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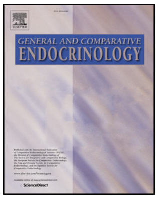

Immunomodulation by testosterone and corticosterone in toads: Experimental evidences from transdermal application

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ABSTRACT

Androgens and glucocorticoids play important roles in vertebrate's reproduction and display complex immunomodulatory functions that may affect survival. In anurans, testosterone and corticosterone are correlated to sexual behavior, reproduction, and immune function. Male toads (*Rhinella jimi*) were treated with acute doses of testosterone (T) and corticosterone (CORT) and immune variables (plasma bacterial killing ability [BKA], swelling after phytohemagglutinin [PHA] injection and the time point of maximum PHA swelling response) were measured. Transdermal T and CORT application increased androgen (T-DHT) and CORT plasma levels after 1 h of treatment, respectively, without a dose-specific effect. Transdermal T treatment did not affect BKA or PHA swelling response. Individuals treated with transdermal CORT showed an earlier maximum PHA swelling response and a tendency of lower BKA 15 h after treatment. Our results indicated that an acute experimental increase of CORT plasma levels diminished time for inflammatory resolution and suppressed non-cellular innate response.

1. 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; 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 (Peters, 2000; Casto et al., 2001), and inflammatory immune response (Belliure et al., 2004). Otherwise, some studies found 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, transdermal application of T increased the swelling response to PHA in the tree frog, *Hyla arborea* (Desprat et al., 2015) 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 and McEwen, 1996, 1999; Bowers et al., 2008), chronically elevated GC levels usually result in immunosuppression (Dhabhar and McEwen, 1999; Dhabhar, 2000, 2009; Sapolsky et al., 2000). A positive correlation of calling rates with plasma corticosterone (CORT) levels has been found in natural choruses of anurans (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 24 h,

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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 *Rhinella 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 positive covariation between plasma steroid levels (androgens and CORT) and immune function 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 (PHA swelling response and number of circulating leukocytes) 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 immune function, this study investigated the immune consequences of experimental acute elevation of T and CORT in *Rhinella jimi* toads from the Caatinga region. 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 in a dose dependent manner. To test these predictions, we assessed: plasma CORT and dihydrotestosterone (T-DHT) levels at baseline, as well as 1 h and 15 h after transdermal application; the BKA immune response at baseline as well as 1 h and 15 h after treatment; swelling response to a PHA challenge; and the time-point of maximum swelling response to the PHA challenge.

2. Materials and methods

2.1. 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 (elevation 95 m, 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 (Arzabe, 1999; Madelaire and Gomes, 2016). Adult *Rhinella jimi* males (N = 39) in foraging activity were collected between April 1st to April 24th 2015. All individuals were found by visual inspection.

Once they were captured, we used heparinized 1 mL syringes with 26G × 1/2 in. needle to obtain a 50 µL blood sample taken by cardiac puncture less than 3 min 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 h, and centrifuged for 5 min at 217 g (mini centrifuge 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.

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

2.2. 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 (Belliure et al., 2004; Wack et al., 2010; Assis et al., 2015). For testosterone 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. For the 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. Steroid solutions remained in an open vial overnight to allow ethanol evaporation.

Twenty four hours after capture, males were randomly divided into 5 experimental groups: Placebo (N = 9), 5.0 µL of sesame oil; T-Low (N = 7), 12.0 µg of testosterone (3 µL of testosterone working solution); and T-High (N = 7), 20.0 µg of testosterone (5 µL of testosterone working solution); CORT-Low (N = 7), 7.0 µg of corticosterone (5 µL of corticosterone working solution); CORT-High (N = 7), 14.0 µg of corticosterone (10 µL of corticosterone working solution). A drop containing the correspondent amount of working solution was applied on the dorsal area of each toad, using a micropipette and to guarantee the hormone absorption, we remove the water pot from their individual plastic containers for 1 h. Transdermal hormone applications were always performed in the lab at 8:30 pm, 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 1 h and 15 h after applications. These blood samples were subsequently processed for plasma hormone level determination as well as BKA assays. On the same night, after hormonal treatment and the obtainment of the 1 h blood sample, animals were submitted to a PHA skin-swelling assay.

2.3. Phytohemagglutinin (PHA) immunological challenge

To assess cell-mediated immunity, individuals were submitted to a PHA immunological challenge, 1 h 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 a cutaneous inflammatory immune response (Brown et al., 2011; Clulow et al., 2015). 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 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 12 h and 24 h 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 percentage swelling in response to PHA was calculated by dividing the maximum swelling value after injection by the first measure minus one [$(S_{\mathrm{final}}/S_{\mathrm{initial}}) - 1$].

