Oocyte maturation in the toad *Rhinella arenarum* (Amphibia, Anura): Evidence of cAMP involvement in steroid production and action

Ana J. Arias Torres1,2 | José B. Páez3 | Liliana I. Zelarayán1,3

1 Instituto Superior de Investigaciones Biológicas (INSIBIO)-CONICET-UNT, San Miguel de Tucumán, Tucumán, Argentina
2 Instituto de Ambiente de Montaña y Regiones Áridas (IAMRA), Universidad Nacional de Chilecito (UNdeC), Chilecito, La Rioja, Argentina
3 Facultad de Bioquímica, Química y Farmacia, UNT, San Miguel de Tucumán, Tucumán, Argentina

Correspondence
Liliana Isabel Zelarayán, Instituto de Biología, Facultad de Bioquímica, Química y Farmacia, INSIBIO-UNT. Chacabuco 461, 4000 San Miguel de Tucumán, Tucumán, Argentina.
Email: lzelarayan@fbqf.unt.edu.ar

Funding information
Secretaría de Ciencia, Arte e Innovación Tecnológica (SCAIT), PIUNT No. 26/D/541, Universidad Nacional de Tucumán

In this work, we describe the participation of the adenylate cyclase/3′-5′-cyclic adenosine monophosphate (cAMP) pathway in the seasonal follicular secretion of progesterone (P4) and testosterone (T), and its relationship with the maturation of *Rhinella arenarum* oocytes. Under gonadotropin stimulation, P4 secretion was the dominant steroid produced during the reproductive period, resulting in 100% germinal vesicle breakdown (GVBD) in oocytes in vitro; in contrast, T and estradiol (E2) secretion increased (∼16 nM/20 follicles and ∼80 pM/20 follicles, respectively) during the non-reproductive period, but only yielded 50% GVBD. Treatment of the follicles with dibutyryl-cAMP or forskolin induced a significant increase in T secretion during both periods, but P4 secretion did not significantly change and GVBD did not occur. These results suggest that high cAMP levels in the oocyte maintain meiotic arrest and prevent the induction effect of follicular steroids. An increase in cAMP levels in denuded oocytes, however, negatively regulated T-induced maturation since treatment with increasing db-cAMP or forskolin inhibited their maturation. Therefore, we hypothesize that an elevation in T during the non-reproductive period favors its aromatization to E2, leading to follicle growth. During the reproductive period, P4 production might promote oocyte maturation when environmental conditions are favorable for reproduction. Together, the results indicate that steroidogenesis is seasonal and depends on gonadotropic activity in *R. arenarum*.

**KEYWORDS**
amphibian, follicles, gonadotropins, meiosis, steroids

1 | INTRODUCTION

In amphibians, reproduction is a process whose synchronization requires coordination between environmental (light, temperature, and humidity), physiological, and behavioral signals (Vu & Trudeau, 2016).

**Abbreviations**: AC, adenylate cyclase; AR, amphibian Ringer solution; cAMP, 3′-5′-cyclic adenosine monophosphate; db-cAMP, dibutyryl cAMP; E2, estradiol; ECLIA, electrochemiluminescence immunoassay; GVBD, germinal vesicle breakdown; hCG, human chorionic gonadotropin; MIS, maturation-inducing steroid; MPF, M-phase promoting factor; P4, progesterone; PKA, protein kinase A; T, testosterone.

The secretion of hypophysal gonadotropins in females stimulates steroidogenesis and oogenesis in a seasonal manner (Norris & Lopez, 2011). These gonadotropins promote ovarian steroidogenesis by the activation of signaling mechanisms that increase intracellular oocyte levels of 3′-5′ cyclic adenosine monophosphate (cAMP) (Nagahama & Yamashita, 2008)—one of many nucleotides that regulate the cell cycle in fish, amphibians, and mammals (Das, Pal, & Maitra, 2016; Nader, Courjaret, Dib, Kulkarni, & Machaca, 2016; Thomas, 2012). Oocyte maturation is directly regulated by gonadotropins, maturation-inducing steroid (MIS), and the M-phase promoting factor (MPF), which,
respectively, affect the ovarian follicular cells, the oocyte surface, and the oocyte cytoplasm (Figure 1). During the reproductive period, an increase in gonadotropins induces follicular cells to produce MIS, leading to meiotic resumption or maturation that is evidenced by germinal vesicle breakdown (GVBD). Oocytes arrested in prophase I complete the first meiotic division, and then arrest again in metaphase II (Marteil, Richard-Parpaillon, & Kubiak, 2009). Thus, the amphibian ovary regulates the development and release of mature oocytes ready for successful fertilization under tight gonadotropic control.

Oocyte maturation is a consequence of the interaction between MIS and the oocyte membrane that activates G protein signaling. In Xenopus laevis oocytes, Gs ensures meiotic arrest by activating adenylate cyclase (AC) to maintain elevated cAMP levels (Levin & Hammes, 2016; Romo, Hinrichs, Guzman, & Olate, 2002). In Rhinella arenarum, a South American anuran amphibian with a wide geographical distribution whose reproduction and oviposition take place in spring and summer (September–February), which overlaps with the rainy season in Argentina (Canosa, Pozzi, Rosembil, & Ceballos, 2003), activation of a Goi by the peptide analog Mastoparan-7 (Higashijima, Burnier, & Ross, 1990; Zelarayán, Ajmat, Bonilla, & Bühler, 2013) causes a rapid decrease in cytoplasmic cAMP levels by the inhibition of AC, which consequentially inhibited Protein kinase A (PKA) and leads to oocyte maturation during the reproductive and non-reproductive periods (Zelarayán et al., 2013). PKA inhibition by H-89 also induced maturation in R. arenarum oocytes (Zelarayán et al., 2013). This signaling cascade used by R. arenarum oocytes is analogous to the pathway reported for sea urchin oocytes (Voronina & Wessel, 2004), in which meiosis resumption seems to depend on the activation of a Goi. Yet even though AC activity is stimulated by forskolin, Goi activation by Mas-7 is not able to induce meiotic resumption in denuded R. arenarum oocytes (Zelarayán et al., 2013), suggesting that intracellular cAMP level in oocytes does not decline in the absence of follicle cells.

