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RESEARCH PAPER

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Effect of glucocorticoids on androgen biosynthesis in the testes of the toad *Rhinella arenarum* (Amphibia, Anura)

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Abstract

In rat Leydig cells, glucocorticoids (GCs) inhibit testosterone production through the interaction with the glucocorticoid receptor (GR). However, the sensitivity of those cells to GCs is regulated by the enzyme 11β-hydroxysteroid dehydrogenase Type 1 (11β-HSD1). In the testes of the toad *Rhinella arenarum*, the presence of an 11β-HSD similar to type 2 and a cytosolic GR has also been described. However, there is a lack of information regarding the effects of GCs on amphibian testicular steroidogenesis. In this study, the effects of corticosterone on androgen production, and the activity of two steroidogenic enzymes in toad testes were reported. Corticosterone inhibits androgen production via the GR because the GR antagonist RU486 prevents corticosterone-induced inhibition of testosterone. Corticosterone also reduced the activity of the cytochrome P450 17-hydroxylase, C17,20-lyase ($Cyp450_{c17}$) without affecting the 3β-hydroxysteroid dehydrogenase/isomerase activity. This effect on $Cyp450_{c17}$ was likewise inhibited by RU486. On the other hand, corticosterone had no effect on the amount of steroidogenic acute regulator protein. These results suggest that GCs inhibit steroidogenesis in toad testes by reducing of $Cyp450_{c17}$ activity via a GR-mediated mechanism.

HIGHLIGHTS

- GCs inhibit steroidogenesis in toad testes by the suppression of $Cyp450_{c17}$ via GR-mediated mechanism.
- Corticosterone reduced the activity of the $Cyp450_{c17}$ without affecting 3β-HD/I.
- Corticosterone has no effect on the amount of StAR protein.

KEYWORDS
amphibians, androgens, glucocorticoids (GC), steroidogenesis, testes

1 | INTRODUCTION

Glucocorticoids (GC) are steroid hormones synthesized in the vertebrate adrenal/interrenal glands. These hormones exert a wide variety of actions in virtually all organs, through the interaction with at least two different intracellular receptors belonging to the nuclear hormone receptor superfamily, Type I or mineralocorticoid receptor and the Type II or glucocorticoid receptors (GR) (Funder, 1997). Rapid nongenomic actions of GCs have also been proposed for mammals, avian, fish, and amphibians (Borski, 2000; Breuner & Orchinik, 2009; Evans, Murray, & Moore, 2000; Evanson, Herman, Sakai, & Krause, 2010; Jiang, Liu, & Tasker, 2014; Orchinik, Matthews, & Gasser, 2000; Orchinik, Murray, & Moore, 1991). The GR intracellular receptors for GCs have been described in several organs such as liver, kidney, skin,

and testis (Denari & Ceballos, 2006; Lange & Hanke, 1988; Lange, Hanke, & Morishige, 1989; Orchinik et al., 2000; Regueira, Sassone, Scaia, Volonteri, & Ceballos, 2013). In the anuran *Rhinella arenarum*, GR has been shown to be activated by corticosterone, the most abundant GC synthesized by the interrenal gland of this species (Ceballos, Cozza, & Lantos, 1983).

GC are the main peripheral effectors of the stress system and are regulated by the hypothalamic–pituitary–adrenal axis (Chrousos, 2009; Sapolsky, Romero, & Munck, 2000). When released in response to stressful stimuli, GCs induce a variety of physiological effects, among them, highly disrupting effects on reproductive physiology (Ing et al., 2014; Lin et al., 2014; Sapolsky, 1985; Sapolsky et al., 2000; Witorsch, 2016). Several studies have demonstrated that Leydig cells are one of the targets of GCs (Bambino & Hsueh, 1981; Hardy et al., 2005; Hu et al., 2008; Monder, Hardy, Blanchard, & Blanchard, 1994; Monder, Miroff, Marandici, & Hardy, 1994; Welsh, Bambino, & Hsueh, 1982). In these cells, a GC-induced inhibition of steroidogenesis is elicited by interaction with the intracellular GR and also through a rapid, nongenomic action (Dong et al., 2004; Hu et al., 2008). Just as in other vertebrates (Witorsch, 2016), intracellular levels of GCs in *R. arenarum* (Denari & Ceballos, 2005) are regulated by the expression of the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) in Leydig cells. In rats, Leydig cells exhibit 11β-HSD NADPH/$NADP^+$ dependence (Gao, Ge, Lakshmi, Marandici, & Hardy, 1997; Gao, Shan, Monder, & Hardy, 1996; Ge & Hardy, 2000; Ge et al., 2005), its expression being induced by luteinizing hormone (Wang, Zhang, & Gao, 2009). In contrast, toad Leydig cells express an 11β-HSD NADH/NAD^+-dependent with predominantly oxidative activity (Denari & Ceballos, 2005).

