RESEARCH ARTICLE

Validation of Electrochemiluminiscence Immunoassay for Ovarian Steroid Determination in *Rhinella arenarum*

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ABSTRACT In this work, we describe the validation of an electrochemiluminescence immunoassay (ECLIA) that allowed us for the first time to determine the levels of progesterone (P_4) and testosterone (T) secreted by *Rhinella arenarum* follicles during the preovulatory (POP) and reproductive (RP) periods. We also verified the relation between P_4 and T levels and oocyte maturation. Moreover, we demonstrated that the extraction protocol developed for the determinations of P_4 and T by ECLIA proved to be efficient and reproducible since the efficacy of the extraction was above 95% in all assays conducted. The results indicate that in the validation process the variation coefficient (CV) between assays is compatible with the analytical procedures based on automated immunoassays (CV $<$ 8%) and that the adaptation proposed for the samples allows the determination of T and P_4 with the Cobas e-411 analyzer. Our results indicate that in basal conditions the levels of T released by *R. arenarum* follicles were higher than those of P_4 during POP and RP. In these conditions, steroid secretion failed to induce germinal vesicle break down (GVBD) in the follicles. Under gonadotropin stimulation, steroidogenesis showed a remarkable increase in both periods, especially during POP. This increase was correlated with a high maturation percentage in the follicles incubated in vitro (GVBD = 72 ± 16%) during POP. During RP, human Chorionic Gonadotropin (hCG) induced 81.75 ± 9.1% GVBD. This study is the first report of the seasonal steroidogenic activity in the ovary of *R. arenarum* in situ using an ECLIA-modified protocol developed in our laboratory. *J. Exp. Zool. 00:1–9*, 2016. © 2016 Wiley Periodicals, Inc.

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INTRODUCTION

Meiotic maturation events in amphibian oocytes are similar to those that take place in most vertebrates. They include movement of the germinal vesicle, breakdown of the nuclear envelope, resumption of meiosis, and its stoppage in Metaphase II. Gonadotropins activate steroid production by follicle cells, and these steroids act on the oocyte to induce maturation (Bayaa et al., 2000; Lutz et al., 2003; Haccard et al., 2012) and ovulation during the reproductive period.

In amphibians, progesterone (P_4) has long been considered the physiological inducer of maturation (Maller and Krebs, '80; Zelarayán et al., 2004, 2012). Nowadays it has been suggested that other steroids such as androgens might be involved in this process in *Xenopus laevis* (White et al., 2005) and *Rhinella arenarum* oocytes (Arias Torres et al., 2015).

In most anurans, reproductive cycles are influenced by numerous environmental factors capable of activating neuroendocrine pathways that control the synthesis and release of hypophysial gonadotropins whose action regulates two closely related processes: gametogenesis and steroidogenesis. Thus, the steroids secreted by the ovary are released into the follicular environment and their levels vary according to seasonal changes. These variations have been studied in anuran species such as *Rana esculenta* (d'Istria et al., '74; Fasano et al., '89), *Rana pipiens* (Wada et al., '76), *Rana catesbeiana* (Licht et al., '83), *Bufo japonicus* (Itoh and Ishii, '90), *Hynobius nigrescens* (Hasumi et al., '93), and *B. arenarum* (Medina et al., 2004). However, the relation between steroid secretion and the reproductive cycle of amphibian females is not yet known in depth. Although the seasonal variation in plasma steroids (Medina et al., 2004) and the effect of exogenous sex steroids on in vitro oocyte maturation (Arias Torres et al., 2015) have been studied in *R. arenarum* (ex *Bufo arenarum*), no data exist connecting the effects of the endogenous steroids released by ovarian follicles on meiosis resumption.

Great advances have been made in methods for the quantitative determination of steroids in biological samples. Steroids, which have a molecular mass of 300 Da, are mainly quantified by competitive immunoassay, a determination in which the antibody provides the specificity for the target steroid. Radio immunoassay (RIA), which was developed in the 1970s on the basis of competitive union, is a standard method used for the direct quantification of plasma hormones whose drawback is the need for prolonged incubation periods. Another standard method such as high-performance liquid chromatography (HPLC) affords precise results but has disadvantages such as high costs and the need for specialized technical staff. Both RIA and HPLC provide specific results, but require laborious preanalytical procedures (Jafri et al., 2011). These methods provide analytical precision (expressed as variation coefficient) and are highly sensitive to the matrix of the calibrators and of the samples (accuracy or systematic error), which can affect the steroid-antibody reaction and the quantification of the signal. In all analytical procedures, the pattern matrix should be constitutively similar to the sample matrix.