The interplay between PKA and MPF activation has been described in fishes (Thomas, 2012) and toads (Ferrell, 1999), but the link between PKA inactivation and MPF activation requires deeper analysis (Eyers et al., 2005; Khan & Maitra, 2013). Several lines of evidence suggest that fish oocyte maturation is accompanied by a decrease in cAMP and in PKA activity induced by MIS (Cerdá, Petrino, Landin, & Lin, 1997; Khan & Maitra, 2013; Thomas, 2012), implicating a relationship between cAMP and steroids in the regulation of oocyte maturation. Indeed, steroid-induced oocyte maturation in amphibians and fishes can be prevented by an increase in cAMP levels or by inhibitors of cAMP degradation (e.g., isobutyl xanthine) (Khan & Maitra, 2013). Therefore, cAMP appears to possess dual functionality in the ovarian follicle: in response to gonadotropins, cAMP levels increase in follicle cells, which promotes steroidogenesis, but must decrease in the oocyte for meiotic resumption to occur (Eyers et al., 2005; Ferrell, 1999; Rasar & Hammes, 2006; Zelarayán et al., 2013).

Progesterone (P4) was considered the most-relevant MIS for meiotic resumption in amphibian oocytes for several decades (Smith, 1989). In X. laevis oocytes, P4-induced maturation was proposed based on a drop in intracellular cAMP levels during the first few minutes of action of the hormone, thereby reducing PKA activity (Khan & Maitra, 2013), which exerts a tight control on oocyte maturation (Ferrell, 1999). More recently, however, testosterone (T) produced by ovarian follicles was demonstrated to trigger GVBD in X. laevis (Deng et al., 2009; Sen, Prizant, & Hammes, 2011). We previously demonstrated that P4 is capable of inducing maturation in R. arenarum oocytes, and that androgens were less-efficient maturation inducers (Arias Torres, Bühler, & Zelarayán, 2016). Using an electrochemiluminescence immunoassay (ECLIA), we determined that R. arenarum follicles in basal conditions release more T than P4 during the preovulatory and reproductive periods, but these steroids failed to induce GVBD. Under gonadotropin stimulation, however, steroidogenesis remarkably increased in both periods, and resulted in a high in vitro maturation percentage (72 ± 16% GVBD in follicles from the preovulatory period, 81.75 ± 9.1% GVBD from the reproductive period) (Arias Torres, Páez, & Zelarayán, 2016). Given that the downstream signaling involved in this androgen-induced GVBD have not yet been described for toads, we aimed to study the participation of the AC/cAMP signaling in T-induced R. arenarum oocyte maturation as well as follicular steroid secretion, and its relationship with oocyte maturation.

2 | RESULTS

2.1 | Effect of cAMP and forskolin on T-induced maturation

In previous work, we demonstrated that both spontaneous and P4-induced maturation of R. arenarum oocytes can be modulated by
cAMP intracellular levels (Zelarayán, Oterino, Sánchez Toranzo, & Bühler, 2000). Low doses of dibutyryl cAMP (db-cAMP) (0.001 and 0.01 mM) did not affect P4-induced maturation, whereas doses above 0.1 mM did (Zelarayán et al., 2000). Therefore, db-cAMP doses higher than 0.01 mM (0.01–1.0 mM) were assayed for their effects on T (1 μM)-induced oocyte maturation. A significant reduction in GVBD percentage occurred, starting at 0.2 mM db-cAMP (14 ± 3% decrease) and reaching complete inhibition at 1.0 mM (Figure 2a).

Considering that intracellular concentrations of cAMP result from the interplay between its production by AC and its later degradation by phosphodiesterase, we analyzed the effect of the AC activator forskolin (0.01–10 μM) on oocyte maturation. In previous experiments, we showed that doses of 0.02 μM forskolin inhibited P4-induced nuclear maturation in a dose-dependent manner in R. arenarum oocytes (Zelarayán et al., 2013). Therefore, forskolin doses higher than 0.01 μM were assessed here (0.01–10 μM). As observed with db-cAMP, forskolin-dependent AC activation dose-dependently inhibited T-induced oocyte maturation, with 1 μM forskolin almost completely blocking maturation (3.5 ± 3% GVBD) (Figure 2b).

2.2 | Effect of a transient increase in follicular cAMP levels on oocyte maturation

Hypophyseal gonadotropins regulate the secretion of ovarian steroids through signaling mechanisms that involve the activation of the AC/cAMP pathway, so we asked if variations in follicular cAMP levels induced MIS secretion, thereby mimicking the action of gonadotropins. Batches of whole follicles from five animals obtained during the reproductive and non-reproductive periods were incubated with db-cAMP or forskolin under different experimental time frames. Control groups incubated for 24 hr in amphibian Ringer solution (AR) did not undergo maturation, whereas all oocytes in T-treated follicles underwent meiotic resumption (100% GVBD) (Table 1, left). The presence of db-cAMP for 24 hr failed to promote the maturation of T-treated follicles after a total 24-hr as well as the presence of db-cAMP for pulses of 8, 16, or 24 hr followed by transfer to AR (Table 1, left). Similarly, follicles incubated in the presence of T plus forskolin or preincubated in forskolin for different durations (8, 16, or 24 hr) and then transferred to AR failed to resume meiosis (Table 1, right). Thus, incubation of follicles with db-cAMP or forskolin (8, 16, 24 hr), chronically or transiently, failed to trigger meiotic resumption. Although Sánchez Toranzo, Bonilla, Zelarayán, Oterino, and Bühler (2004) reported that Phosphodiesterase inactivation with theophylline inhibited maturation in a dose-dependent manner (0.25–1.00 μM) in R. arenarum oocytes, our data (Table 1) suggest that addition of cAMP hydrolysis inhibitors is not needed during experiments to achieve a similar effect.