2.4. 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 non-pathogenic *Escherichia coli* (Microbio-Logics,

#24311-ATCC 8739) working solution ($\sim 10^4$ 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 were transferred in duplicate to a 96 wells microplate, which was incubated at 37 °C for 2 h. 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: [100 − (optical density of sample/optical density of positive control)], which represents the percentage of killed microorganisms in the samples compared to the positive control.

2.5. Hormonal assay

Plasma samples were extracted with ether, according to Mendonça et al. (1996) and Madelaire and Gomes (2016). Each plasma sample was added to 3 mL of ether, which was then vortexed for 30 s 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 24 h). 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 dihydrotestosterone (DHT), therefore the results refer to plasma testosterone plus dihydrotestoeterone (T-DHT) levels.

2.6. 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).

2.7. Statistical analysis

Descriptive statistics were conducted for all variables, when samples for different variables (BKA and hormone levels) could not be obtained from all individuals and time points, it is indicated on the tables and figures. The scope of plasma hormones levels was calculated by dividing the plasma hormone concentration (HC) after treatment (T = 1 h and 15 h) by hormone concentration in the field (basal) minus one $[(HC_T/HC_{Basal}) - 1]$. The variation in BKA (ΔBKA) was calculated by subtracting the BKA of the plasma collected on the field (basal) from the BKA displayed 1 h and 15 h after the hormonal transdermal application $(BKA_T - BKA_{Basal})$. The index of body condition was calculated as the residuals of a standard least squares linear regression using snout-vent length as the independent variable and body mass as the dependent variable. The ANOVA and Chi-Square was carried out using IBM SPSS Statistics, Version 20.0 (IBM corp., 2011) and the mixed linear models (Bates et al., 2015) were run in R software, version 3.5.1, after data had been transformed to Log10 or Sine to improve the data normality (Log_{10}: Body mass, Hormone plasma concentration, PHA, BKA, Sine: Delta BKA and Scope of hormone plasma concentration).

ANOVA was performed to compare body mass among groups. Independent t-tests were performed to compare the percentage swelling from the feet injected with PHA and saline at different time-points (12 h and 24 h after the challenge). Given that some individuals displayed their maximum PHA swelling response at different times (12 h or 24 h 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 PHA swelling response, we performed a qui-square test assuming equal proportions for different time-points (12 h and 24 h) 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 androgens (T-DHT) or CORT levels from field (basal), as well as 1 h and 15 h after hormonal application. To verify any differences in plasma hormone levels among treatment and Placebo groups, we performed ANOVA followed by Bonferroni corrections, comparing field baseline levels, 1 h and 15 h after transdermal application. To understand how transdermal treatment affected immune response, we modeled the dependent variables (BKA 1 h and 15 h after treatment, ΔBKA 1 h and 15 h after treatment, and PHA swelling response) as a function of treatment (categorical with two levels: placebo and hormonal treatment), hormone variables (hormone plasma levels 1 h and 15 h after treatment and Scope of plasma levels 1 h and 15 h after treatment), body condition and a random factor (individual). 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 fixed factors significance from each selected model were considered (Burnham and Anderson, 2002; Bates et al., 2015). When the significance of fixed factors in the selected models were $P > 0.05$ (Luke, 2017), we consider the immune response were not explained by any of the studied variables. The model comparisons are shown in Table 1.

3. Results

All descriptive statistics of studied variables are shown in Table 2. Body mass did not differ among groups ($F_{4,31} = 2.11$, $P = 0.10$). For all groups, the PHA injected right foot showed a swelling response 12 h or 24 h after the injection when compare to saline injected left foot (12 h: $t = 6.89$, $df = 72$, $P < 0.0001$; 24 h: $t = 4.74$, $df = 72$, $P < 0.0001$). Maximum PHA swelling response (%) in the right foot occurred 12 h or 24 h 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, 1 h and 15 h after treatment time-points ($F_{2,16} = 1.45$, $P = 0.26$) (Fig. 1A). One hour after treatment, mean T-Low and T-High showed 3.7 and 9.2 fold higher plasma T-DHT levels *versus* baseline, respectively ($F_{2,12} = 12.12$, $P < 0.001$ for T-Low;

Table 1
Constructed models to test the relation between immune variables and explanatory variables treatment, hormone plasma levels and body condition index (BC).