During the past few years, ECLIA (electrochemiluminescence immunoassay), an alternative method based on an automated enzymatic immunoassay, was developed for the determination of plasma steroids (protein matrix) in the routine clinical biochemistry lab.

ECLIA is a method with a larger reportable concentration range than RIA, so that it is of great clinical and experimental usefulness. This fast automated assay has stable calibration curves that do not require calibration for each batch of samples (Sánchez-Carbayo et al., '98).

This work analyzes for the first time the participation of ovarian follicles of *R. arenarum* as a source of sex steroids and their possible relationship with oocyte maturation using the ECLIA test, which was chosen on the basis of its sensitivity, rapidity, and reproducibility of results.

On the basis of our previous studies and considering that in *R. arenarum* maturation has a seasonal variation, the aim of the present work was twofold:

1. To adapt steroid samples secreted by follicles in a saline incubation medium to the protein matrix of the ECLIA immunoassay.
2. To use the validated ECLIA test in the determination of P_4 and testosterone (T) secreted in vitro by *R. arenarum* ovarian follicles and analyze their relation with meiosis resumption.

MATERIALS AND METHODS

Animals

Adult female *R. arenarum* specimens were collected in Tucumán, northwestern Argentina. They were kept in captivity for a few hours at room temperature. The animals were collected in July (preovulatory period (POP)) and in September–December (reproductive period (RP) for three consecutive years. Ovaries were extracted immediately in order to minimize the effect of captivity on steroid basal levels.

In Vitro Culture of Ovarian Follicles

Amphibian Ringer solution (AR; saline matrix) (6.60 g NaCl/L, 0.15 g $CaCl_2$/L, and 0.15 g KCl/L) with penicillin G-sodium (30 mg/L) and streptomycin sulfate (50 mg/L), pH 7.4, was used as a culture medium in all routine incubations.

Full-grown intact follicles (1.5–1.6 mm) were isolated from ovarian tissues using watchmaker's forceps after 1–2 hr of equilibration at room temperature (22–25°C). In each follicle, the oocyte was surrounded by follicular cells, theca cells, and external epithelium.

The incubations were performed for 6 hr at 24–26°C in multiwell culture dishes (Costar 3524, Cambridge, MA, USA) with randomized samples of 20 follicles distributed into separate wells containing 1 mL of AR or AR + human Chorionic Gonadotropin (hCG) (10 UI/mL). Duplicates in two wells were run in each experimental group.

After 6 hr, the incubation medium was separated and stored or kept at −20°C until steroid determination with ECLIA.

Meiosis resumption (GVBD) was monitored in the follicles at 20 hr.

Reagents

Rat packs of reagents labeled PROGII or TESTOII (Roche Diagnostics GmbH, Germany) were used. The PROGII pack consists of the following reagents: M, streptavidin-coated microparticles (6.5 mL); R1, antiprogesterone ~ biotin antibody (10 mL) (antiprogesterone monoclonal biotinylated antibodies (mouse) 0.15 mg/mL; phosphate buffer 25 mmol/L pH 7,0; preservative); R2, progesterone peptide~Ru(bpy) $^{2+}{}_{3}$ (8 mL) (progesterone bound to a synthetic peptide labeled with a ruthenium chelate 10 ng/mL; phosphate buffer 25 nmol/L, pH 7,0; preservative).

The TESTOII pack consists of the following reagents: M, streptavidin-coated microparticles (6.5 mL); R1, antitestosterone ~ biotin antibody (10 mL) (antitestosterone monoclonal biotinylated antibodies (sheep) 40 ng/mL; releasing reagent 2-bromoestradiol; buffer MES ($C_6H_{13}NO_4S$) 50 mmol/L pH 6,0; preservative); R2, testosterone-peptide ~ Ru(bpy) $^{2+}{}_{3}$ (9 mL) (testosterone labeled with ruthenium chelate 1.5 ng/mL; buffer MES 50 nmol/L, pH 6.0; preservative).