2.3 | Effect of gonadotropic stimulation on follicular steroidogenesis and maturation

The gonadotropic effect on the follicular secretion of T, P4, and E2 and on meiotic resumption was analyzed using experiments designed to mimic the action of gonadotropins (Figure 1). Intact follicles were incubated in AR (control) or with hCG (10 UI/ml) for 6, 9, or 12 hr, at which points each steroid concentration was determined in the supernatant by ECLIA. Follicles were then kept in AR for an additional period (20 hr total incubation) to determine if the secreted steroids induced meiotic resumption. Secretion of P4, T, and E2 varied during the two reproductive periods, both under basal conditions (AR) and hCG stimulation, as did the biological response of the oocytes (Figure 3).

![FIGURE 2](image-url) Effects of cAMP and forskolin on T-induced oocyte maturation. Oocyte samples (20) were preincubated for 1 hr with (a) db-cAMP (0.01–1.0 mM) or (b) forskolin (0.01–10 μM). Maturation was then induced with T (1 μM). Control samples were incubated in AR alone, 10 μM T, or 1 mM db-cAMP or 10 μM forskolin. GVBD was assessed after 20 hr of incubation. Each bar represents the mean ± standard error (n = 7), performed in duplicate with different animals. Means with different letters are significantly different (p < 0.05)
Significant differences ($p < 0.05$) were observed in the basal secretion of T, which peaked at a threefold increase in concentration between 9 and 12 hr during the reproductive period; however, no GVBD was observed at any of the periods analyzed (Figure 3a). Under gonadotropic stimulation (10 UI/ml), follicular T secretion increased significantly in both periods. During the non-reproductive period, T concentration was approximately 10–15 times greater than in basal conditions (−15 nM between 6 and 12 hr of incubation). This remarkable hCG-associated increased secretion correlated with a high maturation percentage (50–75% GVBD between 6 and 12 hr) (Figure 3a). During the reproductive period, stimulation with hCG caused an increase in T secretion of up to twofold during the first 9 hr of incubation with respect to basal values, followed by a decrease at 12 hr of incubation (Figure 3a). HCG also induced the maximum biological response (85% and 100% GVBD at 6 and 12 hr, respectively) (Figure 3a).

Under basal conditions, P4 secretion remained below 1 nM per 20 follicles after 12 hr of incubation in both reproductive and non-reproductive periods, and no GVBD was observed (Figure 3b). When whole follicles were stimulated with hCG, however, P4 secretion and biological response increased significantly during both periods; in fact, P4 secretion increased several times that measured at basal conditions at 9 hr of incubation, and achieved maximal GVBD (100%) during the reproductive period.

The increased follicular secretion of T during the non-reproductive period suggested its transformation into E2 by the action of aromatase (CYP19). Indeed, significant differences were found in E2 follicular secretion between the reproductive and non-reproductive periods (Figure 3c). Basal follicular secretion of E2 during the non-reproductive period (51.0 ± 3.4 pM/20 follicles) was significantly higher than during the reproductive period (18.0 ± 2.8 pM/20 follicles), but no biological response was observed (0% GVBD) (Figure 3c). Under gonadotropic stimulation, the non-reproductive period, secretion of E2 significantly increased (77.5 ± 5.6 pM/20 follicles) with respect to basal levels during the non-reproductive period, without an effect on GVBD (Figure 3c). However, no differences were observed in the follicular secretion of E2 under gonadotropic stimulation (17.0 ± 3.7 pM/20 follicles) or in its absence (18.0 ± 2.8 pM/20 follicles during the reproductive period, even though oocyte maturation occurred (100% GVBD) (Figure 3c).

### TABLE 1  Effect of a transient increase in cAMP levels on T-induced follicle maturation

| db-cAMP incubation paradigm                        | Forskolin incubation paradigm | GVBD (%) |
|---------------------------------------------------|-------------------------------|----------|
| AR only (24 hr)                                   | AR only (24 hr)               | 0        |
| 1 µM T (24 hr)                                    | 1 µM T (24 hr)                | 100      |
| 1 µM T + 0.5 mM db-cAMP (24 hr)                   | 1 µM T + 10 µM forskolin (24 hr) | 88.7 ± 11.3 |
| 0.5 mM db-cAMP (8 hr) → AR (16 hr)                | 10 µM forskolin (8 hr) → AR (16 hr) | 0        |
| 0.5 mM db-cAMP (16 hr) → AR (8 hr)                | 10 µM forskolin (16 hr) → AR (8 hr) | 0        |
| 0.5 mM db-cAMP (24 hr)                            | 10 µM forskolin (24 hr)       | 0        |

Whole follicle samples were cultured as indicated, and GVBD was assessed at the end of the incubation period. Values are means ± standard error of experiments performed in duplicate with different animals ($n = 5$).

### 2.4 | Role of AC in the follicular secretion of T and P4

Considering that the effects of gonadotropins on steroidogenesis are accompanied by the activation of AC and increases in cAMP in follicular cells, we quantified the levels of T and P4 released by the follicles of *R. arenarum* in the presence of forskolin (10 µM). This AC activator induced a significant increase in T secretion compared to the levels found in basal conditions in both the reproductive and non-reproductive periods (Figure 4a). A tenfold increase was observed starting after 6 hr incubation, especially during the non-reproductive period (20 ± 4.8 nM/20 follicles), although incubation for longer periods (9 or 12 hr) did not change T levels (Figure 4a). During the reproductive period, T secretion induced by AC activation was threefold greater than basal levels, but no biological effect was observed (Figure 4a). Conversely, no significant stimulatory effect of forskolin was observed for P4 secretion from follicles isolated during either period (Figure 4b).

