explained 49.9% of the variance, and was related to the variation of total leukocyte numbers, monocyte numbers and N/L ratio in the same direction and eosinophil numbers in the opposite direction. Component 2 explained 24.4 % of the variance and was related to basophil numbers.

1.4.2. Model selection

Morphological components

For *R. jimi*, animals with larger kidney mass (second morphological component) were characterized by higher T-DHT and CORT plasma levels (Fig. 1A, 1B; Table 2). High values for the second morphological component, along with high T-DHT and CORT plasma levels, occur especially when animals are calling (Fig. 1A, 1B; Table 2). Additionally, males that displayed lower mass of the fat bodies (third morphological component) were characterized by higher CORT plasma levels (Fig. 1C; Table 2). Variation in spleen, stomach content mass, and body index in *R. jimi* (first morphological component) was not explained by any of the studied explanatory variables (period, parasite load, CORT, or T-DHT).

For *R. granulosa*, individuals with larger kidneys, stomach content mass, and body index (second morphological component) displayed higher T-DHT plasma levels (Fig. 2A; Table 2). Values for the second morphological component and T-DHT plasma levels were especially high when males were calling (Fig. 2A; Table 2). Additionally,

one of the selected models indicates a relationship between kidney mass, stomach content mass, body index (second morphological component), and parasite load (Table 2), but the respective plot shows only a relationship characterized by a flat line (Fig. 2C). Individuals with smaller spleens and a lower mass of the fat bodies (first morphological component) showed higher CORT plasma levels (Fig. 2B; Table 2). During the dry period, individuals of *P. diplolister* were aestivating and showed a higher mass of the fat bodies, lower stomach content mass (first morphological component), lower body condition index (second morphological component), as well as heavier parasite load (Fig. 3A, 3B; Table 2). Additionally, individuals of *P. diplolister* that displayed larger kidney mass (third morphological component) showed higher parasite load and lower plasma levels of T-DHT (Fig. 3C; Table 2). CORT plasma levels did not explain the variation of any morphological components of *P. diplolister*.

Leukocyte Components

During the breeding period, when males of *R. jimi* were calling, individuals displayed a high number of basophils and monocytes in their bloodstream (first leukocyte component) along with high levels of CORT in the plasma (Fig. 1D; Table 3). Also during the breeding period, *R. jimi* males displayed a higher N/L ratio and total numbers of leukocytes (second leukocyte component) along with high plasma levels of T-DHT (Fig. 1E; Table 3). Furthermore, the *R. jimi* males that displayed a higher number of eosinophils in their bloodstream (third leukocyte component) also had a heavier parasite load and higher plasma levels of T-DHT (Fig. 1F; Table 3) and CORT (Fig. 1G; Table 3).

Males of *R. granulosa* with higher total numbers of leukocytes and monocytes and a higher N/L ratio in the bloodstream (first leukocyte component) also showed higher plasma T-DHT level s (Fig. 2D; Table 3). During the breeding period, when males were calling, individuals presented higher total leukocyte and monocyte counts, higher N/L ratio (first leukocyte component), and higher plasma levels of CORT (Fig. 2E; Table 3). The general linear models showed that number of circulating basophils and eosinophils of *R. granulosa* (second leukocyte component) is explained by period, T-DHT plasma levels, and parasite load (Table 3), but the respective plots did not show a clear relation between these variables (Fig. 2F, 2G). During the breeding event, when

males of *P. diplolister* were calling, individuals displayed a higher number of circulating leukocytes and monocytes and N/L ratio and lower eosinophil counts (first leukocyte component) along with high T-DHT plasma levels (Fig. 3D; Table 3). During the breeding period, *P. diplolister* males with a higher number of circulating basophils (second leukocyte component) also showed higher T-DHT plasma levels (Fig. 3E; Table 3).

1.4.3. PHA Immunological Challenge

During the reproductive period, the PHA injection caused a significant swelling compared to saline for all three species (*R. jimi*: t = 6.734, df = 16, P = 0.0001; *R. granulosa*: t = 3.401, df = 18, P = 0.003; *P. diplolister*: t = 2.201, df = 18, P = 0.041; Fig. 4). During the dry period, only the nonaestivating species presented edema in response to PHA injection (*R. jimi*: t = 3.194, df = 8, P = 0.013; *R. granulosa*: t = 2.413 df = 6, P = 0.052; *P. diplolister*: t = 0.737, df = 2, P = 0.538; Fig. 4). Additionally, *R. jimi* showed a stronger response to the PHA challenge during the reproductive period compared to the dry period (*R. jimi*: t = 2.193, df = 12, P = 0.049; *R. granulosa*: t = 1.587, df = 12, P = 0.138; *P. diplolister*: t = 1.189, df = 10, P =0.262; Fig. 4).

1.5. Discussion

1.5.1. Seasonal Variation in Steroid Plasma Levels, Circulating Leukocytes, and Inflammatory Response

As expected, anurans from the Brazilian semiarid Caatinga showed a seasonal pattern of variation in the plasma levels of steroids and immune parameters. During the reproductive season, and particularly when engaged in calling activity, males from three different species showed higher gonadal and interrenal steroid plasma levels along with indexes of higher immune function. Breeding males had a higher number of circulating leukocytes and a stronger response to immunological challenge, indicating a stronger cell-mediated immunity. These data do not corroborate previous studies emphasizing an androgen-mediated immunosuppressive effects associated to reproductive season (Peters, 2000; Roberts et al. 2004; Deviche and Cortez 2005; Foo et al. 2016) but point instead to an increased immune responsiveness during periods of breeding activity (Ruiz et al. 2010; Desprat et al. 2015). Moreover, the ability to respond to the PHA challenge decreases during the dry season, when steroid plasma

levels and number of circulating leukocytes are low. Particularly, the response to the PHA challenge was absent in *Pleurodema diplolister* during aestivation, indicating that the inhibition of inflammatory response during the dry season might be associated with differences in access to alimentary resources during this period (French et al. 2007a; French and Moore 2008; Ruiz et al. 2010).

The *Rhinella* species showed larger kidneys, and all species displayed a smaller mass of the fat bodies during the reproductive period and breeding event, when androgen and CORT plasma levels were high (Figs. 1A, 1B, 2A, 3C). Increased kidney mass during the reproductive period might be related to an enhanced production of lymphocytes, since this organ has lymphopoietic activity (Hansen and Zapata 1998; Flajnik and Du Paskier 2003; Rollins-Smith and Woodhams 2011). An increased consumption of proteins could also contribute to the larger kidney size during this period (Hammond and Janes 1998). Further studies are necessary to test these hypotheses. Lower mass of the fat bodies associated with higher CORT levels in *Rhinella* species (Figs. 1C, 2b) is evidence for an energy recruiting role by this steroid in order to sustain reproductive activity (Leary et al. 2008).

Males of *Rhinella granulosa* with higher body condition and larger stomach content mass had high plasma levels of T-DHT (Fig. 2A). These results corroborate the positive association between body condition and androgen plasma levels previously observed for many other ectothermic vertebrates (Aubret et al. 2002; Bonnet et al. 2002; King and Bowden 2013; Leary et al. 2015; Lind and Beaupre 2015). This indicates that males characterized by higher body condition might also attain greater reproductive success. A positive association between body condition and size with mating success has been previously observed in red-sided garter snakes (*Thamnophis sirtalis*; Shine et al. 2000). However, the implications of body condition and testosterone plasma levels for mating success remain to be tested in toads from the Caatinga. Unlike the two studied *Rhinella* species, *P. diplolister* males collected during aestivation had no stomach content mass, lower body condition index, and higher mass of the fat bodies (Fig. 3A, 3B) when compared to those collected during the breeding period. These results indicate that these animals do not feed during the dry season but use the fat reserves accumulated during the previous rain season to survive the aestivation period

and emerge for the following breeding event (Carvalho et al. 2010; Madelaire and Gomes 2016).

We observed higher total number of circulating leukocytes and N/L ratio during the reproductive period for all studied anuran species. Frequently, increased total number of circulating leukocytes during the reproductive period occurred with increased specific leukocyte counts, specifically monocytes in *R. granulosa* and *P. diplolister* and basophils in *P. diplolister*. As predicted, all leukocyte variables were positively associated with T-DHT plasma levels in the studied anurans, as well as with CORT in toads, across periods. Earlier studies in vertebrates support the immunostimulatory role of elevated androgen and CORT levels. Thus, Ross et al. (2009) found that heterophil/lymphocyte ratio was positively correlated with testosterone plasma levels in the peacock *Pavo cristatus*. In rats, acute stress induces increased levels of CORT, and experimental treatment with CORT stimulates the inflammatory response, likely due to the glucocorticoid-induced activation of the lymph nodes and leukocyte migration to the area of inflammation (Dhabhar et al. 1996). Other studies in vertebrates support the role of CORT in leukocyte distribution during an immune challenge (Dhabhar et al. 1996; Sapolsky et al. 2000; Goessling et al. 2015).

Elevated numbers of circulating leucocytes may have implications for the immune response and explain a stronger response to PHA challenge in anurans, as previously shown for other vertebrate species. Thus, Zhang and Zhao (2015) demonstrated that the proportion of neutrophils in the bloodstream is positively correlated to PHA swelling and serum bacterial capacity in wild rodents (*Cricetulus barabensis*). Additionally, Bílková et al. (2015) found a positive correlation between circulating number of basophils and lymphocytes within the cellular infiltrate of a PHA challenge in zebra finch males. In anurans, response to acute stressors and treatment with corticosterone also promote increased neutrophil to lymphocyte ratios in the blood stream (Assis et al. 2015; Falso et al. 2015). Therefore, the increased number of circulating leukocytes during the breeding period of anurans from the Caatinga might be mediated by CORT and androgen plasma levels, and related to higher immune responsiveness.

As we predicted, *Rhinella jimi* and *P. diplolister* displayed increased swelling in response to the PHA challenge during the reproductive period, when T-DHT and CORT plasma levels were higher. Our results are in accordance with a recent study showing that transdermal application of testosterone increases the PHA response in the tree frog Hyla arborea (Desprat et al. 2015). In fact, previous studies have demonstrated that androgens exert a proinflammatory effect by increasing interleukins and the infi ltr ation of monocytes into wound areas (Angele et al. 1999; Ashcroft and Mills 2002; Lai et al. 2009).

The absence of a response to the PHA challenge in *P. diplolister* during aestivation, along with a lower number of circulating leukocytes, indicates immunosuppression during the metabolically suppressed aestivating state. These results are similar to those from other studies in seasonally aestivating or hibernating tetrapods, in varying ecological contexts. Hibernation, for example, was associated with a lower circulating number of leukocytes in the wild rodent *Glis glis* (Havenstein et al. 2016), prehibernation was associated with reduced bacterialkilling capacity in the turtle *Chrysemys picta* (Refsnider et al. 2015), and hibernating frogs showed lower immunoglobulin titers (Pratihar and Kundu 2010) and lower serum antibody titers (Cooperetal.1992). Seasonal variation inimmuneresponse could be associated with different ecological dynamics (Nelson et al.2002; Mølleretal.2003), including variationinabundance/shortage of energetic resources (French et al. 2007a; French and Moore 2008). Diet restriction can affect individuals by decreasing growth rates, reproductive potential, and immune response (Stearns 1992; Demas et al. 2012). Accordingly, food supplementation enhances immune responses in tetrapods (Buchanan et al. 2003; Roberts et al. 2009; Ruiz et al. 2010; Husak et al. 2016). It is noteworthy that the interspecific variation in the level of immune suppression during the dry period compared to the reproductive season is directly related to the levels of activity of different anurans during drought in the Caatinga. The immunosuppression associated with the dry season is lower in the two species of toads that remain foraging, when compared to the aestivating *P. diplolister*. It is probable that the rainy season in the Caatinga is characterized by higher availability of alimentary resources, allowing anurans to present improved body conditions (Figs. 1A, 2b) and, consequently, higher immune responsiveness (French et al. 2007a; Husak et al. 2016).

Our data suggest that anurans from the Brazilian semiarid region present an increased immune responsiveness during reproductive seasons and breeding activity, when plasma steroid levels are elevated, and do not support the hypothesis of a trade-off between investment in reproduction and immunocompetence (Folstad and Karter 1992). Although many studies have shown immunosuppressive effects of testosterone (Deerenberg et al. 1997; Suzuki et al. 1997; Peters 2000; Casto et al. 2001; Oppliger et al. 2004; Deviche and Cortez 2005; Pap et al. 2010), the effects of androgens on the immune system are controversial (Hasselquist et al. 1999; Saha et al. 2002; Buchanan et al. 2003; Greenman et al. 2005; Roberts et al. 2007; Desprat et al. 2015). Furthermore, acutely elevated plasma levels of CORT, as displayed by these anurans when they are calling, could also enhance the immune response (Dhabhar et al. 1995; Martin et al. 2005; Merril et al. 2014). Investment in a more active immunological system during a period of high energy demand, such as the reproductive season, can actually be adaptive by increasing survival of the individuals during this critical life-history stage (Goldman 2001; Nelson 2004; Martin et al. 2008).

1.5.2. Relationships between Helminth Intensity and Circulating Eosinophils

Seasonal variation of steroid plasma levels and the number of circulating eosinophils were associated with differences in the helminth parasite load, possibly reflecting eosinophil activation during helminth infestation (Cadman and Lawrence 2010). While these physiological variables are positively associated in *R. jimi*, a species that maintains foraging activity during the dry season in the locality studied, the aestivating *P. diplolister* showed a different pattern. In *R. jimi*, eosinophil counts positively correlated with the parasite loads, and a significant albeit less clear association between the eosinophil count and intensity of the helminth infection was found in *R. granulosa*. Despite lower values for most leukocyte parameters during aestivation, eosinophil counts were higher in *P. diplolister* during this period, along with a higher helminth load. These results emphasize that variation in helminth intensity might be more closely related to specific aspects of the immune response, such as eosinophilia, high immunoglobulin E, and mastocytosis (Cadman and Lawrence 2010; Desfuli et al. 2015), rather than seasonal variation in steroid plasma levels, total circulating leukocytes, and the inflammatory response to PHA. The fact that T-DHT was positively related to parasite load in *R. jimi*, as well as our earlier finding that *P.*

diplolister does not have parasites during the reproductive period (Madelaire et al., forthcoming), could also indicate that T-DHT can affect the immune response to helminths in species-specific ways (Ezenwaetal.2012; Fuxjager et al. 2011). Furthermore, higher parasite load and increased numbers of eosinophils in *P. diplolister*, despite low levels of other leukocyte parameters during aestivation, may indicate that parasites impose an energetic demand during this critical period. The energetic cost of immune response to helminths remains to be investigated.

1.6. Conclusions

Our results support a hypothesis of a strong seasonal pattern of covariation among leukocyte parameters, spleen and kidney mass, and steroid (CORT and T-DHT) plasma levels in males of three anuran species from the Brazilian semi-arid Caatingas region, with higher values measured during the reproductive season, particularly during calling activity. Moreover, anurans from the Caatinga show a stronger response to PHA challenge during the reproductive period, indicating an elevated immune responsiveness when steroid plasma levels are high. These results do not corroborate the hypothesis of steroid-mediated immunosuppression during reproduction. On the contrary, anurans from the Caatinga show attenuated immune responsiveness during the drought, when steroid plasma levels are low. The immunosuppression was particul arly evident fo r aestivating *P. diplolister*, indicating that the availability of energetic resources might be an important factor determining seasonal variation in the ability to mount an inflammatory response. Helminth parasite intensity was associated with eosinophil counts, which are higher during aestivation in *P. diplolister*, when steroid plasma levels are low, and during the reproductive season in *R. jimi*, when steroid plasma levels are high. These data emphasize that variation in helminth intensity might be more closely related to specific aspects of the immune response than to general seasonal patterns of variation in steroid plasma levels, total circulating leukocytes and the inflammatory response.

1.7. Acknowledgements

We are grateful to F. Assis, B. Titon Jr., and B. O. Cassettari for their support during the fieldwork and L. Crabtree for English review. The authors declare no competing or financial interests. C.B.M. and F.R.G. conceived the study, designed the experiments, and contributed substantially to interpreting the data. C.B.M. and I.S. collected the data; C.B.M. analyzed the data; C.B.M., F.R.G., and I.S. wrote the manuscript and take full responsibility for the content of the article. This research was supported by the State of São Paulo Science Foundation (FAPESP) through grant 2010/52290-4 led by F.R.G. and grant 2014/50643-8 led by F.R.G. and I.S. and a Master's Fellowship awarded to C.B.M. (2011/03030-2).

1.8. Table

Table 1. Constructed models to test the relation between morphological and leukocyte variables and explanatory variables period, plasma hormone levels, parasite.

TESTED MODELS
Variable ~ null
Variable ~ period
Variable ~ hormone levels
Variable ~ parasite load
Variable ~ period + hormone levels
Variable ~ period + parasite load
Variable ~ hormone levels + parasite load
Variable ~ hormone levels*parasite load
Variable ~ period + hormone levels + parasite load
Variable ~ period + hormone levels *parasite load

Table 2. Selected models for explaining variance in morphological components as a function of period in the life cycle (Reproductive season, Breeding event, and Dry season), parasite load, and plasma levels of androgens and corticosterone. *Rhinella jimi* [T-DHT (N = 21) and corticosterone (N = 18)]; *Rhinella granulosa* [T-DHT (N = 13) and corticosterone (N = 15)]; *Pleurodema diplolister* [T-DHT (N = 14) and corticosterone (N = 6)]. AICc = Akaike's information criterion for small samples; gl = number of parameters; dAICc = difference of AICc between any model and the best model; Weight = weight for each selected model. Par_load = parasite load; M1 = 1st morphological componente; M2 = 2nd morphological componente; M3 = 3rd morphological componente

		Selected model	AICc	gl	dAICc	Weight
R. jimi	T-DHT	M2 ~ T-DHT + period	58.4	5	0.0	0.67
	CORT	M2 ~ CORT + period	58.4	5	0.0	0.67
		M3 ~ null model	56.6	2	0.0	0.36
		M3 ~ CORT	57.8	3	1.1	0.20
		M3 ~ period	58.0	4	1.3	0.19
R. granulosa	T-DHT	M2 ~ T-DHT	40.5	3	0.0	0.29
		M2 ~ null model	40.6	2	0.0	0.29
		M2 ~ period	40.6	4	0.1	0.28
	CORT	M1 ~ CORT	51.0	3	0.0	0.67
		M2 ~ null model	49.0	2	0.0	0.35
		M2 ~ par_load	49.1	3	0.1	0.33
P. diplolister	T-DHT	M1 ~ period	42.1	3	0.0	0.34
		M1 ~ period + par_load	43.1	4	1.0	0.21
		M2 ~ null model	44.5	2	0.0	0.48
		M2 ~ par_load	46.1	3	1.6	0.21
		M3 ~ period + par_load	42.4	4	0.0	0.37
		M3 ~ T-DHT + par_load	43.1	4	0.6	0.27
		M3 ~ null model	44.2	2	1.7	0.15

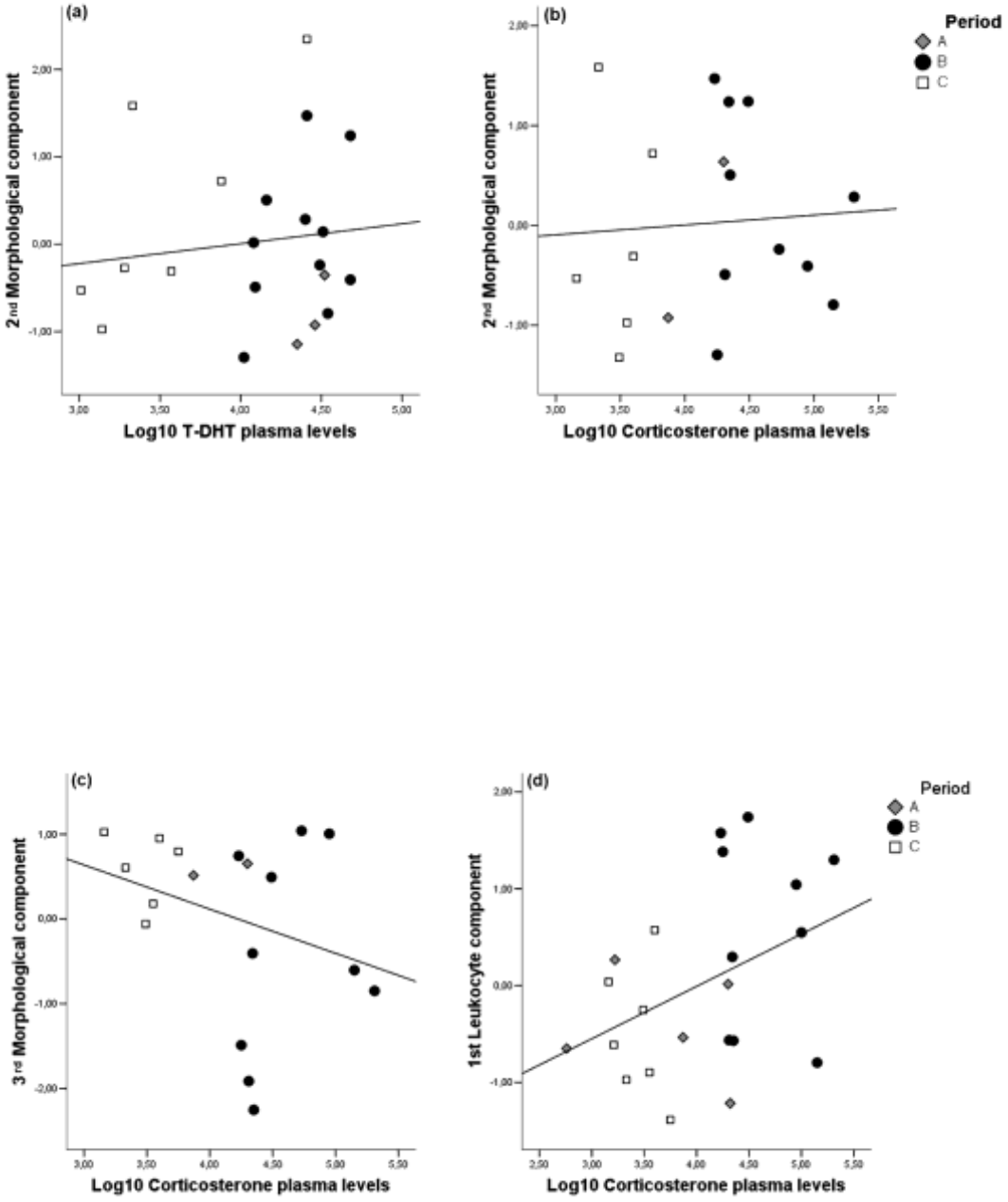
Table 3. Selected models for explaining variance in morphological components as a function of period in the life cycle (Reproductive season, Breeding event, and Dry season), parasite load, and plasma levels of androgens and corticosterone. *Rhinella jimi* [T-DHT (N = 24) and corticosterone (N = 27)]; *Rhinella granulosa* [T-DHT (N = 15) and corticosterone (N = 18)]; *Pleurodema diplolister* [T-DHT (N = 16) and corticosterone (N = 9)].

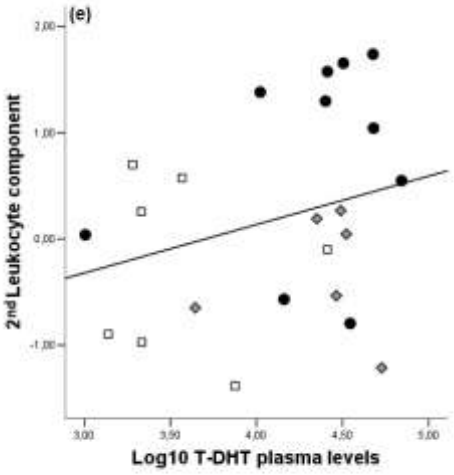
		Selected model	AICc	gl	dAICc	Weight
R. jimi	T-DHT	L1 ~ period	62.5	4	0.0	0.59
		L2 ~ null	71.7	2	0.0	0.25
		L2 ~ period	71.8	4	0.1	0.24
		L2 ~ period + T-DHT	71.9	5	0.2	0.22
		L3 ~ T-DHT	52.7	3	0.0	0.45
		L3 ~ T-DHT + par_load	54.0	4	1.3	0.23
	CORT	L1 ~ period	76.1	4	0.0	0.51
		L1 ~ period + CORT	78.0	5	1.8	0.21
		L2 ~ null model	80.0	2	0.0	0.36
		L2 ~ period	80.8	4	0.9	0.23
		L2 ~ par_load	81.9	3	1.9	0.13
		L3 ~ period + CORT*par_load	57.8	7	0.0	0.31
		L3 ~ CORT*par_load	58.8	5	1.1	0.18
R. granulosa	T-DHT	L1 ~ null model	41.5	2	0.0	0.52
		L1 ~ T-DHT	42.9	3	1.4	0.26
		L2 ~ T-DHT	43.8	4	0.0	0.78
	CORT	L1 ~ CORT	27.0	3	0.0	0.59
		L1 ~ period	28.4	4	1.4	0.28
		L1 ~ null model	26.3	2	0.0	0.57
		L2 ~ period	55.7	4	0.0	0.49
		L2 ~ period + par_load	57.5	5	1.8	0.20
P. diplolister	T-DHT	L1 ~ period	26.7	3	0.0	0.41
		L1 ~ T-DHT	27.5	3	0.8	0.30
		L2 ~ null model	46.7	2	0.0	0.25
		L2 ~ T-DHT	46.8	3	0.0	0.25
		L2 ~ period	47.3	3	0.6	0.19
	CORT	L1 ~ period	27.0	3	0.0	0.59
		L1 ~ period + par_load	28.4	4	1.4	0.28

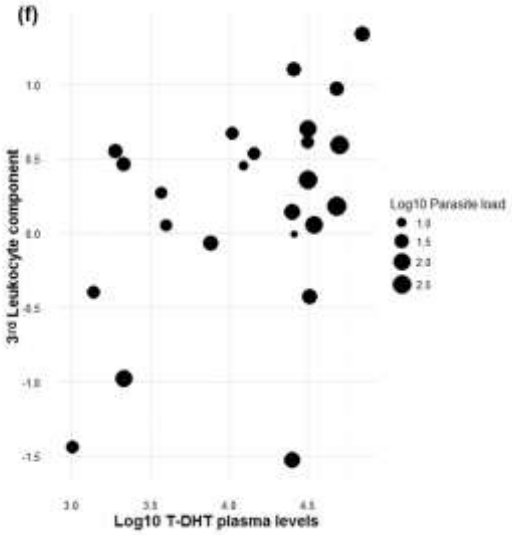
AICc = Akaike's information criterion for small samples; gl = number of parameters; dAICc = difference of AICc between any model and the best model; Weight = weight for each selected

model. Par_load = parasite load; L1 = 1st leukocyte component; L2 = 2nd leukocyte component; L3 = 3rd leukocyte component.