Tested models
1) Immune variable ~ Null Model
2) Immune variable ~ Treatment
3) Immune variable ~ Treatment + Hormone variable
4) Immune variable ~ Treatment * Hormone variable
5) Immune variable ~ Treatment + BC
6) Immune variable ~ Treatment + Hormone variable + BC
7) Immune variable ~ Treatment * Hormone variable + BC

Table 2

Descriptive analysis of steroid plasma levels, immunological and morphological variables of *Rhinella jimi* males from Placebo (5.0 µL of sesame oil), T-Low (12.0 µg of testosterone), T-High groups (12.0 µg of testosterone), Testosterone (T-Low and T-high clustered), CORT-Low (7.0 µg of corticosterone), CORT-High (14.0 µg of corticosterone) groups and Corticosterone (CORT-Low and CORT-High clustered).

Variables	Placebo		T-Low		T-High		Testosterone		CORT-Low		CORT-High		Corticosterone	
	N	Median (Min–Max)	N	Median (Min–Max)	N	Median (Min–Max)	N	Median (Min–Max)	N	Median (Min–Max)	N	Median (Min–Max)	N	Median (Min–Max)
T-DHT basal (ng/ml)	9	2.3 (1.0–3.6)	7	0.8 (0.4–2.9)	7	0.7 (0.3–2.2)	14	0.7 (0.3–2.9)	–	–	–	–	–	–
T-DHT 1 h (ng/ml)	9	1.8 (0.4–3.1)	7	3.8 (1.6–8.9)	7	8.9 (2.5–19.9)	14	7.5 (1.6–19.9)	–	–	–	–	–	–
T-DHT 15 h (ng/ml)	9	1.4 (0.4–3.2)	7	1.3 (0.9–2.2)	7	1.7 (0.7–3.5)	14	1.6 (0.7–3.5)	–	–	–	–	–	–
Scope T1 h	9	−0.4 (−0.7–2.1)	7	3.9 (0.3–17.2)	7	5.6 (1.3–47.3)	14	5.2 (0.3–47.3)	–	–	–	–	–	–
Scope T10 h	9	−0.2 (−0.9–0.3)	7	1.2 (−0.6–1.7)	7	0.5 (0–6.3)	14	0.6 (−0.6–6.3)	–	–	–	–	–	–
CORT basal (ng/ml)	9	2.5 (0.6–4.2)	–	–	–	–	–	–	6	6.2 (1.5–10.0)	5	0.7 (0.4–15.2)	11	5.2 (0.4–15.2)
CORT 1 h (ng/ml)	9	8.8 (3.0–27.8)	–	–	–	–	–	–	5	42.2 (16.8–211.3)	5	59.8 (47.0–98.2)	10	57.5 (16.8–211.3)
CORT 15 h (ng/ml)	9	12.4 (3.0–21.2)	–	–	–	–	–	–	6	10.5 (4.4–72.2)	6	25.5 (3.3–55.0)	12	15.9 (3.3–72.2)
Scope CORT 1 h	9	3.4 (−0.1–37.5)	–	–	–	–	–	–	5	10.4 (1.3–39.8)	5	99.1 (5.5–150.5)	10	35.2 (1.3–150.5)
Scope CORT 15 h	9	−0.8 (−1.0–0.1)	–	–	–	–	–	–	6	1.8 (−0.2–6.3)	5	18.5 (−0.6–84.5)	11	2.7 (−0.6–84.5)
PHA SW (%)	9	7.7 (2.5–10.3)	7	7.9 (2.5–16.9)	7	4.0 (1.7–11.2)	14	5.8 (1.7–16.9)	7	4.4 (0.9–6.8)	7	8.7 (2.1–20.8)	14	5.0 (0.9–20.8)
BKA basal (%)	9	84 (0–100)	7	82 (0–100)	7	95 (0–100)	14	88.6 (0–100)	7	93.0 (86–100)	7	10.0 (0–90)	14	87.9 (0–100)
BKA 1 h (%)	9	91 (2–100)	7	59 (0–97)	7	84 (10–98)	14	76.7 (0–97.7)	7	87.0 (0–100)	7	82.0 (0–100)	14	85.2 (0–100)
BKA 15 h (%)	9	88 (0–100)	7	41 (0–94)	6	78 (0–95)	13	62.8 (0–95.3)	7	88.0 (0–100)	7	60.0 (2.0–94.0)	14	69.0 (0–100)
ΔBKA 1 h	9	9.3 (−11.6–88.9)	7	3.9 (−85.4–92.6)	7	0.06 (85.8–17.3)	14	1.6 (85.8–92.7)	7	−4.4 (−91.9–7.0)	7	19.1 (−89.7–84.5)	14	0.4 (−91.9–84.5)
ΔBKA 15 h	9	4.7 (−85.3–88.9)	7	−4.4 (−80.1–86.8)	6	19.8 (97.1–9.3)	13	4.4 (97.1–86.8)	7	−7.0 (−97.7–14.0)	7	13.2 (87.8–84.5)	14	−2.7 (−97.7–84.5)
Body mass (g)	9	103.1 (82.8–141.1)	7	118.7 (86.4–143.5)	7	91.6 (74.5–121.3)	14	111.6 (74.5–143.5)	7	129.1 (93.0–138.8)	7	104.4 (75.5–120.1)	14	109.6 (75.5–138.8)
SVL (mm)	9	103.7 (97.5–116.6)	6	111.2 (109.4–121.2)	7	100.3 (94.9–109.3)	13	109.3 (94.9–121.2)	7	109.0 (47.2–114.8)	7	103.3 (94.4–107.9)	14	103.8 (47.2–114.8)