### 2.5 | Role of cAMP on the follicular secretion of T and P4

Incubation of *R. arenarum* follicles in the presence of db-cAMP (0.5 mM) resulted in an increase in follicular T secretion (Figure 5a) in a manner similar to stimulation with forskolin, but less efficiently (Figure 4a). Addition of db-cAMP to the incubation medium induced a significant increase in T follicular secretion during the non-reproductive period compared to basal conditions, and this increase was observed at up to 9 hr of incubation (Figure 5a). During the reproductive period, T secretion also reached maximum levels at 9 hr (6 ± 0.46 nM/20 follicles). Follicular secretion of P4 did not change with respect to basal levels in the presence of db-cAMP during either period studied (Figure 5b). No meiotic resumption (0% GVBD) was observed during any db-cAMP condition (Figure 5).

### 3 | DISCUSSION

Several publications describe the influence of hypophysal hormones in the secretion of ovarian steroids in amphibians (Norris & Lopez, 2011), but few specifically address this for *R. arenarum*. Steroid concentrations in the plasma of adult females are known (Medina, 2011), but few specifically address this for *R. arenarum*.
Ramos, Crespo, Gonzalez Calvar, & Fernandez, 2004), as well as in the ovary in situ (Arias Torres, Páez, et al., 2016). Here, we extended this knowledge base by tracking the seasonal secretion of sex steroids from full-grown *R. arenarum* follicles. Basal follicular secretion of T remained below 1 nM per 20 follicles during the non-reproductive period, and showed a significant increase during the reproductive period; however, this treatment did not induce resumption (Figure 3a). Following hCG administration, however, T level rose with a concomitant induction of meiotic resumption, showing a partial biological response during the non-reproductive period (50–70% GVBD) versus a full response during the reproductive period (100% GVBD) (Figure 3a). A similar hCG-dependent effect was observed for P4 production by follicles (Figure 3b). Although the absolute

**FIGURE 3** Seasonal follicular secretion of T, P4, and E2 under gonadotropic stimulation. Whole follicles from reproductive (RP) and non-reproductive (NRP)-period animals were incubated for 6, 9, or 12 hr in 1 ml of AR, or AR plus hCG (10 UI/ml). Steroids—(a) T, (b) P4, and (c) E2—were extracted from the incubation medium at the end of the incubation period. Concentrations of each analyte in the medium were determined by ECLIA after 20 hr of incubation; follicles were fixed for determining GVBD rates. Each bar represents the mean concentration (mean ± standard error) of analyte secreted by 20 follicles. Independent assays were performed in duplicate on different animals (n = 4 per reproductive period). Means with different letters are significantly different (p < 0.05)

**FIGURE 4** Seasonal follicular secretion of T and P4 in the presence of forskolin. Whole follicles from reproductive (RP) and non-reproductive (NRP) period animals were incubated for 6, 9, or 12 hr in 1 ml of AR, or AR plus forskolin (10 µM). Steroids—(a) T and (b) P4—were extracted from the incubation medium at the end of the incubation period. Concentrations of each analyte in the medium were determined by ECLIA after 20 hr of incubation; follicles were fixed for determining GVBD rates. Each bar represents the mean concentration (mean ± standard error) of analyte secreted by 20 follicles. Independent assays were conducted in duplicate with different animals (n = 4 per reproductive period). Means with different letters are significantly different (p < 0.05)
Seasonal follicular secretion of T and P4 in the presence of cAMP. Whole follicles from reproductive (RP) and non-reproductive (NRP) period animals were incubated for 6, 9, or 12 hr in 1 ml of AR, or AR plus dB-cAMP (0.5 mM). Steroids—(a) T and (b) P4—were extracted from the incubation medium at the end of the incubation period. Concentrations of each analyte in the medium were determined by ECLIA after 20 hr of incubation; follicles were fixed for determining GVBD rates. Each bar represents the mean concentration (mean ± standard error) of analyte secreted by 20 follicles—were extracted from the incubation medium at the end of the incubation period. Concentrations of each analyte in the medium were determined by ECLIA after 20 hr of incubation; follicles were fixed for determining GVBD rates. Each bar represents the mean concentration (mean ± standard error) of analyte secreted by 20 follicles. Independent assays were conducted in duplicate with different animals (n = 4 per reproductive period). Means with different letters are significantly different (p < 0.05).

FIGURE 5 Seasonal follicular secretion of T and P4 in the presence of cAMP. Whole follicles from reproductive (RP) and non-reproductive (NRP) period animals were incubated for 6, 9, or 12 hr in 1 ml of AR, or AR plus dB-cAMP (0.5 mM). Steroids—(a) T and (b) P4—were extracted from the incubation medium at the end of the incubation period. Concentrations of each analyte in the medium were determined by ECLIA after 20 hr of incubation; follicles were fixed for determining GVBD rates. Each bar represents the mean concentration (mean ± standard error) of analyte secreted by 20 follicles. Independent assays were conducted in duplicate with different animals (n = 4 per reproductive period). Means with different letters are significantly different (p < 0.05).

concentration of T was greater than P4, gonadotropin stimulation during the reproductive period caused a remarkably greater increase in P4 (~sixfold) compared to T secretion (~twofold) over basal ovarian secretion. Such differential follicular response in T versus P4 production support our previous work demonstrating that R. arenarum oocytes mature under the action of different steroids, wherein androgens can induce oocyte maturation (Arias Torres, Bühler, et al., 2016) but P4 proved to be the most effective (Arias Torres, Páez, et al., 2016). Therefore, oocyte maturation in this species might be more sensitive to fold-change increases in P4 secretion rather than to absolute concentrations.