Reproductive physiology in vertebrates can be disrupted by GCs through a number of mechanisms. Several studies suggest that basal concentrations of GCs are insufficient to affect reproduction, but GC-mediated stress was considered to inhibit it at several levels not only in mammals (Sapolsky et al., 2000), but also in other vertebrates (Breuner, Patterson, & Hahn, 2008; F.L. Moore & Miller, 1984; F.L. Moore & Zoeller, 1985; F.L. Moore, Boyd, & Kelley, 2005). During breeding, many amphibians display a significant rise in plasma GCs (Denari & Ceballos, 2005; Michael Romero, 2002) implying that GCs may facilitate reproduction in these species. Nevertheless, there is a lack of information regarding the direct effect of GCs in a nonmammalian testicular function (Tesone, Regueira, & Ceballos, 2012), even when a positive correlation between corticosterone and reproduction has been reported for many amphibian (Michael Romero, 2002; I.T. Moore & Jessop, 2003).

The toad *R. arenarum* is a seasonal breeder; its breeding season is restricted to the springtime in the Southern hemisphere, the period between September and December. During breeding, males show low levels of circulating androgens (Canosa & Ceballos, 2002a) and high concentrations of plasma GCs (Denari & Ceballos, 2005). In this context, the main objective of the current study was to investigate in this animal model, the in vitro effects of corticosterone, the natural GC, on androgen production, and the activity of the steroidogenic enzymes cytochrome P450 17-hydroxylase, C17,20-lyase ($Cyp450_{c17}$) and 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD/I), as well as the expression of the steroidogenic acute regulator protein (StAR).

2 | MATERIALS AND METHODS

2.1 | Animals

Adult male toads of *R. arenarum* (170 ± 15 g) were collected during various nonreproductive seasons (January to August in the Southern hemisphere; 2013 [*n* = 16], 2014 [*n* = 17], 2015 [*n* = 15], and 2018 [*n* = 4]), in a nonagricultural area near Buenos Aires City (Argentina). Nonreproductive toads were selected because they exhibit a high level of $Cyp450_{c17}$, enzyme activity although a very low activity during the reproductive season (Fernández Solari, Pozzi, & Ceballos, 2002). Toads were kept in the animal facilities of the Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina, under natural conditions, with free access to water and fed with crickets and zophobas. Animals were transported to the laboratory 24 hr before experiments. Toads were anesthetized by immersion in 1% neutralized tricaine methanesulfonate (MS222) according to Gentz (2007), their death being the result of the ensuing surgery. This procedure is in accordance with the Guiding Principles for the Care and Use of Research Animals promulgated by the Society for the Study of Reproduction and with the approval of Comisión Institucional para el Cuidado y Uso de Animales de Laboratorio (Protocol N° 21), Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina.

2.2 | Experimental design

Testes were quickly dissected under sterile conditions, placed on Leibovitz's (L15) medium plus 10 mM Hepes, 10% fetal bovine serum plus antibiotic (penicillin, 100 IU/ml and streptomycin, 0.1 mg/ml), and antimycotic (amphotericin B, 0.25 mg/ml; Canosa & Ceballos, 2002b), and fat bodies, mesorchia, and Bidder's organ were removed. Fetal bovine serum was inactivated at 55°C and depleted of steroids by charcoal:dextran treatment before use.

Both testes from each animal, weighing between 190 and 220 mg, were cut into approximately 2-mm-thick slices (20 ± 3 mg), with scalpels.

For androgen production and enzymatic activity determinations, six slices from each testis were individually and randomly transferred to culture plates containing 2 ml of culture medium. Testicular slices were treated for 24 hr with 0, 15, or 150 nM corticosterone (Sigma-Aldrich, MO) with 10 μM RU486 or 1.5 μM glycyrrhetinic acid (GA; Figure 1). The concentrations of corticosterone were in the physiological order of toad plasma corticosterone, corresponding to plasma concentrations of nonreproductive (15 nM), and reproductive (150 nM) seasons (Denari & Ceballos, 2005). Both, GA and RU486 were supplied by Sigma-Aldrich. GA was used as an 11β-HSD Type 2 (11β-HSD2) inhibitor (Brem, Matheson, Conca, & Morris, 1989; Denari & Ceballos, 2005), whereas RU486 as an antagonist of the GR

(Bygdeman, Swahn, Gemzell-Danielsson, & Svalander, 1993; Denari & Ceballos, 2006).

After treatments, media were discharged, replaced with 1-ml Krebs-Ringer-glucose solution containing 10 mM Hepes (KRGH), pH 7.4, and treated during 1 hr with or without 20 IU human chorionic gonadotropin (hCG) (stimulated or basal condition, respectively). After incubation, each fragment was individually harvested and immediately processed for enzymatic activity as describes below. Androgen production was measured in KRGH media.

For StAR protein quantification, both testes from each toad were cut into six slices. Two slices, one from each testis, were randomly transferred to culture plates containing 4-ml culture medium and incubated for 24 hr with 0, 15, and 150 nM of corticosterone. After treatments, media were discharged, and each duplicate was incubated during 1 hr in 1 ml KRGH medium either with or without 20 IU hCG (stimulated or basal condition, respectively; Supporting Information Figure 2). StAR protein quantification was performed by the western blot technique. The concentration of hCG was selected according to Pozzi and Ceballos (2000).