N = Valid N; Min = Minimum; Max = Maximum; CORT = corticosterone plasma levels; T-DHT = androgens plasma levels; basal = collected in the field; 1 h = 1 h after hormonal treatment; 15 h = 15 h after hormonal treatment; Scope T = [(T-DHT concentration at 1 h or 15 h/T-DHT Concentration at Basal) − 1]; Scope CORT = [(CORT concentration at 1 h or 15 h/CORT Concentration at Basal) − 1]; PHA SW = PHA swelling response; BKA = bacterial killing ability; ΔBKA = variation on BKA (BKA at 1 h or 15 h – BKA at Basal); SVL = snout vent length.

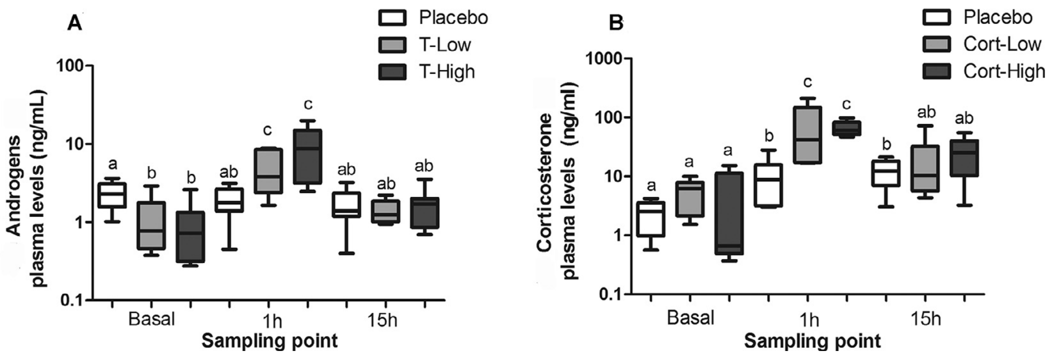

Fig. 1. Variation in androgens (A) and corticosterone (B) plasma levels basal, 1 and 15 h after transdermal treatment in males of *R. jimi* from Placebo (N = 9), T-Low (N = 7), T-High (N = 7), CORT-Low (N = 7), CORT-High (N = 7) represented as a box (25th and 75th percentiles with median line) and whisker (1.5× interquartile range or max value if less). The Y axis is Log_{10} transformed. Different letters indicates significant difference.

$F_{2,12} = 23.83$, $P < 0.0001$ for T-High). After 15 h, all groups had similarly low plasma T-DHT levels ($F_{2,20} = 0.06$, $P = 0.94$) (Fig. 1A). Despite the fact that baseline plasma T-DHT levels of the Placebo group were slightly higher than the mean of T-Low and T-High groups ($F_{2,20} = 6.59$, $P < 0.007$; Bonferroni $P < 0.05$), T transdermal application increased plasma T-DHT levels after 1 h in the T-Low and T-High groups, *versus* Placebo ($F_{2,20} = 10.87$, $P < 0.001$; Bonferroni $P < 0.05$) (Fig. 1A). Groups T-Low and T-High did not differ in plasma T-DHT levels at 1 h time-point after treatment (Bonferroni $P > 0.05$), therefore the immune variables from each group were clustered for model selection analyses.

