RESEARCH ARTICLE

Oocyte Maturation in the Toad *Rhinella arenarum* (Amphibia, Anura): Evidence of cAMP Involvement in Steroid Production and Action†

Running Title: Follicular Steroidogenesis in *Rhinella arenarum*

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Abbreviations: AC, adenylate cyclase; AR, amphibian Ringer solution; cAMP, 3´- 5´ cyclic adenosine monophosphate; db-cAMP, dibutyryl cAMP; E_2, estradiol; ECLIA, electrochemiluminescence immunoassay; GVBD, germinal vesicle breakdown; hCG, human Chorionic gonadotropin; MIS, Maturation-inducing steroid; MPF, M-phase promoting factor; P_4, progesterone; PKA, Protein kinase A; T, testosterone.

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Abstract

In this work, we describe the participation of the Adenylate cyclase/3'-5'-cyclic adenonsine monophosphate (cAMP) pathway in the seasonal follicular secretion of progesterone (P_4) and testosterone (T), and its relationship with the maturation of *Rhinella arenarum* oocytes. Under gonadotropin stimulation, P_4 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 (E_2) 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 P_4 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 E_2, leading to follicle growth. During the reproductive period, P_4 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*.

Key words: Follicles – Steroids- Gonadotropins- Amphibian - Meiosis

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1 Introduction

In amphibians, reproduction is a process whose synchronization requires coordination between environmental (light, temperature and humidity), physiological, and behavioral signals (Vu and Trudeau, 2016). The secretion of hypophyseal gonadotropins in females stimulates steroidogenesis and oogenesis in a seasonal manner (Norris and López, 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 and Yamashita, 2008) – one of many nucleotides that regulate the cell cycle in fish, amphibians, and mammals (Thomas, 2012; Das et al., 2016; Nader et al., 2016). 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 (Fig. 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 et al., 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, Gαs ensures meiotic arrest by activating Adenylate cyclase (AC) to maintain elevated cAMP levels (Romo et al., 2002; Levin and Hammes, 2016). 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 et al., 2003), activation of a Gαi by the peptide analog Mastoparan-7 (Higashijima et al., 1990; Zelarayán et al., 2013) causes a rapid decrease in cytoplasmic cAMP levels by the inhibition of AC, which consequently 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 and Wessel, 2004), in which meiosis resumption seems to depend on the activation of a Gαi. Yet even though AC activity is stimulated by forskolin, Gαi 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 and 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 et al., 1997; Thomas, 2012; Khan and Maitra, 2013), 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 and 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 (Ferrell, 1999; Eyers et al., 2005; Rasar and Hammes, 2006; Zelarayán et al., 2013).

Progesterone (P_4) was considered the most-relevant MIS for meiotic resumption in amphibian oocytes for several decades (Smith, 1989). In *X. laevis* oocytes, P_4-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 and 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 et al., 2011). We previously demonstrated that P_4 is capable of inducing maturation in *R. arenarum* oocytes, and that androgens were less-efficient maturation inducers (Arias Torres et al., 2016a). Using an electrochemiluminescence immunoassay (ECLIA), we determined that *R. arenarum* follicles in basal conditions release more T than P_4 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 et al., 2016b). 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 P_4-induced maturation of *R. arenarum* oocytes can be modulated by cAMP intracellular levels (Zelarayán et al., 2000). Low doses of dibutyryl cAMP (db-cAMP) (0.001 and 0.01 mM) did not affect P_4-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 P_4-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 5 animals obtained during the reproductive and non-reproductive periods were incubated with db-cAMP or forkolin under different experimental time frames. Control groups incubated for 24 h 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 h failed to promote the maturation of T-treated follicles after a total 24-h as well as the presence of db-cAMP for pulses of 8, 16, or 24 h 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 h) and then transferred to AR failed to resume meiosis (Table 1, right). Thus, incubation of follicles with db-cAMP or forskolin (8, 16, 24 h), chronically or transiently, failed to trigger meiotic resumption. Although Sánchez Toranzo et al. (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, P_4, and E_2 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 h, at which points each steroid concentration was determined in the supernatant by ECLIA. Follicles were then kept in AR for an additional period (20 h total incubation) to determine if the secreted steroids induced meiotic resumption. Secretion

of P_4, T, and E_2 varied during the two reproductive periods, both under basal conditions (AR) and hCG stimulation, as did the biological response of the oocytes (Figure 3).