For short-term treatments, slices were incubated during 2 hr in 2-ml L15 medium plus 10 mM Hepes, with or without the previously described concentrations of corticosterone. After treatments, media were discharged, and slices were incubated with or without 20 IU hCG as mentioned above.

2.3 | Radioimmunoassay

Basal and hCG-stimulated androgen biosynthesis were analyzed in incubation media by assaying immunoreactive testosterone by radioimmunoassay using [1,2,6,7(n)-^{3}H]testosterone (75.5 Ci/mmol; PerkinElmer, MA; Pozzi & Ceballos, 2000). Testosterone antibody was obtained from The Colorado State University (CO) and was used in a 1:125,000 dilution. The buffer used was 10 mM phosphate-buffered saline, 1% gelatin, and 20 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4. Because the cross reactivity of testosterone antibody with dihydrotestosterone was 35%, results were expressed as androgen production (Fernández Solari et al., 2002). The sensitivity of the assay was 10 pg. Steroids were assayed in triplicate. Intra and interassay coefficients of variation were 7.6% and 9.4%, respectively. Androgen production was expressed as pg per ml of the medium.

2.4 | Enzymatic assays

After incubation, each testis fragment was individually harvested and homogenized in 750 µl of 10 mM Tris-HCl, pH 7.4, containing 20% glycerol, 0.4 mM β-mercaptoethanol, and 0.1 mM EDTA (T-buffer). Protein concentration was estimated by the method of Bradford using bovine serum albumin as standard (Bradford, 1976). The activity of 3β-HSD/I was assayed in a volume equivalent to 30 µg protein in 1-ml T-buffer (pH 7.4, 28°C) with 0.5 mM NAD^+, and 25 µM [7(n)-^{3}H] pregnenolone (PerkinElmer); samples were incubated for 20 min (Pozzi, Lantos, & Ceballos, 1997). The activity of $Cyp450_{c17}$ was determined by incubating a volume of the homogenate containing 50-µg protein for 10 min in the presence of 5 µM [7(n)-^{3}H]pregnenolone in 1-ml 50 mM potassium phosphate buffer, pH 7.4, containing 0.1 mM EDTA, 0.4 mM β-mercaptoethanol, 5 mM glucose-6-phosphate, 0.5 mM NADPH, and 1 IU/ml glucose-6-phosphate dehydrogenase (Fernández Solari et al., 2002). The reaction was stopped with cold dichloromethane, and media were extracted twice. Substrates and products were separated by thin layer chromatography with methylene chloride:acetone (82:18, v/v) as solvent systems for all the enzymes. The amount of [^{3}H]progesterone produced from 3β-HSD/I assays, as well as the amount of [^{3}H]3β, 17-dihydroxy-5-pregnen-20-one plus [^{3}H]dehydroepiandrosterone produced from $Cyp450_{c17}$ assays, was quantified after chromatography. The 17-hydroxylase activity was calculated as the sum of [^{3}H]3β, 17-dihydroxy-5-pregnen-20-one and [^{3}H]dehydroepiandrosterone, whereas 17,20-lyase activity was obtained by using the amount of [^{3}H]dehydroepiandrosterone. The specific activities of enzymes were expressed as nmol of product*min/mg protein.

2.5 | Radioactivity

Scintillation counting was carried out with a Wallac 1409 DSA equipment (Wallac, Turku, Finland) in which quenching was corrected individually for each sample through automated optimal energy-window opening. The scintillation cocktail for all samples was OptiPhase-Hi safe 3 (Wallac).

2.6 | Western blot analysis

After treatments, samples were homogenized with the ice-cold buffer (0.25 M sucrose, 10 mM Tris-HCl, 0.4 mM, β-mercaptoetanol, and 0.1 mM EDTA, pH 7.4) and centrifuged at 400*g* for 10 min to sediment debris. Supernatants were separated and centrifuged at 800 g for 10 min to obtain postnuclear fractions. These fractions were centrifuged at 15,000*g* for 20 min, and the pellet resuspended in 25 µl of ice-cold buffer to obtain mitochondrial fractions (Pozzi et al., 1997). All steps were carried out at 4°C. Protein concentration was estimated by Qubit Fluorometer (Invitrogen, CA). For electrophoresis, 80 µg of mitochondrial fractions were mixed with sample buffer (40 mM Tris-HCl, 2% sodium dodecyl sulfate (SDS) (w/v), 4% glycerol (v/v), 0.001% bromophenol blue (w/v), and 2 mM β-mercaptoethanol, pH-6.8). Proteins were electrophoresed at 110 V in a 12% SDS-polyacrylamide gel. After transference, membranes were incubated in the phosphate-buffered solution containing 0.1% Tween-20 (PBS-T) and 3% peroxide hydrogen for 10 min and blocked for 1.5 hr at room temperature in PBS-T containing 5% low-fat powdered milk. Incubations with primary antibodies were performed overnight at 4°C in PBS-T-2% low-fat powdered milk with 0.2 µg/ml anti-StAR antibody (Santa Cruz Biotechnology, CA), according to Kim, Oha, Ahn, Soh, and Kwona (2009), and 2 µg/ml mouse monoclonal antiglyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Ambion-Applied Biosystems, CA). Membranes were then incubated with the secondary antibody coupled to horse radish peroxidase (0.2 µg/ml goat