Baseline plasma CORT levels in all groups showed similar values ($F_{2,16} = 0.93$, $P = 0.41$). Mean of plasma CORT levels from Placebo group were 3.2 times higher at 1 h after treatment and 4.0-fold increased at 15 h after treatment, *versus* the mean of baseline levels ($F_{2,16} = 13.19$, $P < 0.001$; Bonferroni $P < 0.05$) (Fig. 1B). Corticosterone transdermal application increased means of plasma CORT levels after 1 h in the CORT-Low by 12.5 fold and CORT-High by 12.3 fold ($F_{2,8} = 15.89$, $P < 0.002$ for CORT-Low; $F_{2,6} = 25.9$, $P < 0.002$ for CORT-High). After 15 h, individuals from CORT-Low and CORT-High groups showed decreased plasma CORT levels, at a level similar to those observed in the Placebo group ($F_{2,18} = 0.97$, $P = 0.40$) (Fig. 1B). Considering the CORT plasma levels from both CORT-Low and CORT-High did not differ at any time-points (Bonferroni $P > 0.05$), the immune variables from each group were clustered for the following analysis.

Corticosterone treatment affected the time-point of the maximum PHA swelling response. A higher percentage (78.6%) of individuals in this group displayed a maximum PHA swelling response 12 h after the challenge ($X^2 = 4.57$, df = 1, $P = 0.03$). The other treatments (Placebo and Testosterone) did not affect the time of maximum PHA swelling response (Placebo $X^2 = 0.11$, df = 1, $P = 0.74$, Testosterone $X^2 = 1.14$, df = 1, $P = 0.29$) (Fig. 2).

Although selected models include a relation between immune variables (BKA, ΔBKA and PHA swelling response) and the explanatory variables (treatment, androgens plasma levels and body condition) in the animals treated with testosterone (Table 3), the fixed factors from each selected model were not significant ($P > 0.05$).

In individuals treated with CORT, the BKA 15 h after treatment was lower in individuals with higher corticosterone plasma levels 1 h after the treatment (Fig. 3, Table 4 and 5). After removing an individual that had exceptionally high CORT levels from the analysis, the effect of treatment and CORT plasma levels 1 h after treatment was not significant (Fig. 3, Table 4). For PHA swelling response, BKA 1 h after treatment, ΔBKA 1 h and 15 h after treatment, the selected models included a relation with the explanatory variables (corticosterone treatment, corticosterone plasma levels and body condition) (Table 3), still the fixed factors in each model were not significant ($P > 0.05$).

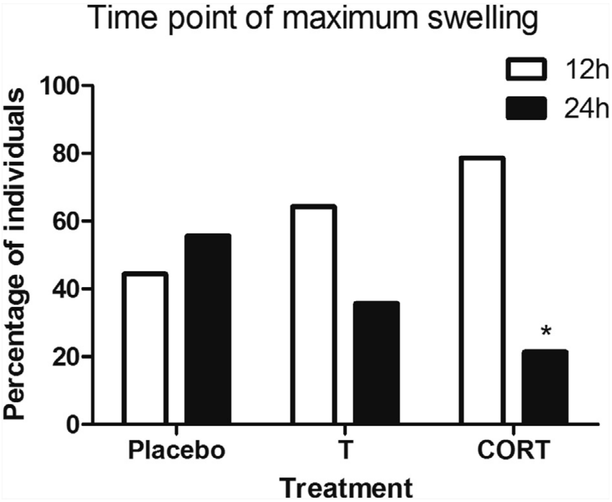

Fig. 2. Proportion of males of *R. jimi* displaying maximum swelling response 12 and 24 h after injection of PHA. Individuals were treated with transdermal application of Sesame oil (Placebo – N = 9), Testosterone (N = 14) and Corticosterone (N = 14) 1 h before PHA injections. Asterisks indicate significant difference of proportions within groups.

4. 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. Although animals were collected during a period of the year when toads are usually under reproductive condition in this region, basal androgen plasma levels were abnormally low, even when compared to those androgen levels previously measured for this species during the dry season (Madelaire and Gomes, 2016). While T treatment raised plasma T-DHT levels to those previously measured for this species during the dry season (Madelaire and Gomes, 2016), CORT treatment increased plasma CORT to levels similar to those when males are calling (Madelaire and Gomes, 2016). Transdermal testosterone did not affect the PHA swelling response, time-point of the maximum PHA swelling response or BKA. CORT treatment accelerated the time of the maximal swelling response indicating CORT may accelerate inflammatory resolution. Additionally, individuals that showed higher CORT plasma levels 1 h after treatment displayed a tendency of lower BKA 15 h after treatment.