The following material was also used: progesterone II CalSet (for 4 × 1 mL) or testosterone II CalSet II (for 4 × 1 mL), PreciControl Universal (for 2 × 3 mL), diluent estradiol/progesterone (2 × 22, mL of diluent for samples), common laboratory equipment, ProCell (6 × 380 mL buffer, CleanCell (6 × 380 mL of detergent solution measuring cell), Elecsys SysWash (1 × 500 mL wash water additive), SysClean adapter, Elecsys 2010 AssayCup (60 × 60 test tubes), Elecsys 2010 Assay Tip (30 × 120 pipette tips).

Universal Diluent (UD = protein matrix) (Roche).

Chemiluminescence Enzyme Immunoassay

T and P_4 levels in AR solutions were measured using a competitive chemiluminescence enzyme immunoassay. This assay is based on the principle of competitive union using a monoclonal antibody with reagents for the Cobas e-411 immunology analyzer (Roche). During the immunological reaction, the sample steroid and the analog compete for binding to the antibody since the latter is in a 1:2 molar ratio with respect to the analog. As in other competitive immunoassays, the response level (counts per second) decreases as steroid concentration increases in the sample.

In this analytical system, the reporting range for P_4 is 0.095–191.0 nM and for T is 0.087 nM at 52.0 nM.

The specificity of the monoclonal antibody was obtained from the Roche Diagnostic Elecsys, 2010 guide for T and from the Roche Diagnostic Elecsys 2013 guide for P_4, where it was determined by measuring the percentages of cross reaction with steroids related to T or P_4. For the antibody used in the determination of T, the percentages of cross reaction with other related androgens were as follows: androstenodione ≤2.5%, DHEA-S (dehydroepiandrosterone sulfate) ≤0.003%, testosterone propionate ≤2.46%, 5-α-androsteno-3β,17β-diol ≤2.11%, 5-α-dihydrotestosterone ≤0.86%, 11-ceto-testosterone ≤3.22%, 11-β-hydroxytestosterone ≤18.0%, and nondetectable progesterone (Roche Diagnostic Elecsys, 2010).

For the antibody used in the determination of P_4, the following cross reactions were obtained: medroxyprogesterone 0.812%; 5-β-dihydroprogesterone 20.7%; 17-α-hydroxyprogesterone 1.30%; 17-α-hydroxypregnenolone 0.018%, and testosterone 0.020% (Roche Diagnostic Elecsys, 2013).

Validation of the Analytical Step

Since the analytical system ECLIA is validated for a human serum protein matrix, it was necessary to validate the analytical step to measure steroids in the saline incubation medium of *R. arenarum* ovarian follicles.

The validation of the samples included the following assays:

- *Adaptation*: 0.7 mL of sample from the incubation of follicles in AR (saline matrix) was placed in a glass pipe, and 0.5 mL of dichloromethane was added in order to extract the steroids from the sample. Then the pipe contents underwent reversible emulsion in a vortex mixer for 5 min and centrifugation for 3 min at 10,000 rpm, and the upper aqueous layer was aspirated. The pipes were left uncovered overnight to allow the solvent to evaporate at room temperature. The dry extract obtained was reconstituted with 0.3 mL of universal diluent (UD). It was centrifuged again, this final solution being the right one for the analytical system to be used.
- *Matrix change assays*: The systematic errors of the adaptation process of the samples (matrix change) to the quantitative analytical system (ECLIA) were evaluated as follows: One milliliter of calibration solution of P_4 (53.0 ng/mL) (provided by the Roche kit) was placed in a glass pipe, and 0.5 mL of dichloromethane was added; then the tube was agitated and centrifuged, and the aqueous layer was aspirated as in the adaptation section.

  Once the steroid dried extract was obtained from the calibration solution of P_4, in a second step, it was reconstituted in different matrices separately, with 0.3 mL of AR (saline matrix) or in 0.3 mL of UD (protein matrix), and incubated at 37°C for 10 min with intermittent agitation. It was centrifuged again and then determined by ECLIA. The same procedure was

followed with a calibration solution of T (11.50 ng/mL). The reconstitution assay was conducted with each calibration solution of P_4 and of T in different matrices in quintuplicate.