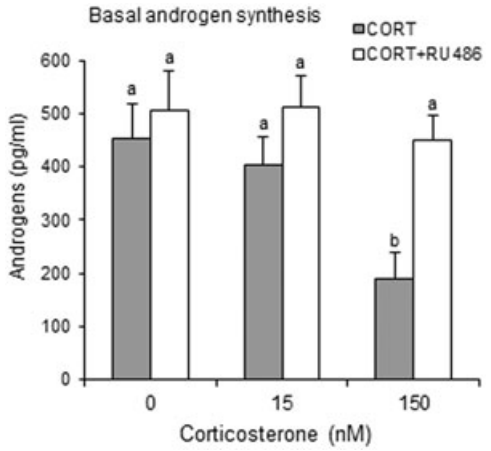


FIGURE 1 Effects of corticosterone on basal androgen production. Fragments of testis were cultured with different concentrations of corticosterone alone (gray bars) or in the presence of 10 μM RU486 (white bars). After 24-hr incubation, media were changed and basal androgen production (pg/ml medium) in each individual fragment was determined by RIA, after 1-hr incubation in KRGH, pH 7.4. Results from the six treatments are shown as six couples to emphasize the biological meaning and expressed as mean ± *SE*. Different letters mean significant differences among treatments, $n = 10$, $F_{7,63} = 18.25$, $p < 0.0001$. KRGH: Krebs-Ringer-glucose solution containing 10 mM Hepes; RIA: radioinmunoassay; SE: standard error of mean

antirabbit IgG, Chemicon International, for StAR and 0.2 μg/ml horse anti-mouse IgG, Vector Laboratories, CA, for GAPDH). Immunopositive bands were visualized by using enhanced chemiluminescent detection reagent (1.25 mM luminol, 0.198 mM cumaric acid, 0.038 v/v peroxide hydrogen, and 100 mM Tris-HCl buffer) in a Fujifilm LAS-1000 chemiluminescent detection system. Densitometric analysis of corresponding bands was performed with ImageGauge software (Fuji Photo Film, CO). To validate the semiquantitative method, the relationship between protein concentration and optical density was analyzed. This ratio was linear in the range of the concentration used (30–100 μg of protein).

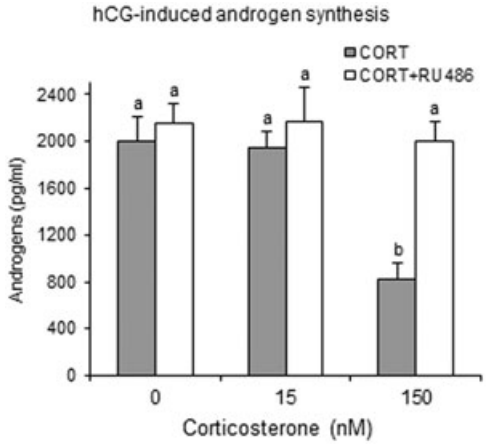


FIGURE 2 Effects of corticosterone on hCG-induced androgens synthesis. Fragments of testis were cultured with corticosterone alone (gray bars), or in the presence of 10 μM RU486 (white bars). After 24 hr, androgen synthesis was induced with 20 IU hCG during 1 hr in KRGH pH 7.4. Results from the six treatments are shown as three couples to emphasize the biological meaning and expressed as mean ± *SE*. Different letters mean significant differences among treatments, $n = 10$, $F_{7,63} = 19.04$, $p < 0.0001$. hCG: human chorionic gonadotropin; *SE*: standard error of mean

2.7 | Statistical analysis

Results were expressed as mean ± standard error of mean, and analyzed and compared by using analysis of variance (ANOVA) test followed by Tukey's multiple comparisons tests to detect significant differences among treatments (Steel & Torrie, 1980). Western blot results were compared by means of a randomized block ANOVA design. Androgen production and enzymatic activity assays results were compared using a generalized randomized block ANOVA design, in which there is more than one observation per treatment within each block. In all the cases each toad was considered as a block. Statistical analyses were performed with STATISTICA 6.0 (StatSoft, Inc., OK). Differences were considered significant with $p < 0.05$. Before statistical analysis, data were tested for normality and homoscedasticity by mean of Lilliefors and Bartlett's test, respectively. Data were not transformed because they satisfied the ANOVA assumptions.