1.9. Figures


(a)
2nd Morphological component
Log10 T-DHT plasma levels
(b)
Period
A
B
C
2nd Morphological component
Log10 Corticosterone plasma levels
(c)
3rd Morphological component
Log10 Corticosterone plasma levels
(d)
Period
A
B
C
1st Leukocyte component
Log10 Corticosterone plasma levels


(e)
2nd Leukocyte component
Log10 T-DHT plasma levels


(f)
3rd Leukocyte component
Log10 T-DHT plasma levels
Log10 Parasite load

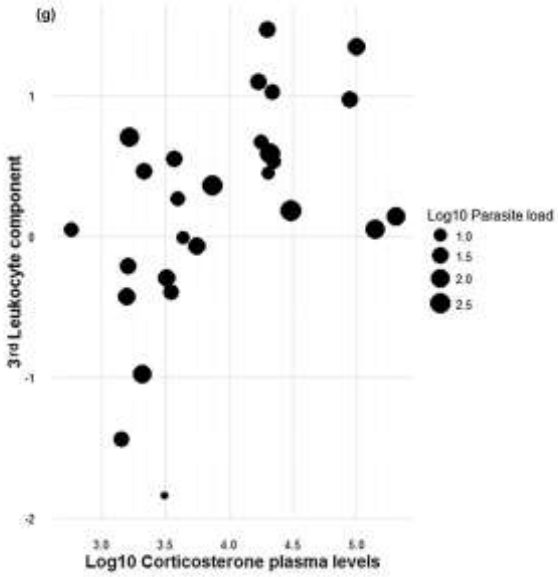
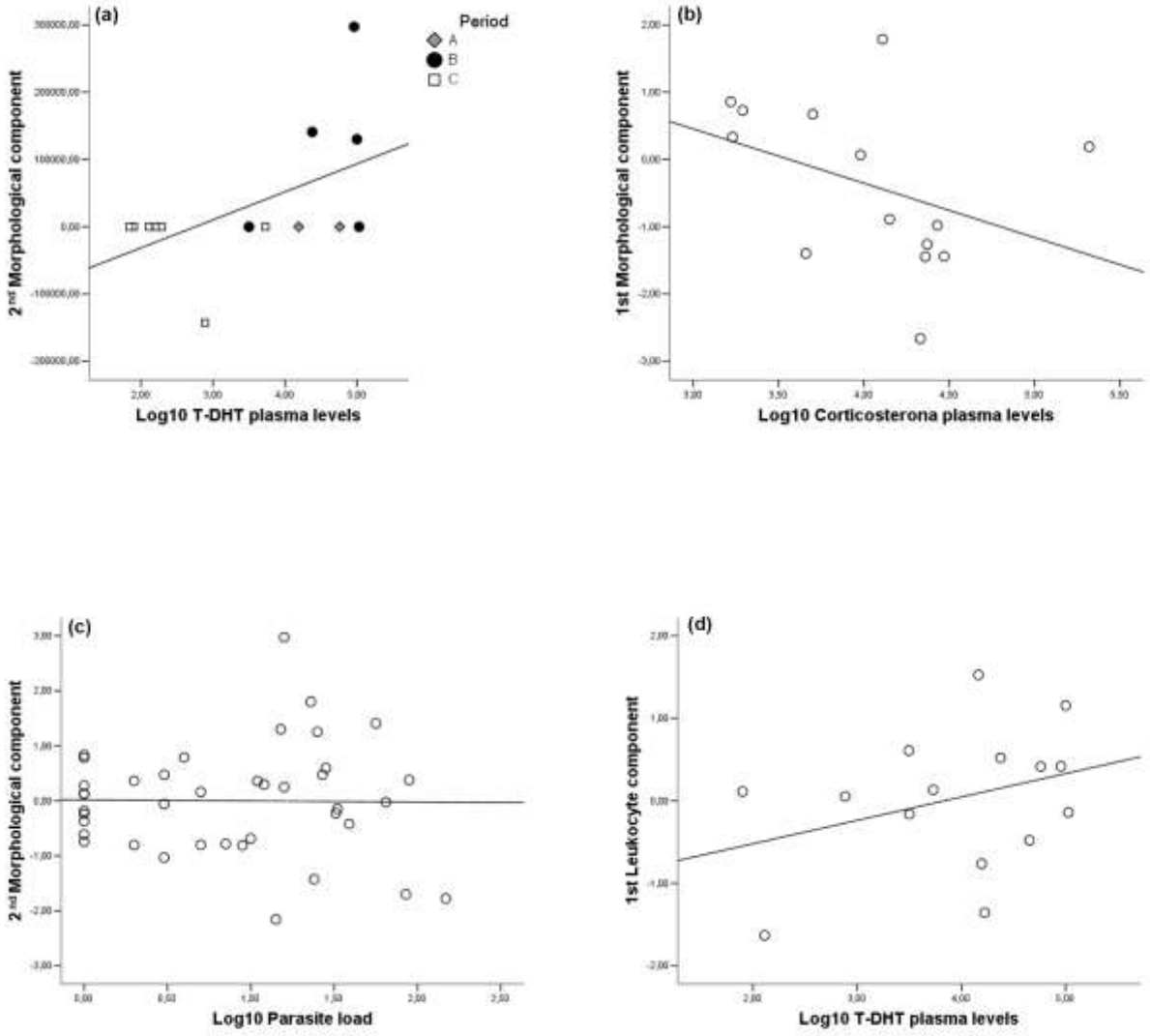
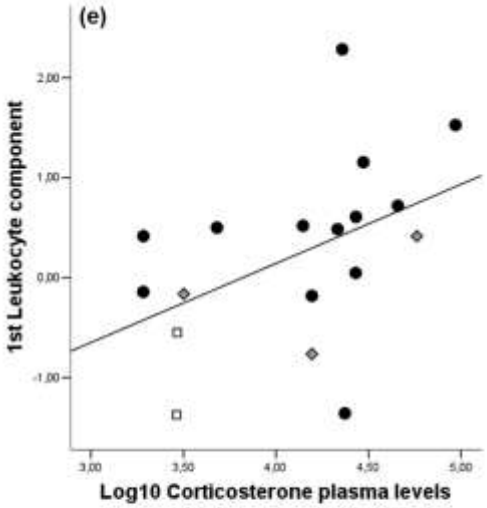
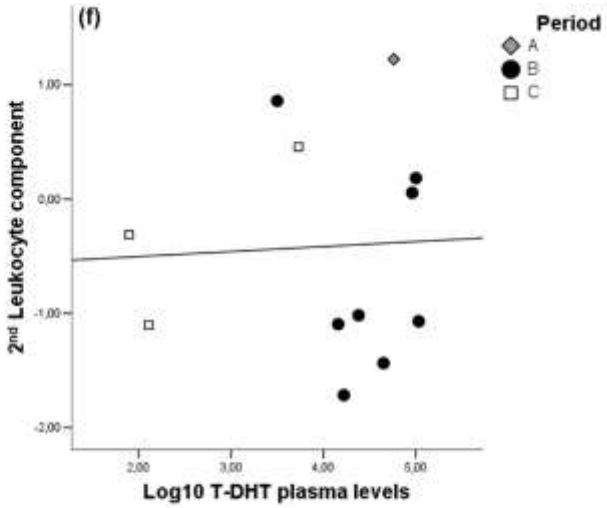

(g)
3rd Leukocyte component
Log10 Corticosterone plasma levels
Log10 Parasite load
1.0
1.5
2.0
2.5

Fig 1. Relation between morphological and leukocyte components, androgens (T-DHT) and corticosterone plasma levels, period and parasite load in *Rhinella jimi*: **(a)** relation between 2nd smorphological component (Kidneys mass) and T-DHT plasma levels; **(b)** relation between 2nd morphological component (Kidneys mass) and corticosterone plasma levels; **(c)** relation between 3rd morphological component (fat bodies mass) and corticosterone plasma levels; **(d)** relation between 1st leukocyte component (basophil and monocyte numbers), CORT plasma levels and period; **(e)** relation between 2nd leukocyte component (N/L rate and total number of leukocytes), T-DHT plasma levels and period; **(f)** relation between 3rd leukocyte component (number of eosinophil), T-DHT plasma levels and parasite load; **(g)** relation between 3rd leukocyte component (number of eosinophils), CORT plasma levels and parasite load. Lines shown indicate best fit.


(a)
Period
A
B
C
2nd Morphological component
Log10 T-DHT plasma levels
(b)
1st Morphological component
Log10 Corticosterona plasma levels
(c)
2nd Morphological component
Log10 Parasite load
(d)
1st Leukocyte component
Log10 T-DHT plasma levels


(e)
1st Leukocyte component
Log10 Corticosterone plasma levels


(f)
Period
A
B
C
2nd Leukocyte component
Log10 T-DHT plasma levels

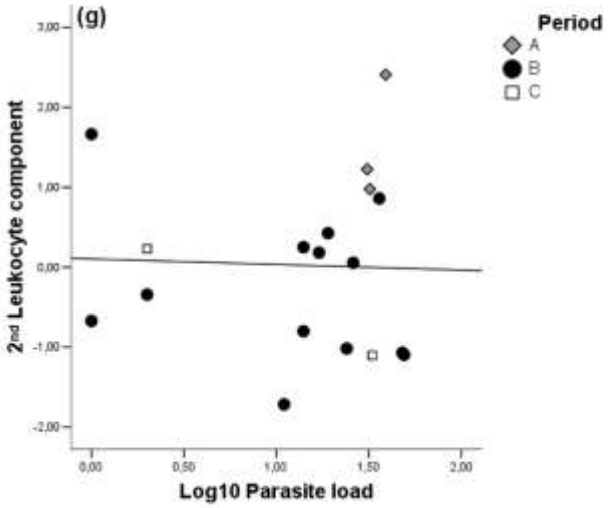
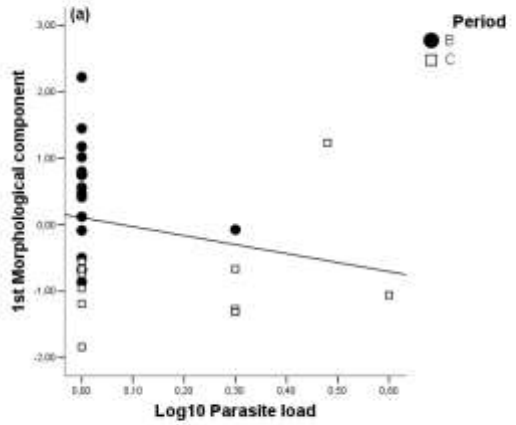
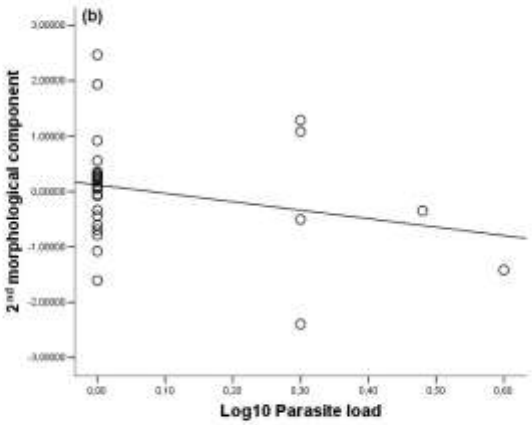
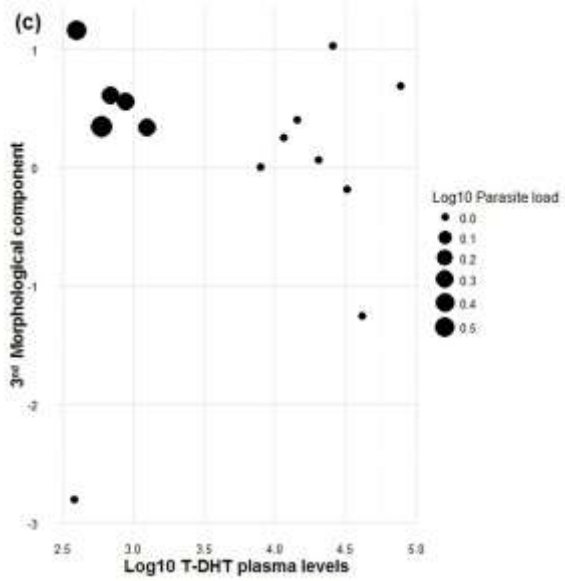
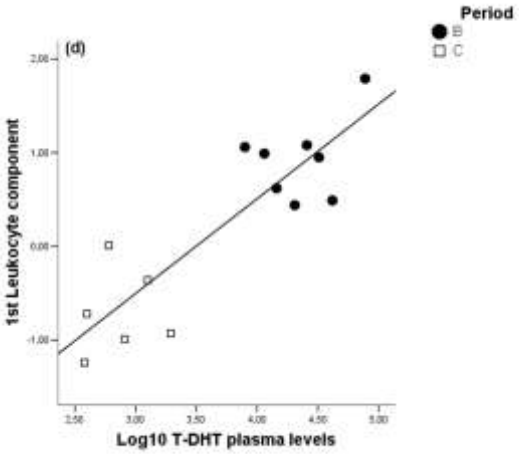

(g)
Period
A
B
C
2nd Leukocyte component
Log10 Parasite load

Fig 2. Relations between morphological and leukocyte components, T-DHT and corticosterone plasma levels, period and parasite load in *Rhinella granulosa*: **(a)** 2nd morphological component (kidneys, stomach content masses and body index), T-DHT plasma levels and period; **(b)** 1st morphological component (spleens and fat bodies masses) and corticosterone plasma levels; **(c)** 2nd morphological component (kidneys, stomach content masses and body index) and parasite load; **(d)** 1st leukocyte component (total number of leukocytes, monocyte number and N/L rate) and T-DHT plasma levels; **(e)** 1st leukocyte component (total number of leukocytes, monocyte number and N/L rate), corticosterone plasma levels and period; **(f)** 2nd leukocyte component (Eosinophil, -Basophil), T-DHT plasma levels and period; **(g)** 2nd leukocyte component (Eosinophil, -Basophil), period and parasite load. Lines shown indicate best fit.


(a)
Period
B
C
1st Morphological component
Log10 Parasite load


(b)
2nd morphological component
Log10 Parasite load


(c)
3rd Morphological component
Log10 T-DHT plasma levels
Log10 Parasite load
0.0
0.1
0.2
0.3
0.4
0.5


(d)
Period
B
C
1st Leukocyte component
Log10 T-DHT plasma levels

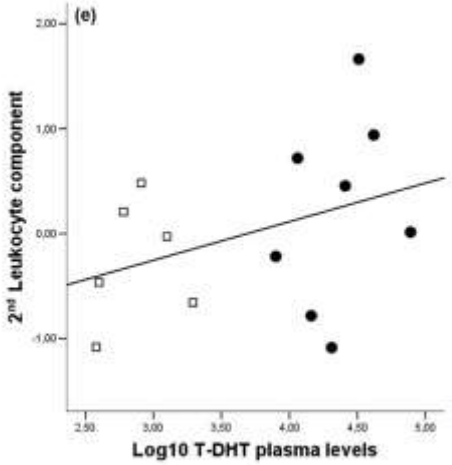

(e)
2nd Leukocyte component
Log10 T-DHT plasma levels

Fig 3. Relation between morphological and leukocyte components, T-DHT and CORT plasma levels, period and parasite load in *Pleurodema diplolister*: **(a)** 1st morphological component (-Fat bodies, stomach content masses) and parasites load; **(b)** 2nd morphological component (body condition index) and parasites load; **(c)** 3rd morphological component (Kidneys masses), T-DHT plasma levels and parasites load; **(d)** 1st leukocyte component (-number of eosinophils, total number of leukocytes, number of monocytes), T-DHT plasma levels and period; **(e)** 2nd leukocyte component (number of basophils), T-DHT plasma levels and period. Lines shown indicate best fit.

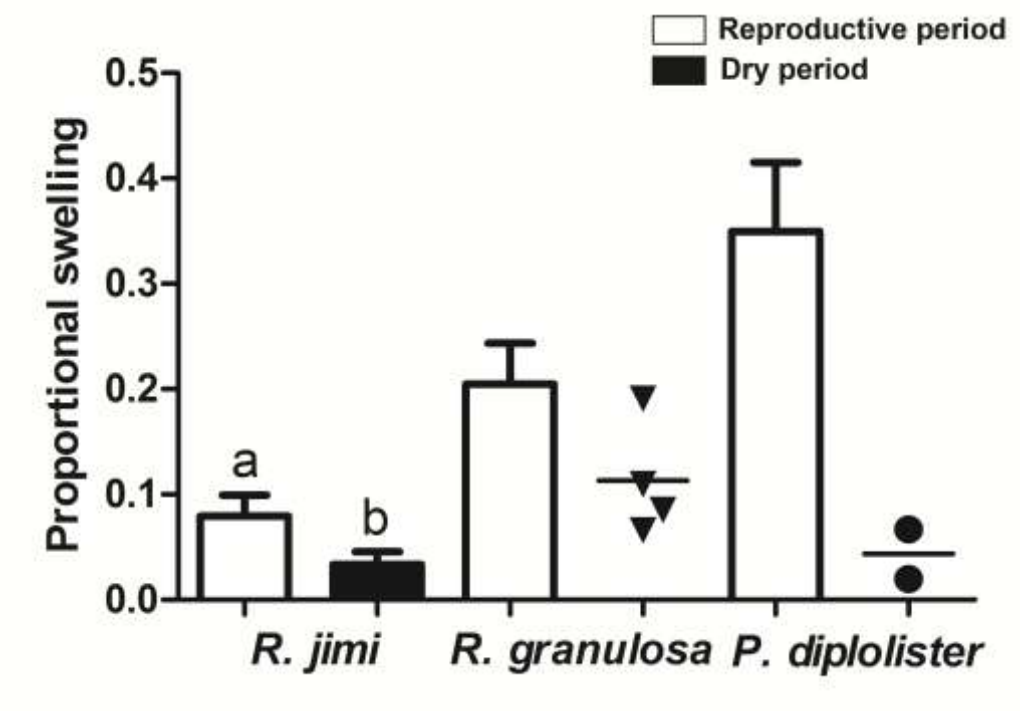


Fig 4. Proportional swelling from PHA challenge during reproductive and dry period for **(a)** *R. jimi*, **(b)** *R. granulosa* and **(c)** *P. diplolister*. Mean + standard error. Different letters indicates proportional swelling differences between periods ($P<0.05$).

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1.11. Appendix

Table A1. Descriptive analysis of morphological, leukocyte variables, number of total parasites and steroid plasma levels for *Rhinella jimi*, *Rhinella granulosa*, *Pleurodema diplolister*, during reproductive period (A), Breeding (B) and dry period (C).

	Valid N	*R. jimi*			Valid N	*R. granulosa*			Valid N	*P. diplolister*	
Period		**Reproductive**	**Breeding**	**Dry**		**Reproductive**	**Breeding**	**Dry**		**Breeding**	**Dry**
Mass (g)	42	335,47 ± 98,39	262,04 ± 54,54	121,16 ± 43,05	59	11.0 ± 4.30	6.93 ± 2.22	5.33 ± 1.36	40	4,07 ± 0,77	2,40 ± 0,72
SVL (mm)	45	154,75 ± 78,07	144,72 ± 12,27	106,71 ± 10,92	59	48,20 ± 6,60	43,85 ± 4,19	40,01 ± 3,22	38	31,02 ± 2,49	26,22 ± 3,64
Stomach content (g)	47	3,05 ± 6,34	0,37 ± 0,67	1,05 ± 1,87	59	0,12 ± 0,11	0,10 ± 0,12	0,08 ± 0,09	38	0,101 ± 0,103	0,006 ± 0,005
Fat bodies (g)	47	15,95 ± 8,39	10,46 ± 11,85	4,36 ± 3,46	59	0,47 ± 0,37	0,114 ± 0,15	0,21 ± 0,08	39	0,048 ± 0,046	0,105 ± 0,062
Kidneys (g)	47	2.07 ± 0.27	2.00 ± 0.72	0.94 ± 0.49	58	0.056 ± 0.018	0.057 ± 0.036	0.031 ± 0.011	38	0.016 ± 0.007	0.006 ± 0.004
Spleen (g)	44	0,12 ± 0,09	0,14 ± 0,07	0,09 ± 0,08	42	0,003 ± 0,002	0,001 ± 0,002	0,003 ± 0,004	-	-	-
Parasites (un)	47	50.00 ± 82.78	82.00 ± 103.26	36.2 ± 42.36	59	39.54 ± 44.67	25.13 ± 25.96	6.01 ± 8.88	41	0.08 ± 0.40	1.07 ± 1.43
Total leukocytes (un/µl)	40	5.34 ± 3.12	2.63 ± 1.32	2.11 ± 6.59	50	2.26 ± 1.08	2.12 ± 1.34	0.73 ± 0.48	28	1.98 ± 1.19	0.58 ± 0.27
N/L ratio	27	0,56 ± 0,24	0,45 ± 0,13	0,49 ± 0,15	26	0,55 ± 0,22	0,63 ± 0,23	0,55 ± 0,14	21	0,58 ± 0,15	0,46 ± 0,13
Eosinophils (un)	27	13,70 ± 6,45	14,90 ± 4,90	6,80 ± 4,20	26	12,80 ± 9,50	4,50 ± 5,38	4,25 ± 4,09	21	0,72 ± 1,27	2,50 ± 2,27
Basophils (un)	27	1,71 ± 1,38	5,00 ± 3,30	2,30 ± 1,80	26	2,00 ± 1,41	8,40 ± 4,17	4,5 ± 2,13	21	2,72 ± 1,61	1,90 ± 1,19
Monocyte (un)	27	2,86 ± 1,86	3,30 ± 2,20	1,40 ± 1,40	26	2,29 ± 1,98	4,80 ± 3,49	2,5 ± 1,41	21	3,54 ± 1,43	0,90 ± 1,28
Testosterone (ng/ml)	26	28.90 ± 15.87	30.28 ± 17.92	5.70 ± 8.36	19	73.29 ± 66.79	14.55 ± 10.48	0.14 ± 0.65	20	26.74 21.98	0.87 0.52
Corticosterone (ng/ml)	23	10.06 ± 9.76	65.25 ± 61.66	3.06 ± 1.50	24	4.47 ± 3.72	52.69 ± 70.09	2.90 ± 0.69	9	25.78 22.93	2.12 0.63

Table A2. Component score result from the principal component analysis (PCA) performed on morphological variables for *Rhinella jimi* (N = 37), *Rhinella granulosa* (N = 39), *Pleurodema diplolister* (N = 29).

	R. jimi			*R. granulosa*		*P. diplolister*		
	Morphological component			Morphological component		Morphological component		
	1	2	3	1	2	1	2	3
Fat bodies mass	0.032	-0.047	**0.957**	**0.780**	0.371	**-0.774**	0.411	0.243
Kidneys mass	0.147	**0.933**	-0.079	-0.381	**0.600**	0.044	-0.113	**0.958**
Spleen mass	**-0.621**	0.504	0.361	**0.797**	0.339	-	-	-
Body condition index	**0.756**	0.213	0.284	-0.201	**0.605**	0.003	**0.939**	-0.129
Stomach content mass	**0.672**	0.029	-0.068	-0.362	**0.578**	**0.842**	0.250	0.265
% Variance explained	28.8	25.5	20.6	31.3	26.4	34.8	27.2	25.6
Total Variance		74.9%			57.7%		87.6%	

Table A3. Component score result from the principal component analysis (PCA) performed on leukocyte variables for *Rhinella jimi* (N = 27), *Rhinella granulosa* (N = 22), *Pleurodema diplolister* (N = 20).
N/L = neutrophil/lymphocyte ratio

	R. jimi			*R. granulosa*		*P. diplolister*	
	Leukocyte component			Leukocyte component		Leukocyte component	
	1	2	3	1	2	1	2
Basophil	**0.832**	-0.196	0.193	0.395	**-0.570**	0.014	**0.950**
Monocyte	**0.764**	0.298	-0,222	**0.766**	0.012	**0.805**	0.295
N/L ratio	0.000	**0.716**	-0.156	**0.761**	-0.238	**0.766**	-0.284
Total leukocyte	0.045	**0.733**	0.303	**0.661**	0.350	**0.898**	0.051
Eosinophil	0.002	0.045	**0.936**	0.223	**0.806**	**-0.654**	0.420
% Variance explained	26.8	22.8	21.1	36.3	23.0	49.9	24.4
Total Variance		**70.7**			**59.3**		**72.8**

2. Muscle maintenance and energy status of anurans living in an extremely seasonal semi-arid environment, the Brazillian Caatinga

RUNNING TITLE: Anuran muscle maintenance and energy status

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Key words: AKT; eIF2α; AMPK, Heat shock proteins, cytochrome c oxidase; aestivation

Summary statement

We studied seasonal variation of key metabolic regulators in the muscles of anurans that lives under drastic environmental changes and differ in their seasonal activity patterns.

Submitted to the Journal of Experimental Biology in 17th August, 2017.

2.1. Abstract

Strongly seasonal environments pose challenges for performance and survival of animals, especially when resource abundance seasonally fluctuates. We investigated the seasonal variation of expression of key metabolic regulators in the muscles of three species of anurans from the Brazilian semi-arid area, Caatinga, which displays drastic seasonal changes in environmental conditions. The three studied anuran species (*Rhinella jimi*, *R. granulosa* and *Pleurodema diplolister*) differ in their seasonal activity patterns. We examined the expression of proteins regulating energy turnover (AMP-activated protein kinase [AMPK] and protein kinase B [AKT]), protein synthesis and homeostasis (total and phosphorylated eukaryotic initiation factor 2α [eIF-2α and p-eIF-2α] and chaperone proteins [HSP 60, 70, and 90]) in muscles related to reproduction and locomotion. Cytochrome c oxidase (COX) activity was also assessed as an index of the muscle aerobic capacity. Our results point to the importance of metabolic regulators mediating survival during the drastic seasonal variation. The toads that remain active during the drought maintain muscles through more energy extensive pathways including elevated protein synthesis, while the estivating species employs energy conservation strategy suppressing protein synthesis, decreasing chaperone expression and increasing expression of AMPK. All three studied species activate cell survival pathways during the drought to prevent muscle atrophy, and maintain the muscle capacity throughout the year, despite the resource limitation. These strategies are important considering the unpredictability of the reproductive event and high demand on muscular activity during the reproductive season.

2.2. Introduction

Strongly seasonal environments characterized by large changes in biotic and abiotic conditions such as temperature and food availability, pose challenges to the organisms that need to adjust their functions to survive and complete their life cycles (Storey and Storey, 2005; Navas and Carvalho, 2010). These physiological adjustments are dependent on biochemical regulators and cell signaling pathways involved in mediating cell survival, ensuring cell and organ integrity and prioritizing the use of energy to meet the (often conflicting) needs of survival, growth and reproduction (Storey and Storey, 2005; Storey and Storey, 2010). In vertebrate ectotherms, the skeletal muscle physiology and performance are directly related to the overall fitness due to the muscles' involvement in courtship, territorial defense, foraging, escape from predators, mating interactions and migration (Navas et al., 2006). Understanding of the regulatory mechanisms that ensure the maintenance and functionality of the skeletal muscles in seasonally fluctuating environments can provide essential insights into the physiological adjustments that support survival and performance (Storey and Storey, 2005). Additionally, trade-off between the muscles related to survival and reproduction might be imposed by energy restriction (Stearns, 1989). Physiological and molecular mechanisms that optimize muscle maintenance and function under conditions of environmental stress and resource limitation are not well understood and require further investigation.

Anurans from the Brazilian semi-arid area, the Caatinga, face drastic seasonal changes in environmental conditions (Abe, 1995; Rodrigues, 2003; Carvalho et al., 2010). In the Caatinga, anurans depend on unpredictable heavy rain events occurring during the short rain season (January to April) to reproduce. Small showers that may occur during the rainy season are not enough to trigger breeding, yet make water and food are more easily available to the anurans. The rest of the year is the dry season, characterized by scarce water and food resources. In some years, there is no rain and these animals had to survive until the next reproductive opportunity. Anurans from the Caatinga show interspecific variation in behavioral strategies to survive the drought. Some species such as *Rhinella jimi* and *R. granulosa* remain active and foraging around humid areas (Madelaire and Gomes, 2016). In contrast, *Pleurodema diplolister* aestivates and does not feed until the first rains start (Carvalho et al., 2010; Madelaire et

al., 2017). These anuran species display adjustments in reproductive (Madelaire and Gomes, 2016) and immune physiology (Madelaire et al., 2017) throughout the year. During the reproductive period, they display elevated plasma levels of androgens and higher immunological profile and response. Corticosterone plasma levels are also high during episodes of calling activity. During the drought, anurans present lower steroid plasma levels and lower immune parameters and performance, with the strongest suppression observed in the aestivating species (Madelaire and Gomes, 2016; Madelaire et al., 2017). These findings indicate that anurans must also regulate energy metabolism and muscle function to deal with the energetic and physiological challenges due the seasonally variable demands of reproduction, resource acquisition and metabolic depression during aestivation.

There is an extensive literature on physiological and biochemical adjustments associated to metabolic depression in environments characterized by different levels of predictability of seasonal climatic variation (Heldmaier and Elvert, 2004; Storey and Storey, 2005; Navas and Carvalho, 2010). However, possible biochemical adjustments displayed by anurans that remain active during the periods of severe environmental stress and resource limitation, such as some species of anuras in the Caatinga, are not well understood. Furthermore, it is unknown whether seasonal adjustments differ between different muscle types in anurans, reflecting preferential investment into the maintenance or reproductive effort. In this study, we investigated the seasonal variation of expression of key metabolic regulators in the muscles of males from three species of anurans from Brazilian semi-arid Caatinga that differ in their seasonal activity patterns: *Rhinella jimi* and *R. granulosa* that remain foraging during drought and *P. diplolister*, which aestivates burrowed during this period. We studied muscles predominantly specialized on reproduction or locomotion, including the trunk and larynx muscles used to sustain calling activity (Pough et al., 1992; Sullivan et al., 1995); the flexor carpi radialis used in amplexus (grasping) behavior (Melichna et al., 1972); and plantaris used in locomotion (Astley and Roberts, 2012).