The role of steroids in the reproduction of amphibian females has not been wholly elucidated. P4 was initially postulated as the physiological maturation inducer in amphibian oocytes, but data from X. laevis suggesting that T is the dominant in vivo steroid stimulant of oocyte maturation have questioned the generalizability of this hypothesis (Hammes, 2004; Rasar & Hammes, 2006; Sen et al., 2011; White, Jammongjit, Gill, Lutz, & Hammes, 2005). Indeed, some publications indicate that T may be related to ovulation (Fortune, 1983), to maturation (Miedlich, Taya, Young, & Hammes, 2017), or to ovarian follicle growth via its aromatization to estrogens (Gohin, Bodinier, Fostier, Bobe, & Chesnel, 2011; Sen et al., 2014). A role for estrogens in meiotic resumption is also controversial. In fishes, estrogens are associated with inhibition of maturation competence of oocytes, although they can also activate the synthesis of growth factors involved in oocyte development (Jalbert & Fostier, 1984; Kamangar, Gabillard, & Bobe, 2006; Pang & Thomas, 2009). Moments before ovulation, however, a change in the steroidogenic capacity of the full-grown follicle decreases E2 plasma levels and increases MIS abundance (Lubzens, Young, Bobe, & Cerdá, 2010). E2 is known to inhibit oocyte maturation in amphibians (Lin & Schuetz, 1983, 1985; Pickford & Morris, 1999; Schuetz, 1972). The observed elevation of E2 during the R. arenarum non-reproductive period (Figure 3c) could maintain meiotic arrest when environmental conditions are unfavorable for maturation or before the oocyte has completed development. Given that E2 is also related to vitellogenin uptake in growing follicles, as demonstrated in R. arenarum oocytes (O’Brien, Salicioni, Cabada, & Arranz, 2010), T-to-E2 conversion would allow follicles in late vitellogenesis to enter the reproductive period ready to mature when environmental conditions are favorable. Consistent with data herein, studies carried out in Rana nigromaculata demonstrated a relationship between follicle size and the levels of specific steroids secreted in vitro: at the beginning of their development, follicles mainly secrete E2, but as development progresses, they secrete T and, when full development is attained, they secrete P4 (Gohin et al., 2011; Kwon et al., 1993). In R. arenarum, Medina et al. (2004) demonstrated that during the preovulatory period, oocytes complete development in the ovary; as oocyte maturation and ovulation approach, serum levels of P4 increase; and post-ovulation, serum levels of P4 and T remain low, coinciding with the re-initiation of ovarian follicle growth in vivo.

The activity of T and E2 produced by and acting on amphibian follicles may not be mutually exclusive; instead, they could be intimately related. CYP17 is a key enzyme that mediates androgen production from C21 steroids, both through the Δ4 pathway and through the Δ5 pathway of steroidogenesis (Yang, Lutz, & Hammes, 2003). Gohin, Bobe, and Chesnel (2010) demonstrated seasonal changes in cyp17a1 and cyp19a1 mRNA abundance in amphibian females, with lower levels observed before meiotic resumption in X. laevis follicles—which may reflect the maturity of the oocytes. hCG-induced Cyp17 activation is accompanied by an increase in T secretion in the R. arenarum ovary, and its inhibition with spironolactone leads to a significant decrease in T secretion and an increase in P4 together with the biological response (data not shown). T (10–16 nM/20 follicles) and E2 (~80 pM/20 follicles) concentration also increased in tandem when stimulated with hCG during the non-reproductive period (Figure 3), further implicating their interconversion. This conclusion is consistent with observations that secretion of E2 by R. arenarum follicles increased more during the
non-reproductive period following gonadotropic stimulation, supporting a role for conversion of T to E2 during late vitellogenesis (e.g., 1.5–1.6 mm diameter follicles) (Figure 3); the aromatase inhibitor Anastrazole causes a decrease in this follicular E2 secretion during the non-reproductive period of R. arenarum (data not shown). Furthermore, direct application of E2 to follicles does not induce maturation of R. arenarum oocytes (data not shown). Therefore, in contrast to X. laevis, T likely serves to promote R. arenarum follicular growth by acting as a precursor steroid for estrogen synthesis.

The oocyte-maturation outcome of gonadotropins on steroidogenesis may occur through the AC/cAMP signaling, via the aromatization of T into E2 during the non-reproductive period (Lin & Schuetz, 1983, 1985; Pickford & Morris, 1999; Schuetz, 1972). We previously determined that cAMP signaling, via activation of Gαi, is involved in P4 stimulation of R. arenarum oocyte maturation (Zelarayán et al., 2013). Here, treatment of denuded oocytes with increasing doses of db-cAMP or forskolin, both of which dose-dependently inhibited G(α)i2 induction of GVBD induced by T (Figure 2). Treatment also significantly stimulated T secretion during both seasonal periods, whereas P4 abundance did no change under the same conditions; cAMP-related stimulant did not promote oocyte maturation (Figures 4 and 5). These results together indicate that elevated cAMP in the follicle promotes steroidogenesis, but also prevents R. arenarum oocytes from resuming meiosis. Furthermore, a decrease in intracellular cAMP by the action of Phosphodiesterase led to GVBD after activation of the pre-MPF in R. arenarum oocytes (Sánchez Toranzo, Bonilla, Zelarayán, Oterino, & Bühler, 2006), further indicating the participation of cAMP signaling in amphibian oocytes. Retention of the oocyte in an immature state could result from the transfer of cAMP from follicle cells to the oocytes and/or the increased follicular secretion of E2 during the non-reproductive period, which would maintain the oocyte in a state of growth. These results are also consistent with the original studies in X. laevis that defined a role for steroids in oocyte maturation, implicating the involvement of a transient, P4 receptor-dependent decrease in intracellular cAMP caused by AC inhibition (Sadler & Maller, 1982).

cAMP plays a different role in follicular cells compared to oocytes. In Plecoglossus altivelis follicles, Yamamoto and Yoshizaki (2008) demonstrated that a gonadotropin-dependent increase in cAMP levels in follicular cells activates key enzymes for steroidogenesis and, consequently, promotes the release of MIS. Yet, a transient decrease in cAMP concentration in the oocyte is still required for maturation to occur under MIS stimulation. They proposed a cAMP model for the follicle to account for the dual functions of cAMP: in follicular cells, cAMP stimulates MIS production whereas in oocytes, it maintains meiotic arrest. Our results are consistent with this hypothesis, and reiterate data presented for R. pipiens (Kwon & Shuetz, 1985).