3 | RESULTS

3.1 | Effects of GCs on androgen biosynthesis

Testicular fragments were exposed to 0,15, and 150 nM corticosterone for 24 hr, with or without RU486, and basal production of androgens was determined after treatments. As shown in Figure 1, after 24-hr incubation, 150 nM of corticosterone inhibited androgen production ($F_{7,63} = 18.25$, $p < 0.0001$). To evaluate whether the effect of GCs on steroidogenesis is mediated by the GR, testicular slices were incubated with either of the two concentrations of corticosterone mentioned above, in the presence of 10 μM RU486, an antagonist of mammalian GR that also binds with high affinity to toads testicular GR (Denari & Ceballos, 2006). The addition of RU486 completely inhibited the effect of corticosterone, suggesting that the action of GCs on androgen production is due to its interaction with the GR (Figure 1). In addition, 150 nM corticosterone inhibited the hCG-induced androgen increase ($F_{7,63} = 19.04$, $p < 0.0001$) (Figure 2) without modifying the ratio hCG-stimulated/basal androgen production ($F_{3,63} = 0.84$, $p = 0.5817$) (Figure 3). The addition of RU486 alone without corticosterone neither affects basal nor hCG-induced androgen biosynthesis (Figures 1 and 2). Furthermore, short-term incubations with corticosterone did not affect the production of androgens (data not shown).

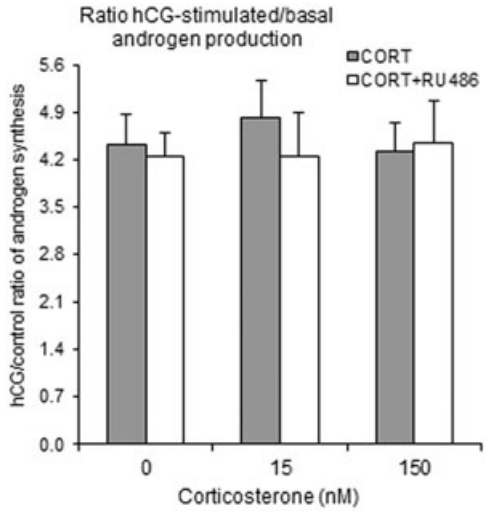


FIGURE 3 Effects of corticosterone on the ratio of hCG-induced and basal androgens synthesis. Fragments of testis were cultured with corticosterone alone (gray bars) or with (white bars) 10 μM RU486. After 24 hr, basal- and hCG-induced androgen productions were evaluated after 1-hr incubation in KRGH pH 7.4. The concentration of hCG was 20 IU hCG. Results from the six treatments are shown as three couples to emphasize the biological meaning and expressed as mean ± standard error. Androgen production was normalized to the corresponding control value without hCG. There were no significant differences among treatments, $n = 10$, $F_{3,63} = 0.84$, $p = 0.5817$. hCG: human chorionic gonadotropin

3.2 | Effect of 11β-HSD inhibition on GC action

The role of 11β-HSD in the modulation of corticosterone action was assayed by incubating testicular slices along with corticosterone, either with or without 1.5 μM GA, an inhibitor of toad 11β-HSD. As shown in Figure 4, the inhibitor increases the sensitivity of testes to GCs ($F_{7,63} = 32.41$, $p < 0.0001$), but does not modify androgen production in the absence of corticosterone (data not shown).

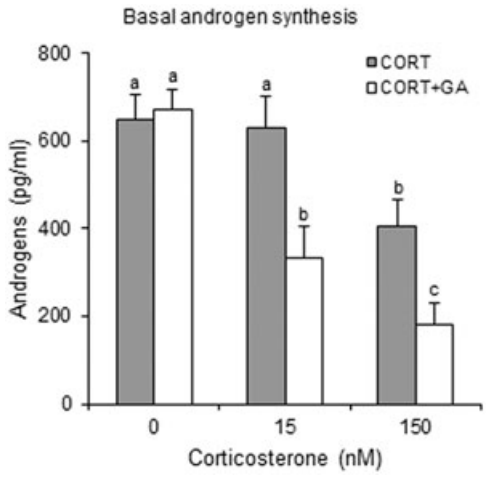


FIGURE 4 Effects of corticosterone and glycyrrhetinic acid (GA) on basal androgen synthesis. Fragments of testis were cultured with corticosterone alone (gray bars) or in the presence of 1.5 μM GA (white bars). After 24 hr, incubation media were processed as described in Figure 1. Results from the six treatments are shown as three couples to emphasize the biological meaning and expressed as mean ± *SE*. Different letters mean significant differences among treatments, $n = 10$, $F_{7,63} = 32.41$, $p < 0.0001$. *SE*: standard error of mean

3.3 | Effects of corticosterone on Cyp450$_{c17}$ and 3β-HSD/I activities

With the purpose of analyzing whether GC action on androgen production is due to the inhibition of one or more steroidogenic enzymes, activities of Cyp450$_{c17}$ and 3β-HSD/I were determined after 24 hr of treatment. Figure 5a shows that 150 nM corticosterone significantly decreased the activity of the key enzyme for androgen biosynthesis, the Cyp450$_{c17}$ ($F_{7,49} = 4.17$, $p = 0.00114$). This result is consistent with the decrease in basal androgen production in the presence of GCs. The effect of corticosterone on the cytochrome was inhibited by the addition of RU486 (Figure 5a). Neither the ratio lyase/17-hydroxylase ($F_{7,49} = 0.2$, $p = 0.9562$) (Figure 5b), nor the activity of 3β-HSD/I ($F_{3,49} = 1.24$, $p = 0.2977$; Figure 5c) were affected by any of the GC concentrations used.