We investigated the expression of two protein kinases that play a key role in the regulation of energy turnover in the muscle (the AMP-activated protein kinase and protein kinase B) and key proteins involved in the regulation of the protein synthesis and homeostasis (eukaryotic initiation factor 2α and chaperone proteins). The

cytochrome c oxidase (COX) activity was also assessed as an index of the mitochondrial density and aerobic capacity in the tissue (Hardewig et al., 1999). The AMP-activated protein kinase (AMPK) is an energy sensor of the cell responding to the AMP:ATP ratio (Hue and Rider, 2007; Hardie, 2011). During periods that animals need to save energy, AMPK is activated to regulate catabolic *versus* anabolic metabolism increasing ATP synthesis and suppressing ATP consuming pathways (Hue and Rider, 2007; Hardie, 2011). Protein kinase B (AKT) plays a central role in metabolism and cell survival stimulating glucose uptake, glycogen synthesis, lipogenesis and protein synthesis, and regulating the cell cycle and apoptosis (Brazil et al., 2004). Eukaryotic initiation factor 2α regulates protein synthesis and plays a key role in the stress response and suppression of the ATP-consuming protein synthesis under low energetic budget scenarios (Holcik and Sonenberg, 2005). Heat shock proteins are involved in the general stress response acting as molecular chaperones and regulating folding of newly synthesized proteins or those damaged by stressors (Sørensen et al., 2003). Considering their key roles in regulation of the muscle integrity and function, we we expect to see different patterns of activation of the signaling proteins across the season in different species. We also anticipate that the stress-related pathways involved in energy conservation are upregulated and the aerobic capacity (measured as the COX activity) suppressed in anuran muscles during the drought, when the species that remain active (*R. jimi* and *R. granulosa*) are facing food and water shortage, and *P. diplolister* is aestivating.

2.3. Materials and Methods

2.3.1. Field collections

Field work was conducted at Fazenda São Miguel near the city of Angicos, in the State of Rio Grande do Norte, Brazil (5º30'43"S. 36º36'18''W). The area is in the domain of Brazilian Caatinga, and is characterized by high temperatures. January is the hottest month with an average temperature of 27.4ºC (minimum: 22.8ºC, maximum: 32.0ºC), and July is the coldest month, with an average temperature of 24.3ºC (minimum: 20.3ºC, maximum: 28.3ºC) (http://pt.climate-data.org/location/312354/). The annual average temperature from 1950 to 2000 is 26.6 ºC (worldclim.org) and there are two distinct seasons: a rainy season (January to April, 96.4 mm of precipitation/month) and a dry season (August to November, 2.5 mm of

precipitation/month). The months of June, July and December can be consider dry or rainy months depending on extension of the drought in the specific year. In response to the challenges of the dry season, anurans from this locality have adopted different behavioral strategies. *Rhinella granulosa* and *Rhinella jimi* remain active, foraging close to humid areas and artificial water sources (Madelaire et al. 2017), while *Pleurodema diplolister* aestivate borrowed in the sandy soil under the beds of temporary rivers (Carvalho et al., 2010). For this study, animals were collected during two different periods in 2015: (A) during the reproductive season (March, 5–12th, 2015); (B) during the dry season (August, 10–16th, 2015).

During the reproductive period, males of *R. granulosa* (N = 13), *R. jimi* (N = 11) and *P. diplolister* (N = 16) were found by visual inspection. During the dry period, males of *R. granulosa* (N = 6), *R. jimi* (N = 7) were found by visual inspection, and *P. diplolister* (N = 8) was found by excavating the known burrowing sites in the sandy soil. Anurans were collected and individually maintained in plastic containers with access to water, except the individuals of *P. diplolister* collected during the dry period, which were maintained in plastic containers filled with humid sand collected in the location they were found. After two days, animals were weighted (0.01g), euthanized with an injection of sodium thiopental solution (25 mg/ml) (Thiopenthax) and immediately placed in a container maintained in ice cold water until death. To dissect the muscles, individuals were placed on top of frozen gel pack. The dissected muscles were plantaris from the posterior limb, flexor from anterior limbs, larynx and trunk. Muscle samples for the immunoblot analyses were immediately frozen in liquid nitrogen. For COX activity, small muscle biopsies were incubated for 1 to 2 minutes in a cryopreservation medium (10 mM EGTA, 1.3 mM $CaCl_2$, 20 mM imidazole, 20 mM taurine, 49 mM K-MES, 3 mM K_2HPO_4, 9.5 mM $MgCl_2$, 5 mM ATP, 15 mM phosphocreatine, 10 mg/ml fatty acid-free BSA, 20% glycerol, pH 7.1) (Kuznetsov et al., 2003) and frozen in liquid nitrogen. Tissues were stored in liquid nitrogen until their transport to the University of North Carolina at Charlotte (UNC Charlotte), NC, USA on dry ice. At UNC Charlotte the muscle samples were stored at -80ºC until analyses. Fieldwork, maintenance of animals, and transport of samples were conducted under the approved permissions of Comissão de Ética no Uso de Animais do IB (CEUA) (Protocol number: 181/2013) and Ministério do Meio Ambiente, ICMBio, SISBio

(License to collect and transport animals: N°29896-1; Export License number: 15BR017888/DF).

2.3.2. Immunoblotting

Each muscle was weighed and homogenized (1:10 w:v) in ice-cold buffer (100 mM Tris, pH = 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-X, 10% glycerol, 0.1% sodium dodecylsulfate (SDS), 0.5% deoxycholate, 0.5 μg mL^{-1} leupeptin, 0.7 μg mL^{-1} pepstatin, 40 μg mL^{-1} phenylmethylsulfonyl fluoride (PMSF), and 0.5 μg mL^{-1} aprotinin). The homogenate was sonicated three times for 10 s each (output 69 Watts; Sonicator 3000, Misonix Inc.) and centrifuged at 14000 × g for 5 min at 4°C. The protein content of the supernatant was measured using Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA, USA) with the bovine serum albumin (BSA) as a standard. Protein-containing supernatant was mixed 3:1 (v:v) with a solution containing 4 parts of 4x Laemmli buffer and 1 part of 1 M dithiothreitol (DTT), boiled for 5 minutes and frozen in -20ºC until further analysis.

Samples (20-50 μg protein per lane, depending on the antibody) were loaded into 10% polyacrylamide gels and run at 72V for 3 hours at room temperature. After the run, the gels were incubated for 30 min in 96·mmol·l^{-1} glycine, 12·mmol·l^{-1} Tris and 20% methanol (v/v). The proteins were transferred to a nitrocellulose (for HSP60, HSP70 and HSP90) or polyvinylidene difluoride (PVDF) membrane (for all other antibodies) using a Trans-Blot semi-dry cell (Thermo Fisher Scientific Inc., Portsmouth, NH, USA). Membranes were blocked for one hour in 3% non-fat milk in Tris-buffered saline, pH 7.6 with 0.1% Tween 20 (TBST) at room temperature, and incubated overnight at 4ºC with the primary antibodies diluted 1:1000 in 5% BSA in TBST. After washing off the primary antibody with TBST, the membranes were probed with the polyclonal secondary antibodies conjugated with horseradish peroxidase (Jackson Immuno Research, West Grove, PA, USA) diluted 1:1000 with 3% non-fat milk in TBST for one hour at the room temperature. After washing off the secondary antibody, the proteins were detected using enhanced chemiluminescence according to the manufacturer's instructions (Amersham Biosciences, Pierce, Rockford, IL, USA). The signals were captured on X-ray film and relative optical density of protein bands was digitalized with an image analysis software (Gel Doc EZ Imager, Bio-Rad, Hercules, CA, USA) and quantified using Image Lab™ software (Bio-Rad Laboratories Inc.,

Hercules, CA, USA). The loading order of samples in the gels was randomized. The protein loads per lane were identical for all muscle types and for both studied seasons except for p-eIF2α in *R. granulosa* collected during the dry season where the original load of 30 µg per lane did not produce a signal, and 50 µg per lane was then tested. A single sample (used as an internal control) was loaded on each gel and used to standardize the expression of the target proteins in order to minimize the effects of gel-to-gel variation. The following antibodies were used: AKT (AKT rabbit polyclonal IgG; Cell Signalling Technology, cat. #9272, Danvers, MA, USA), total AMP-activated protein kinase (AMPK) (AMPKα, Thr172, Rabbit mAb, Cell Signalling Technology, cat. #2535, Danvers, MA, USA), phospho-EIF- 2α (Ser51) (no. 07-760, Millipore, cat. #07-760, Temecula, CA, USA), EIF-2α (no. AHO1182, Life Technology, Grand Island, NY, USA), HSP 60 (HSP60 (insect) polyclonal antibody, Enzo Life Science, cat.#ADI-SPA-805-D,Farmingdale, NY, USA), HSP 70 (Heat Shock Protein 70 (HSP70) Ab-2, Mouse Monoclonal Antibody, Thermo Fisher Scientific Inc., cat. # MA3-006, Portsmouth, NH, USA), HSP 90 (Anti-HSP90 antibody, Rat (monoclonal), cat. #SPA-835, Stressgen Bioreagents, Ann Arbor, MI, USA). All antibodies produced a single band of the expected length (Fig. S1).

2.3.3. Measurements of cytochrome c oxidase capacity

Cryotubes containing the frozen plantaris or trunk muscles were incubated for ~ 2 min at 35ºC until the cryopreservation medium was completely thawed. The muscle fibers were immediately washed in ice-cold medium containing 120 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, 2 mM KH_2PO_4, 20 mM HEPES, 1mM EGTA Ca-free, 10µg mL^{-1} PMSF and homogenizes in 1-2 ml of the same media with several passes of a Potter-Elvenhjem homogenizer and a loosely fitting Teflon pestle at 200 rpm. The homogenate was centrifuged at 2000 × g and 4ºC for 8 min to remove cell debris, and the supernatant containing mitochondria was used to measure activity of cytochrome c oxidase (COX).

COX activity was determined by measuring the oxygen consumption of mitochondria-containing supernatant at 23°C in the presence of 5 µM antimycin A, 5 mM ascorbate and 10 mM *N*,*N*,*N*′,*N*′-Tetramethyl-*p*-phenylenediamine (TMPD) as an electron donor (Kuznetsov and Gnaiger, 2010). Oxygen consumption was measured using a fiber optic oxygen sensor connected to the Microx TX3 oxygen monitor with

temperature correction (Precision Sensing, Dussel-dorf, Germany) and Oxy Micro ver. 2.00 software (World Precision Instruments, Sarasota, FL). A two-point calibration was performed prior to each measurement with saturated Na_2SO_3 solution and air-saturated assay media serving as 0% and 100% calibration points, respectively. To correct for the potential autooxidation of TMPD, oxygen consumption was measured after addition of 25 mM KCN to inhibit COX, and the difference in the oxygen consumption rates in the presence and absence of KCN was used to calculate COX activity. Concentrations of oxygen in the respiration chamber were monitored using Logger Pro 3.2 with a Vernier LabPro interface (Vernier Software and Techi8nology, Beaverton, OR). Protein concentrations in mitochondrial isolates were measured using a Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA) in the presence of 0.1% Triton X-100 used to solubilize mitochondrial membranes, with BSA as the standard. Respiration rates were expressed as µmol O_2 min^{-1} g^{-1} mitochondrial protein. Each biological replicate represented an individual isolate obtained from the pooled tissues of 2–3 animals.

2.3.4. Statistical analysis

Variables were log_{10} transformed to improve normality. For the immunoblotting results, a two-way ANOVA was used to compare the effects of the season, gel and their interaction on protein expression (measured as a densitometric signal). When the effects of the gel and/or gel x season interactions were not significant, a one-way ANOVA was used to test for the effect of the season. To compare different muscles within a season, a one-way ANOVA was used followed by Bonferroni post-hoc test. For COX activity, a two-way ANOVA was used to compare the effects of species and muscles (COX ~ species + muscle); and species and season in each muscle (COX_{muscle} ~ species + season). Assumptions of the ANOVA and t test (normality and equal variance) were met and statistical significance was set at $P < 0.05$. All tests were run in R software, version 2.10.0 (R Development Core Team, 2010). Data are shown as means ± S.E.M..

2.4. Results

2.4.1. Metabolic signaling

Total AMPK showed higher expression during the dry period compared to the reproductive period in all studied muscle types of *R. jimi* (larynx - $F_{1,6} = 0.25$, $P = 0.001$; trunk - $F_{1,12} = 0.68$, $P = 0.001$; flexor - $F_{1,9} = 0.13$, $P = 0.04$; plantaris - $F_{1,12} = 0.65$, $P = 0.003$) (Fig. 1). A similar trend was seen in *P. diplolister*, albeit it was only

significant in the plantaris muscle ($F_{1,10}$ = 0.14, P = 0.002) (Fig. 1C). *R. granulosa* displayed similar AMPK expression levels across the two studied seasons in all muscle types (Flexor: $F_{1,8}$ = 2.74, P = 0.14; trunk: $F_{1,12}$ = 0.84, P = 0.38; plantaris: $F_{1,12}$ = 0.34 P = 0.57). When compared within the same season, AMPK levels in different muscle types were similar within *R. jimi* and *R. granulosa* (P > 0.05, Table 1). In *P. diplolister*, AMPK levels were similar in different muscle types during the dry period (Table 1). During the reproductive period, trunk muscles of *P. diplolister* showed higher AMPK expression compared to other muscles ($F_{2,20}$ = 4.23, P = 0.03, Bonferroni P = 0.03) (Fig. S2).

Expression of the protein kinase B (AKT) tended to be higher during the dry period in all studied species and muscle types (except flexor for all species and the larynx of *R. jimi;* Fig. 1E-H). This trend was significant in the larynx muscles from *P. diplolister* ($F_{1,8}$ = 0.58, P = 0.001), the trunk muscles from *R. granulosa* and *P. diplolister* ($F_{1,7}$ = 1.55, P = 0.002; $F_{1,6}$ = 0.79, P = 0.006), and the plantaris muscle from all three studied species (*R. granulosa* - $F_{1,7}$ = 0.24, P = 0.03; *R. jimi* - $F_{1,6}$ = 0.25, P = 0.001; *P. diplolister* - $F_{1,11}$ = 1.21, P = 0.002) (Fig. 1E-G). There were no significant differences of AKT expression between seasons in the flexor muscle in the three studied species (*R. granulosa* - $F_{1,6}$ = 3.57, P = 0.11; *R. jimi* - $F_{1,8}$ = 0.19 , P = 0.68; *P. diplolister* - $F_{1,4}$ = 5.58, P = 0.08). AKT levels were the same across different muscle types when compared within the same season in all three studied species (P > 0.05, Table 1).

2.4.2. Protein homeostasis

Total expression levels of the eukaryotic initiation factor 2α (eIF2α) were lower during the dry period than in the reproductive period in the larynx and flexor muscles from *P. diplolister* ($F_{1,7}$ = 0.079, P = 0.016; $F_{1,4}$ = 0.110, P = 0.04) (Fig. 2A-D), and in the trunk and plantaris muscles from *R. granulosa* ($F_{1,14}$ = 1.06, P = 0.002; $F_{1,12}$ = 0.132, P = 0.02) (Fig. 2A-D). In all other muscle types (including the trunk and plantaris of *P. diplolister,* the larynx and flexor of *R. granulosa*, and all muscle types of *R. jimi*), season had no significant effect on eIF2α expression (P > 0.05). In *R. granulosa*, the flexor muscle had the highest levels of total eIF2α during the dry period, and the flexor and trunk muscles had elevated levels of eIF2α during the reproductive period, compared to the other studied muscle types (Table 1; Fig. S3A,B; Bonferroni P = 0.001

and 0.028, respectively). For *R. jimi* collected in the reproductive period, trunk and plantaris muscles showed lower total eIF2α expression compared to the larynx and flexor (Table 1; Fig. S3C; Bonferroni P = 0.042). No differences in total eIF2α levels were found between different muscle types of *R. jimi* during the dry period, or of *P. diplolister* during the dry and reproductive periods (Table 1).

Phosphorylated eukaryotic initiation factor 2-α (p-eIF2α) showed lower expression during the dry period in trunk, flexor and plantaris muscles ($F_{1,13}$ = 0.94, P = 0.003; $F_{1,9}$ = 0.23, P = 0.006; $F_{1,10}$ = 0.47, P = 0.004, respectively) (Fig. 2E-H), but not in the larynx from *R. jimi* ($F_{1,4}$ = 3.52, P = 0.13). In contrast, p-eIF2α levels were elevated in the muscles of *P. diplolister* during the dry period compared to the reproductive one (Fig. 2E-H), and this trend was significant in the trunk ($F_{1,6}$ = 0.30, P = 0.003) and marginally significant in the flexor muscle ($F_{1,1}$ = 83.38 P = 0.069) but not in the larynx ($F_{1,8}$ = 0.673, P = 0.436) or plantaris ($F_{1,12}$ = 0.506 P = 0.49). Notably, p-eIF2α levels were below the detection limit in all muscles from *R. granulosa* during the dry period (at 50 µg of total protein per lane) (Fig. 2E-H). Comparisons between different muscle types within each species and each study season showed similar p-eIF2α expression among different muscle types in *R. jimi and R. granulosa* during the dry and reproductive periods, and in *P. diplolister* during the reproductive period (Table 1). However, during the dry period the trunk muscle of *P. diplolister* had significantly higher levels than flexor and plantaris, and levels of p-eIF2α in the plantaris tissue was below that in all other studied muscle types (Table 1, Fig. S3D).

The ratio of p-eIF2α to total eIF2α levels was lower during the dry period in all muscle types of *R. granulosa* (reflecting the non-detectable levels of p-eIF2α during the dry period) and in the flexor and plantaris muscles *R. jimi* ($F_{1,3}$ = 0.518, P = 0.02; $F_{1,4}$ = 0.257, P = 0.034), but not *P. diplolister* (P > 0.05) (Fig. 2I-L). The ratio of p-eIF2α to total eIF2α did not significantly vary among the different muscle types when compared within the same season in *P. diplolister* and *R. granulosa* (Table 1). In *R. jimi* during the dry period, larynx muscle showed higher p-eIF2α/ eIF2α ratio when compared to the other muscles (Fig. S3E; Bonferroni P = 0.05); this difference was not significant during the reproductive period (Table 1).

Heat shock protein 60 (HSP60) showed lower expression during the dry period in the flexor from *R. jimi* ($F_{1,11}$ = 1.12, P = 0.001), and in the larynx and trunk of *P. diplolister* ($F_{1,7}$ = 0.33, P = 0.047; $F_{1,8}$ = 0.11, P = 0.008) (Fig. 3A-D). In all other studied tissue/species combinations, no significant differences in HSP60 levels were found between the reproductive and dry periods (P > 0.05). Notably, the trunk muscles of *R. jimi* and *R. granulosa* had lower HSP60 levels compared to other muscle types in the reproductive season (Table 1; Fig. S4A,C). During the dry period, HSP60 levels in the trunk muscle of *R. jimi* were similar to that in the larynx and flexor while HSP60 levels in the plantaris muscle were higher than in the larynx and flexor (Table 1; Bonferroni P = 0.052) (Fig. S4B). In *P. diplolister,* HSP60 were similar among different muscle types within each respective studies season (P > 0.05).

Heat shock protein 70 (HSP70) showed lower expression during the dry period compared to the reproductive season in the larynx and trunk of *P. diplolister* ($F_{1,9}$ = 0.42, P = 0.03; $F_{1,13}$ = 0.191, P = 0.02) (Fig. 3E-H). Similarly, the trunk muscles of *R. jimi* had lower HSP70 levels during dry period compared to the reproductive one ($F_{1,7}$ = 0.19, P = 0.02). The flexor muscle of *R. jimi* showed higher HSP70 expression during the dry period ($F_{1,6}$ = 10.27, P = 0.019), while larynx displayed similar HSP70 expression along the year ($F_{1,5}$ = 2.05, P = 0.21) (Fig. 3E-H). *R. granulosa*‘s trunk muscles (but not the plantaris, flexor or larynx) showed higher HSP70 levels during the dry period compared to the reproductive season ($F_{1,8}$ = 8.35, P = 0.02) (Fig. 3E-H). Within each species and study season, only *R. jimi* showed differences in HSP70 levels among different muscle types during the reproductive season, with the highest levels in the trunk and the lowest levels in the plantaris muscle (Table 1; Fig. S4D; Bonferroni P = 0.006).

Heat shock protein 90 (HSP 90) showed lower expression during the dry period compared to the reproductive period in the larynx and flexor muscles of *R. jimi* ($F_{1,8}$ = 0.27, P = 0.007; $F_{1,11}$ = 0.37, P = 0.001) and in the larynx and trunk muscles from *P. diplolister* ($F_{1,1}$ = 1.02, P = 0.03; $F_{1,6}$ = 0.44, P = 0.042) (Fig. 3I-L). In contrast, in the plantaris muscle of *R. jimi* lower levels of HSP90 were found during the reproductive period compared to the dry one ($F_{1,2}$ = 0.13 P = 0.045). HSP90 could not be detected in *R. granulosa*. Comparison among different muscle types within the same season showed elevated HSP90 levels in the larynx and flexor compared to the trunk and

plantaris of *R. jimi* during the reproductive period (Bonferroni P = 0.013), and in the larynx of *P. diplolister* compared to all other tissue types, also during the reproductive period (Bonferroni P = 0.007). (Fig. S4E,F, Table 1). In all other studied species/season combinations, no significant differences in HSP90 levels were found between different muscle types (P > 0.05).

2.4.3. Aerobic capacity

Activity of cytochrome c oxidase (COX), which can serve as a marker of the mitochondrial density and thus aerobic capacity of the tissue, was higher in the trunk and plantaris muscles of *R. jimi* compared to *R. granulosa* and *P. diplolister* ($F_{2,22}$ = 7.000, P = 0.005 and $F_{2,43}$ = 9.616, P = 0.001, for the dry and the reproductive period respectively) (Fig. 4). Notably, COX activity tended to be lower in the trunk muscle than in the plantaris muscle across the species during the dry period (by 40-45% in *R. granulosa* and *P. diplolister* and by 68% in *R. jimi),* although this difference was significant only for *R. jimi* ($F_{1,22}$ = 5.334, P = 0.03). Comparing seasons within species, COX activity was marginally higher during reproductive season in the trunk *R. granulosa* (t = 2.194, df = 5, P = 0.07), but not in the plantaris (t = 0.132, df = 1, P = 0.90). *Rhinella jimi* and *P. diplolister* did not display COX activity differences among seasons (P > 0.05).

2.5. Discussion

Our results point to the importance of metabolic regulators mediating survival during the drastic seasonal variation faced by the desert anurans. The toads that remain active during the dry period maintain muscles through more energy extensive pathways including elevated protein synthesis, while the estivating frog employs energy conservation strategy that involves suppression of protein synthesis, decrease in the chaperone expression and higher expression of AMPK. These adjustments are consistent with their lower metabolic rates (Carvalho et al., 2010) and need for saving energy during aestivation. All three studied species activate cell survival pathways during the dry period in the muscles likely to prevent muscle atrophy. All three studied species thereby maintain the muscle capacity throughout the year, despite the resource limitation. These strategies are important considering the unpredictability of the

reproductive event and high demand on muscular activity during the reproductive season.

2.5.1. Cellular survival and protein synthesis pathways in the muscle

The protein kinase B (AKT) is an important signaling protein involved in cellular survival pathways in the muscle (Manning and Cantley, 2007). AKT expression was elevated during the dry period in the trunk and plantaris muscles from all studied species, with the most pronounced increases in an aestivating species, *P. diplolister*. Similarly, elevated expression of AKT was reported in the foot muscle and hepatopancreas of estivating snails (Ramnanan et al., 2007) and in the liver of a frog *Rana sylvatica* exposed to freezing (Zhang and Storey, 2013). Given that AKT promotes cell survival, downregulates pro-apoptotic factors (Datta et al., 1999; Ramnanan et al., 2007; Zhang and Storey, 2012; Zhang and Storey, 2013; Gerber et al., 2016), and activates the cascade involved in cell cycle arrest and quiescence (Burgering and Medema, 2003), the upregulation of AKT in these anurans during the dry period could be important for preventing the muscle atrophy when resources are limited.

Notably, the upregulation of AKT during the dry period goes hand-in-hand with an increase of the phosphorylated form of eIF-2α (p-eIF-2α) in the trunk muscle and/or a decrease of the total eIF-2α in the larynx and flexor muscles of the estivating species, *P. diplolister*. This agrees with the earlier findings showing that phosphorylated eIF-2α can facilitate AKT activation thereby promoting cell survival (Rajesh et al., 2015). Furthermore, the eIF-2α is an essential initiation factor in protein translation which controls the translation rates and becomes inactivated by phosphorylation (Hershey, 1989). Thus, low levels of eIF-2α and/or elevated expression of p-eIF-2α indicate suppression of the protein synthesis in the muscles of *P. diplolister* during estivation. Suppression of the protein synthesis is a common energy-saving mechanism in estivating species (Pakay et al., 2003) and has been observed in desert amphibians *Neobatrachus centralis* (Fuery et al., 1998) and in estivating snails *Otala lactea* (Pakay et al., 2002; Ramnanan et al., 2009). Earlier studies on estivating frogs (*Cyclorana alboguttata)* showed that muscles are protected against atrophy during prolonged (9 months) estivation with no decline in muscle mass, cross-sectional area or fiber number (Symonds et al., 2007). Our study in *P. diplolister* suggests a possible mechanism for

this protection involving the coordinated suppression of the protein synthesis to conserve energy reserves and activation of the cell survival pathways to prevent loss of the muscle cells.

Unlike *P. diplolister*, the protein synthesis was activated during the dry period in the muscles of *R. jimi* and *R. granulosa* (two species that remain active throughout the year) as indicated by a decline in the amount of inactive p-eIF-2α in all studied muscle types. This was especially notable in *R. granulosa* where p-eIF-2α levels were below the detection limits of immunoblotting during the dry period. Increased protein synthesis along with the activation of the cell survival pathways may help building the muscle mass in preparation for the reproductive period in the two active frog species that are less resource-limited during the dry period compared to their estivating counterpart, *P. diplolister*. The maintenance of the muscle mass during the dry period is important for the Caatinga anurans, which start reproduction immediately after fairly unpredictable rainfall events (Madelaire and Gomes, 2016). The reproductive behavior involves strenuous calling activity (which engages the trunk muscles) in all three studied species and, in *P. diplolister*, males must also energetically beat legs to build foam nests for eggs deposition (Carvalho et al., 2010). Despite some variation in the AKT, eIF-2α and p-eIF-2α levels among the muscle types, the seasonal patterns of expression of these proteins were generally consistent in different muscles within each studied species. These results indicate that molecular mechanisms involved in the muscle maintenance during the resource-limited dry season are similar in the locomotory muscle (i.e. plantaris) and the reproductively-related muscles (such as trunk, flexor and larynx).

2.5.2. Indices of energy status

Elevated expression of AMPK in muscle tissues (indicative of the cellular energy stress) was observed during the dry period in all muscles of *R. jimi* and in plantaris muscle of *P. diplolister*. An increase in AMPK levels is common during the resource- and energy-limited periods in many organisms including hibernating mammals (Zhang et al., 2015; Rider, 2016) and frogs exposed to hypothermia, hypoxia, freezing, dehydration or anoxia (Bartrons et al., 2004; Rider et al., 2006; Rider, 2016). Activation of AMPK stimulates ATP-producing pathways, increases uptake of glucose and fatty acids (Barnes et al., 2002) and suppresses energy-demanding metabolic

processes such as protein synthesis (Kahn et al., 2005; Hardie, 2011). Furthermore, AMPK induces cell cycle arrest (Hardie, 2011; Rider, 2016), which also contributes to energy savings. The increase of AMPK expression in anurans from the Caatinga during the dry period could thus contribute to coping with the resource limitations (such as lower prey availability in an active species and lack of feeding in the estivating frog). No change in AMPK levels between the reproductive and dry season was found in the muscles of *R. granulosa*. The reasons for these differences in AMPK response between the two non-estivating active species are not known, but may be related to a smaller size (and thus lower absolute resource requirements) of *R. granulosa* compared with *R. jimi*. During reproductive period, AMPK expression was particularly elevated in trunk of *P. diplolister* compared to other muscle types, which might indicate high energy demand of the trunk muscles due to calling activity. Calling occurs in the highly synchronized, explosive fashion following the rain event in *P. diplolister* compared to the more protracted response of the other two species (Madelaire and Gomes, 2016).

COX activity (indicative of the mitochondrial density) was considerably higher in the muscles of the largest of the three studied species (*R. jimi)* compared to *P. diplolister* or *R. granulosa*. COX activity was especially high in the plantaris muscle of *R. jimi* which is consistent with higher locomotory capacity of large toads from *Rhinella marina* group of species (Bocxlaer et al., 2010). Generally, the mitochondrial COX capacity of the frogs' muscles was maintained at the same level in the reproductive and dry period except for a small but significant decline in the COX activity in the trunk muscle of *R. granulosa* during the dry period. This indicates that the aerobic capacity of the locomotor as well as the reproductive muscles is maintained throughout the year despite the energy and resource limitation in the dry season.

2.5.3. Expression of molecular chaperones

Molecular chaperones involved in the folding of nascent and damaged proteins (including a mitochondrial HSP60 and cytosolic HSP70 and HSP90) were expressed at lower levels in the muscles of the estivating *P. diplolister* during the dry period. Similarly, a decrease in HSP expression during quiescence has been previously found in other estivating species such as land snails (Reuner et al., 2008; Mizrahi et al., 2012). A decrease in HSP expression in estivating frogs goes hand-in-hand with the suppressed

protein synthesis in the muscles and may reflect lower protein turnover rates during estivation (Pakay et al., 2002; Storey and Storey, 2004).