Specifically, transient incubation of whole R. arenarum follicles (obtained during both non-reproductive and reproductive periods) with forskolin or db-cAMP stimulated P4 and T secretion (Figures 4 and 5), but inhibited oocyte maturation (Table 1). Therefore, high cAMP levels in the oocyte maintain meiotic arrest and counteract any inducer effect of the steroids released by the follicular cells under the action of forskolin or db-cAMP.

In conclusion, the results obtained herein demonstrate that R. arenarum follicles secrete steroids in a seasonal manner under hCG stimulation or when AC/cAMP/PKA signaling is activated. Oocytes responded to these steroid secretions by elevating cAMP concentrations, which blocked meiotic resumption. These in situ data suggest that, during the non-reproductive period, T secretion is associated with the synthesis of E2, which is involved with aromatization and follicle growth in the ovary, whereas during the reproductive period, P4 secretion is responsible for oocyte maturation and ovulation during favorable environmental conditions.

4 MATERIALS AND METHODS

4.1 Hormones and reagents

Forskolin (Sigma, St. Louis, MO) and testosterone (Sigma) were dissolved in absolute ethanol to obtain a stock solution of 4 mM and 10 μM, respectively. db-cAMP (Sigma) was dissolved in AR, and various doses were added to the culture medium at a constant volume (5 μl). hCG (ELEA, Argentina) was dissolved in distilled water to obtain a stock solution of 2 UI/μL. AR (6.60 g NaCl/L, 0.15 g CaCl2/L, and 0.15 g KCl/L, pH 7.4) with penicillin G-sodium (30 mg/L) and streptomycin sulfate (50 mg/L) was used as a culture medium in all routine incubations.

4.2 Animals

Adult, female R. arenarum specimens were collected for 3 consecutive years from Tucumán, Northwestern Argentina, during September to February (reproductive period) and March to August (non-reproductive period). They were kept in captivity for a few hours at room temperature, in accordance with the Guiding Principles for the Care and Use of Research Animals of the Society for the Study of Reproduction.

Ovaries were extracted soon after animal capture to minimize the effect of captivity on basal steroid levels. Full-grown, intact follicles (1.5–1.6 mm) were isolated from ovarian tissues using watchmaker’s forceps after 1–2 hr of stabilization at room temperature (22–25°C) in AR. The oocyte of these follicles was surrounded by follicular cells, theca cells, and external epithelium. Denuded oocytes were obtained by removing the theca and the follicular epithelium with watchmaker’s forceps, and then gently shaking the oocytes with follicular cells for 5 min (100 oscillations per min) (Zelarayán, Oterino, & Bühler, 1995) to detach the follicular cells, leaving the oocyte intact surrounded by its vitelline envelope.

4.3 Maturation assays

Incubations were performed at 24–26°C in multi-well culture dishes (Costar 3524, Cambridge, MA) with randomized samples of 20 oocytes or follicles distributed into separate wells containing 2 ml of AR. At the end of the incubation period (24 hr), treated oocytes or follicles were fixed, and GVBD was determined.
4.3.1 Denuded oocytes

Denuded oocytes were preincubated for 60 min with increasing concentrations of db-cAMP (0.01–1.0 mM) or forskolin (0.1–10 µM), and then maturation was induced with 1 µM T. Batches of oocytes were incubated in AR; AR + 1 µM T; or AR + 1 mM db-cAMP or 10 µM forskolin.

4.3.2 Whole follicles

Batches of 20 follicles were incubated in AR solution for 24 hr; 1 µM T for 24 hr; 1 µM T + 0.5 mM db-cAMP or 10 µM forskolin for 24 hr; or 0.5 mM db-cAMP or 10 µM forskolin for 8, 16, or 24 hr. At the end of the incubation period, the follicles from the db-cAMP- or forskolin-only batches (8, 16, or 24 hr) were washed thoroughly and then transferred to an AR solution for the remaining time (total of 24 hr).

4.4 Steroid secretion assays

Follicles were obtained from animals collected during both reproductive periods. Batches of 20 whole follicles were incubated at a controlled 26°C in plastic multi-well dishes containing 1 ml of AR in the presence of hCG (10 UI/ml), db-cAMP (0.5 mM), or forskolin (10 µM) for different time periods (6, 9, and 12 hr); follicles incubated in AR (basal condition) were used as controls. At the end of the incubation period, the supernatant was removed and kept at −20°C until the time of extraction for ECLI. Meiotic resumption (GVBD) was determined in the follicles at 20 hr.

4.5 Extraction and quantification of steroids for ECLI A

Steroids were extracted from the incubation medium, and quantified using ECLI A with the method validated previously by our group (Arias Torres, Páez, et al., 2016). In this analytical system, the reporting range for P₄ is 0.095–191 nM, for T is 0.087–52.0 nM, and for E₂ is 18.4–15781.0 pM. Packs of PROG II, TESTO II, and ESTRADIOL II reagents (Roche Diagnostics GmbH, Germany) were used as standards.