- *Recovery assays*: After verifying that UD is the adequate matrix to measure the steroids from samples in a saline matrix using ECLIA, we determined the systematic error of the adaptation procedure (matrix change) at two concentration levels: at high concentration and at low concentration (Table 3). As reference solutions, two calibration solutions of P_4 (provided by the Roche kit) were used: a calibration solution of P_4 at low concentration (0.95 ng/mL) and a calibration solution of P_4 at high concentration (5.2 ng/mL).

The percentage of P_4 recovery at both concentration levels (high and low) was obtained after extracting the hormone from the calibration solutions with dichloromethane, reconstituting the dry extract with UD and measuring with ECLIA. Finally, the values obtained were compared with the values of the calibration solutions. The P_4 recovery assays at two concentration levels were performed in quintuplicate, and the error generated at random in these assays was determined (CV).

The same procedure was used to determine the T recovery percentages at two concentration levels. Calibration solutions of T (provided by the Roche kit) were used as reference solutions: a calibration solution of T at low concentration (0.88 ng/mL) and a calibration solution of T at high concentration (4.8 ng/mL).

In these analytical methods, recovery was optimal: between 90% and 110%.

Statistical Method

Significant differences were determined by Student's test when two means were compared. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Validation of ECLIA for P_4 and T Determination

The validation of ECLIA to determine P_4 and T in samples obtained from the incubation of ovarian follicles of *R. arenarum* in a saline matrix (AR) was successful.

In the analytical systems, the matrix effect is a parameter that largely determines the quality of the determinations. The error generated in the adaptation of the samples (matrix change) to the quantitative analytical system ECLIA was evaluated as described below. We determined the P_4 concentration (in quintuplicate) in the P_4 calibration solution (53.0 ng/mL) in two different matrices: with AR (saline matrix) or in UD (protein matrix) (Table 1). After recovering P_4 from the P_4 calibration solution (53.0 ng/mL) in a saline matrix (AR), the P_4 concentration obtained was 34.7 ± 3.90 ng/mL ($n = 5$) whereas the P_4 concentration obtained in a protein matrix (UD) was 51.0 ± 3.60 ng/mL ($n = 5$). The results show that the concentration of the hormone measured in a protein matrix (51.0 ± 3.60 ng/mL) is close to the P_4 concentration in the reference calibration solution (53.0 ng/mL).

Table 1. Recovery of P_4 from a calibration solution (53.0 ng/mL) in different matrices (AR or UD) in quintuplicate ($n = 5$)

	Progesterone calibration solution (53.0 ng/mL)	
Diluent	Ringer (AR)	Universal diluent (UD)
n	5	5
Medium (ng/mL)	34.7	51.0
CV (%)	3.90	3.60
$P < 0.01$*		

Asterisk indicates significant differences between the diluents ($P < 0.05$).

Table 2. Recovery of T from a calibration solution (11.50 ng/mL) in different matrices (AR or UD) in quintuplicate ($n = 5$)

	Testosterone calibration solution (11.50 ng/mL)	
Universal	Ringer (AR)	Universal diluent (UD)
n	5	5
Medium (ng/mL)	7.90	11.30
CV (%)	4.30	3.80
P < 0.01*		

Asterisk indicates significant differences between the diluents ($P < 0.05$).

In the case of T, after the recovery of the hormone from the T calibration solution (11.5 ng/mL) in a saline matrix (AR), the concentration obtained was 7.9 ± 4.3 ng/mL ($n = 5$) whereas the concentration obtained in a protein matrix (UD) was 11.3 ± 3.8 ng/mL ($n = 5$) (Table 2). The results show that the T concentration measured in a protein matrix (11.3 ± 3.8 ng/mL) is similar to the T concentration in the reference calibration solution (11.5 ng/mL).

The statistical test used shows that the P_4 concentration of the reconstituted calibration solution in a saline matrix is significantly different ($P < 0.01$) from the P_4 concentration of the reconstituted calibration solution in a protein matrix (UD) (Table 1). The same result ($P < 0.01$) was found when measuring the T concentration in two different matrices (Table 2).