The higher expression of HSPs in the muscles larynx and trunk of *P. diplolister* during the reproductive period might be attributed to the stress caused by the exercise from calling behavior and high steroid receptor expression levels (Kregel, 2002; Storey and Storey, 2011; Evgen'ev et al., 2014). Calling is a highly energetic demanding aerobic exercise for anuran males (Bevier, 1997; Wells, 2001), and calling effort is positively correlated with plasma levels of androgens and corticosterone (Emerson and Hess, 2001; Moore et al., 2005; Assis et al., 2012). Furthermore, anurans show increased expression of steroid hormones during reproductive season (Moore et al., 2005; Carr, 2011), which are associated with HSP in the inactive state (Pratt and Toft, 1997; Falkenstein et al., 2000; Sapolsky et al., 2000). Thus, elevated expression of HSPs during the reproductive season in *P. diplolister* might also reflect a compensatory response of HSPs to inactivation by high levels of steroids. This latter explanation, however, appears less likely as no consistent increase of the HSP levels was found in the muscles of *R. jimi* or *R. granulosa* males that also display high steroid levels during the reproductive season. Overall, HSP levels of in the muscle tissues in the two anurans species that are year-round active showed relatively little variation and no consistent pattern of seasonal change between different muscle types. This indicates the lack of strong unfolded protein response and thus maintenance of the protein homeostasis during the period of reproductive activity as well as during times of resource limitation in these species.

2.6. Conclusions

Cell regulatory strategies employed by the three studied species of desert anurans reflect differences in their life habit and activity levels as well as the common need to maintain the muscle capacity in an extremely seasonal environment of the Caatinga. Activation of the cell survival pathways is the most consistent response in the muscles of all three studied species during the dry period likely playing a role in preventing muscle atrophy during the resource limitation. Expression of the regulators of protein homeostasis (including chaperones and regulators of the protein synthesis) reflect different levels of resource limitation, so that the less resource-limited active species upregulate protein synthesis and maintain high levels of chaperones during the dry periods in the muscle while a severely resource-limited aestivating species shuts down the protein synthesis to conserve energy. Notably, an active *R. jimi* and aestivating *P. diplolister* (but not an active species *R. granulosa*) express elevated AMPK levels in the muscles during the dry period indicating that the former two species are more susceptible to the resource limitation than *R. granulosa*. Against our prediction, we do not observe differential metabolic regulation or trade-off between reproductive and locomotor muscles. Future studies are needed to determine whether the muscle maintenance during the dry period is prioritized over that of other tissues and whether potential trade-offs exist between the support of the muscle capacity throughout the year and other fitness-related functions in desert frogs.

2.7. Acknowledgements

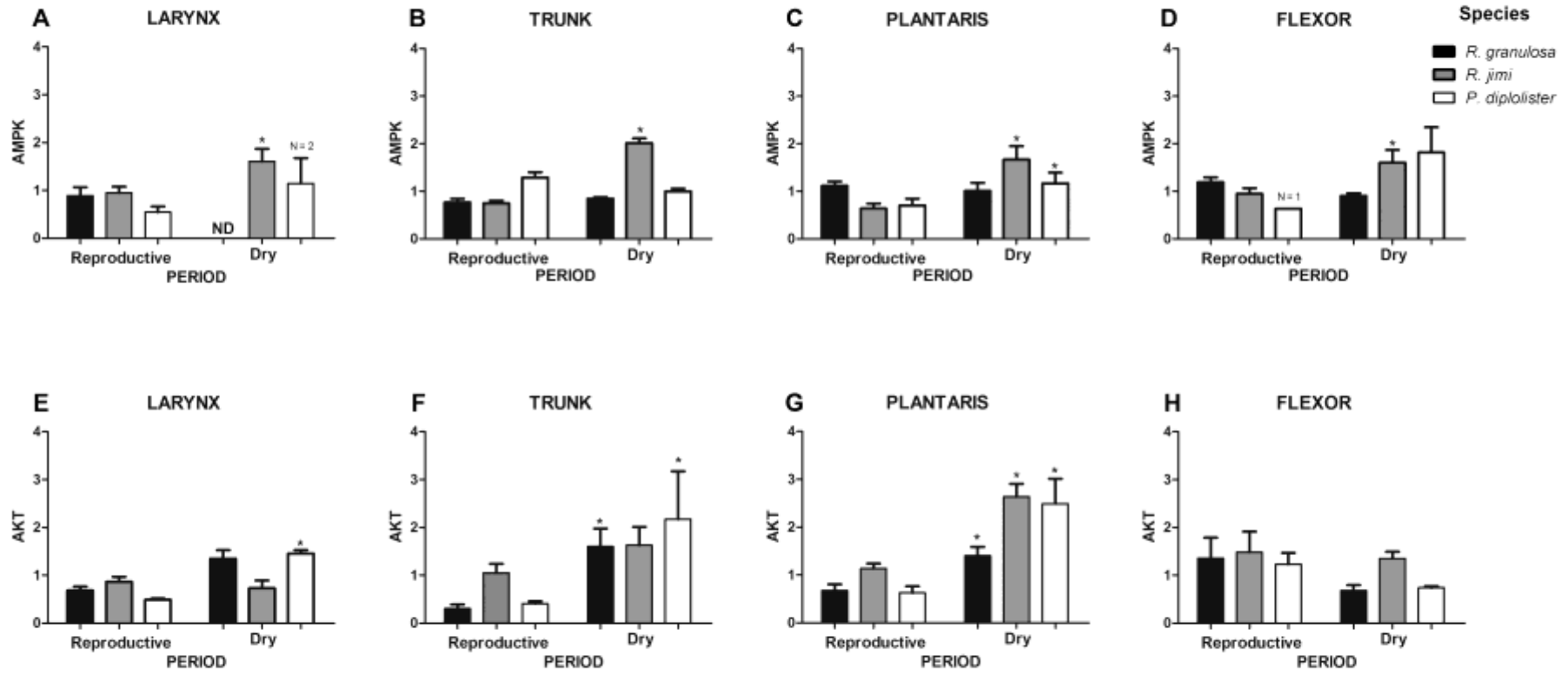
We are grateful to Assis, F. and Cassettari, B.O. for support in the fieldwork; and Ivanina, A.V. and Martins, A.N. for sharing their time, knowledge and expertise. This research was supported by the State of São Paulo Science Foundation (FAPESP) through grant (2014/50643-8) led by F.R.G. and I.S. and PhD Fellowship for Research Internships Abroad (BEPE) awarded to C.B.M. (2015/02484-0).

2.8. Table

Table 1. ANOVA: comparison of protein expression in different muscles within a season. F ratios (with the degrees of freedom and the error shown as a subscript), P values are given, and significant effects ($P < 0.05$) are highlighted in bold. Missing analysis are either protein detection bellow limit or lack of samples.

	Rhinella jimi	*Rhinella granulosa*	*Pleurodema diplolister*
AKT	**Reproductive**	**Reproductive**	**Reproductive**
	$F_{3,13} = 3.69$ P = 0.04	$F_{3,13} = 3.35$ P = 0.053	$F_{3,11} = 0.91$ P = 0.47
	Dry	**Dry**	**Dry**
	$F_{3,6} = 4.56$ P = 0.06	$F_{3,5} = 0.34$ P = 0.80	$F_{3,10} = 1.39$ P = 0.30
AMPK	**Reproductive**	**Reproductive**	**Reproductive**
	$F_{3,18} = 2.44$ P = 0.10	$F_{3,13} = 1.70$ P = 0.20	**$F_{3,17} = 3.42$ P = 0.04**
	Dry	**Dry**	**Dry**
	$F_{3,18} = 2.35$ P = 0.11	$F_{2,7} = 0.72$ P = 0.52	**$F_{1,10} = 0.14$, P = 0.002**
p-eIF2-α	**Reproductive**	**Reproductive**	**Reproductive**
	$F_{3,14} = 2.21$ P = 0.13	$F_{3,6} = 0.87$ P = 0.51	$F_{3,15} = 1.59$ P = 0.24
	Dry	**Dry**	**Dry**
	$F_{3,14} = 0.74$ P = 0.55	-	**$F_{3,6} = 11.53$ P = 0.01**
eIF2-α	**Reproductive**	**Reproductive**	**Reproductive**
	$F_{3,15} = 11.18$ P = 0.001	**$F_{3,18} = 23.82$ P = 0.001**	$F_{3,19} = 2.89$ P = 0.06
	Dry	**Dry**	**Dry**
	$F_{3,5} = 26.67$ P = 0.002	$F_{3,6} = 1.19$ P = 0.39	$F_{3,5} = 1.02$ P = 0.46
p-eIF2-α/ eIF2-α	**Reproductive**	**Reproductive**	**Reproductive**
	$F_{3,10} = 1.94$ P = 0.19	$F_{3,5} = 0.17$ P = 0.92	$F_{3,12} = 1.28$ P = 0.32
	Dry	**Dry**	**Dry**
	$F_{3,6} = 11.30$ P = 0.007	-	$F_{3,4} = 3.02$ P = 0.16
HSP60	**Reproductive**	**Reproductive**	**Reproductive**
	$F_{3,22} = 9.44$ P = 0.001	**$F_{3,15} = 3.70$ P = 0.04**	$F_{2,13} = 1.46$ P = 0.27
	Dry	**Dry**	**Dry**
	$F_{3,12} = 13.90$ P = 0.001	$F_{2,6} = 0.002$ P = 0.99	$F_{3,5} = 2.65$ P = 0.16
HSP70	**Reproductive**	**Reproductive**	**Reproductive**
	$F_{3,16} = 10.36$ P = 0.001	$F_{3,9} = 0.47$ P = 0.71	$F_{2,25} = 0.92$ P = 0.41
	Dry	**Dry**	**Dry**
	$F_{3,6} = 0.90$ P = 0.50	$F_{3,8} = 1.16$ P = 0.38	$F_{3,8} = 2.63$ P = 0.12
HSP90	**Reproductive**	**Reproductive**	**Reproductive**
	$F_{3,12} = 28.07$ P = 0.001	-	**$F_{2,8} = 40.87$ P = 0.001**
	Dry	**Dry**	**Dry**
	$F_{3,14} = 1.45$ P = 0.27	-	$F_{1,4} = 0.003$ P = 0.96

2.9. Figures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LARYNX
AMPK
ND
N = 2
Reproductive
Dry
PERIOD
B
TRUNK
C
PLANTARIS
D
FLEXOR
N = 1
Species
R. granulosa
R. jimi
P. diplolister
E
LARYNX
AKT
F
TRUNK
G
PLANTARIS
H
FLEXOR

Fig 1. Expression of AMPK and AKT in different muscle types of *R. granulosa*, *R. jimi*, and *P. diplolister* during reproductive and dry periods. Data are means ± S.E.M., n = 3–10, except when otherwise indicated. AMPK expression is shown for (A) larynx, (B) trunk, and (C) plantaris (D) flexor muscles. AKT expression is shown for (E) larynx, (F) trunk, (G) plantaris and (H) flexor muscles. Asterisks indicate significant difference between the reproductive and dry periods ($P < 0.05$). ND - not determined because of the lack of samples.

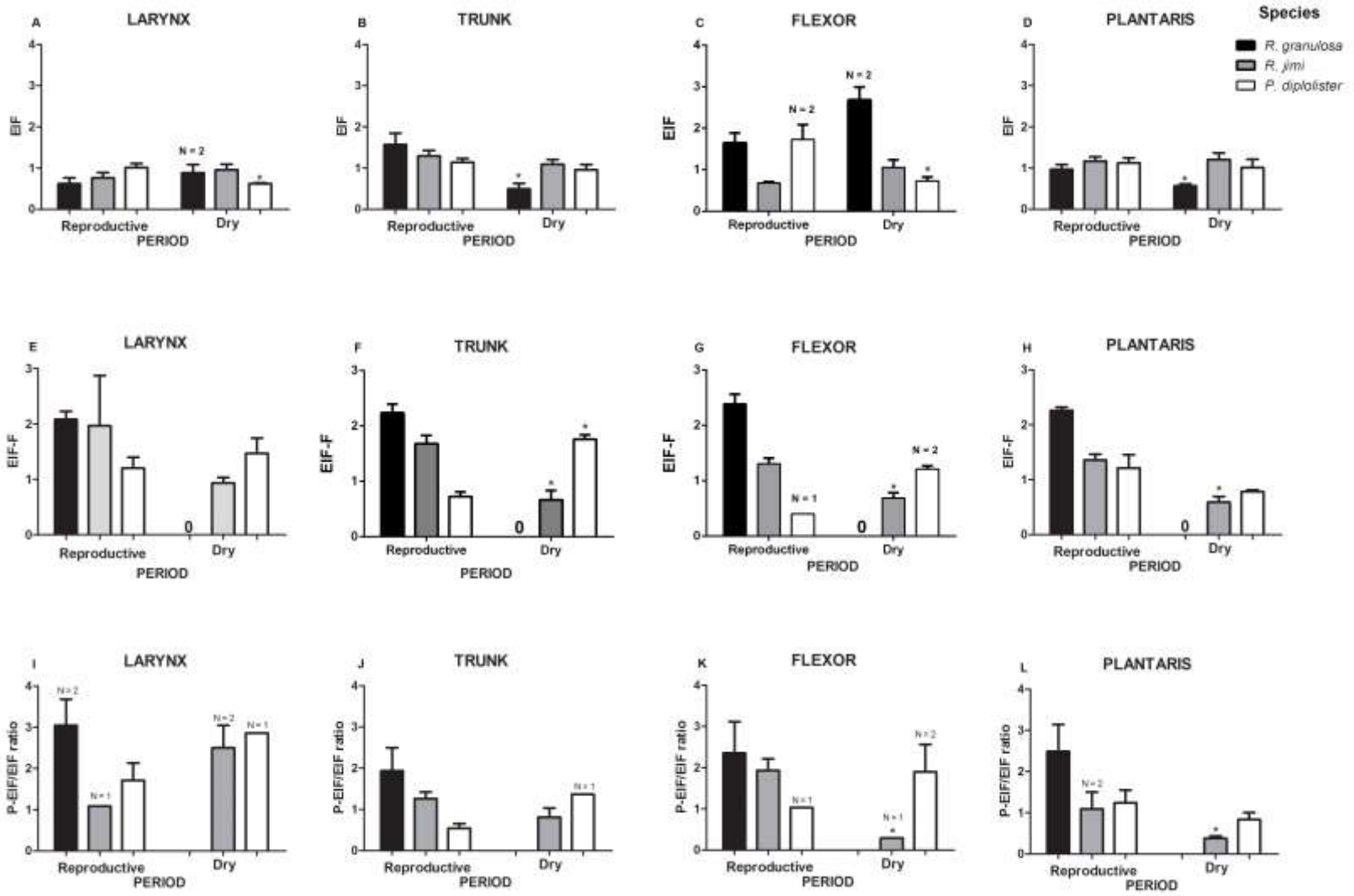
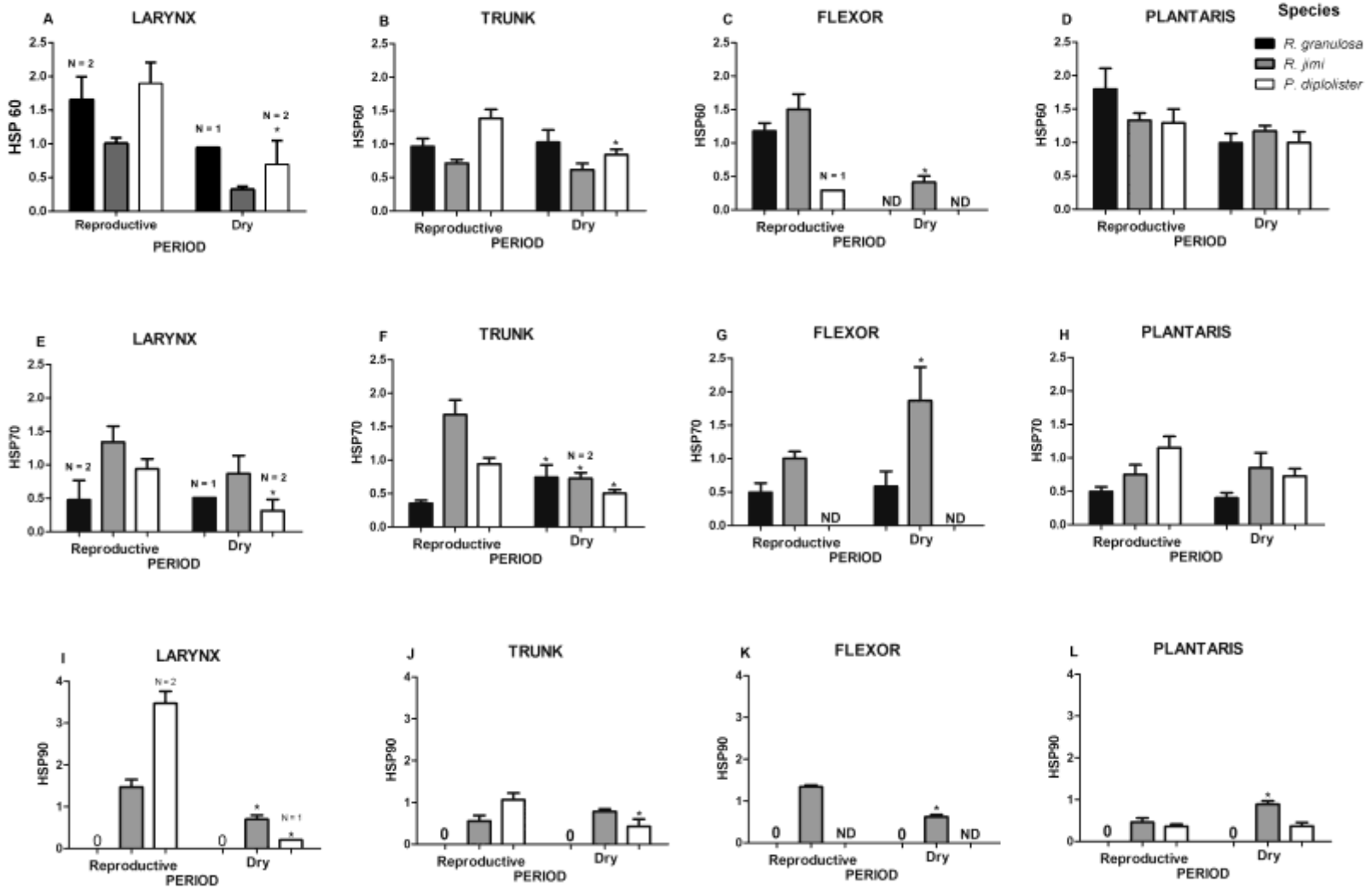

A
LARYNX
B
TRUNK
C
FLEXOR
D
PLANTARIS
Species
R. granulosa
R. jimi
P. diplolister
EIF
E
LARYNX
F
TRUNK
G
FLEXOR
H
PLANTARIS
EIF-F
I
LARYNX
J
TRUNK
K
FLEXOR
L
PLANTARIS
P-EIF/EIF ratio
N = 2
N = 1
0
*
Reproductive
Dry
PERIOD

Fig 2. Relative intensity of EIF-2α protein levels (A) larynx, (B) trunk, (C) flexor and (D) plantaris; phosphorylate EIF-2α protein levels (E) larynx, (F) trunk, (G) flexor and (H) plantaris; phosphorylate EIF-2α/ EIF-2α ratio (I) larynx, (J) trunk, (K) flexor and (L) plantaris in *R. granulosa*, *R. jimi*, and *P. diplolister* during reproductive and dry period. Data are means ± S.E.M., n = 3–10, except when it is indicated. Zero indicates protein detection bellow limit. Asterisks indicates significant difference between reproductive and dry periods ($P < 0.05$).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LARYNX
HSP 60
N = 2
N = 1
N = 2
*
Reproductive
Dry
PERIOD
B
TRUNK
HSP60
*
C
FLEXOR
HSP60
N = 1
ND
*
ND
D
PLANTARIS
Species
R. granulosa
R. jimi
P. diplolister
E
LARYNX
HSP70
F
TRUNK
HSP70
G
FLEXOR
HSP70
H
PLANTARIS
HSP70
I
LARYNX
HSP90
J
TRUNK
HSP90
K
FLEXOR
HSP90
L
PLANTARIS
HSP90

Fig 3. Relative intensity of HSP 60 protein levels in (A) larynx, (B) trunk, (C) flexor, (D) plantaris, HSP 70 protein levels in (E) larynx, (F) trunk, (G) flexor, (H) plantaris, HSP 90 protein levels in (I) larynx, (J) trunk, (K) flexor and (L) plantaris in *R. granulosa*, *R. jimi*, and *P. diplolister* during reproductive and dry period. Data are means ± S.E.M., n = 3–11, except when indicated. ND indicates not determined because of the lack of samples. Zero indicates protein detection bellow limit. Asterisks indicates significant difference between reproductive and dry periods ($P < 0.05$).

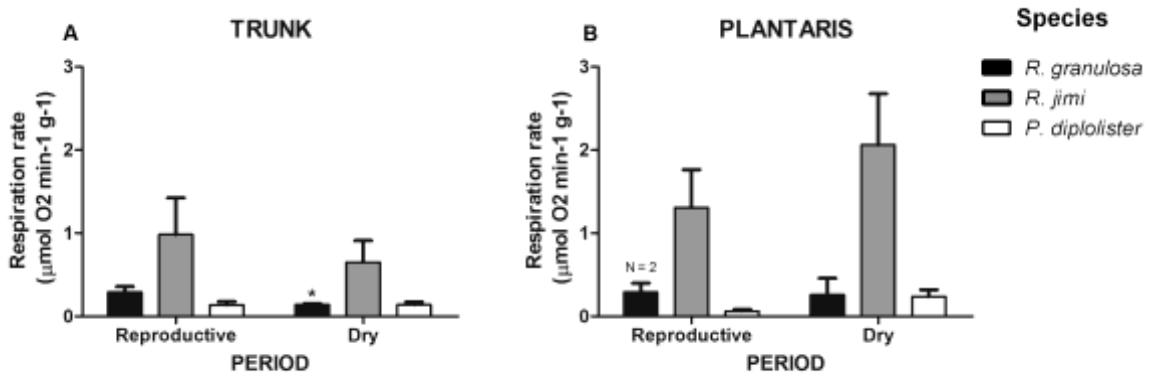

Fig 4. Activity of cytochrome c oxidase (COX) in trunk (A) and plantaris (B) muscles during reproductive and dry periods in *R. granulosa*, *R. jimi*, and *P. diplolister*. Data are means ± S.E.M., n = 3–11, except when indicated. Asterisk indicates significant difference between periods ($P < 0.05$).

2.10. Supplementar figures

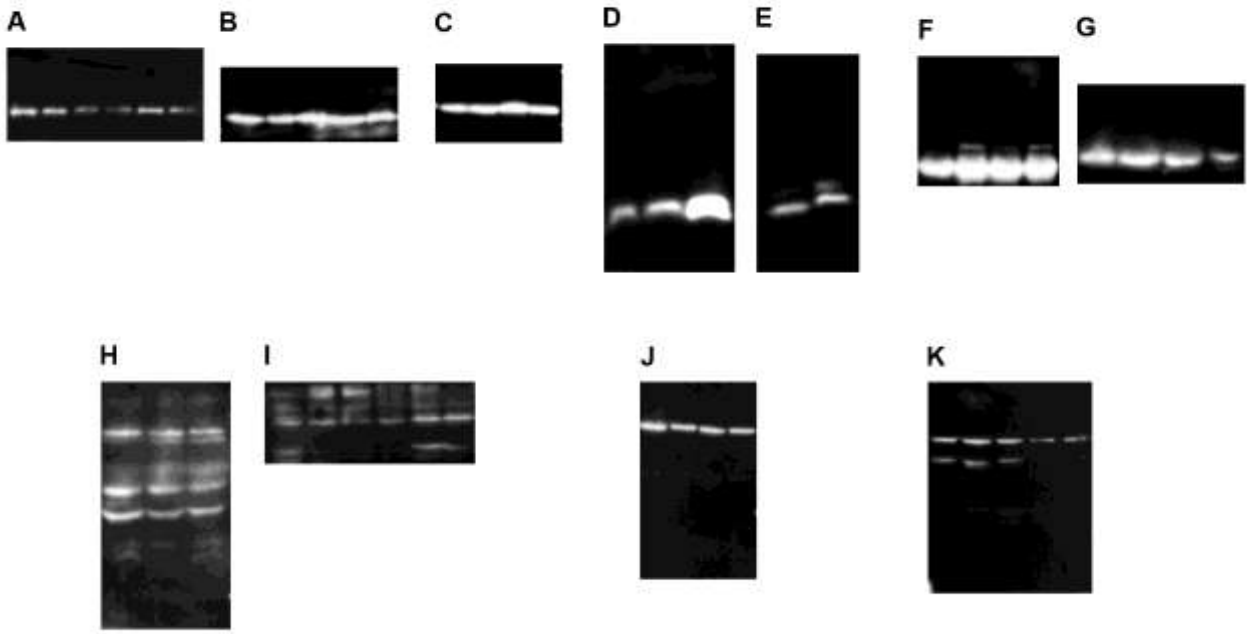

Fig S1. AMPK expression during (A) reproductive and (B) dry period for *R. jimi*, AKT expression during (C) reproductive period for *R. jimi*; Total EIF-2α expression during (D) reproductive and (E) dry period for *P. diplolister;* Phosphorylated EIF-2α expression during (F) reproductive and (G) dry period for *R. jimi*; HSP60 expression during (H) reproductive and (I) dry period for *R. jimi*; HSP70 expression during (J) reproductive period for *R. jimi*; and HSP90 expression during (K) reproductive period for *R. jimi*.

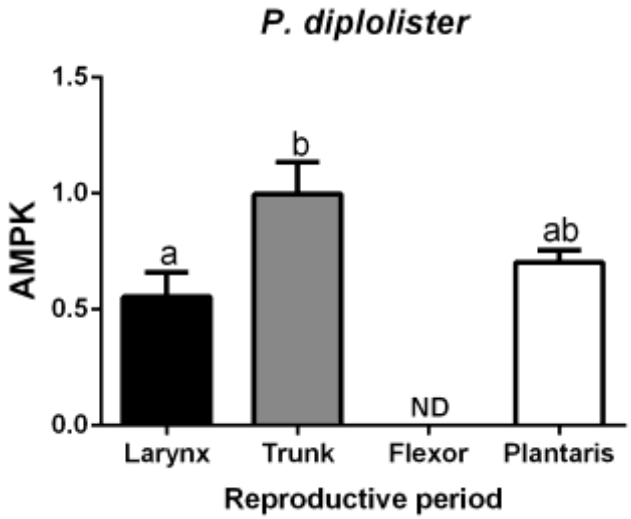

Fig S2. Relative intensity of AMPK protein levels among different muscles in *P. diplolister* during reproductive period. Data are means ± S.E.M., n = 7–10. ND indicates not determined because of the lack of samples. Different letters indicates significant difference ($P < 0.05$).