4.6 Statistical method

Data of GVBD and steroid levels were analyzed by Student’s t-test and two-way analysis of variance (ANOVA). p < 0.05 was considered statistically significant.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the financial assistance of The Secretaría de Ciencia, Arte e Innovación Tecnológica (SCAIT) of the Universidad Nacional de Tucumán and a Doctoral Fellowship from CONICET given to Ana Josefa Arias Torres. The authors wish to thank the various anonymous reviewers for their valuable suggestions, which helped us improve this manuscript.

REFERENCES

Arias Torres, A. J., Bühler, M. I., & Zelarayán, L. I. (2016). In vitro steroid-induced meiosis in Rhinella arenarum oocytes: Role of pre-MPF activation. Zygote, 24(2), 252–258.

Arias Torres, A. J., Páez, J. B., & Zelarayán, L. I. (2016). Validation of electrochemiluminescence immunoassay for ovarian steroid determination in Rhinella arenarum. Journal of Experimental Zoology Part A: Ecological Genetics and Physiology, 325(4), 265–273.

Canosa, L. F., Pozzi, A. G., Rosemblit, C., & Ceballos, N. R. (2003). Steroid production in toads. Journal of Steroid Biochemistry and Molecular Biology, 85, 227–233.

Cerdá, J., Petrino, T. R., Landin, A. M., & Lin, W. P. (1997). Effects of isoquinolinesulfonamide H-8 on Fundulus heteroclitus ovarian follicles: Role of cyclic nucleotide-dependent protein kinases on steroidogenesis and oocyte maturation in vitro. Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology, 117(1), 75–81.

Das, D., Pal, S., & Maitra, S. (2016). Releasing prophase arrest in zebrafish oocyte: Synergism between maturational steroid and IGF1. Reproduction, 151(1), 59–72.

Deng, J., Carabajal, L., Evaul, K., Rasar, M., Jamnongjit, M., & Hammes, S. R. (2009). Nongenomic steroid-triggered oocyte maturation: Of mice and frogs. Steroids, 74, 595–601.

Eyres, P. A., Liu, J., Hayashi, N. R., Lewellyn, A. L., Gautier, J., & Maller, J. L. (2005). Regulation of the G(2)/M transition in Xenopus oocytes by the cAMP-dependent protein kinase. Journal of Biological Chemistry, 280(26), 24339–24346.

Ferrell, J. E., Jr. (1999). Xenopus oocyte maturation: New lessons from a good egg. Bioessays, 21(10), 833–842.

Fortune, J. E. (1983). Steroid production by Xenopus ovarian follicles at different developmental stages. Developmental Biology, 99(2), 502–509.

Gohin, M., Bobe, J., & Chesnel, F. (2010). Comparative transcriptomic analysis of follicle-enclosed oocyte maturational and developmental competence acquisition in two non-mammalian vertebrates. BMC Genomics, 11, 18.

Hammes, S. R. (2004). Steroids and oocyte maturation. A new look at an old story. Molecular Endocrinology, 18(4), 769–775.

Higashijima, T., Burnier, A. C., & Fostier, A. (1984). The follicular sensitivity in vitro to mastoparan, related amphiphilic peptides and hydrophobic amines. Journal of Biological Chemistry, 236(4), 72–77.

Jalabert, B., & Fostier, A. (1984). The follicular sensitivity in vitro to mastoparan, related amphiphilic peptides and hydrophobic amines. Journal of Biological Chemistry, 259(24), 14187–14186.

Khan, P. P., & Maitra, S. (2013). Participation of cAMP-dependent protein kinase and MAP kinase pathways during Anabas testudineus oocyte maturation. General and Comparative Endocrinology, 181, 88–97.

Kwon, H. B., & Shuetz, A. W. (1985). Dichotomous effects of forskolin on somatic and germ cell components of the ovarian follicles: Evidence of cAMP involvement in steroid production and action. Journal of Experimental Zoology, 236, 219–228.
Kwon, H. B., Ahn, R. S., Lee, W. K., Im, W. B., Lee, C. C., & Kim, K. (1993). Changes in the activities of steroidogenic enzymes during the development of ovarian follicles in Rana nigromaculata. General and Comparative Endocrinology, 92(2), 225–232.

Levin, E. R., & Hammes, S. R. (2016). Nuclear receptors outside the nucleus: Extraneuronal signaling by steroid receptors. Nature Reviews Molecular Cell Biology, 17(12), 783–797.

Lin, Y. W., & Schuetz, A. W. (1983). In vitro estrogen modulation of pituitary and progesterone-induced oocyte maturation in Rana pipiens. Journal of Experimental Zoology, 224(2), 281–291.

Lin, Y. W. P., & Schuetz, A. W. (1985). Intrafollicular action of estrogen in regulating pituitary-induced ovarian progesterone synthesis and oocyte maturation in Rana pipiens: Temporal relationship and locus of action. General and Comparative Endocrinology, 58, 421–435.

Lubzens, E., Young, G., Bobe, J., & Cerdà, J. (2010). Oogenesis in teleosts: How eggs are formed. General and Comparative Endocrinology, 165(3), 367–389.

Martel, G., Richard-Parpaillon, L., & Kubiak, J. Z. (2009). Role of oocyte quality in meiotic maturation and embryonic development. Reproductive Biology, 9, 203–224.

Medina, M., Ramos, I., Crespo, C. A., Gonzalez Calvar, S., & Fernandez, S. N. (2004). Changes in serum sex steroid levels throughout the reproductive cycle of Bufo arenarum females. General and Comparative Endocrinology, 136, 143–151.

Miedlich, S. U., Taya, M., Young, M. R., & Hammes, S. R. (2017). Paxillin and embryonic PolyAdeynlation Binding Protein (ePAPB) engage to regulate androgen-dependent Xenopus laevis oocyte maturation—A model of kinase-dependent regulation of protein expression. Molecular and Cellular Endocrinology, 448, 87–97.

Nader, N., Courijaret, R., Dib, M., Kulkarni, R. P., & Machaca, K. (2016). Release from Xenopus oocyte prophase I meiotic arrest is independent of a decrease in cAMP levels or PKA activity. Development, 143(11), 1926–1936.