For T-treated individuals, mean androgen plasma levels increased, up to 9.2 fold 1 h post-application. However, due to abnormally low basal T-DHT levels for this period of the year (mean ± sd Placebo 2.3 ± 0.9, T-Low 1.1 ± 0.9, T-High 1.0 ± 0.8), compared to 2011

Table 3
Selected models for explaining variance in immune as a function of body condition and hormonal variables after testosterone treatment in males of *Rhinella jimi*.

Hormonal variable	Immune variable	Selected model	AICc	gl	ΔAICc	Weight
1 h after treatment	BKA 1 h	Φ ~ null	46.5	3	0.0	0.30
		Φ ~ T	47.5	4	1.0	0.18
		Φ ~ T * H	47.5	6	1.0	0.17
		Φ ~ T * H + IC	48.0	7	1.5	0.14
1 h after treatment	BKA 15 h	Φ ~ null	58.2	3	0.0	0.34
		Φ ~ T	58.4	4	0.2	0.31
		Φ ~ T + H	60.2	5	2.0	0.12
15 h after treatment		Φ ~ T + H	55.9	5	0.0	0.36
		Φ ~ T * H	57.2	6	1.3	0.18
		Φ ~ T + H + BC	57.8	6	1.9	0.14
Scope after 1 h	ΔBKA 1 h	Φ ~ null	50.4	3	0.0	0.43
		Φ ~ T * H	52.4	6	1.9	0.16
		Φ ~ T	52.4	4	2.0	0.15
Scope after 1 h	ΔBKA 15 h	Φ ~ T	36.8	4	0.0	0.43
		Φ ~ T + H	38.3	5	1.5	0.20
		Φ ~ T + IC	38.8	5	2.0	0.16
Scope after 15 h		Φ ~ T * H	35.3	6	0.0	0.40
		Φ ~ T * H + BC	36.8	7	1.5	0.19
		Φ ~ T	36.8	4	1.6	0.18
1 h after treatment	PHA SW	Φ ~ T + BC	8.6	5	0.0	0.57
		Φ ~ T + H + BC	10.6	6	2.0	0.21
15 h after treatment		Φ ~ T + BC	8.6	5	0.0	0.51
		Φ ~ T + H + BC	10.6	6	1.4	0.26
Scope after 1 h		Φ ~ T + BC	8.6	5	0.0	0.39
		Φ ~ T + H + BC	9.2	6	0.6	0.29
		Φ ~ T * H + BC	9.9	7	1.3	0.21
Scope after 15 h		Φ ~ T + BC	8.6	5	0.0	0.51
		Φ ~ T + H + BC	10.6	6	2.0	0.19

1 h after treatment = hormone plasma levels 1 h after treatment, 15 h after treatment = hormone plasma levels 15 h after treatment, Scope after 1 h = Scope of hormone plasma concentration 1 h after treatment, Scope after 15 h = Scope of hormone plasma concentration 15 h after treatment, BKA 1 h = bacterial killing ability 1 h after treatment, BKA 15 h = Bacterial killing ability 15 h after treatment, ΔBKA 1 h = variation in BKA 1 h after treatment, ΔBKA 15 h = variation in BKA 15 h after treatment, PHA SW = PHA swelling response, Φ = response variable, T = Treatment; H = hormonal variable; BC = index of body condition; AICc = Akaike's information criterion for small samples; gl = number of parameters; ΔAICc = difference of AICc between any model and the best model; Weight = weight for each selected model.

(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 (T-Low 5.1 ± 3.1 ng/ml, T-High 10.0 ± 6.4 ng/ml at 1 h post-treatment). In fact, mean androgen plasma 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). Interannual variation in plasma T levels has been previously 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 the year considered in the study (Todd et al., 2010). In this specific year, one or more environmental 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 require further investigation.

Androgen plasma levels achieved after the transdermal T treatment had no effect on BKA, intensity of response or the time-point of the maximum PHA swelling response. In agreement, T treatment had no effect on wound healing in salamanders (*Desmognathus ochrophaeus*) (Thomas and Woodley, 2017). The spectrum of testosterone effects on immune variables display a large variation among different studies. Tree frog males (*Hyla arborea*) with higher body mass treated with transdermal testosterone displayed higher swelling response to the PHA challenge (Desprat et al., 2015). In the other hand, testosterone treatment suppressed humoral immunity in male lizards (*Psammodromus algirus* and *Acanthodactylus erythrurus*) (Belliure et al., 2004) and decreased blood bacterial-killing capacity in the house sparrows (Pap et al., 2010). The T-DHT levels achieved after treatment in *R. jimi* toads were similar to those found during dry period, which might have contributed to the lack of effect on immune parameters. The consequences of elevation of T-DHT to levels similar to those found during reproductive period on immune parameters of *R. jimi* remain to be tested.