From the P value ($P < 0.01$), we can deduce that the means are different, indicating the need to use UD as an adequate matrix to determine the steroids released by the follicles into the incubation media using ECLIA (Tables 1 and 2).

After determining that the protein matrix (UD) was appropriate to measure P_4 and T released by the follicles with ECLIA, we analyzed the systematic error of the adaptation of the samples to the matrix change at two concentration levels, at a high and at a low concentration level both for P_4 and for T

Table 3. Analysis of the systematic error generated by the adaptation of saline samples to a protein matrix at high and low concentration values of the calibrators of P_4 and of T

	Low level		High level	
Concentration value	Expected (ng/mL)	Recovery (%)	Expected (ng/mL)	Recovery (%)
P_4	0.95	96 ± 5.1	5.20	101 ± 4.6
T	0.88	95 ± 5.0	4.80	96 ± 5.4

(Table 3) as follows: The progesterone concentration was determined in a P_4 calibration solution at low concentration (0.95 ng/mL) and in a P_4 calibration solution at high concentration (5.2 ng/mL), which after extraction were reconstituted with a protein matrix (UD). The P_4 recovery percentage in a protein matrix at both concentration levels was above 95% with CV lower than 8% (low level = 96 ± 5.1%; high level = 101 ± 4.6%) (Table 3).

The same procedure was used to determine the percentages of recovery of T at two concentration levels. We used T calibration solutions as reference solutions, one at low concentration (0.88 ng/mL) and another at high concentration (4.8 ng/mL), which after extraction were reconstituted with a protein matrix (UD). The percentage of recovery of T in a protein matrix at both concentration levels was ≥95% with CV lower than 8% (low level = 95 ± 5%; high level = 96 ± 5.4%) (Table 3).

The recovery levels of both steroids in a protein matrix were high (>95%) compared to the minimum and maximum concentration values of the calibrators of P_4 and of T, and the CV between assays (quintuplicates) were not above 8% (Table 3).

The recoveries of 100% indicate that after adaptation to the protein matrix, the steroid concentration values in the calibrators were very close to the steroid concentration values declared by Roche (Table 3). The exactitude (systematic error) expressed as recovery percentage (>95% for all assays) and precision expressed in CV between assays (<8%) in the results found in this work are compatible with those of the validation of the Cobas e-411 system for human serum.

The above results validate the adaptation procedure for the determination of steroids from saline solutions (AR) in a protein matrix. Table 3 shows the analytical CV for quintuplicates, which are optimal considering that there is a manual and an automated stage in the validation procedure. This procedure, which includes extraction, matrix adaptation, and quantification, allowed high recovery levels for both hormones.

Follicle Secretion of P_4 and T and Their Effect on Meiosis Resumption

After validating the methodology used to determine P_4 and T in an amphibian saline solution (AR) by ECLIA, we determined their variation at two stages of the reproductive cycle of *R. arenarum* and analyzed their relation with meiosis resumption. Fully grown follicles from recently captured females during both periods (POP and RP) were incubated in AR solution or in AR + hCG solution (10 UI/mL). After incubation for 6 hr, P_4 and T levels in the incubation medium were determined by ECLIA. The follicles were kept in AR to determine whether the steroids secreted were capable of inducing meiosis resumption at 20 hr of incubation.

The secretion of follicular steroids P_4 and T showed variations during the POP and RP periods, in basal conditions (AR) and under stimulation with hCG (Fig. 1). In basal conditions, significant differences ($P < 0.05$) were found in steroid levels in both the POP and the RP period. Figure 1A shows that, in these conditions, T follicle secretion was higher than P_4 secretion in both periods ($P < 0.05$). During RP, the follicles secreted four times more P_4 (0.166 ± 0.12 nM) than during POP (0.037 ± 0.01 nM), whereas for T a lower increase in secretion was observed during the RP period (RP = 1.067 ± 0.74 nM and POP = 0.807 ± 0.09 nM). Under these conditions, the secretion of the steroids assayed failed to induce GVBD in the follicles.