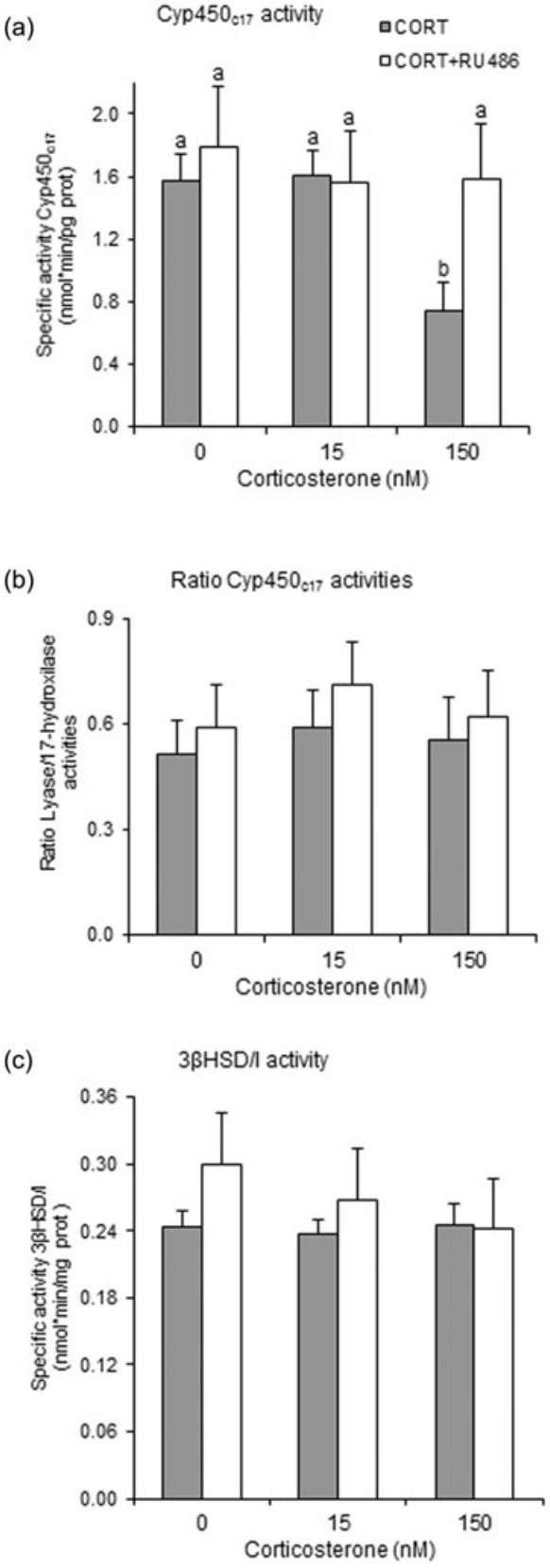
3.4 | Effects of corticosterone on StAR protein level

The effect of corticosterone on StAR expression was studied by Western blot analysis. A 24-hr treatment with corticosterone did not produce any significant decrease in the amount of basal ($p = 0.3530$; Figures 6a,b) nor hCG-stimulated StAR protein ($p = 0.3420$; Figures 6c,d).

4 | DISCUSSION

As in other nonmammalian vertebrates, in several amphibians there is an increase in the concentration of plasma GCs during breeding (Denari & Ceballos, 2005; Michael Romero, 2002; I.T. Moore & Jessop, 2003). Nonetheless, exogenous GC administration and/or natural rise in GCs have been found to be inversely correlated with circulating androgen levels in anuran and urodelean species (Burmeister, Somes, & Wilczynski, 2001; Licht, McGreery, Barnes, & Pang, 1983; Orchinik, Licht, & Crews, 1982; Woodley & Lacy, 2010). The question arising from these results is whether this inverse correlation between GCs and androgens is to a certain extent due to the effect of GCs on the steroidogenic activity of Leydig cells.