Twenty-four hours of captivity increased the mean of CORT plasma 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, 1995; Narayan et al., 2011, 2012; Assis et al., 2015; Titon et al., 2017). 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 24 h of captivity without movement restriction.

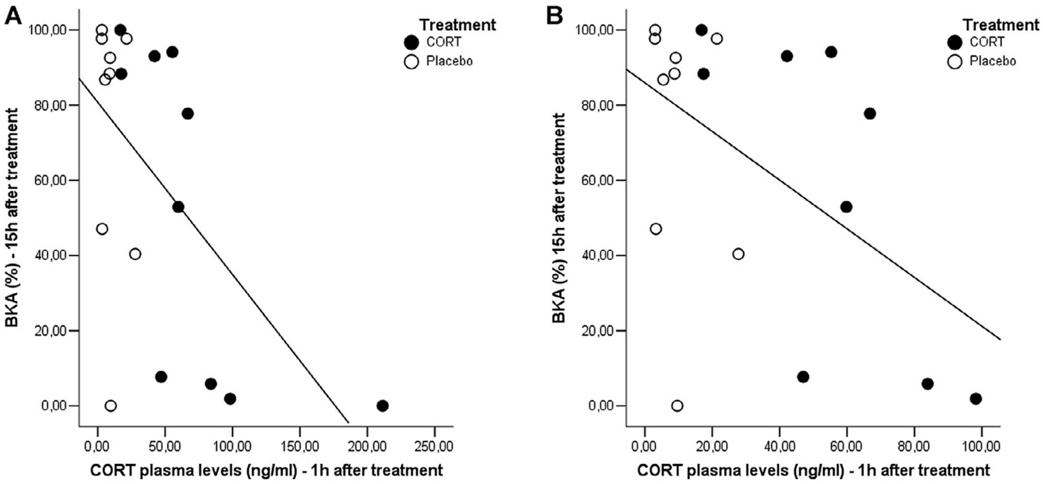

Fig. 3. (A) Corticosterone plasma levels 1 h after treatment and BKA 15 h after treatment in individuals treated with transdermal sesame oil (Placebo – N = 9) or corticosterone (CORT – N = 10). In (B), one outlier (CORT = 211.33 ng/ml) was removed. Line shown indicates best fit.

Table 4
Selected models for explaining variance in immune as a function of body condition and hormonal variables after corticosterone treatment in males of *Rhinella jimi*.

Hormonal variable	Immune variable	Selected model	AICc	gl	ΔAICc	Weight
1 h after treatment	BKA 1 h	Φ ~ T + H	45.9	5	0.0	0.42
		Φ ~ T * H	47.5	6	1.6	0.19
		Φ ~ T + H + BC	47.9	6	2.0	0.16
1 h after treatment	BKA 15 h	Φ ~ T * H	41.5	6	0.0	0.43
		Φ ~ T + H	43.0	5	1.4	0.21
		Φ ~ T * H + IC	43.5	7	2.0	0.16
1 h after treatment * without outlier		Φ ~ null	38.5	3	0.0	0.35
		Φ ~ T + H	39.7	5	1.2	0.19
		Φ ~ T	40.2	4	1.7	0.15
		Φ ~ T * H	40.2	6	1.8	0.14
15 h after treatment		Φ ~ T + H	47.4	5	0.0	0.41
		Φ ~ T + H + IC	49.0	6	1.6	0.18
		Φ ~ T * H	49.2	6	1.9	0.16
Scope after 1 h	ΔBKA1h	Φ ~ null	44.6	3	0.0	0.42
		Φ ~ T	46.5	4	1.9	0.16
Scope after 1 h	ΔBKA15h	Φ ~ T + H	42.6	5	0.0	0.38
		Φ ~ T * H	44.1	6	1.5	0.18
		Φ ~ null	44.5	3	1.9	0.15
Scope after 15 h		Φ ~ null	47.5	3	0.0	0.44
		Φ ~ T * H	49.4	4	1.9	0.17
1 after treatment	PHA SW	Φ ~ null	15.5	3	0.0	0.31
		Φ ~ T + H	16.1	5	0.7	0.22
		Φ ~ T * H	17.2	6	1.8	0.13
		Φ ~ T	17.4	4	1.9	0.12
15 h after treatment		Φ ~ null	17.5	3	0.0	0.53
		Φ ~ T	19.3	4	1.8	0.22
Scope after 1 h		Φ ~ null	15.2	3	0.0	0.49
		Φ ~ T	17.2	4	2.0	0.18
Scope after 15 h		Φ ~ null	17.3	3	0.0	0.51
		Φ ~ T	19.3	4	1.9	0.20