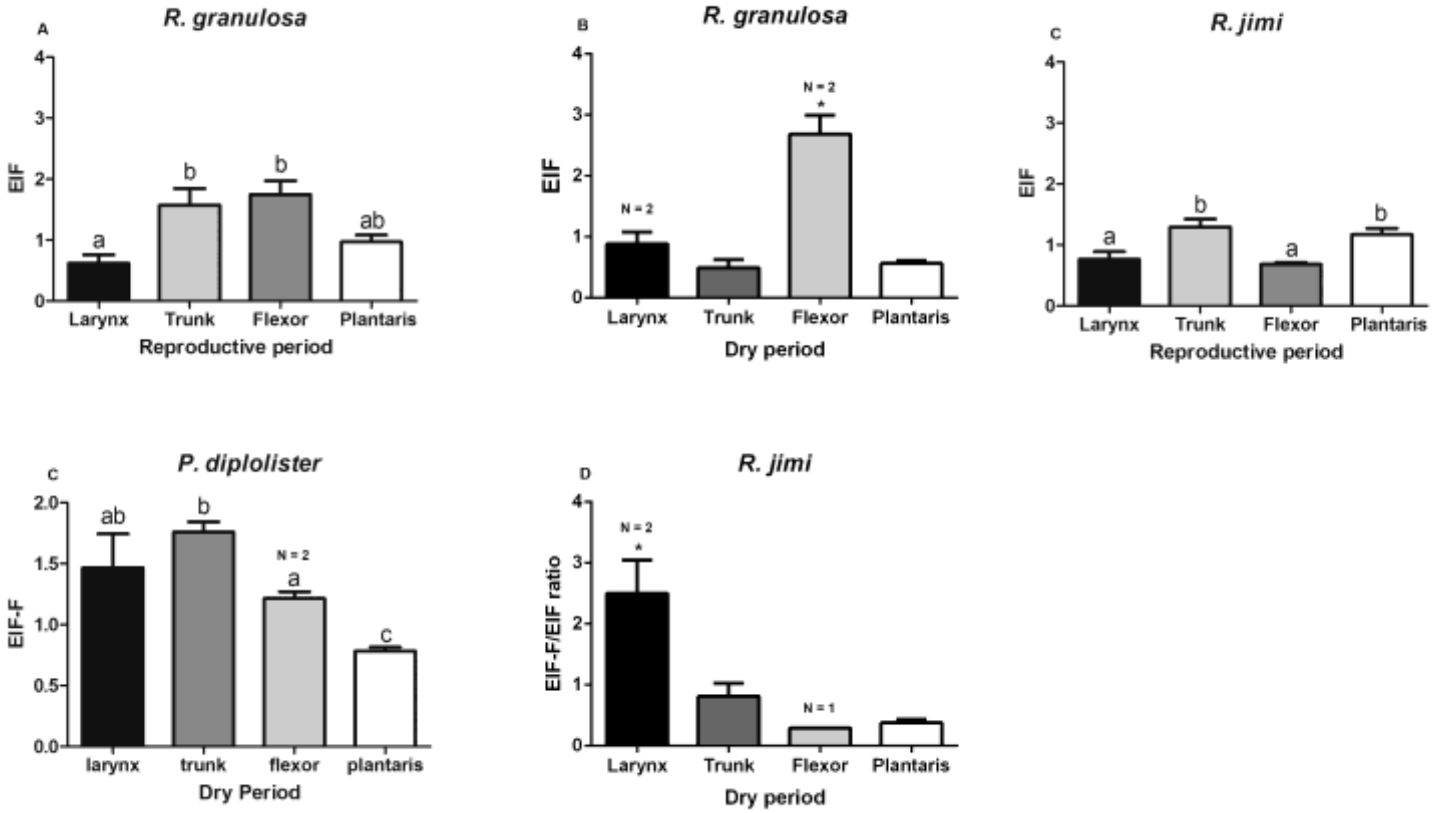

Fig S3. Relative intensity of EIF-2α protein levels among different muscles in *R. granulosa* during (A) reproductive and (B) dry period and *R. jimi* during (C) reproductive period; phosphorylate EIF-2α among different muscles in *P. diplolister* during (D) dry period; and phosphorylate EIF-2α/EIF-2α ratio among different muscles in *R. jimi* during (E) reproductive period. Data are means ± S.E.M., n = 3–10, except when otherwise indicated. Asterisks and different letters indicates significant difference ($P < 0.05$).

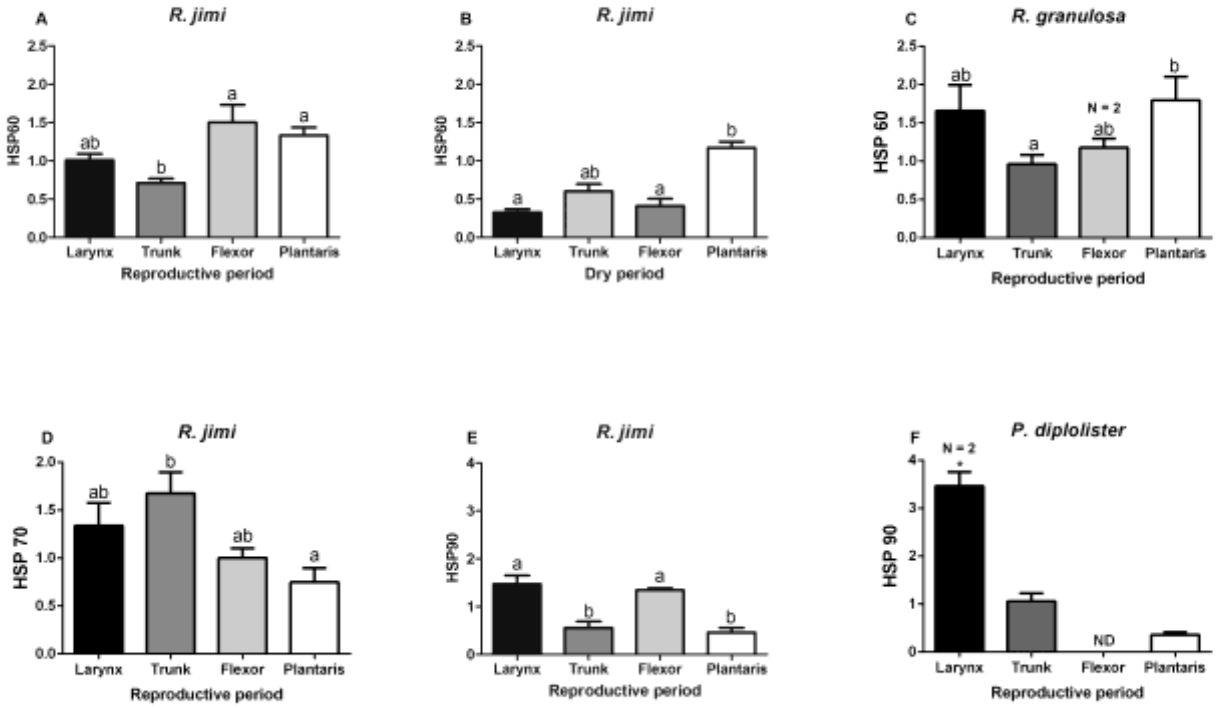

Fig S4. Relative intensity of HSP 60 protein levels among different muscles in *R. jimi* during (A) reproductive and (B) dry period and in *R. granulosa* during (C) reproductive period; HSP 70 protein levels in *R. jimi* during (D) reproductive period; and HSP 90 in *R. jimi* during (E) reproductive period and in *P. diplolister* during (F) reproductive period. Data are means ± S.E.M., n = 4–8, except when otherwise indicated. ND indicates not determined because of the lack of samples. Asterisks and different letters indicates significant difference ($P < 0.05$).

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3. Immunomodulation by testosterone and corticosterone in toads: experimental evidences from transdermal application

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Keywords: bacterial killing ability; PHA; anura; Caatinga

Highlights

- We study the immunomodulation of acute elevation of T and CORT in toads
- T treatment lowered the swelling response to phytohemagglutinin
- T and CORT application did not affect the plasma bacterial killing ability
- CORT treatment did not affect the intensity of swelling response to PHA
- Acute doses of CORT abbreviated the time of inflammatory process (PHA)

Submitted to the special number of the General and Comparative Endocrinology (*Neotropical Vertebrates*) in 4th October, 2017.

3.1. Abstract

The steroids, testosterone (T) and corticosterone (CORT), play important roles in vertebrate reproduction and display complex immunomodulatory functions that may affect survival. We investigated the immune consequences of acute experimental elevation of T and CORT in *Rhinella jimi* toads during the reproductive season. Due to abnormally low plasma T levels before treatment, transdermal T application increased androgen plasma levels to those values typicaly observed during the dry, non-reproductive period. Transdermal T application lowered the swelling response to phytohemagglutinin (PHA) challenge, which is consistent with previous results found for this species in the wild, during drought. Additionally, transdermal T and CORT application did not affect the intensity of the plasma bacterial killing ability (BKA). However, although CORT treatment did not affect the intensity of swelling response to PHA, it abbreviated the process, suggesting an increased efficiency of inflammatory resolution. Although plasma CORT levels are positively correlated to BKA at baseline, individuals characterized by higher basal plasma CORT levels, and at 1h after transdermal CORT, showed a lower BKA at 1h and 10h after treatment. These results indicate that lower androgen levels suppress the inflammatory response, whilst acute doses of CORT increase immune efficiency and high CORT doses can be immunossupressive, possibly *via* the regulation of the complement system.

3.2. Introduction

High androgen plasma levels coordinate the development of primary sexual characteristics, as well as the expression and maintenance of male mating behavior, for most vertebrate seasonal breeders (Hau, 2007). For anurans, testosterone (T) is responsible for the development of many secondary sexual characteristics and shows permissive effects on the expression and maintenance of calling behavior (Norris and Lopez, 2011; Assis et al. 2012). In addition to expression of sexual characteristics, several studies have emphasized the potential immunosuppressive effects of elevated plasma androgen levels (Folstad and Karter, 1992; Fraive et al. 2002; Opplinger et al. 2004; Cox and John-Alder, 2007). However, evidence in the literature points to more complex immunomodulatory effects of androgens (Roberts et al. 2004). Some experimental and correlative studies have shown an association of higher plasma T levels with decreased blood bacterial-killing capacity (Pap et al. 2010), antibody response (Peter et al. 2000; Casto et al. 2001), and inflammatory immune response (Belliure et al. 2004). However, other studies show increased T levels in association with enhanced antibody and inflammatory response in immunologically challenged males (Evans et al. 2000; Peters, 2000; Roberts et al. 2007; Pap et al. 2010). In anurans, Madelaire et al. (2017) found that males from three different species show concomitantly higher plasma androgen levels and swelling response to (phytohemagglutinin) PHA challenge during the reproductive, *versus* non-reproductive, season. Moreover, Desprat et al. (2015) showed that the transdermal application of T increases the swelling response to PHA in the tree frog, *Hyla arborea*, but had no effect on wound healing in the salamander, *Desmognathus ochrophaeus* (Thomas and Woodley, 2017).

In addition to androgens, several groups of vertebrates show higher plasma levels of glucocorticoids (GC) during the reproductive season that may enhance the mobilization of energy substrates and facilitate reproduction (Moore and Jessop, 2003). The elevation of plasma GC can exert complex immunomodulatory effects (Dhabhar, 2009; Assis et al. 2015; Thomas and Woodley, 2015). Whilst acutely elevated plasma GC levels frequently enhance immune responses, including the increased trafficking and infiltration of leukocytes (Dhabhar et al. 1996; 1999; Bowers et al. 2008), chronically elevated GC levels usually result in immunosuppression (Dhabhar and

McEwen, 1999; Dhabhar, 2000; Sapolsky et al. 2000; Dhabhar, 2009). For anurans, evidence indicates a positive correlation of calling rates with plasma corticosterone (CORT) levels in natural choruses (Moore et al. 2005; Assis et al. 2012). Madelaire et al. (2017) found anuran males, during calling activity, to have higher plasma CORT levels and higher numbers of circulating leukocytes in the blood. Graham et al. (2012) and Assis et al. (2015) observed that movement restriction stress (12 and 24h, respectively) increased plasma CORT levels and decreased plasma bacterial killing ability (BKA), whilst acute transdermal CORT treatment not only increased plasma CORT levels but also levels of phagocytosis in the toad, *R. icterica* (Assis et al. 2017). Moreover, chronic daily CORT treatment: decreases the levels of circulating eosinophils in the anurans, *Litoria caerulea* and *Lithobates catesbeianus* (Kaiser et al. 2015); decreases plasma BKA in the toad *R. icterica* (Assis et al. 2015); delays cutaneous wound healing in the salamander, *Desmognathus ochrophaeus* (Thomas and Woodley, 2015); and enhances *Batrachochitrium dendrobatidis* (Bd) fungus infection abundance in red-legged salamander, *Plethodon shermani* (Fonner et al. 2017).

Anurans living in the Brazilian semiarid zone, the Caatinga, depend on unpredictable rain events occurring during the short rainy season (January to May) to reproduce (Abe, 1995; Rodrigues, 2003; Madelaire and Gomes, 2016). Male anurans from the Caatinga show elevated plasma levels of androgens throughout this reproductive period, whilst plasma CORT levels increase only during calling activity, which occurs only during heavy rain falls (Madelaire and Gomes, 2016). We have been following the natural endocrinological, immunological and reproductive patterns of anuran species from the Caatinga since 2011. The seasonal pattern of covariation between plasma steroid levels (androgens and CORT) and immunocompetence in anurans from this area does not corroborate the hypothesis of steroid-mediated immunosuppression during reproduction. On the contrary, anurans from the Caatinga show attenuated immune responsiveness during the drought, when plasma steroid levels are low (Madelaire et al. 2017).

In order to understand the functional correlation between plasma steroids levels and immunocompetence, this study investigated the immune consequences of experimental acute elevation of T and CORT in *Rhinella jimi* toads from the Caatinga region during the reproductive period. We predicted that increasing androgens and CORT to plasma physiological levels observed during calling activity, *via* T and CORT

transdermal application respectively, would improve immune response. To test these predictions, we assessed: plasma CORT, T-and dihydrotestosterone (DHT) levels at baseline, as well as 1h and 10h after transdermal application; the BKA immune response at baseline as well as 1h and 10h after treatment); edema response to PHA injection after hormonal treatment; and the time-point of maximum swelling response to the PHA challenge.

3.3.Material and Methods

3.3.1. Field characterization and procedures

The field study was conducted at Fazenda São Miguel, a private area located near the city of Angicos, in the State of Rio Grande do Norte, Brazil (5°30'43"S. 36°36'18"W), within the domain of the Brazilian Caatingas. During the rainy season, anurans reproduce only when heavy rain occurs (150mm or higher – personal observation; Arzabe, 1999; Madelaire and Gomes, 2016). Adult *Rhinella jimi* males (N = 39) were collected between April 1st to April 24th 2015, during the reproductive period in that region (Madelaire and Gomes, 2016), but were not displaying calling activity. All individuals were found by visual inspection.

Once they were captured, we used heparinized 1 mL syringes with 26G×1/2 inch needle to obtain a 50µL blood sample taken by cardiac puncture less than 3 minutes after capture to avoid any interference of manipulation stress on plasma steroid levels (Romero and Reed, 2005). Blood samples were maintained on ice for up to 2 hours, and submitted to centrifugation for 5 min at 217 g (mini centrifuge for 6 tubes, MiniStar). Plasma samples were stored in cryogenic tubes and were kept in liquid nitrogen until they could be transferred to a -80°C freezer in the University of São Paulo, for hormone and bacterial killing ability assays, as described below ("Hormone plasma levels determination" and "Bacterial killing ability assay").

All individuals were weighted (precision 0.01g) and had their snout-vent length measured (0.01mm precision). Thereafter, toads were individually maintained in plastic containers (4.3L – 27.5 x 17.8 x 15.0 cm), with holes in the lid allowing air circulation. All animals had free access to water for 24 hours. After this period, hormonal transdermal treatment were applied.

3.3.2. Hormonal solution and transdermal application

Hormonal solutions were prepared as established by Assis et al. (2015). Working concentrations were defined on the basis of the steroid levels commonly found on *R. jimi* males during the breeding season and showing breeding activity (Madelaire and Gomes 2016) as well as being based on previous studies that performed transdermal steroids applications in ectotherms (Beillure et al. 2004; Wack et al. 2010; Assis et al. 2015). CORT working solution was prepared using 2.1 mg of corticosterone (Sigma - 27840) diluted in 210 µL of 99% ethanol. Afterwards, 1500 µL of sesame oil was added to the mix, which was homogenized to obtain a 1.4 µg/µL of CORT solution. For the T solution (4 µg/µL), 2 mg of testosterone propionate (Sigma - T1875) was added to 200 µL of 99% ethanol and 500 µL of sesame oil, resulting in a 4 µg/µL T solution. Steroid solutions remained in an open vial overnight to allow ethanol evaporation.

24 hours after capture, males were randomly divided into 5 experimental groups: Placebo (N = 9), 5.0 µL of sesame oil; CORT-Low (N = 7), 7.0 µg of corticosterone (5µL of corticosterone working solution); CORT-High (N = 7), 14.0 µg of corticosterone (10µL of corticosterone working solution); T-Low (N = 7), 12.0 µg of testosterone (3µL of testosterone working solution); and T-High (N = 7), 20.0 µg of testosterone (5µL of testosterone working solution). Transdermal hormone applications were always performed in the lab at 8:30pm, a time when males are usually calling on nights of reproductive activity (Madelaire and Gomes 2016; Madelaire et al. 2017). Blood samples were obtained by cardiac puncture 1h and 10h after applications. These blood samples were subsequently processed for plasma hormone level determination as well as BKA assays. Access to water was prevented one hour before and one hour after the treatment, in order to guarantee hormonal absorption. On the same night, after hormonal treatment and the obtainment of the 1h blood sample, animals were submitted to a PHA skin-swelling assay.

3.3.3. Phytohemagglutinin (PHA) immunological challenge

To assess cell-mediated innate immunity, individuals were submitted to a PHA immunological challenge, 1h after hormonal treatment. This assay uses a lectin derived from the red kidney bean (*Phaseolus vulgaris*) to elicit localized inflammation that reflects an organism's capacity to mount an immune response (Brown et al. 2011, Clulow et al. 2015). Hind limbs were measured using a thickness gauge (Digimess®, 0.01mm precision), and the hind fleshy base of the right foot was then injected with 10

µl of a 20 mg/ml solution of PHA (Sigma L8754) in saline using a 10 µl glass syringe and 30Gx1/2" needle. As a control, the hind fleshy base of the left foot of the same individual was injected with 10 µl of saline. The thickness of both feet was measured 12h and 24h after the injections, as established for this species in the study of Madelaire et al. 2017. Each measurement was repeated at least three times at each measurement time, and the mean of these values was used for subsequent calculations. The proportional swelling in response to PHA was calculated by dividing the maximum swelling value after injection by the first measure minus one [($S_{final}/S_{initial}$)-1].

3.3.4. Bacterial killing ability (BKA)

To assess individual humoral innate immune response based on soluble proteins, plasma samples were submitted to a BKA assay following the protocol of Assis et al. (2013). Each plasma sample was diluted in Ringer's solution (10 µl plasma: 190 µl Ringer) and mixed with 10 µl of *E. coli* working solution (~10^4 microorganisms). The negative control consisted of 210 µl of Ringer's solution, whilst the positive control was a mixture of 10 µl of *E. coli* working solution diluted in 200 µl of Ringer's solution. All samples and controls were incubated for 60 min at 37°C and, after the incubation period, 500 µl of tryptic soy broth (TSB) was added. Bacterial suspensions were thoroughly mixed and 300 µl of each was transferred in duplicate to a 96 wells microplate, which was incubated at 37°C for 2 hours. Subsequently, the samples' optical densities were measured hourly in a plate spectrophotometer (wavelength 600 nm), giving a total of 4 readings. The BKA was evaluated at the beginning of the bacterial exponential growth phase using 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.

3.3.5. Hormonal assay

Plasma samples were extracted with ether, according to Mendonça et al. (1996) and Madelaire et al. (2016). Each plasma sample was added to 3 mL of ether, which was then vortexed for 30 seconds and centrifuged (4°C, 9 min, at 217 g). Following this, samples were allowed to decant in a -80°C freezer for 7 min and the liquid phase was transferred to another tube. These tubes were kept in a laminar flow hood at room temperature, until all ether had evaporated (approximately 24h). The samples were re-suspended in EIA buffer and androgens and CORT were assayed using EIA kits

(Cayman Chemical® - Testosterone: item 582701; Corticosterone: item 500655), according to manufacturer's instructions. Intra-assay variation was estimated to be 13.02% for testosterone kits and 10.0% for CORT. Inter-assay variation was estimated using the average of four intermediate values from the standard curve (as recommended by the kit instructions) and was 19.3% for testosterone and 34.9% for CORT kits. Sensitivities of CORT and testosterone assays were 30 pg/mL and 6 pg/mL, respectively. The Cayman ELISA kit for testosterone display 27.4% cross reactivity with DHT, therefore the results refer to plasma testosterone plus dihydrotestoeterone (T-DHT) levels.

3.3.6. Ethic note

After experimental procedures, animals were euthanized with an intraperitoneal injection of sodium thiopental solution (25 mg/ml) (Thiopenthax®). All the experiments and fieldwork were conducted with the approval of the Comissão de Ética no Uso de Animais do IB (CEUA) (Protocol number: 181/2013), and Ministério do Meio Ambiente, ICMBio, SISBio (License number: N°29896-1).

3.3.7. Statistical analysis

Descriptive statistics were conducted for all variables. ANOVAs, t-tests, Qui-Square and Pearson correlation were carried out using IBM SPSS Statistics, Version 20.0 (IBM corp., 2011), after data had been transformed to meet the assumptions of data normality ($\log_{10}$; or $[\log_{10}(\text{data}+1)]$ for BKA), except for scope of plasma hormone levels and ΔBKA. The scope of plasma hormones levels was calculated by dividing the plasma hormone concentration (HC) after treatment (T = 1h and 10h) by hormone concentration on the field (basal) minus one $[(HC_T/HC_{BASAL})-1]$. The variation in BKA (ΔBKA) was calculated by subtracting the BKA presented by the plasma collected on the field (basal) from the BKA displayed 1h and 10h after the hormonal transdermal application ($BKA_T - BKA_{BASAL}$).

ANOVA was performed to compare body mass among groups. Independent t-tests were performed to compare the proportional edema from the feet injected with PHA and saline at different time-points (12h and 24h after the challenge). Given that some individuals displayed their maximum swelling response at different times (12h or 24h after treatment), we also performed independent t-tests to compare the maximum response in the right foot (PHA) *versus the* maximum response in left foot (saline).

Additionally, in order to verify if hormone treatment affected the time of the maximal swelling response to PHA, we performed a qui-square test assuming equal proportions for different time-points (12h and 24h) for the maximum swelling responses within groups (Placebo, T and CORT).

For each experimental group receiving transdermal application, repeated measures ANOVA followed by Bonferroni corrections were performed to evaluate differences among values of BKA, androgens (T-DHT) or CORT levels from field (basal), as well as 1h and 10h after hormonal application. To verify any differences in plasma hormone levels, as well as BKA and PHA response among treatment and Placebo groups, we performed ANOVA followed by Bonferroni corrections or t-tests between groups, comparing field baseline levels, 1h and 10h after transdermal application.

Additionally, we performed Pearson correlation tests to investigate the relationship between plasma hormone levels and immunological responses for each group. The variables placed in the matrix were: plasma hormone levels (basal, 1h and 10h after treatment), scope of plasma hormone levels (1h and 10h after treatment), maximum proportional swelling response to PHA, BKA (basal, 1h and 10h after treatment), and ΔBKA (1h and 10h after treatment). We also performed Pearson correlation tests for BKA and plasma T-DHT levels from the field (basal), as well as for BKA and plasma CORT levels from the field (basal).

3.4. Results

All descriptive statistics of studied variables are shown in Table 1. Body mass showed no differences among groups ($F_{4,36} = 2.37$, $P = 0.07$). For all groups, the PHA injected right foot, *versus* saline injected left foot, showed a swelling response 12h or 24h after the injection (12 hours: $t = 6.89$, $df = 72$, $P < 0.0001$; 24 hours: $t = 4.74$, $df = 72$, $P < 0.0001$). Maximum swelling response (%) in the right foot (PHA) occurred 12h or 24h after the injection, being higher than the maximum swelling response (%) in the left foot (saline) ($t = 6.88$, $df = 72$, $P < 0.0001$).

Plasma androgen (T-DHT) levels of the Placebo group did not differ between baseline, 1h and 10h after treatment time-points ($F_{2,26} = 1.45$, $P = 0.26$) (Figure 1). One hour after treatment, T-Low and T-High showed 3.7 and 9.2 fold higher plasma T-DHT levels, *versus* baseline respectively ($F_{2,20} = 12.43$, $P = 0.001$ for T-Low; $F_{2,20} = 25.01$, P

< 0.0001 for T-High). After 10h, all groups had similarly low plasma T-DHT levels ($F_{2,22}$ = 0.04, P = 0.96) (Figure 1). Despite the fact that baseline plasma T-DHT levels of the Placebo group were slightly higher than the T-Low and T-High groups ($F_{2,22}$ = 7.89, P = 0.003; Bonferroni P < 0.05), testosterone transdermal application increased plasma T-DHT levels after 1h in the T-Low and T-High groups, *versus* Placebo ($F_{2,22}$ = 10.82, P = 0.0007; Bonferroni P < 0.05) (Figure 1). Additionally, T-Low and T-High groups did not differ in plasma T-DHT levels at the1h time-point after treatment (Bonferroni P > 0.05).

Baseline plasma CORT levels in all groups showed similar values ($F_{2,19}$ = 1.57, P = 0.23). Plasma CORT levels from Placebo group were 3.2 times higher at 1h after treatment and 4.0-fold increased at 10h after treatment, *versus* baseline levels ($F_{2,26}$ = 13.19, P < 0.001; Bonferroni P < 0.01) (Figure 2). Individuals from CORT-Low and CORT-High treatments showed similar plasma CORT levels at 1h after hormonal application (t=0.19, df = 9, p=0.85; Figure 2), with plasma CORT levels raised by 12.5 and 12.3 fold respectively, *versus* baseline levels. After 10 hours, individuals from CORT-Low and CORT-High groups showed decreased plasma CORT levels, at a level similar to those observed in the Placebo group ($F_{2,20}$ = 0.95, P = 0.40) (Figure 2). Considering that plasma CORT levels from both CORT-Low and CORT-High did not differ at any time-points, these groups were clustered for correlation analyses and the time-point of maximal swelling response.

The proportional swelling response to the PHA challenge displayed marginally significant differences for testosterone treatment group only (Corticosterone treatment: $F_{3,33}$ = 1.57, P = 0.66; Testosterone $F_{2,20}$ = 3.30, P = 0.057, not indicated by Bonferroni). A t-test was subsequently performed, which indicated that individuals from the T-High treatment presented with a smaller swelling response, compared to Placebo (t = 1.79, df = 14 P = 0.04) (Figure 3). Corticosterone treatment affected the time-point of the maximum swelling response to the PHA challenge. A higher proportion (78.6%) of individuals in this group displayed a maximum swelling response 12h after the PHA challenge (X^2 = 4.57, df = 1, P = 0.03). The other treatments (Placebo and Testosterone) did not affect the time of maximum swelling (Placebo X^2 = 0.11, df = 1, P = 0.74, Testosterone X^2 = 1.14, df = 1, P = 0.29) (Figure 4).

Baseline plasma BKA did not differ between groups ($F_{4,35}$ = 2.22, P = 0.09). Plasma BKA did not change over the time of sampling for all treatments (Placebo: $F_{2,23}$ = 2.82,

P = 0.81; T-Low: $F_{2,20}$ = 0.05, P = 0.95; T-High: $F_{2,20}$ = 1.32, P = 0.30; CORT: $F_{2,20}$ = 2.85, P = 0.09; CORT-High: $F_{2,20}$ = 0.71, P = 0.51) (Figure 5A and 5B).

The swelling response to PHA, plasma BKA and ΔBKA did not have any correlation with T-DHT hormone variables (plasma hormone levels basal, 1h and 10h, nor with the scope of plasma hormone levels at 1h and 10h) (P > 0.05).

When Placebo, CORT Low and CORT high groups were analyzed together, individuals showing higher plasma CORT levels also presented with higher BKA at baseline (Pearson: 0.443, P = 0.025) (Figure 6A). Individuals showing higher plasma CORT levels, basally and 1h after treatment, were characterized by a lower BKA response 10h after the treatment (ΔBKA) (Pearson: -0.637, P = 0.035; Pearson: -0.612, P = 0.045, respectively) (Figure 6B and 6C). The scope of the plasma CORT levels at 1h after treatment was also negatively correlated with BKA 1h after treatment in the Placebo group (Pearson: -0.825, P = 0.006) (Figure 6D). PHA did not show any correlation with CORT hormone variables (plasma hormone levels basally, 1h and 10h after treatment, as well as the scope of the plasma hormone plasma levels at 1h and 10h) (P > 0.05).

3.5. Discussion

Both hormonal treatments (T and CORT) successfully increased plasma T-DHT and CORT levels, respectively. Additionally, captivity was a meaningful stressor that increased CORT plasma levels of individuals from the Placebo group. Individuals treated with a higher dose of T raised plasma T-DHT levels, achieving values similar to those measured for this species during the dry season. Moreover, individuals treated with the higher dose of T also showed lower swelling response to the PHA challenge. Transdermal testosterone did not affect the time-point of the swelling response to PHA and plasma BKA. CORT treatment had no impact on BKA or PHA proportional swelling, but accelerated the time of the maximal swelling response. Whilst individuals showed a positive correlation between basal BKA and CORT, the correlation pattern reversed after treatment, with individuals displaying a higher plasma CORT plasma level and 1h after treatment, showing a lower BKA response (ΔBKA) 1h and 10h after treatment.

For T-treated individuals, plasma androgen levels increased, up to 9.2 fold 1h post-application. However, due to abnormally low basal T-DHT levels in this reproductive season (Table 1), compared to 2011 (mean±sd 30.3±17.9 ng/ml, Madelaire et al. 2017), 2013 (26.1±26.9 ng/ml), and 2014 (26.7±23.0 ng/ml) (Madelaire C.B. unpublished data), the plasma T-DHT levels after treatment did not achieve typical reproductive levels (Table 1). In fact, plasma androgen levels after treatment were comparable to those measured for this species during the dry season (2011: 5.7±8.4 ng/ml, Madelaire et al. 2017). Inter-annual variation in plasma T levels has previously been reported for ectotherms (Schuett et al. 2005; Lind and Beaupre, 2014), suggesting that low plasma T-DHT basal levels may be also attributed to some atypical environmental condition in that year (Tood et al. 2010). Factors such as temperature, rainfall, social environment and barometric pressure can influence reproductive behavior (Burmeister and Wilczynski, 2000; Oseen and Wassersug, 2002; Saenz et al. 2006) and plasma T levels (Rastogi et al. 1978; Sun et al. 2011). In this specific year, one or more of such factors may have disrupted the reproductive cycle of these *R. jimi* individuals. Environmental cues that can modulate reproductive behavior and plasma T levels in anurans from the Caatinga requires investigation.