Trying to confirm the hypothesis ensuing from the former question, androgen production regulation by GCs was studied in toad testes. The results obtained in herein corroborate that a physiological concentration of GCs—corresponding to the breeding season—is able to inhibit the synthesis of androgens in testes of animals collected during the nonreproductive season. The inhibition elicited by corticosterone is blocked by a GR antagonist (RU486), confirming that the effect of GCs on androgen biosynthesis is mediated by this receptor. The role of RU486 as a GR antagonist has



been previously reported for other amphibian species (Crespi & Denver, 2004; Orchinik et al., 2000). Furthermore, in toad testes, it has been demonstrated that RU486 displaces the binding of dexamethasone and corticosterone from the GR (Denari & Ceballos, 2006). The inhibition of 11β-HSD with GA increases the sensitivity of Leydig cells to corticosterone, suggesting that the activity of this enzyme modulates the action of this natural GC. The intratesticular localization of the GR and its association with 11β-HSD activity is

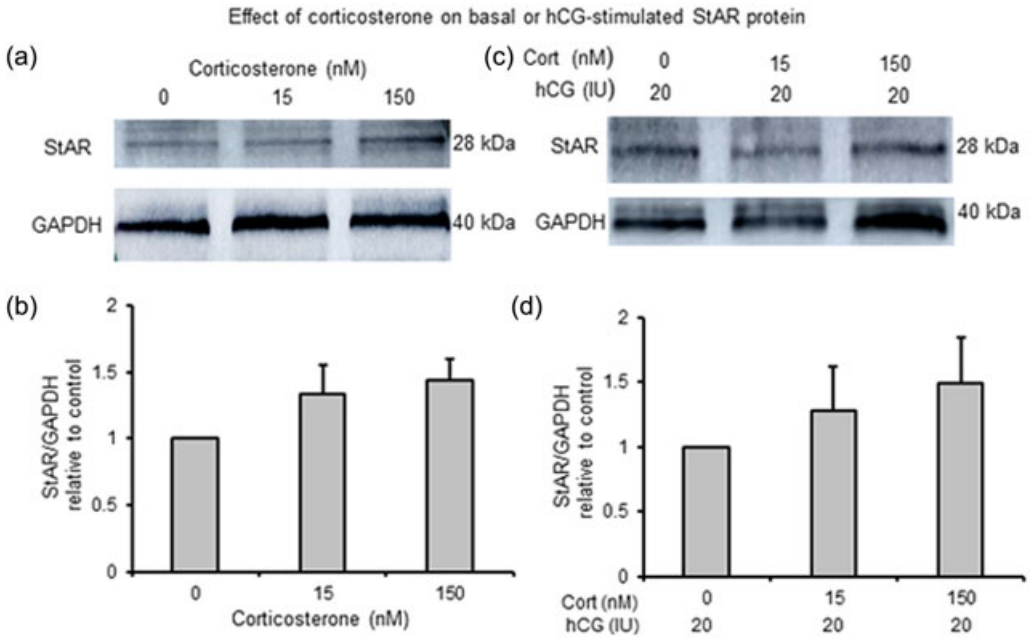


FIGURE 6 Effects of corticosterone on StAR protein levels. Testes were cultured with different concentrations of corticosterone. After 24-hr testes were processed as described under Methods. The amount of StAR protein was measured by Western blot. Panels a and b correspond to basal StAR; Panels c and d correspond to hCG-stimulated StAR protein. (a,c) Membrane corresponding to one representative experiment. (b,d) Densitometric analysis of StAR-immunoreactive bands. Values of StAR were normalized to the corresponding GAPDH level and expressed as the ratio treatment/control. Results correspond to mean of $n = 6$ animals ± *SE* to basal StAR protein and $n = 6$ animals ± *SE* to hCG-stimulated StAR protein There were no significant differences among treatments, basal $p = 0.3530$ and hCG-stimulated $p = 0.3420$. GAPDH: glyceraldehyde-3-phosphate dehydrogenase; hCG: human chorionic gonadotropin; SE: standard error of mean; StAR: steroidogenic acute regulator protein [Color figure can be viewed at wileyonlinelibrary.com]

another outstanding aspect to take into account. In the toad, GR is located in Leydig cells as well as in Sertoli cells (Denari & Ceballos, 2006), a dual localization comparable with the intratesticular distribution described in the rat (Evain, Morera, & Saez, 1976; Levy et al., 1989). Because 11β-HSD2 decreases intracellular concentration of active GCs, the coexpression of GR with 11β-HSD2 would be crucial in determining the sensitivity to GCs. In fact, as previously described in the rat (Gao et al., 1996; Gao et al., 1997), the inhibition of 11β-HSD with GA in *R. arenarum* renders Leydig cells more susceptible to GCs action, increasing the responsiveness of the testes to lower concentrations of corticosterone (15 nM). In most GC target tissues, such as liver and lung, 11β-hydroxysteroid dehydrogenase Type 1 (11β-HSD1) operates mainly as a reductase (Seckl & Walker, 2001), but in rat Leydig cells the redox potential favors oxidative activity (Ge & Hardy, 2000). Experiments performed by Hu et al. (2008) have suggested that there is a coupling between the dehydrogenase activity of 11β-HSD, and the NADPH-dependent enzymes involved in testosterone biosynthesis. The $NADP^+$ generated by the latter could stimulate the oxidative activity of 11β-HSD1. It would not be applicable in the toad because 11β-HSD expressed in Leydig cells is a NAD^+-dependent isoform similar to the mammalian Type 2 enzyme (Denari & Ceballos, 2005).