1 h after treatment = hormone plasma levels 1 h after treatment, 15 h after treatment = hormone plasma levels 15 h after treatment, Scope after 1 h = Scope of hormone plasma concentration 1 h after treatment, Scope after 15 h = Scope of hormone plasma concentration 15 h after treatment, BKA 1 h = bacterial killing ability 1 h after treatment, BKA 15 h = Bacterial killing ability 15 h after treatment, ΔBKA 1 h = variation in BKA 1 h after treatment, ΔBKA 15 h = variation in BKA 15 h after treatment, PHA SW = PHA swelling response, Φ = response variable, T = Treatment; H = hormonal variable; BC = index of body condition; AICc = Akaike's information criterion for small samples; gl = number of parameters; ΔAICc = difference of AICc between any model and the best model; Weight = weight for each selected model.

Table 5
T and P values of fixed effects from selected models explaining variance in immune as a function of body condition and hormonal variables after corticosterone treatment in males of *Rhinella jimi*. Values in bold indicate significant effect.

Model	Fixed effects	t-value	p-value
BKA 15 h ~ T * H	Intercept	4.20	> 0.01
	Treatment	−2.08	**0.055**
	CORT 1 h	−2.94	**0.011**
	Treatment * CORT 1 h	1.72	0.111

BKA 15 h = Bacterial killing ability 15 h after treatment, T = Treatment; H = hormonal variable, CORT 1 h = corticosterone plasma levels 1 h after treatment.

Transdermal CORT application increased CORT plasma levels to the mean values evident when males are calling during breeding activity (Table 2) (2011: 65.3 ± 61.7 ng/ml, data from Madelaire et al., 2017). The treatment resulted significant acceleration of the time of exhibition of maximum swelling response to PHA, which could be associated to diminished time for inflammatory resolution (Serhan and Savill, 2005; Serhan et al., 2007). An early maximum infiltration of leukocytes during acute inflammation initiates an early clearance of cellular debris and apoptotic neutrophils (Serhan and Savill, 2005; Serhan et al., 2007; Schwab et al., 2007; Winsauer and de Martin, 2007; Serhan and Petasis, 2011). Additionally, increased CORT inhibits nocturnal melatonin secretion from pineal gland during the pro-inflammatory response in rats (Fernandes et al., 2017), 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***, 1 h after adrenocorticotropic hormone. These mechanisms might contribute to an accelerated swelling response to the PHA challenge observed in *R. jimi* treated with CORT, associated with higher efficiency on the inflammatory resolution process (Serhan and Savill, 2005; Serhan et al., 2007; Winsauer and de Martin, 2007; Serhan and Petasis, 2011).

The increase of CORT plasma levels 1 h after treatment resulted in a tendency of decreasing BKA. Similarly to our results, *Rhinella icterica* after being restrained with movement restriction increased CORT levels in 9 fold and showed decreased BKA (Assis et al., 2015). The 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). The diminished time for PHA swelling response along with the reduced activity of complement system proteins 15 h after treatment may be congruent effects of anti-inflammatory response after CORT application, however, this hypothesis remains to be tested. Moreover, calling effort is usually positively correlated with plasma CORT (Emerson and Hess, 2001; Assis et al., 2012; Narayan et al., 2013; Leary, 2014) and Owen-Ashley et al. (2004) demonstrated the interaction of CORT treatment on T-DHT plasma levels and immune function. Future studies are necessary to understand the impact of the cross reaction between these steroids and immune response in anurans.

In summary, toads treated with transdermal T application showed increased T-DHT plasma levels 1 h after treatment, but no effects were observed on immune response. Transdermal CORT treatment brought CORT plasma levels to those normally observed during calling activity, associated with accelerated maximum PHA swelling response and a tendency of lower BKA. Our results indicated that an acute experimental increase of CORT levels diminished time for inflammatory resolution and suppressed the response related to proteins of complement system.

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