Under gonadotropin stimulation (10 UI/mL), steroidogenesis increased remarkably during both periods, especially during POP (Fig. 1B). In this period, an increase was found in P_4 (3.368 ± 2.39 nM) that was almost 100 times greater than under basal conditions. In the case of T, the increase was of about 20 times (T = 14.82 ± 1.30 nM) compared to basal values. This remarkable increase in steroid secretion during POP, stimulated by hCG, was correlated with a high percentage of maturation of the follicles incubated in vitro (GVBD = 72 ± 16%) (Table 4).

During PR, under gonadotropin stimulus, P_4 secretion increased 20 times and T secretion about three times compared to basal values (P_4 = 3.662 ± 2.16 nM; T = 3.666 ± 2.30 nM). During the RP, the variation in the endogenous secretion levels of both steroids caused by hCG (close to 10^{-8} M) induced 81.75 ± 9.1% GVBD in follicles incubated in vitro (Table 4). These results agree with those previously found by our work team (Arias Torres et al., 2015). We demonstrated that the addition of exogenous P_4 or T (10^{-8} M) induces maturation in whole follicles in a similar way. The exogenous addition of both steroids could cause synergistic effects, and GVBD percentages could come close to the values reached when follicles are stimulated with hCG; and both steroids are released into the incubation medium, thus affecting GVBD.

Table 4. Seasonal effect on in vitro induction of maturation in *R. arenarum* follicles

			Steroids nM/20 follicles (mean ± SD)		
Period	*n*	Treatment	P_4	T	% GVBD
POP	8	AR	0.037 ± 0.01	0.807 ± 0.09	0
POP	8	hCG	3.368 ± 2.39	14.823 ± 1.30	72 ± 16.02
RP	21	AR	0.166 ± 0.12	1.067 ± 0.74	0
RP	11	hCG	3.662 ± 2.16	3.666 ± 2.30	81.75 ± 9.10

Note: Fully grown follicles were cultured in the presence of hCG (10 UI/mL) or absence (AR), and P_4 and T levels were determined with ECLIA. Values are means ± SD.

DISCUSSION

This work describes and validates an ECLIA method that allowed us to determine the levels of P_4 and T secreted by the follicles of *R. arenarum* during the POP and RP periods. It also reports for the first time steroid production in a saline solution at the level of the ovarian follicles and its impact in connection with meiosis resumption in *R. arenarum* females.

This study is the first evaluation of the seasonal steroidogenic activity in the ovary of *R. arenarum* in situ using a modified ECLIA protocol developed in our laboratory.

In previous studies, RIA was the method of choice for the determination of steroid hormones in amphibians in plasma and at the level of the ovarian follicles. In this sense, the studies of Fortune ('83) in *X. laevis* and of Kwon et al. ('93) in *Rana nigromaculata* showed the variation in steroids released during the different stages of follicle development, although in those cases the determinations were conducted with RIA. Up to now, there is only one published work that describes the variation in plasma steroids in *R. arenarum* females (Medina et al., 2004). In this work, the authors studied the variations in plasma T, DHT (dihydrotestosterone), P_4, and E_2 (estradiol) levels in *R. arenarum* females throughout the year as indicators of ovarian and oviductal stages.

This work provides an alternative methodology faster than RIA, which is made easier by the use of an automated immunoassay that makes unnecessary the use of radioisotopes and affords a wide range of reportable concentrations (e.g., the reporting range for P_4 is 0.095–191 nM), thus allowing a minimum number of sample dilutions at high concentrations (Sánchez-Carbayo et al., '98). This is also favored by the fact that the calibration curve for ECLIA remains stable for at least 2 months, in contrast with RIA, which requires a new calibration curve for each protocol.

Our results indicate that the protocol developed for hormone determination by ECLIA is adequate for the follow-up of its seasonal variation.

Since the results of the recovery in an analytical procedure are optimal, when they are within the range of 90–110%, the extraction protocol developed to determine P_4 and T is effective and reproducible given that the extraction efficiency was above 95% for all assays conducted for the two hormones under study. Besides, both in the validation of P_4 and in that for T, the variability of the replicates, expressed as CV, is compatible with the analytical procedures based on automated immunoassays (<8%) (Sánchez-Carbayo et al., '98; Molinia et al., 2007).

The adaptation proposed for the target samples, with extraction-recovery and use of protein matrix, made possible T and P_4 determination by ECLIA with the Cobas e-411 analyzer.