Nagahama, Y., & Yamashita, M. (2008). Regulation of oocyte maturation in fish. Development Growth & Differentiation, 1, 195–219.

Norris, D. O., & Lopez, K. H. (2011). Hormones and reproduction of vertebrates (1st ed. Vol. 2). Amphibians. London, UK: Academic Press is an imprint of Elsevier Inc.

O’Brien, E. D., Salicioni, A. M., Cabada, M. O., & Arranz, S. E. (2010). Vitellogenesis in Bufo arenarum: Identification, characterization and immunolocalization of high molecular mass lipovitellin during oogenesis. Comparative Biochemistry and Physiology Part B, Biochemistry and Molecular Biology, 155(3), 256–265.

Pang, Y., & Thomas, P. (2009). Involvement of estradiol-17β and its membrane receptor, G protein coupled receptor 30 (GPR30) in regulation of oocyte maturation in zebrafish, Danio rerio. General and Comparative Endocrinology, 161(1), 58–61.

Pickford, D. B., & Morris, I. D. (1999). Effects of endocrine disrupting contaminants on amphibian oogenesis: Methoxychlor inhibits progesterone-induced maturation of Xenopus laevis oocytes in vitro. Environmental Health Perspectives, 107, 285–292.

Rasar, M. A., & Hammes, S. R. (2006). The physiology of the Xenopus laevis ovary. Methods in Molecular Biology, 322, 17–30.

Romo, X., Hinrichs, M. V., Guzman, L., & Olate, J. (2002). Gas levels regulate Xenopus laevis oocyte maturation. Molecular Reproduction and Development, 63, 104–109.

Sadler, S. E., & Maller, J. L. (1982). Identification of a steroid receptor on the surface of Xenopus oocytes by photoaffinity labeling. Journal of Biological Chemistry, 257, 355–361.

Sánchez Toranzo, G., Bonilla, F., Zelarayán, L., Oterino, J., & Bühler, M. I. (2004). Effect of insulin on spontaneous and progesterone-induced GVBD on Bufo arenarum denuded oocytes. Zygote, 12(3), 185–195.

Sánchez Toranzo, G., Bonilla, F., Zelarayán, L., Oterino, J., & Bühler, M. I. (2006). Activation of maturation promoting factor in Bufo arenarum oocytes: Injection of mature cytoplasms and germinal vesicle contents. Zygote, 14, 305–316.

Schuetz, A. W. (1972). Estrogens and ovarian follicular functions in Rana pipiens. General and Comparative Endocrinology, 18(1), 32–36.

Sen, A., Prizant, H., & Hammes, S. R. (2011). Understanding extraneuronal (nonenguin) androgen signaling: What a frog oocyte can tell us about human biology. Steroids, 76, 822–828.

Sen, A., Prizant, H., Light, A., Biswas, A., Hayes, E., Lee, H. J., . . . Hammes, S. R. (2014). Androgens regulate ovarian follicular development by increasing follicle stimulating hormone receptor and microRNA-125b expression. Proceedings of the National Academy of Sciences of the United States of America, 111(8), 3008–3013.

Smith, L. D. (1989). The induction of oocyte maturation: Transmembrane signaling events and regulation of the cell cycle. Development, 107(4), 685–699.

Thomas, P. (2012). Rapid steroid hormone actions initiated at the cell surface and the receptors that mediate them with an emphasis on recent progress in fish models. General and Comparative Endocrinology, 175(3), 367–383.

Voronina, E., & Wessel, G. M. (2004). Betagamma subunits of hetero-trimeric G-proteins contribute to Ca2+ release at fertilization in the sea urchin. Journal of Cell Science, 117(Pt 25), 5995–6005.

Vu, M., & Trudeau, V. L. (2016). Neuroendocrine control of spawning in amphibians and its practical applications. General and Comparative Endocrinology, 234, 28–39.

White, S., Jamnongjit, M., Gill, A., Lutz, L., & Hammes, S. R. (2005). Specific modulation of nongenomic androgens signaling in the ovary. Steroids, 70, 352–360.

Yamamoto, Y., & Yoshizaki, G. (2008). Heterologous gap junctions between granulosa cells and oocyte in ayu (Plecoglossus altivelis): Formation and role during luteinizing hormone-dependent acquisition of oocyte maturation competence. Journal of Reproduction and Development, 54, 1–5.

Yang, W. H., Lutz, L. B., & Hammes, S. R. (2003). Xenopus laevis ovarian CYP17 is a highly potent enzyme expressed exclusively in oocytes. Evidence that oocytes play a critical role in Xenopus ovarian androgen production. Journal of Biological Chemistry, 278, 9552–9559.

Zelarayán, L. I., Oterino, J., & Bühler, M. I. (1995). Spontaneous maturation in Bufo arenarum oocytes: Follicle wall involvement, respiratory activity and seasonal influences. Journal of Experimental Zoology, 272(5), 356–362.

Zelarayán, L., Oterino, J., Sánchez Toranzo, G., & Bühler, M. I. (2000). Involvement of purines and phosphoinositides in spontaneous and progesterone-induced nuclear maturation of Bufo arenarum oocytes. Journal of Experimental Zoology, 287(2), 151–157.

Zelarayán, L. I., Ajmat, M. T., Bonilla, F., & Bühler, M. I. (2013). Involvement of G protein and purines in Rhinella arenarum oocyte maturation. Zygote, 21(3), 221–230.

How to cite this article: Arias Torres AJ, Páez JB, Zelarayán LL. Oocyte maturation in the toad Rhinella arenarum (Amphibia, Anura): Evidence of cAMP involvement in steroid production and action. Mol Reprod Dev. 2018;85:137–145. https://doi.org/10.1002/mrd.22944