Our results also show that GCs inhibit both $Cyp450_{c17}$-associated activities in the toad testes. Similar results were obtained in adult rat testes, where an increase of corticosterone induced by acute immobilization stress inhibited the activities of both 17-hydroxylase and 17,20-lyase, and impaired testosterone production (Orr & Mann, 1992; Orr, Taylor, Bhattacharyya, Collins, & Mann, 1994). Alternatively, these effects were not reported for rat progenitor Leydig cells (Xiao, Huang, Hardy, Li, & Ge, 2010). In the toad, the treatment with corticosterone had no significant effect on 3β-HSD/I, differing from what has been described for rat progenitor Leydig cells, where treatment with dexamethasone decreases the level of messenger RNA as well as the activity of 3β-HSD/I (Xiao et al., 2010). Furthermore, treatment of rat adrenals with dexamethasone enhances the expression of Type II 3β-HSD/I (Feltus

FIGURE 5 Effects of corticosterone on $Cyp450_{c17}$ and 3β-HSD/I activities. Testes were cultured with different concentrations of corticosterone in the absence (gray bars) or presence (white bars) of 10 μM RU486. After 24-hr incubation, enzymatic activity was determined as describe under Methods section. Results from the six treatments are shown as three couples to emphasize the biological meaning and expressed as mean of ten animals ± *SE*. (a) $Cyp450_{c17}$ specific activity is expressed as nmol/min × mg protein. Different letters mean significant differences among treatments, $F_{7,49} = 4.17$, $p = 0.00114$. (b) Ratio between lyase and 17-hydroxylase activities. Results correspond to mean of ten animals ± *SE*. There were no significant differences among treatments, $F_{7,49} = 0.2$, $p = 0.9562$. (c) 3β-HSD/I specific activity is expressed as nmol*min/mg protein. Results correspond to mean of ten animals ± *SE*. There were no significant differences among treatments, $F_{3,49} = 1.24$, $p = 0.2977$. *SE*: standard error of mean

et al., 2002), suggesting that the effect of GCs on this enzyme is variable and depends not only on the species, but also on the steroidogenic gland.

In *Bufo melanostictus*, in vivo corticosterone treatment resulted in the inhibition of testicular 17β-hydroxysteroid dehydrogenase (17β-HSD) activity and a decrease in serum testosterone levels (Biswas, Chaudhurt, Sarkar, & Bengupta, 2000). This reduction in serum testosterone and testicular 17β-HSD could also have been due to the reduction in pituitary gonadotropin secretion evoked by corticosterone (Biswas et al., 2000). In the tree frog, *Hyla cinerea*, in vivo treatments with exogenous corticosterone also resulted in decreased androgen levels, but it is not clear whether this was a direct effect on the testes, or through another hormonal system affected by GCs (Burmeister et al., 2001). By analyzing the in vitro effects of GCs, our study is the first report showing a direct effect of GCs on amphibian testicular steroidogenesis.

Another possible explanation for the GC-induced decrease in androgens production is that, as it was demonstrated in rat Leydig cells, exogenously administered corticosterone could induce apoptosis and a concomitant decrease in Leydig cells number in the interstitium (Gao et al., 2002). However, 3β-HSD/I activity, another steroidogenic enzyme present in Leydig cells was not impaired by GC treatment in the toad, suggesting that the decrease in androgens is not due to an apoptotic effect but to direct inhibition of $Cyp450_{c17}$.

In *R. arenarum*, testicular steroidogenesis switches from androgen production during the nonbreeding season to the synthesis of 5α-pregnan-3,20-dione and several 3α and 20α reduced steroids during breeding (Canosa, Pozzi, & Ceballos, 1998). Our results suggest that $Cyp450_{c17}$ could play a pivotal role in the seasonal switch of steroidogenesis because the decrease in this enzyme activity would explain the lower androgen biosynthesis during the breeding season. Follicle stimulating hormone induced a decrease in the activity of $Cyp450_{c17}$ (Canosa & Ceballos, 2002b). However, GCs could also contribute to the regulation of androgen synthesis because they decrease the activity of $Cyp450_{c17}$ without modifying the activity of 3β-HSD/I. In addition, GCs do not seem to affect the response of the tissue to gonadotropins because the ratio hCG-induced androgens/basal androgens is not modified. Further studies are needed to confirm this idea.

In conclusion, we found that GCs in concentrations corresponding to those detected during the breeding season inhibit androgen biosynthesis. This reduction is not associated with a decrease in the sensitivity to hCG nor to a decrease in StAR protein. The impairment of androgen production may be due to the suppression of $Cyp450_{c17}$ activity via a GR-mediated mechanism. These observations also suggest that androgen secretion could increase to pre-reproductive levels after the decrease in GC concentrations.

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SUPPORTING INFORMATION

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