Numerous publications have demonstrated that in amphibians the hormonal changes related to the reproductive activity are strongly influenced by the environment. The environment determines the plasma variations in the hormones related to the hypophysis-gonadal axis. Within them, sex steroids regulate the activity of this axis.

The first studies conducted in *Rana nigromaculata* showed that the levels of steroids secreted by follicles incubated in vitro vary with the size of the follicles. E_2 are produced mainly by medium-sized follicles, T by medium-sized follicles, and P_4 by the largest follicles (Kwon et al., '93, Gohin et al., 2011). This suggests that the steroidogenic capacity of the follicles changes during their development, from the production of E_2 to that of P_4. The follicles of *R. nigromaculata* grow more of less synchronically, and all follicles in the ovary are at the same developmental stage. This type of folliculogenesis differs markedly from the multiple stages of follicular growth that occur simultaneously in other amphibians such as *R. arenarum*. The results obtained in this work for fully grown follicles (1.5–1.6 mm) of *R. arenarum* show a change in steroid secretion between the periods studied, which would result mainly from the influence of the season and from the increase in gonadotropins in the PR.

In *R. arenarum* females, the T levels released by the follicles were higher than those of P_4 in basal conditions and in both periods (POP and RP). The high levels of androgens in the follicles of *R. arenarum* during PR coincide with the patterns of plasma androgens described for females of other amphibians. In *Rana esculenta*, *B. japonicas,* and *Rana catesbeiana*, the androgen

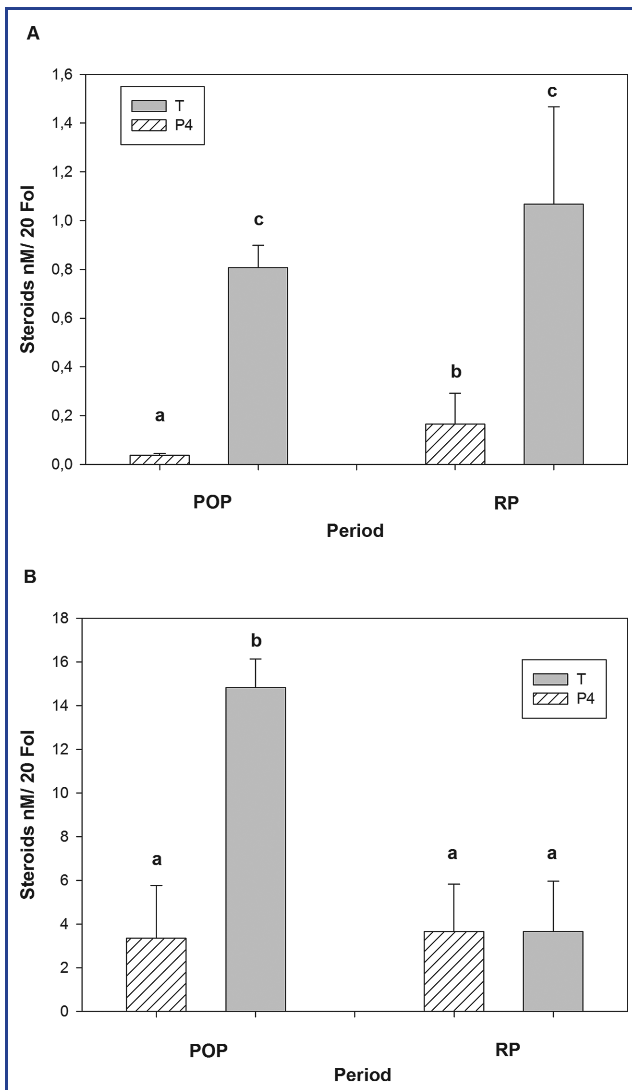

Figure 1. Seasonal P_4 and T production by follicles. Steroids were extracted from follicles at 6 hr of incubation in (A) AR, and P_4 and T concentrations in the medium were measured by ECLIA ($n = 8$ in the POP group; $n = 21$ in the RP group). (B) AR with 10 UI/mL of hCG and P_4 and T concentrations in the medium were measured by ECLIA ($n = 8$ in the POP group; $n = 11$ in the RP group). Each bar represents the average of nM of P_4 and T per 20 follicles (mean ± SD) of experiments performed in duplicate on different animals. Means with different letters are significantly different ($P < 0.05$).

levels in the females are higher during the mating season (D'Istria et al., '74; Licht et al., '83; Polzonetti-Magni et al., '84; Cayrol et al., '85; Itoh et al., '90).