Testosterone treatment decreased the inflammatory response to the PHA challenge, but had no effect on the time of the response (12h or 24h). Previous research is controversial as the effects of androgens and reproductive season on the pro-inflammatory capacity. Desprat et al. (2016) reported a higher swelling response to the PHA challenge after transdermal testosterone application in *Hyla arborea* males with higher body mass; and *R. jimi* displayed a higher swelling response to PHA during the reproductive season, when T-DHT levels were high (Madelaire et al. 2017). The PHA challenge response is driven by increased pro-inflammatory cytokine expression (Vinkler et al. 2014) and the subsequent infiltration of leukocytes into the site of local inflammation (Brown et al. 2011; Vinkler et al. 2012; Clulow et al. 2015). Previous studies investigating the effects of androgens on the inflammatory response indicate an attenuation of cytokine production. However, heightened levels of wound inflammation, including inflammatory cell numbers and pro-inflammatory cytokine levels, have also been reported (Malkin et al. 2003 for review; Giliver, 2010). It is important to note that most leukocytes involved in the PHA response, such as neutrophils and lymphocytes,

express androgen receptors (Giliver, 2010), which may contribute to the variation in the modulatory effect of T on the inflammatory response in different species and contexts.

The effects of testosterone on the immune system may be dose-dependent and/or time-dependent. Macrophages from mice, wall lizards (*Hemidactylus flaviviridis*) and fresh water snakes (*Natrix piscator*) treated with T, decreased nitrite secretion (a marker of macrophage cytotoxicity) in a dose and time-dependent manner (Savita and Rain, 1998; Mondal and Rai, 2002; Tripathi and Singh, 2014). Macrophages from the wall lizards and fresh water snakes treated with T *in vitro* decreased phagocytosis (Mondal and Rai, 2002; Tripathi and Singh, 2014), with this decrease being dose-dependent in the fresh water snake (Tripathi and Singh, 2014). *Rhinella jimi* males maintain high T-DHT levels for months during the reproductive season (Jan to May) and concomitantly a heightened immune response (Madelaire et al. 2017). Tripathi and Singh (2014) found a biphasic effect on the activation of oxidative burst after T treatment. Superoxide anion release by macrophages decreased at 10 $ng.ml^{-1}$ and 1000 $ng.ml^{-1}$ testosterone concentrations, but was unaffected at 100 $ng.ml^{-1}$ (Tripathi and Singh, 2014). These results indicate that very low T levels can be immunosupressive. As already discussed, the plasma T-DHT levels reached in our experiments 1h after treatment were low and comparable to plasma levels previously found in *R. jimi* during the dry period (Madelaire et al., 2017). Moreover, the number of leukocytes and the inflammatory response are lower during the dry season than those measured during the reproductive period (Madelaire et al. 2017). In this way, the decreased inflammatory response following transdermal T application in the present study is consistent with the results naturally found in these toads during the dry, non-reproductive season in the Caatinga.

Twenty four hours captivity increased plasma CORT levels in the Placebo group up to 4.0 fold. Handling and maintenance in captivity are considered a meaningful stress stimulus for anurans (Zerani et al. 1991; Coddington and Cree, 19995; Narayan et al. 2011; 2012; Assis et al. 2015). Despite captivity increasing plasma CORT levels, these levels were not as high as when individuals are calling or following transdermal CORT application (Madelaire and Gomes, 2016; Assis et al. 2017). Additionally, no effects on immunological variables were observed in the Placebo group after the captivity period. These results are in agreement with Assis et al. (2015), who showed that no immunological changes were evident in *R. icterica* over 24h of captivity.

Transdermal CORT application increased plasma CORT levels to the mean values evident when males are calling during breeding activity (Table 1) (2011: 65.3±61.7 ng/ml, data from Madelaire et al. 2017). Corticosterone treatment had no effect on the intensity of edema response, but accelerated the time to maximum swelling. Glucocorticoids can influence function, traffic and transmigration of leukocytes to inflammatory sites, stimulating the clearance of pathogens and foreign elements (Pickford et al. 1971; Dhabhar et al. 1995; Dhabhar et al. 2012, Dhabhar, 2014). The immune enhancing effects of acutely elevated CORT levels include a rapid and robust immune response, mediated *via* an increased cytokine secretion, enhanced leukocyte infiltration in lesioned areas, upregulated maturation and activation of immune cells, as well as the enhanced recruitment of surveillance T cells (Dhabhar and McEwen, 1996; Viswanathan et al. 2005, Dhabhar, 2014). Additionally, CORT inhibits melatonin secretion during the pro-inflammatory response in rats (Markus et al., 2007), which also favors leukocyte transmigration to inflammatory sites (Lotufo et al., 2001; Fernandes et al., 2009). Accordingly, Barsotti et al (2017) found higher plasma CORT levels and concomitantly decreased ocular melatonin levels in the tree frog, *Hypsiboas faber*, 1h after adrenocorticotropic hormone. These mechanisms might contribute to the accelerated swelling response to the PHA challenge observed in *R. jimi* treated with CORT, suggesting an accelerated efficacy of the inflammatory resolution process.

Transdermal CORT treatment had no effect on BKA. The high variation of individual response after CORT treatment (plasma CORT levels ranged from 16.8 to 211.3 ng/ml at 1h post-treatment) may have contributed to the lack of a statistically significant difference regarding CORT effects on BKA across groups. High individual variation in glucocorticoid stress response is reported for many animal groups (Cockrem, 2013), which may be associated with survival in changing environmental conditions (Angelier and Wingfield, 2013). Our results suggest short-term and relatively long-term effects of CORT on the immune response mediated by proteins of complement system. Individuals with higher plasma CORT levels in the field have a higher BKA, indicating that moderately high levels of CORT may stimulate immune responsivity (Bene et al. 2014; Dhabhar, 2014; Muñoz-Durango, 2015; Assis et al. 2017). There is an extensive biomedical literature demonstrating that the pro-inflammatory response can be mediated by mineralocorticoid receptors (MR) activation, with MR antagonists reversing this effect (Muñoz-Durango, 2015 for review). The

moderately high plasma CORT plasma in the field may mediate long-term effects on plasma BKA by interfering with the genomic expression of the complement proteins produced in the liver and by immune cells (Morgan and Gasgue, 1997; Laufer et al. 2001; Buttgereita and Scheffoldon, 2002; Lubbers et al. 2017).

Transiently increased plasma CORT levels, resulting from acute stressors, such as moderate exercise, usually increase immune function (Dhabhar, 2014). Calling behavior is an energetically expensive aerobic activity for male anurans (Taigen and Wells, 1985; Grafe, 1996; Grafe and Thein, 2001; Wells, 2007) with calling effort usually positively correlated with plasma CORT levels (Assis et al., 2012; Narayan et al. 2013; Leary, 2014). Therefore, high plasma CORT levels during calling activity may show an immune-protective effect for anuran males, which are far more exposed to injuries by predators or competitors in these conditions (Ryan et al. 1981). However, individuals with higher plasma CORT plasma under basal conditions, and 1h after treatment, have a lowered BKA response at 1h and 10h following transdermal CORT. These short-term effects of acute CORT doses on BKA may be the result of interference in the complement system regulatory factors, which control complement system activity (Colten and Strunk, 1993; Laufer et al. 2001; Abbas et al. 2014; Lubbers et al. 2017).

In summary, transdermal T treatment acutely increases plasma T-DHT to the levels usually found in these toads during the non-reproductive dry period in the Caatinga. The decreased inflammatory response following transdermal T application is consistent with the results found in wild toads during the dry season. The experimental elevation of T-DHT to reproductive levels, and how this modulates the immune requires investigation. CORT treatment accelerated the maximum swelling response to PHA, which may indicate a glucocorticoid-mediated increase in the efficiency of the immune response. Additionally, CORT treatment did not significantly modulate the response to BKA or the intensity of PHA swelling across groups. However, individuals displaying moderately higher plasma CORT levels at baseline in the field were also characterized by higher plasma BKA. Of note, individuals characterized by higher plasma CORT levels in the field and at 1h after treatment, showed a lower BKA response at 1h and 10h after treatment, indicating possible chronic and acute effects of high plasma CORT

levels in modulating the immune function mediated by the proteins of the complement system.

3.6. Acknowledgements

We are grateful to F. Assis for his support during the fieldwork and to Dr. P.A.C.M. Fernandes for his intellectual contributions to the experimental design. The authors declare no competing or financial interests. All authors conceived the study, designed the experiments, and contributed substantially to interpreting the data. C.B.M. and B.O.C. collected the data; C.B.M. analyzed the data; All author wrote and reviewed the manuscript and take full responsibility for the content of the article. This research was supported by the State of São Paulo Science Foundation (FAPESP) through grant 2013/00900-1 led by F.R.G. and a Doctorate's Fellowship awarded to C.B.M. (2012/24206-4).

3.8. Table

Table 1. Descriptive analysis of steroid plasma levels, immunological and morphological variables of *Rhinella jimi* males from Placebo, T-Low, T-High, CORT- Low and CORT-High groups.

	Placebo		T-Low		T-High		CORT-Low		CORT-High	
Variables	**N**	**Mean±SD**	**N**	**Mean±SD**	**N**	**Mean±SD**	**N**	**Mean±SD**	**N**	**Mean±SD**
T-DHT basal (ng/ml)	9	2.3±0.9	7	1.1±0.9	7	1.0±0.8	-	-	-	-
T-DHT 1h (ng/ml)	9	1.9±0.8	7	5.1±3.1	7	10.0±6.4	-	-	-	-
T-DHT 10h (ng/ml)	9	1.7±0.9	7	1.5±0.5	7	1.7±0.9	-	-	-	-
Scope T after 1h	9	-0.01±0.86	7	6.7±7.0	7	17.0±18.1	-	-	-	-
Scope T after 10h	9	-0.20±0.39	7	0.91±0.88	7	1.8±2.5	-	-	-	-
CORT basal (ng/ml)	9	2.4±1.3	-	-	-	-	6	5.6±3.2	5	4.8±6.5
CORT 1h (ng/ml)	9	10.2±8.7	-	-	-	-	5	74.3±81.3	6	65.4±19.7
CORT 10h (ng/ml)	9	12.0±6.3	-	-	-	-	6	20.4±25.9	6	26.3±18.4
Scope CORT after 1h	9	6.8±11.2	-	-	-	-	5	18.3±17.9	5	88.5±60.9
Scope CORT after 10h	9	-0.71±0.33	-	-	-	-	6	-0.52±0.39	5	-0.48±0.97
PPHA (%)	9	6.7±3.1	7	8.6±4.3	7	4.3±3.3	7	3.9±2.8	7	9.5±6.3
BKA basal (%)	9	58.0±40.8	7	55.3±45.6	7	79.3±35.8	7	93.0±4.7	7	39.0±42.7
BKA 1h (%)	9	73.9±34.0	7	50.0±46.2	7	66.0±37.0	7	66.1±43.5	7	60.7±44.5
BKA 10h (%)	9	72.3±35.0	7	48.2±41.3	6	58.3±46.1	7	54.3±48.5	7	55.1±37.5
Body mass (g)	9	108.7±21.2	7	118.7±17.6	7	82.4±35.8	7	119.1±19.8	7	102.7±15.6
SVL (mm)	9	105.5±6.9	6	113.6±4.8	7	101.3±5.5	7	98.3±23.6	7	102.8±4.1

N = Valid N; SD = standard deviation; CORT = corticosterone plasma levels; T-DHT = androgens plasma levels; basal = colleted in the field; 1h = 1 hour after treatment; 10h = 10 hours after treatment; PPHA = Proportional swelling response to PHA; BKA = bacterial killing ability; SVL = snout vent length.

3.9. Figures

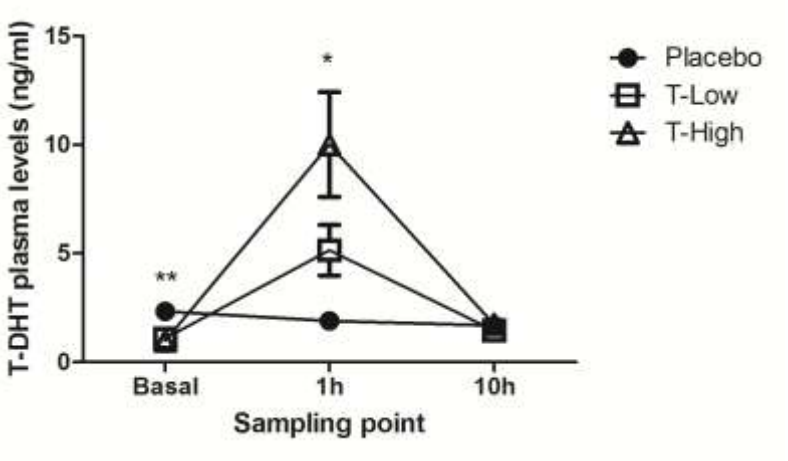

Figure 1. Mean ± standard error of androgens plasma levels basal, 1 and 10 hours after treatment in males of *R. jimi* from Placebo (N = 9), T-Low (N = 7), T-High (N = 7). One asterisk indicates significant difference compared to Placebo. Two asterisks indicates significant difference of Placebo compared to experimental groups.

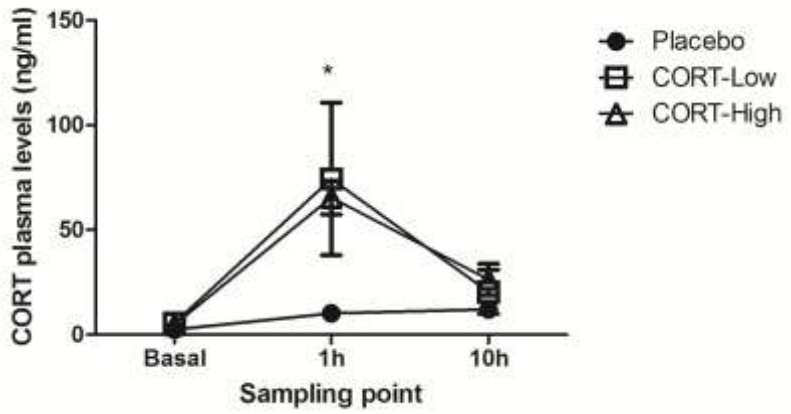

Figure 2. Mean ± standard error of corticosterone plasma levels basal, 1 and 10 hours after treatment in males of *R. jimi* from Placebo (N = 9), CORT-Low (N = 7), CORT-High (N = 7). Asterisk indicates significant difference compared to placebo.

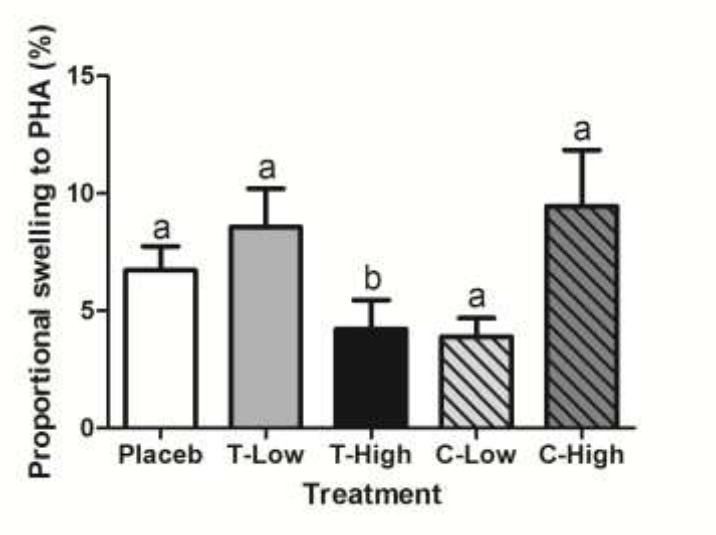

Figure 3. Mean + standard error of proportional swelling response to PHA challenge in males of *R. jimi* from Placebo (N = 9), T-Low (N = 7), T-High (N = 7), CORT-Low (N = 7), CORT-High (N = 7). Different letters indicates significant differences compared to placebo.

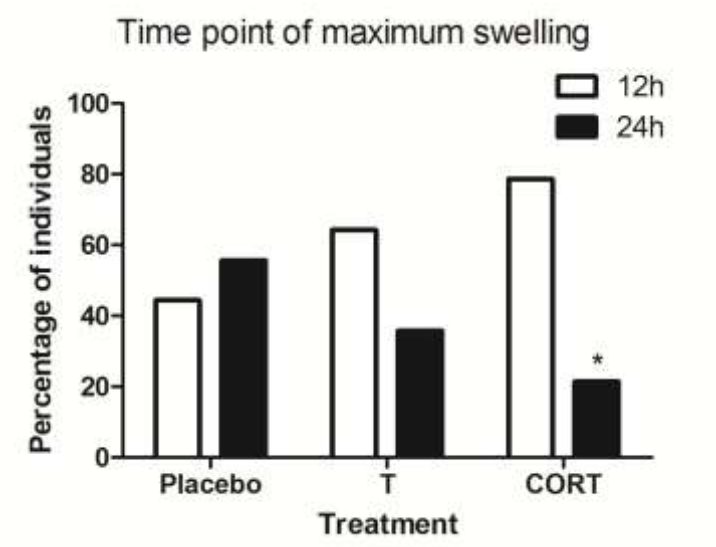

Figure 4. Proportion of males of *R. jimi* displaying maximum swelling response 12 and 24 hours after injection of PHA. Individuals were treated with transdermal application of Saline (Placebo - N = 9), Testosterone (N = 14) and Corticosterone (N = 14) 1 hour before PHA injections. Asterisks indicate significant difference of proportions within groups.

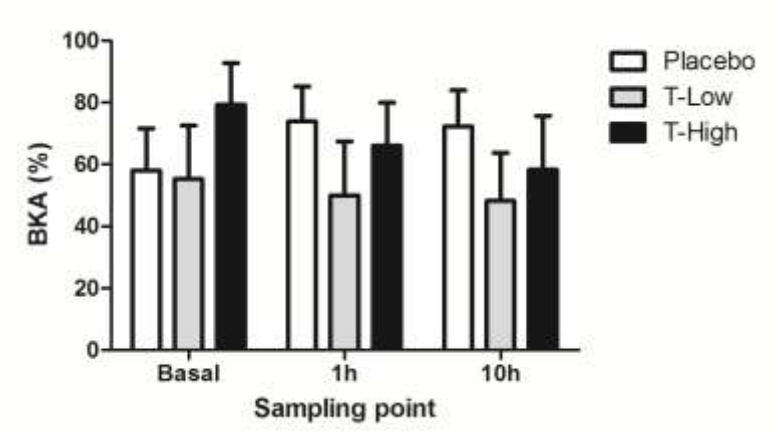

Figure 5A. Mean + standard error of bacterial killing ability basal, 1 and 10 hours after treatment in males of *R. jimi* from Placebo (N = 9), T-Low (N = 7), T-High (N = 7).

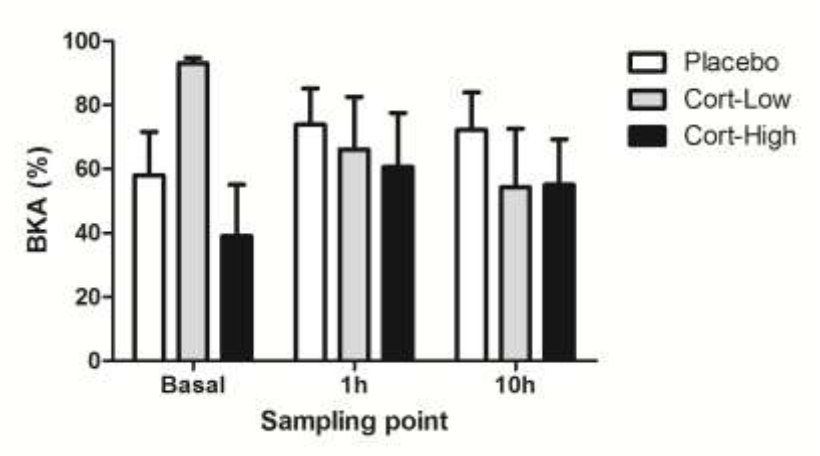

Figure 5B. Mean + standard error of bacterial killing ability basal, 1 and 10 hours after treatment in males of *R. jimi* from Placebo (N = 9), CORT-Low (N = 7), CORT-High (N = 7).

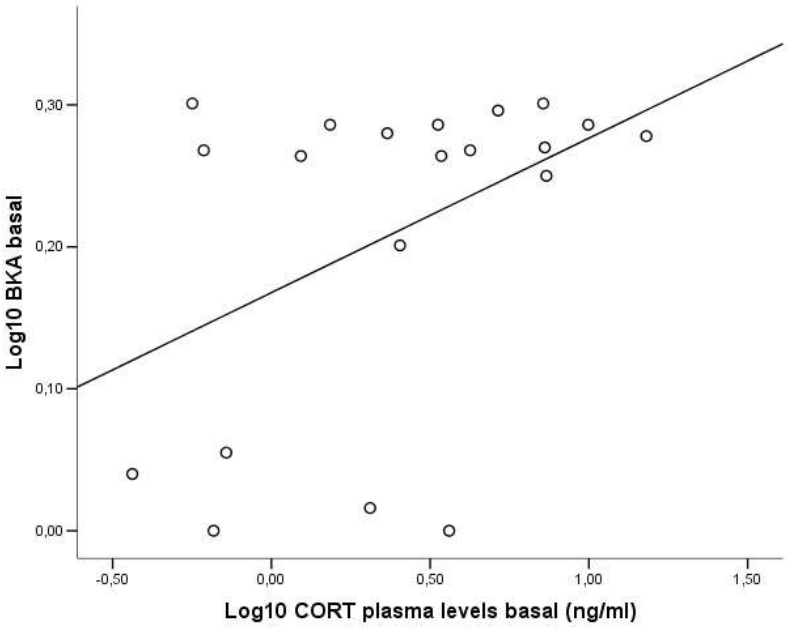

Figure 6A. Correlation between BKA basal and basal corticosterone plasma levels in males of *R. jimi* (N = 23). Line shown indicates best fit. $R^2 = 0.20$.

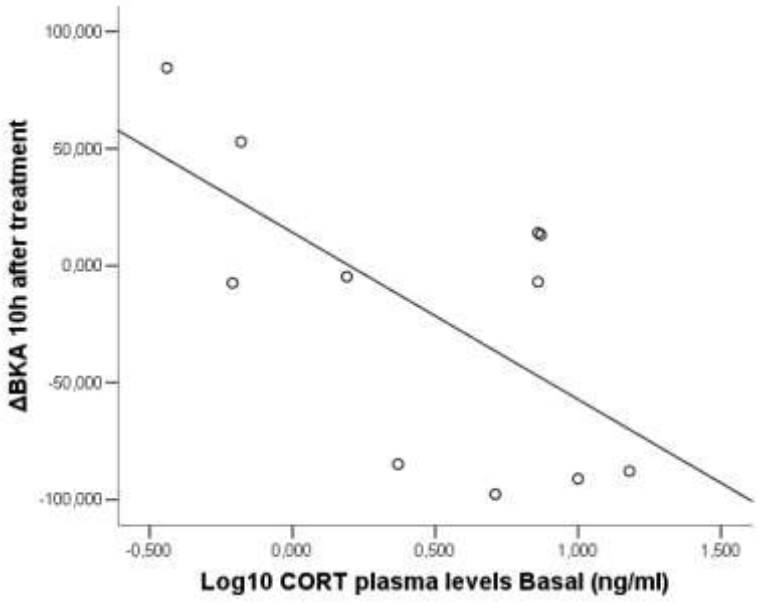

Figure 6B. Correlation between ΔBKA levels ($BKA_{10\ hour\ after\ treatment}$ - BKA_{BASAL}) and basal corticosterone plasma levels in males of *R. jimi* from corticosterone treatment (N = 11). Line shown indicates best fit. $R^2 = 0.406$.

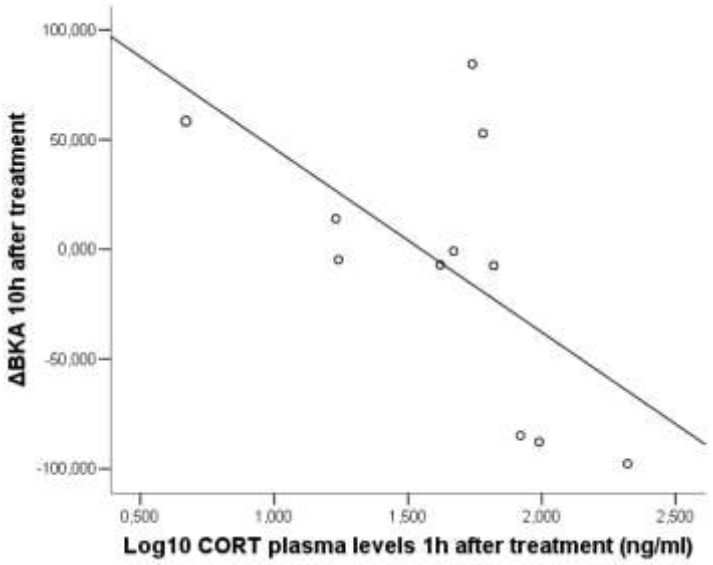

Figure 6C. Correlation between ΔBKA levels ($BKA_{10\ hour\ after\ treatment}$ - BKA_{BASAL}) and corticosterone plasma levels 1h after treatment in males of *R. jimi* from corticosterone treatment (N = 11). Line shown indicates best fit. $R^2 = 0.374$.

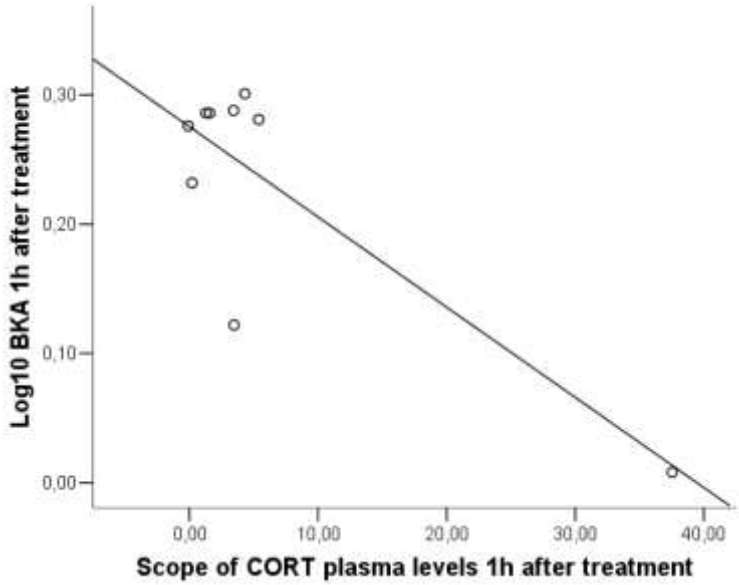

Figure 6D. Correlation between BKA 1h after treatment and scope of corticosterone plasma levels [($CORT_{1\ hour\ after\ treatment}$/$CORT_{BASAL}$)-1] in males of *R. jimi* from placebo group (N = 9). Line shown indicates best fit. $R^2 = 0.681$.

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4.1. Immune response, metabolic cost to inflammation and steroid plasma levels in contrasting annual life-history stages of toads in semi-arid region

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Keywords: standard metabolic rate; bacterial killing ability; PHA; seasonality; anura;

Caatinga

4.2. Abstract

Steroids hormones (e.g. androgens and corticosterone) are involved in complex physiological functions such as reproduction, energy mobilization, metabolism and immune modulation. Fluctuations on environmental energy availability can interact with the effect of these steroids, especially on the immunocompetence. We studied immune parameters (bacterial killing ability, response to phytohemaglutinin [PHA] injection, parasite load), standard metabolic rate (SMR), metabolic rate after PHA challenge and steroid plasma levels in two different phases of the annual life-history cycle (reproductive and non reproductive period) of a toad (*Rhinella jimi*) from a highly seasonal environment (the Brazilian semi-arid, Caatinga). During reproductive season, toads displayed high androgens and corticosterone plasma levels, higher metabolic rate after challenge; higher bacterial killing ability (BKA) compared to the dry period. These results indicate that during reproductive period toad´s immune system may be more activated and do not corroborate a possible trade-off between reproduction and immunocompetence. Individuals showing higher edema and lower metabolic cost to PHA challenge also displayed higher CORT plasma levels, indicating that this hormone could be associated to immunoenhancing effect and a facilitation to immune response process.