The role of androgens in amphibian female reproduction has not been clarified yet, but there is evidence indicating that T may be related to ovulation (Smith and Ecker, '71; Fortune, '83). As to oocyte maturation in *R. arenarum,* it seems probable that the steroid responsible for maturation is P_4 since the increase due to gonadotropin action is remarkably higher for P4 than for T.

Under gonadotropin stimulation, both hormones increased significantly during POP compared to the values found in basal conditions. During RP, under the effect of hCG, we found a 20-fold increase in P_4 secretion with respect to basal values but only a threefold increase in T ($P_4 = 3.66 \pm 2.16$ nM; $T = 3.66 \pm 2.30$ nM). In the case of this species, it seems possible that the variation in hormone secretion in the above conditions is more important than the absolute values reached by the secreted hormones. In short, in *R. arenarum*, under gonadotropin stimulation, P4 levels undergo a significant change with respect to basal values, whereas T levels remain relatively stable. Our results do not rule out the possibility that, as in *X. laevis*, the preovulatory peak of P_4 is later converted into T to stimulate ovulation in females during amplexus (Fortune, '83).

However, on the basis of their experimental results, Lutz et al. (2001) claim that in *X. laevis* androgens would be the main promoters of in vivo maturation. In this species, T, which may derive from P_4 or through the $\Delta 5$ pathway of steroidogenesis through DHEA (dehydroepiandrosterone), is probably the primary physiological inducer, since it is also the most abundant and potent steroid in the plasma and in ovaries of female toads stimulated with hCG. In this case, the authors suggest that the abundance of the steroid determines the strength of its physiological effects.

In *R. arenarum* under stimulation conditions during POP, T secretion (14.82 ± 1.30 nM) was higher than that of P_4 (3.368 $\pm$ 2.39 nM). Similarly, in *X. laevis*, P_4 and T production measured by RIA increased after the follicles were stimulated with hCG (Redshaw and Nicholls, '71; Fortune et al., '75; Fortune and Tsang, '81; Fortune, '83; Lutz et al., 2001), the main steroid produced quantitatively in response to LH (Luteinizing Hormone) being T, which induces in vitro maturation as effectively as P_4 and other steroids such as C19 and C21 (Baulieu, '78).

However, we demonstrated that in experiments conducted with *R. arenarum* follicles incubated in vitro, exogenous P_4 was the most efficient maturation inducer throughout the year, especially during the reproductive season (Arias Torres et al., 2015). Numerous publications show that exogenous P_4 induces maturation (Zelarayán et al., 2012, Arias Torres et al., 2015) and ovulation of amphibian oocytes in the absence of gonadotropins (Schuetz, '71). In *X. laevis* and in *B. vulgaris*, hCG induces the maturation and ovulation of the oocyte by stimulating an increase in endogenous P_4 (Fortune et al., '75).

In our experimental design, it is difficult to determine whether P_4 or another steroid released by the action of hCG is responsible for maturation. As in *Rana pipiens,* P_4 could be metabolized into 5α-reduced derivatives (17OH-P4, AD, or T), which are also effective maturation inducers (Reynhout and Smith, '73). Preliminary experiments of androgen synthesis inhibition conducted in

R. arenarum follicles would allow us to suggest that the conversion of progestins to androgens would not be required during the maturation process of the oocyte of this species (results not shown).

In conclusion, this work deals for the first time with the production and release of steroids at the level of ovarian follicles and with their relation with meiosis resumption in *R. arenarum* females. Our results demonstrate that *R. arenarum* ovaries synthetize P_4 and T and provide the first direct evidence that hCG induces oocyte maturation by stimulating the increase in P_4 and T concentrations in the follicles.

This study is the first determination of the seasonal steroidogenic activity in the ovary of *R. arenarum* in situ using a modified ECLIA protocol developed in our laboratory. The validation and tuning of ECLIA for steroid determination in saline solutions opens interesting perspectives for the in vitro study of steroidogenesis.

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