4.3. Introduction

Steroids hormones, such as androgens and glucocorticoids, are necessary for development and maintenance of male primary and secondary sexual characteristics (Goldey and van Anders 2015) and mobilization of energy stores to cope with the costs of reproduction (Buchanan 2000; Sapolsky et al. 2000). These hormones also play complex roles on the modulation of the immune system performance (Roberts et al. 2004, Dhabhar, 2009).8 For instance, the effects of high testosterone (T) plasma levels in immune response display nuances from immunossupressive (Mills et al. 2010; Pap et al. 2010; Peter et al. 2000; Casto et al. 2001; Belliure et al. 2004; Merril et al. 2015) to immune enhancing effects (Evans et al. 2000; Peters 2000; Roberts et al. 2007; Pap et al. 2010). T can bind to immune cells receptors (Slater et al. 1995) and alter activation and traffic of leukocytes, and release of antibodies and cytokines (Slater et al. 1995; Bebo et al. 1999; Peter et al. 2000; Ashcroft and Mills 2002; Pap et al. 2010; Desprat et al. 2015). More recently, studies have demonstrated that effects of T on the immune response can be dependent on body condition and feeding supplementation, with individuals in better body condition displaying higher T plasma levels and immunocompetence (Lochmiller & Deerenberg 2000; Alonso-Álvarez & Tella 2001; Ruiz et al. 2010; Husak et al. 2016). Glucocorticoids also affect the activation of immune cell (e.g. phagocytosis) and can increase, diminish or cease the release of cytokines, culminating on bidirectional and complex effects on immune performance (DeRijk et al. 2002; Bene et al. 2014; Dhabhar, 2014; Muñoz-Durango, 2015; Assis et al. 2017). Acute high levels of corticosterone (CORT) usually result in enhanced immune response (Dhabhar 1996, Dhabhar and McEwen 1999, Bowers et al. 2008; Dhabhar, 2014). Still, chronically elevated levels of CORT displayed when animals face constant source of adverse conditions, like prolonged environmental stress (French et al.

2010; Graham et al. 2012; Creel et al. 2013), usually result in immunosuppression (Dhabhar and McEwen 1999; Dhabhar 2000; Sapolsky et al. 2000; Dhabhar 2009, French et al. 2010). As previously described for androgens, glucocorticoid effects on immunocompetence can be also dependent on energetic status (French et al. 2007). Wound healing rate is suppressed in female lizards (*Urosaurus ornatus*) under food-restricted diet (French et al. 2007b) and bacterial killing ability decrease in snakes (*Thamnophis elegans*) under chronic stress and food-restricted diet (Neuman-Lee et al. 2015).

The reproductive steroids also can indirectly affect metabolic rates. Experimental evidences for the effect of increased steroid (T, CORT) plasma levels on metabolic rates show contradictory results. Higher T and CORT plasma levels have been shown to increase (Buchanan et al. 2001; DuRant et al. 2008; Preest and Cree, 2008; Wack et al. 2012), decreased (Wikelski et al. 1999; Miles et al. 2007) or display no effects on standard metabolic rates (SMR) (Wikelski et al. 1999; Buttemer and Astheimer, 2000). Otherwise,, many studies have shown that immunological challenges increase rates of oxygen consumption (Ots et al. 2001; Martin et al. 2003). In fact, the energetic cost to mount an immune response can be expensive and might potentially constrain other fitness-relevant phenotypic characteristics (Martin et al. 2003; Sheldon and Verhulst, 1996), such as locomotor performance (Zamora-Camacho et al. 2015), body mass (Bonneaud et al. 2012), growth (van der Most et al. 2011) and reproduction (Martin et al. 2003). However, experiments in the wild and in the laboratory show that available energetic substrates and body condition have a decisive role to determine if trade-off between immune response and other fitness relevant characters will manifest or not (French et al. 2007b; Neuman-Lee et al. 2015; Norris and Evans 2000; Reznick et

al. 2000; Smith et al. 2017). Forbes et al. (2016) found that voles maintained in large outdoor enclosures and receiving food supplementation mounted stronger immune responses against intestinal nematode infections than food-limited voles. Thus, the ecological context and fluctuations in energy resources are relevant challenges faced by individuals. Highly seasonal environments, such as arid and semi-arid areas, usually display a period of shortage of energy resources and individuals reproduce when resources are abundant (Sullivan, 1989; Chesson et al. 2004; Dayton and Fitzgerald, 2005). These systems are especially interesting to study the ecological relevance of energetic trade-offs.

The Brazilian semi-arid, the Caatinga, is a highly seasonal environment. Rain season (January-May) is when the available energetic resources are higher in this environment, and anurans display elevated plasma levels of testosterone and dihydrotestosterone (T-DHT) (Madelaire and Gomes, 2016). However, the reproductive event (when males call to attract females and CORT plasma levels are high) only occurs when heavy rains (100mm) (Madelaire and Gomes, 2016). Rain and consequently anuran reproductive events may not occur in some years in this environment (C.B.M. personal observation). During the dry period (August–November) there is a shortage in water and food sources and plasma levels of both androgens and CORT are low (Madelaire and Gomes, 2016). We studied the SMR, immune response, metabolic cost of an immunological challenge and steroid plasma levels between two different and contrasting phases of the annual life-history cycle (reproductive and non reproductive period) of a toad (*Rhinella jimi*) from the Caatinga. We expected increased metabolic rates after imposing an immunological challenge. We also expected immune response to be more prominent during reproductive period and positively correlated to steroid

plasma levels, when compared to dry season. Although, the effects of elevation of T and CORT plasma levels in SMR have been controversial in the literature (Buttemer and Astheimer, 2000; Wack et al. 2012 for reviews), we expected steroid plasma levels to be positively correlated to standard metabolic rate and metabolic cost to immunological challenge. To test these hypotheses, we assessed steroid plasma levels (CORT and T-DHT), immune parameters (bacterial killing ability, edema response to phytohemaglutinin [PHA] injection, endoparasite load), SMR and metabolic cost of immunological challenge (PHA challenge) in males of the toad (*R.jimi*) from the Caatinga region, during the reproductive season and dry period.

4.4. Material and Methods

Field and animal reproductive status characterization

Field work was conducted at Fazenda São Miguel, a private area located in the Municipality of Angicos, in the State of Rio Grande do Norte, Brazil (5°30'43"S. 36°36'18"W). This area is within the Brazilian semi-arid region, the Caatinga, characterized by high temperatures and seasonally concentrated precipitation. The annual average temperature from 1950-2000 is 26.6ºC (http://worldclim.org). January is the hottest month with an average temperature of 27.4°C (minimum: 22.8°C, maximum: 32.0°C), and July is the coldest month, with an average temperature of 24.3°C (minimum: 20.3°C, maximum: 28.3°C) (http://pt.climate-data.org/location/312354/). The rainy season occurs from January to May (96.4 mm of precipitation/month), while the dry season occurs from August to November (2.5 mm of precipitation/month).

Rhinella jimi males were visually located on the field and hand-collected during 3 different periods. A) within the reproductive season, when androgens (T-DHT) levels are high but CORT plasma levels are low (N= 24; March 21 to April 15, 2013); B)

during a reproductive event, when males are calling, and T-DHT and CORT plasma levels are high (N = 5; February 15 to 18, 2014); and C) outside the reproductive season, which is called dry period and T-DHT/CORT plasma levels are low, (N=8; October 25 to November 5, 2014; N = 8; September 2 to 14, 2014) (Madelaire and Gomes, 2016). We have annually monitored plasma levels of T-DHT and CORT in *R. jimi* during the reproductive and dry periods in 2011-2016. Despite some interannual variability, the plasma levels of T-DHT and CORT were consistent with the seasonal patterns reported above.

Blood sampling

Blood samples were collected by cardiac puncture using heparinized 1 mL syringes with 26G×1/2 inch needle. Blood samples were collected within 3 min of capture to avoid interference of manipulation stress on steroid plasma levels (Romero and Reed 2005). Blood samples were maintained on ice for up to 2 hours and then divided in two aliquots. One aliquot was used for quantification of the steroid plasma levels, and the second aliquot was used to the bacterial killing ability of plasma assay. To obtain plasma, blood was centrifuged for 5 min at 2000 x g (mini centrifuge for 6 tubes, MiniStar). Plasma samples were frozen in liquid nitrogen, transferred to the laboratory in the University of São Paulo and stored at -80°C.

Animal maintenance and metabolic rate measurements

Metabolic rate measurements were obtained only on periods A and C (reproductive and dry period). Animals were individually maintained in plastic containers with access to water for 24 hours after their capture in the field. In order to recover from handling stress, assume a resting posture, individuals were placed into

individual metabolic chambers (750 mL) two hours before measurements started. During this period, humidified air was pumped (100 $mL.min^{-1}$) into the metabolic chambers to prevent dehydration of the animals and to prevent occurrence of hypoxia and/or hypercapnia. Moistened cotton was also placed inside the metabolic chambers to guarantee hydration state during measurements (Gomes et al., 2004). The rates of oxygen consumption were measured using positive-pressure, flow-through respirometry (Withers, 1977). The air was pumped through the chambers at 100 $mL.min^{-1}$ using a Flowbar Multichannel – 8 pump system (Sable Systems; Henderson, NV, USA) to prevent hypoxia and hypercapnia. A control system of air flow with multiple channels (V3-Sable Multiplexer Systems; Henderson, NV, USA) was used to sequentially direct air from metabolic chambers, on the same stream, for an oxygen analyzer (FC-10a O2 analyzer-Sable Systems; Henderson, NV, USA). Data from the O_2 analyzer was recorded on a computer that was equipped with EXPEDATA 1.1.15 software (Sable Systems), via an UI2 interface (Sable Systems). We scrubbed air from the chambers of both water vapor and CO_2 via a column containing drierite/ascarite/drierite prior to gas analysis. We calculated rates of oxygen consumption ($V_{,}. O_2$) as:

$$V,O2 = V, * (FIO2 - FEO2) / (1 - FEO2),$$

where V, is flow rate ($mL.min^{-1}$ STP; Standard Temperature and Pressure) and FIO_2 and FEO_2 are the fractional O_2 concentrations in incurrent and excurrent air, respectively. A baseline measure was recorded 10-min before and after each animal recording. Air room temperature was recorded at 5 min intervals using one HOBO data loggers. V,O_2 was corrected for the mean temperature registered at the moment selected for calculations. EXPEDATA software was programmed to collects data of continuous readings for fractional O_2 concentration at rate of 1 $sample.second^{-1}$. Animals were

weighted to the nearest 0.001g at 0, 24, 36, 48 and 72 hours after the experiment started, and the mean of these values was used to express V,O_2 as $mLO_2.g^{-1}.h^{-1}$. All respirometry tests were videotaped using a webcam and only data correspondent to moments during which toads were at rest were considered for calculations. The room where the experiments were carried was maintained closed and only one person accessed the room (C.B.M.). During the day, the room was maintained with minimum natural light. The room was illuminated with red lamps during the night in order to not disturb the animals.

During the firsts 24 hours of experiment, we obtained 12 registers of 20 minutes each, totalizing 240 minutes for each individual. From each 20 minutes register we selected the 5 minutes period with the lowest continuous readings for fractional O_2 concentration to determine the standard V, O_2. After exclude registers that the individual was moving, the standard metabolic rate (SMR) for each individual consisted of the lowest value of the first 24 hours of measurements.

After the initial 24 hours, individuals were divided in two groups. One group had their right paws injected with phytohemagglutinin and the second group had their right paws injected with saline [method described in the Phytohemagglutinin (PHA) immunological challenge session)]. All individuals were then submitted to the respirometry test as described above for consecutive 50 to 55 hours. During these two days of experiment, we obtained 24 to 30 registers of 20 minutes each, totalizing 480 minutes for each individual. From each 20 minutes register we selected the 5 minutes period with the lowest continuous readings for fractional O_2 concentration to calculate standard V, O_2. The V,O_2 was corrected by temperature and body mass, as described above. Rates of oxygen consumption after saline or PHA injection ($V,O_{2Saline}$; V,O_{2PHA})

for each individual were calculated as highest V,O_2 from these 24 to 30 registers, after excluding registers when individuals were moving.

Phytohemagglutinin (PHA) immunological challenge

To assess cell-mediated innate immunity, hind limbs were measured using a thickness gauge (Digimess—0.01mm precision), and the hind fleshy base of right foot of the toads from the experimental group was then injected with 10 µl of a 20 mg/ml solution of PHA (Sigma L8754) in saline using a 10 µl glass syringes and 30Gx1/2" needles. Animals from the control group had their hind fleshy base of the right foot injected with 10 µl of saline. Injections were carried between 9:00pm to 00:00am. Individuals from each group were injected in sequence, and the order of individuals injected within groups was randomized. Individuals collected during reproductive event (N = 5; February 15 to 18, 2014) were not subjected to respirometry. After 24 hours from capture, they had their hind fleshy base of the right foot injected with 10 µl of PHA and the right foot injected with saline, as a control. The thickness of both feet was measured 12 and 24 hours after the injections. Each measurement was repeated at least three times at each measurement time, and the mean of these values was used for the calculations. Proportional swelling in response to PHA (EdPHA) and saline was calculated dividing the maximum swelling value after injection by the first measure minus one $[(S_{final}/S_{initial})-1]$.

Hormones plasma levels determination

Circulating plasma levels of androgens (T-DHT) and CORT were quantified using enzyme immunoassay kits (Testosterone ELISA Kit 582701; Corticosterone ELISA Kit 500655) from Cayman Chemical (Ann Arbor, MI). Assays were carried out

in accordance with the manufacturer's instructions. Before the assay, hormones were extracted from plasma samples (range from 3 to 20µl) by adding 3ml of diethyl ether ($C_4H_{10}O$), agitating for 30 seconds, and centrifuging for 9 minutes at 4°C (1800 rpm). Samples were then allowed to decant in -80°C freezer for 7 minutes and the liquid phase was poured in a new assay tube (Assis et al., 2015; Madelaire and Gomes, 2016). Samples were run in duplicate and 18 samples were run in each assay plate. Samples collected in animals in reproductive period were diluted 1:250 and 1:500; samples from animals collected in the dry period were diluted 1:20 and 1:40, as established by Madelaire and Gomes (2016). Sensitivity of the assay for testosterone was 6 pg/ml and for corticosterone was 30 pg/ml.

Animal euthanasia and dissection

Individuals were euthanatized with sodium thiopental solution (25 mg/ml) (Thiopenthax®), the snout-vent length was measured to the nearest 0.01 mm and fat bodies were weighted to the nearest 0.001g. All abdominal organs were examined for the presence of endoparasites under stereomicroscope (LM300B, SKU), and the parasites were counted and fixed in warm ethanol, formol, acetic acid (AFA), counted, and stored in individual tubes with 70% ethanol.

Statistical analysis

Descriptive statistics were conducted for all variables, and data was then transformed to $\log_{10}$ to meet the assumptions of data normality. The residuals of standard least-square linear regressions using snout-vent length as the independent variable and body mass, and fat bodies mass as dependent variables were used to calculate the body condition index and fat bodies index, respectively.

To verify if the PHA immunological challenge worked, we performed a t-test to compare the paw measures before the PHA injection, 12 hours and 24 hours after the injection. Additionally, we performed a t-test to compare the maximum proportional edema from animals injected with PHA to the maximum proportional edema from animals injected with saline. A t-test was performed to compare the BKA displayed by individuals during reproductive and dry period.

Additionally, we calculated the metabolic cost to PHA (MC_{PHA}) and saline (MC_{Saline}) injections, subtracting the V,O_{2PHA} and $V,O_{2Saline}$ from the respective SMR. We performed a t-test to compare SMR during reproductive and dry period. Paired t-tests were performed to compare SMR *versus* $V,O_{2PHA;}$ and SMR *versus* $V,O_{2Saline}$. Additionally, we performed t-tests to compare MC_{PHA} *versus* MC_{Saline} from reprodu2ctive; MC_{PHA} *versus* MC_{Saline} from the dry period; and MC_{PHA} *versus* MC_{Saline} between periods. For all t-tests we consider as significant P value < 0.05.

Using general linear models, we tested whether or not BKA, EdPHA, V,O_{2PHA}, MC_{PHA} and parasite load could be explained by period (Reproductive season [A] and Dry season [C]), CORT and androgen plasma levels (T-DHT); or by the additive/ interactive effects of hormones (Table 1). For BKA and EdPHA we included the category Breeding event [B] within the explanatory variable period, since we were able to collect these data when animals were calling (N = 5). Since these models have different number of parameters, we calculated the second-order Akaike information criterion (AICc; Akaike 1974), which penalizes the likelihood of a given model as a function of the number of parameters and corrects for low sample sizes. The AICc value (dAICc < 2.0) and the Akaike weight were used to determine which models had the most support. Akaike weight describes the relative strength of the evidence in support

of a particular model. The best model corresponds to the one with the lowest AICc value, providing a good fit to the data with the fewest parameters (Burnham and Anderson 2002). We also considered the Akaike weight in the power of explanation between competing models with dAICc < 2.0. T-test and ANOVA were carried out using SPSS 5.0 (SPSS, 1992) and general linear models were run in R software, version 2.10.0 (R Development Core Team, 2010).

Ethical note

All the experiments and fieldwork were conducted under approved permission of Comissão de Ética no Uso de Animais do IB (CEUA) (Protocol number: 140/2011), and Ministério do Meio Ambiente, ICMBio, SISBio (License number: 29896-1).

4.5. Results

Descriptive analyses of all variables are presented by season (Table 2).

PHA and saline immunological challenge

Individuals that had their paws injected with PHA displayed a swelling response after 12 hours (Reproductive period: $t = -6.7$, $df = 8$, $P < 0.01$; Dry period: $t = -4.2$, $df = 4$, $P = 0.013$; Breeding event: $t = -5.0$, $df = 4$, $P < 0.01$). When comparing the proportional swelling response to PHA and saline injection after 12 hours, only individuals that were injected with PHA displayed a swelling response (Reproductive period: $t = 5.1$, $df = 14$, $P < 0.01$; Dry period: $t = 4.8$, $df = 6$, $P < 0.01$; Breeding event: $t= -3.7$, $df = 8$, $P < 0.01$). No individuals from PHA or saline group displayed a swelling response after 24 hours of injection ($P > 0.05$).

Metabolic rate before and after PHA/saline challenge

Toads standard metabolic rate (SMR) did not differ between reproductive and dry period ($t = 0.5$, $df = 18$, $P = 0.31$). During both periods, all individuals injected with

PHA (V,O_{2PHA}) and saline ($V,O_{2Saline}$) increased their metabolic rates when compared to SMR (Reproductive: t = 5.3, df = 7, P < 0.001; t = 5.1, df = 3, P < 0.01, respectively; Dry: t = 5.1, df = 4, P < 0.01; t = 4.6, df = 2, P = 0.04) (Fig. 1). The V,O_{2PHA} and $V,O_{2Saline}$ were higher during reproductive period than during the dry period (t = 1.8 df = 16, P = 0.04; t = 2.9 df = 11, P < 0.01; respectively)(Fig. 1). During both periods, the MC_{PHA} was not different from the MC_{saline} (Reproductive: t = 0.3 df = 10, P = 0.4; Dry: t = 0.7 df = 6, P = 0.3) (Fig. 2). The MC_{PHA} was not different between periods (t = 1.0 df = 11, P = 0.2) and the MC_{saline} was higher during reproductive period compared to the MC_{saline} during the dry period (t = 3.1 df = 5, P = 0.01)(Fig. 2).

Bacterial killing ability

Toads displayed higher BKA against *E. coli* during the reproductive period than in the dry period (t_{37} = 2.331; P = 0.013) (Figure 3).

Relations between inflammatory response, parasite load, steroid plasma levels and season

Individuals displaying lower SMR also presented lower T-DHT and CORT plasma levels (Table 3; Figure 4-5). Despite the fact there was a selected model indicating influence of season on SMR (Table 3), a t-test indicated no differences in SMR between periods (results showed above; Figure 1). Individuals displaying lower V,O_{2PHA} also presented low T-DHT and CORT plasma levels (Table 3, Figure 6 and 7). Selected models also indicate influence of the season on the V,O_{2PHA} (Table 3), individuals display higher V,O_{2PHA} during reproductive period. Additionally, the MC_{PHA} was lower in individuals displaying higher T-DHT plasma levels (Fig. 8). The selected models also indicated a tendency of CORT plasma levels influencing MC_{PHA} data variation, although the plot between these variables does not show a clear pattern (Fig. 9). Selected models indicated a tendency of BKA being higher in males with higher T-

DHT levels and lower in individuals displaying higher CORT plasma levels (Table 3). However, the strong seasonal pattern of BKA (Figure 3), could be the predominant explanation, given that the large individual variation of steroid plasma levels did not show a clear pattern regarding BKA (Figure 10 and 11).The EdPHA was higher in individuals displaying higher CORT plasma (Table 3, Figure 12). Individuals with higher parasite load displayed higher T-DHT plasma levels, the selected models also showed a relation with period, although the direction of this relation is not clear (Table 3, Figure 13).

4.6. Discussion

Our results show a annual pattern of variation regarding metabolic responsiveness to immune stimuli and activity of proteins from complement system. During reproductive season, the oxygen consumed after PHA and saline injection, and the BKA was higher compared the dry period. There were no differences in metabolic cost to PHA (MC_{PHA}) challenge between reproductive and dry period, indicating that despite possible lower prey availability during the dry period, individuals are able to fully respond to an immunological challenge. The MC_{saline} was not different from the MC_{PHA} during reproductive and dry period, indicating that both stimuli were sufficient to increase the oxygen consumption. During reproductive period toad´s immune system were more activated, with individuals displaying higher BKA and higher metabolic rates in response to both stimuli: PHA and saline. Additionally, individuals showing higher EdPHA and lower metabolic cost to the inflammation process also displayed higher CORT plasma levels and indicating that this hormone could be associated to both immunoenhancing effect and lowering the cost to the immune response.

The increase of V,O_2 in response to PHA injection corroborates previous studies showing high energetic costs of mounting an immune response (Lochmiller and Deerenberg 2000, Ots et al. 2001, Brace et al. 2017). The PHA injection stimulate an inflammatory response, the influx of lymphocytes, neutrophils, basophils, eosinophils and macrophages to the local of the injury (Martin et al. 2003; Kennedy and Nager, 2006; Tella et al., 2008; Biard et al., 2009; Vinkler et al., 2010; Brown et al., 2011).

Although the metabolic cost to PHA challenge (MC_{PHA}) did not change between periods, V,O_2 in response to PHA challenge and saline was higher during reproductive season suggesting that this species display a more vigilant immune system during this period. BKA was also higher during reproductive period when compared to the dry period, showing that complement proteins and anti-microbial peptides present in the plasma are more expressed and active during reproductive period. Together, our results go in the opposite direction to the possible trade-off between immune response and reproduction. It is possible that the cost of displaying an immune response in detriment of other life history traits, e.g. reproduction, could depend on the amount of resources available (Bonneaud et al. 2003). Interestingly, for ectotherms characterized by low metabolic rates and cost of maintenance (Jorgensen, 1988), environmental resources available during reproductive season seems to be high enough to fade this possible trade-off even in a semi-arid region. In agreement with these results, we found that during reproductive period, individuals display higher fat body index and higher number of circulating leukocytes (Madelaire and Gomes, 2016; Madelaire et al. 2017).

The negative relationship between SMR and T-DHT we found in *R. jimi* was reported experimentally in male white-crowned sparrows (*Zonotrichia leucophrys gambelii*) (Wikelski et al. 1999). According to these authors, individuals displaying higher levels of testosterone invested more energy in locomotion and less energy in basic physiological processes. Additionally, Desprat et al. (2017) found that tree-frogs (*Hyla arborea*) treated with testosterone displayed lower respiration rates in mitochondria from trunk muscles, which could be interpreted as higher mitochondrial efficiency by limiting the proton leakage across the inner membrane. It is possible that males of *R. jimi* in better energetic conditions are able to increase T-DHT during reproductive period, benefiting from higher mitochondrial efficiency and directing higher energetic fluxes to reproduction and immune function. This hypothesis might be investigated through T-DHT treatment as well as by accompanying the relation between T-DHT plasma levels and mitochondrial efficiency at different life history stages. Our results also show a negative relationship between SMR and CORT in *R. jimi*. The relation between CORT plasma levels and SMR has been controversial in the literature (Wikelski et al., 1999; Preest and Cree, 2008; Miles et al. 2007; Wack et al. 2012), although experimentally increased CORT plasma levels decreased SMR of both side-

blotched lizards (*Uta stansburiana*) (Miles et al., 2007) and Coho salmon (Davis and Schreck, 1977). Although these results have been interpreted as adaptive because it might enhance survival during stressful episodes by minimizing energetic costs (Miles et al., 2007), the mechanisms involved on this relationship remain to be studied.

Toad´s oxygen consumption rates increased 5.6 fold and 4 fold in response to PHA challenge on reproductive and dry period, respectively. The consequences of diverting energy from other relevant processes to immune function can be severe 2(Sheldon and Verhulst 1996; Norris and Evans 2000; Bonneaud et al. 2003; Uller et al. 2006). The metabolic cost of the immune response after the PHA challenge in this study is within the magnitude of those associated to other activities such as active foraging, amplexus, and nest building in anurans (Bucher et al. 1982), indicating that immune response might also impose an energetic challenge to concomitant maintenance of other fitness-relevant activities to these animals. The energetic impact of an immune response to fitness related activities in *R. jimi* remains to be tested.

The higher swelling response to PHA challenge in individuals displaying higher CORT plasma levels is in accordance with an immunoenchancing effect of this steroid hormone. Acutely elevated levels of glucocorticoid are known by increase cell-mediated immunity, inflammatory response (Dhabhar 1996, Dhabhar and McEwen 1999), and trafficking of leukocytes (Dhabhar 1996; Bowers et al. 2008, Goessling et al. 2015). Assuming that individuals with a greater swelling response are those characterized by a greater level of immune efficiency (Kennedy and Nager, 2006), the integrative effects of high CORT plasma levels might increase fitness by favoring survival. The tendency that BKA is lower in individuals with higher CORT could be an indication of a possible trade-off between different arms of the immune system. However, a possible differential investment among immune responses remains to be tested.

Our results showing that CORT has role increasing the immune response (EdPHA) and lowering the energetic cost to inflammation resolution process (Fig. 7), facilitating the immune response process. In agreement with these results, Bastos, 2017 found that LPS injection in *R. icterica* induces systemic inflammation led to increased cytokine 1L-1β and concomitantly activates HPI axis, increasing CORT plasma levels by 10 fold. Individuals of *R. jimi* treated with CORT and challenged with a PHA

injection displayed the edema 12h earlier than individuals from placebo group (Madelaire et al. under review), indicating this hormone could also increase the efficiency of the immune response. Our results also show that individuals displaying higher T-DHT plasma levels also show lower energetic cost to the immunological challenge. Testosterone is known to increase immune response (Evans et al. 2000; Peters 2000; Roberts et al. 2007; Pap et al. 2010). However, the physiological mechanisms connecting this hormone to effects on metabolism and the facilitation of the immune response process remain to be elucidated.

Additionally, we found that individuals displaying higher parasite loads also display higher T-DHT plasma levels. Free-living lizards (*Psammodromus algiru*) implanted with silastic tube contained T displayed an increase of ectoparasite load compared to the control (Salvador et al. 1996). Testosterone is known to stimulate territorial behavior (Moore 1986; Sinervo et al. 2000), movement (Olsson et al. 2000; Sinervo et al. 2000; Cox et al. 2005; John-Alder et al. 2009), and hunting behavior success (Desprat et al. 2017). In this way, individuals displaying higher T levels might increase encounter rate with ecto-parasites (Boyer et al. 2010), as well as endo-parasites acquired directly by transdermal penetration and indirectly, by food ingestion. Results from the present study do not indicate immunossupression associated to T-DHT plasma levels on the examined immunological parameters (BKA and PHA), suggesting that better body conditions allows to maintain high T levels despite the high parasite load. Nevertheless, differential investments on immunological parameters could also result in higher BKA and PHA response at the cost of diminishing parasite immune response. These hypotheses remain to be tested in *R. jimi*.

4.7. Conclusions

Our results show a strong season pattern of variation metabolic responsiveness to immune stimuli and activity of proteins from complement system. During reproductive period toad´s immune system is more activated, with individuals displaying higher BKA and increased metabolic rates in response to both stimuli: PHA and saline. The absence of differences of MC_{PHA} between reproductive and dry period may indicate that despite possible lower prey availability during the dry period, individuals are able to fully respond to an immunological challenge. Individuals

showing higher EdPHA also display higher CORT plasma levels and the negative correlation between metabolic cost of immunological challenge and steroid levels may be an indication that these hormones increase and facilitate the immune response. Additionally, individuals displaying higher parasite load also had higher T-DHT plasma levels, along the BKA and EdPHA that show no immunosuppressive effect during reproductive period, may indicate that animals in better body conditions can maintain high T levels despite the high parasite load.

4.8. Acknowledgements

We are grateful to F. Assis for his support during the fieldwork. The authors declare no competing or financial interests. C.B.M. and F.R.G. conceived the study, designed the experiments, and contributed substantially to interpreting the data. C.B.M. and B.O.C. collected the data; C.B.M. analyzed the data; C.B.M. and F.R.G. wrote the manuscript and all authors reviewed it and take full responsibility for the content of the article. This research was supported by the State of São Paulo Science Foundation (FAPESP) through grant 2013/00900-1 led by F.R.G. and a Doctorate's Fellowship awarded to C.B.M. (2012/24206-4).

4.9. Tables

Table 1. Constructed models to test the relation between immunological parameters and explanatory variables period and plasma hormone levels.

TESTED MODELS
Variable ~ null
Variable ~ period
Variable ~ CORT
Variable ~ T-DHT
Variable ~ period + CORT
Variable ~ period + T-DHT
Variable ~ CORT + T-DHT + period
Variable ~ CORT*T-DHT + period

Table 2. Descriptive analyses of morphological, immunological and metabolic variables, parasite load, corticosterone and androgens plasma levels from *Rhinella jimi*, during reproductive, dry period, and breeding event.

Period	N	Reproductive	N	Breeding	N	Dry
Body mass (g)	35	173.88 ± 68.78	5	195.39 ± 77.07	22	128.71 ± 63.38
SVL (mm)	28	127.52 ± 15.17	5	135.35 ± 22.58	22	108.51 ± 16.42
Edema PHA (mm)	17	0.41 ± 0.25	5	0.68 ± 0.19	15	0.40 ± 0.33
Edema PHA (%)	17	0.07 ± 0.05	5	0.11 ± 0.05	15	0.07 ± 0.06
BKA (%)	26	0.82 ± 0.27	5	0.55 ± 0.51	16	0.56 ± 0.38
SMR ($V.O_2.ml.\ g^{-1}.h^{-1}$)	12	0.05 ± 0.03			8	0.04 ± 0.02
V,O_{2PHA} ($V.O_2.ml.g^{-1}.h^{-1}$)	13	0.28 ± 0.12			5	0.16 ± 0.09
$V,O_{2Saline}$ ($V.O_2.ml.g^{-1}.h^{-1}$)	10	0.27 ± 0.12			3	0.10 ± 0.04
MC_{PHA} ($V.O_2.ml.g^{-1}.h^{-1}$)	8	0.21 ± 0.09			5	0.12 ± 0.07
MC_{Saline} ($V.O_2.ml.g^{-1}.h^{-1}$)	4	0.20 ± 0.10			3	0.06 ± 0.02
Parasites (un)	22	38.82 ± 36.74	5	88.80 ± 117.05	14	35.86 ± 60.83
Androgens (ng/ml)	22	26.07 ± 26.92	5	26.74 ± 23.02	15	4.49 ± 5.76
Corticosterone (ng/ml)	22	1.58 ± 1.98	5	14.84 ± 7.58	15	1.22 ± 0.60

SVL = snout vent lenght; Edema PHA = edema in response to PHA chellenge; BKA = plasma bacterial killing ability; SMR = standard metabolic rate; V,O_{2PHA} = oxygen consuption in response to PHA challenge; $V,O_{2Saline}$ = oxygen consumption in response to saline injection; MC_{PHA} = metabolic cost to the PHA challenge; MC_{Saline} = metabolic cost to the saline injection; Androgens = androgens plasma levels; Corticosterone = corticosterone plasma levels.

Table 3. Selected models for immunological and metabolic variables and parasite load of *Rhinella jimi.*

Models	**AICc**	**dAICc**	**df**	**Weight**
SMR ~ CORT	3.1	0.0	3	0.23
SMR ~ CORT + T + P	3.4	0.3	5	0.20
SMR ~ CORT*T + P	3.7	0.5	6	0.18
SMR ~ T + P	4.1	1.0	4	0.14
SMR ~ T	4.6	1.5	3	0.11
SMR ~ CORT + P	4.8	1.7	4	0.10
V,O_{2pha} ~ T + P	5.4	0.0	4	0.56
V,O_{2pha} ~ CORT + T + P	7.4	2.0	5	0.21
MC_{pha} ~ CORT*T + P	12.8	0.0	6	0.39
MC_{pha} ~ CORT + T + P	14.1	1.3	5	0.21
MC_{pha} ~ T + P	14.6	1.8	4	0.16
Parasite load ~ T-DHT	44.6	0.0	3	0.54
Parasite load ~ T-DHT + P	46.5	1.9	5	0.21
EdPHA ~ CORT	-13.8	0.0	3	0.59
BKA ~ CORT*T-DHT + P	-64.2	0.0	7	0.26
BKA ~ CORT + T-DHT + P	-63.1	1.1	6	0.15
BKA ~ T-DHT	-62.8	1.4	3	0.13
BKA ~ P	-62.7	1.6	4	0.12
BKA ~ T-DHT + P	-62.4	1.8	5	0.10
BKA ~ CORT	-62.2	2.0	3	0.09

SMR = standard metabolic rate; V,O_{2PHA} = rate of oxygen consumption in response to PHA challenge; MC_{PHA} = metabolic cost to PHA challenge; EdPHA = Proporcional edema in response to PHA challenge; BKA = Bacterial killing ability; T-DHT = androgens plasma levels; CORT = corticosterone plasma levels; P = period.

4.10. Figures

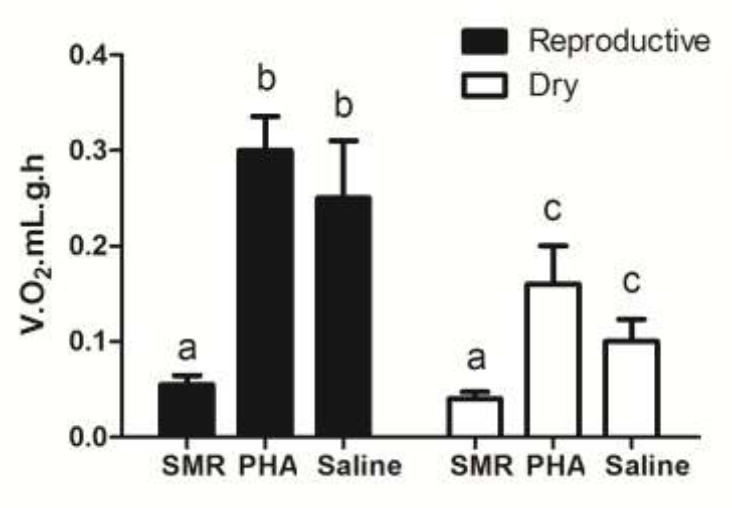


Figure 1. Rate of oxygen consumption before and after PHA immunological challenge and saline injection, during reproductive and dry period (Mean + Standard error). Different letters indicate significant difference within and between seasons ($P < 0.05$).

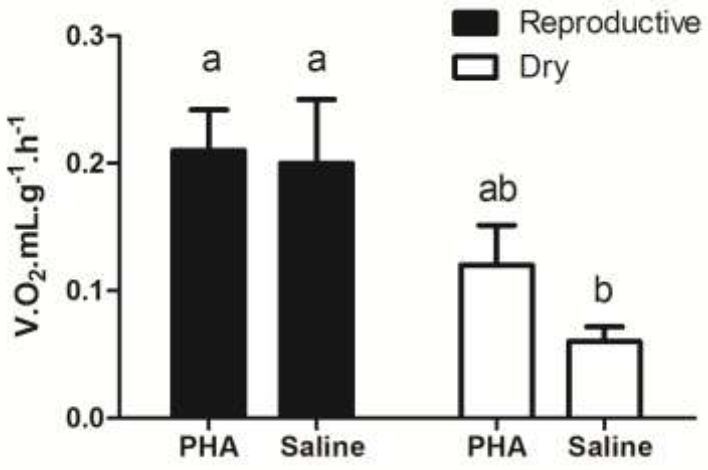


Figure 2. Metabolic cost to PHA challenge and saline injection, during reproductive and dry period (Mean + Standard error). Different letters indicate significant difference within and between seasons ($P < 0.05$).

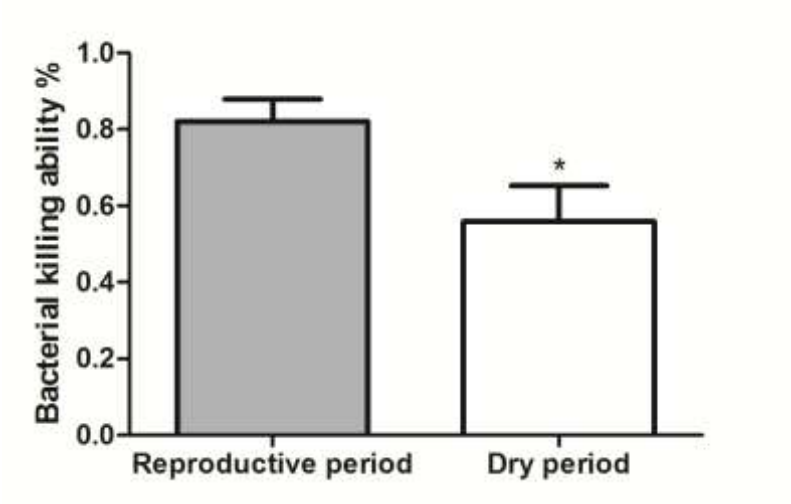


Figure 3. Bacterial killing ability during reproductive and dry period. (Mean + Standard error). Asterisk indicates significant difference ($P < 0.05$).

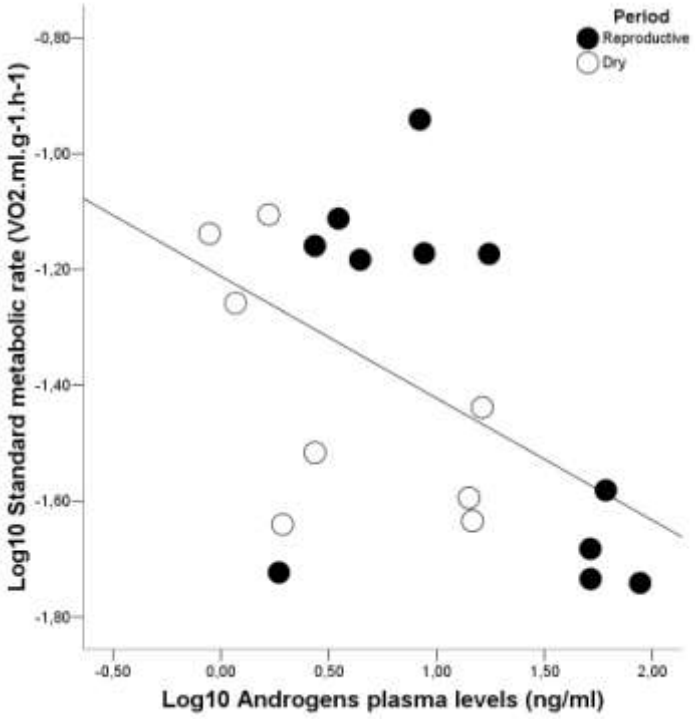


Figure 4. Standard metabolic rate and androgens plasma levels during reproductive and dry period. Line shown indicates best fit.

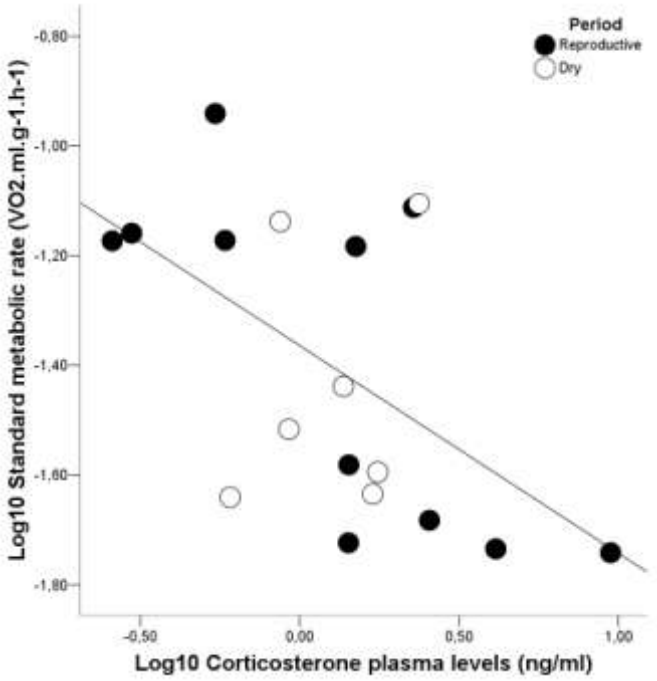


Figure 5. Standard metabolic rate and corticosterone plasma levels during reproductive and dry period. Line shown indicates best fit.

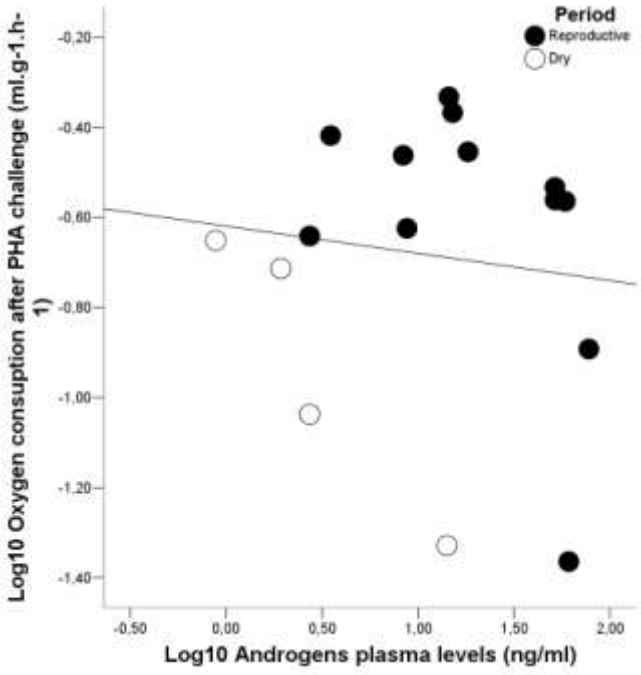


Figure 6. Oxygen consumption in response to the immunological challenge with PHA and androgens plasma levels during reproductive and dry period. Line shown indicates best fit.

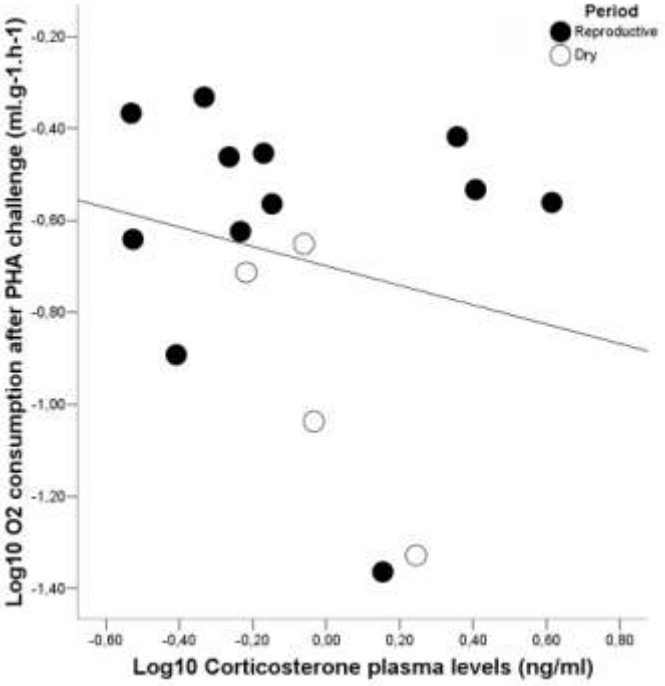


Figure 7. Oxygen consumption in response to injection with PHA and corticosterone plasma levels during reproductive and dry period. Line shown indicates best fit.

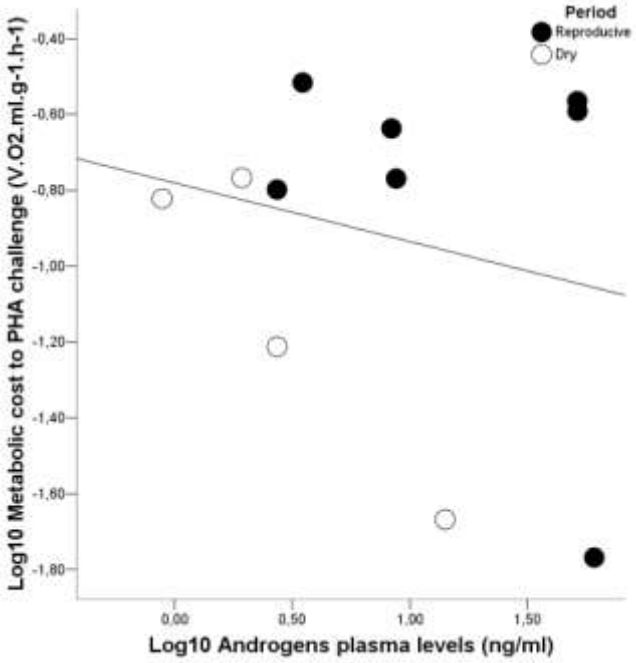


Figure 8. Metabolic cost in response to the immunological challenge with PHA and androgens plasma levels. Line shown indicates best fit.

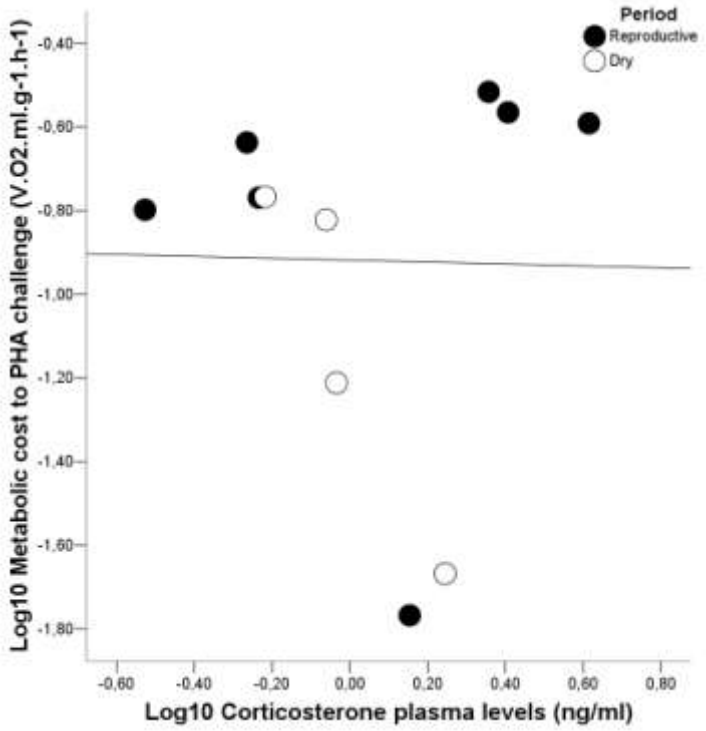


Figure 9. Metabolic cost in response to the immunological challenge with PHA and corticosterone plasma levels. Line shown indicates best fit.

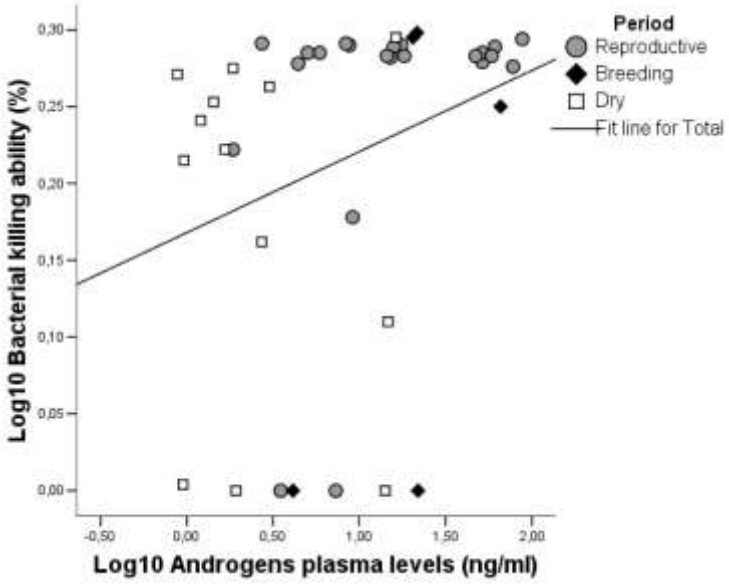
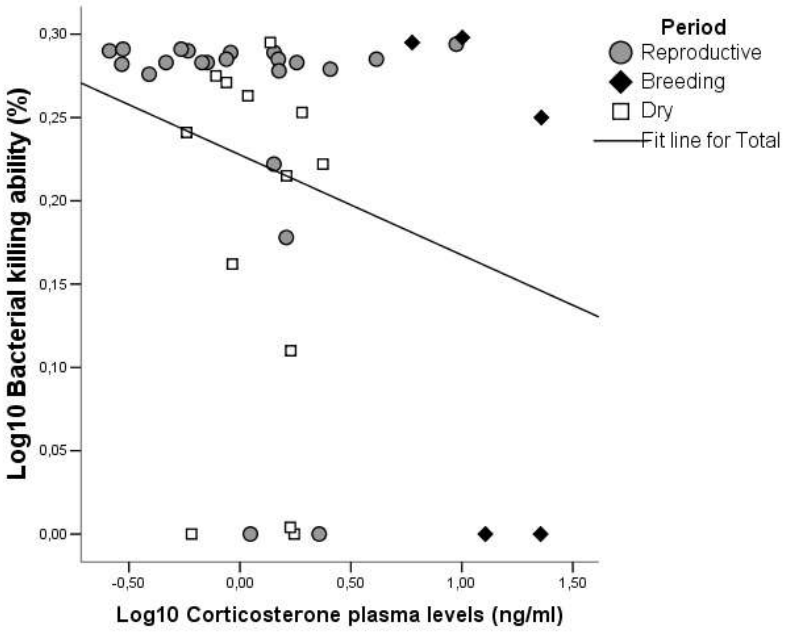


Figure 10. Bacterial killing ability and androgens plasma levels during reproductive, dry period and breeding event. Line shown indicates best fit.



11. Bacterial killing ability and corticosterone plasma levels during reproductive, dry period and breeding event. Line shown indicates best fit.

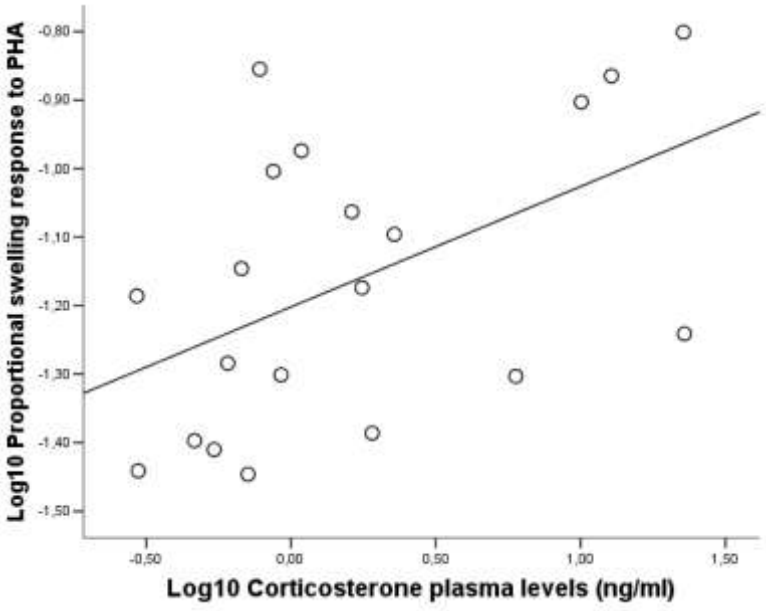


Figure 12. Prorportional swelling response to PHA challenge and corticosterone plasma levels. Line shown indicates best fit.

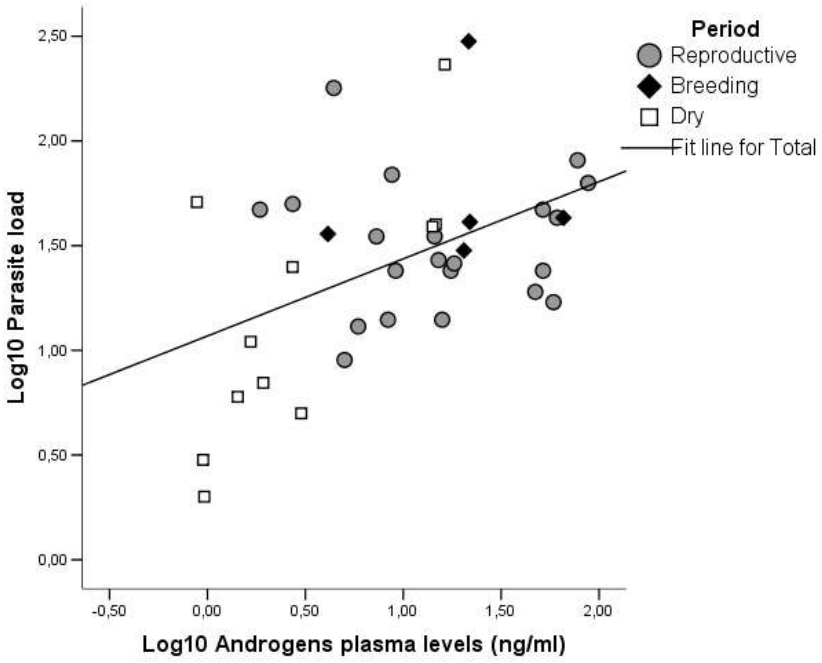


Figure 13. Parasite load and androgens plasma levels during reproductive, dry period and breeding event. Line shown indicates best fit.

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Conclusões Gerais

Nossos resultados indicam que os anuros que vivem na Caatinga apresentam variações fisiológicas sazonais de variáveis imunológicas (parâmetros leucocitários, e resposta inflamatória) associados positivamente à variação plasmática de hormônios esteróides. Sendo a variação mais acentuada na espécie estivadora, *Pleurodema diplolister*. Os indicadores de imunocompetência apresentaram-se mais altos no período reprodutivo, especialmente quando os animais estavam vocalizando e os níveis de corticosterona estavam ainda mais elevados. Assim, esses resultados não corroboram a hipótese de *trade-off* entre reprodução e imunidade.

No tocante à variação sazonal de marcadores moleculares, nossos resultados indicam que a espécie *P. diplolister* durante o período de estivação, apresenta um perfil molecular coerente com um estado de conservação de energia, diminuindo a síntese de proteínas e suprimindo processos que consomem ATP. Adicionalmente, durante o período de seca, as três espécies estudadas (tanto a estivadora quanto as que se mantém ativas nesse período) apresentam ativação de processos para manutenção da integridade de célula e tecidos, mecanismo que pode ser relevante considerando a história natural dessas espécies. A integridade de músculos associados à reprodução mantém essas estruturas prontas para a imprevisibilidade do evento reprodutivo.

O tratamento transdérmico de testosterona e corticosterona na espécie *R. jimi* corroborou as hipóteses de efeitos imunomoduladores desses esteróides. O tratamento agudo de testosterona aumentou a concentração hormonal a níveis observados durante o período não reprodutivo (seca) e diminuiu a resposta inflamatória, corroborando resultados previamente observados (Capítulo 1). O tratamento agudo de corticosterona, por sua vez, aumentou a eficiência da resposta imune, abreviando o processo de resposta inflamatória.

A taxa metabólica em resposta ao desafio de PHA nos indivíduos de *R. jimi* foi mais alta no período reprodutivo, quando os níveis de hormônios esteróides estão elevados. Adicionalmente, machos com maiores níveis plasmáticos de corticosterona apresentaram maior edema em resposta a PHA e menor taxa metabólica em resposta ao desafio de PHA, indicando que este hormônio além de afetar a eficiência da resposta

imune (Capítulo 3) também pode aumentar a resposta inflamatória e diminuir o custo energético associado.

Os resultados dos quatro capítulos desta tese apresentaram resultados inéditos de Fisiologia integrativa, Endocrinologia ambiental e Ecoimunologia das espécies estudadas, contribuindo para o arcabouço teórico de evidências que apontam na direção contrária da hipótese de imunossupressão durante a temporada reprodutiva. De fato, nossos resultados apontam que altos níveis de hormônios esteroides (testosterona e corticosterona) aumentam os paramêtros imunitários, bem como a resposta imune. Adicionalmente, o capítulo 2 traz as primeiras evidências de variação sazonal de reguladores metabólicos em anuros não estivadores, trazendo contribuições significativas para área eco-molecular.