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 h 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 h of incubation). This remarkable hCG-associated increased secretion correlated with a high maturation percentage (50 - 75% GVBD between 6 and 12 h) (Figure 3a). During the reproductive period, stimulation with hCG caused an increase in T secretion of up to twofold during the first 9 h of incubation with respect to basal values, followed by a decrease at 12 h of incubation (Figure 3a). hCG also induced the maximum biological response (85 and 100% GVBD at 6 and 12 h, respectively) (Figure 3a).

Under basal conditions, P_4 secretion remained below 1 nM per 20 follicles after 12 h of incubation in both reproductive and non-reproductive periods, and no GVBD was observed (Figure 3b). When whole follicles were stimulated with hCG, however, P_4 secretion and biological response increased significantly during both periods; in fact, P_4 secretion increased several times that measured at basal conditions at 9 h 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 E_2 by the action of aromatase (CYP19). Indeed, significant differences were found in E_2 follicular secretion between the reproductive and non-reproductive periods (Figure 3c). Basal follicular secretion of E_2 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, during the non-reproductive period, secretion of E_2 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 E_2 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).

2.4 Role of AC in the follicular secretion of T and P_4

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 P_4 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 h incubation, especially during the non-reproductive period (20 ± 4.8 nM/20 follicles), although incubation for longer periods (9 or 12 h) 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 P_4 secretion from follicles isolated during either period (Figure 4b).

2.5 Role of cAMP on the follicular secretion of T and P_4

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 h of incubation (Figure 5a). During the reproductive period, T secretion also reached maximum levels at 9 h (6 ± 0.46 nM/20 follicles). Follicular secretion of P_4 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 hypophyseal hormones in the secretion of ovarian steroids in amphibians (Norris and Lopez, 2011), but few specifically address this for *R. arenarum*. Steroid concentrations in the plasma of adult females are known (Medina et al., 2004), as well as in the ovary in situ (Arias Torres et al., 2016b). Here, we extended this knowledgebase 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 P_4 production by

follicles (Figure 3b). Although the absolute concentration of T was great than P_4, gonadotropin stimulation during the reproductive period caused a remarkably greater increase in P_4 (~sixfold) compared to T secretion (~twofold) over basal ovarian secretion. Such differential follicular response in T versus P_4 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 et al., 2016a) but P_4 proved to be the most effective (Arias Torres et al., 2016b). Therefore, oocyte maturation in this species might be more sensitive to fold-change increases in P_4 secretion rather than to absolute concentrations.

The role of steroids in the reproduction of amphibian females has not been wholly elucidated. P_4 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 has questioned the generalizability of this hypothesis (Hammes, 2004; White et al., 2005; Rasar and Hammes, 2006; Sen et al., 2011). Indeed, some publications indicate that T may be related to ovulation (Fortune, 1983), to maturation (Miedlich et al., 2017), or to ovarian follicle growth via its aromatization to estrogens (Gohin et al., 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 (Jalabert and Fostier, 1984; Kamangar et al., 2006; Pang and Thomas, 2009). Moments before ovulation, however, a change in the steroidogenic capacity of the full-grown follicle decreases E_2 plasma levels and increases MIS abundance (Lubzens et al., 2010). E_2 is known to inhibit oocyte maturation in amphibians (Schuetz, 1972; Lin and Schuetz, 1983, 1985; Pickford and

Morris, 1999). The observed elevation of E_2 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 E_2 is also related to vitellogenin uptake in growing follicles, as demonstrated in *R. arenarum* oocytes (O'Brien et al., 2010), T-to-E_2 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 E_2, but as development progresses, they secrete T and, when full development is attained, they secrete P_4 (Kwon et al, 1993; Gohin et al., 2011). 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 P_4 increase; and post-ovulation, serum levels of P_4 and T remain low, coinciding with the re-initiation of ovarian follicle growth in vivo.

The activity of T and E_2 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 et al., 2003). Gohin et al. (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 P_4

together with the biological response (data not shown). T (10-16 nM/20 follicles) and E_2 (~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 E_2 by *R. arenaru*m follicles increased more during the non-reproductive period following gonadotropic stimulation, supporting a role for conversion of T to E_2 during late vitellogenesis (e.g., 1.5-1.6 mm diameter follicles) (Figure 3); the aromatase inhibitor Anastrazole causes a decrease in this follicular E_2 secretion during the non-reproductive period of *R. arenarum* (data not shown). Furthermore, direct application of E_2 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 E_2 during the non-reproductive period (Schuetz, 1972; Lin and Schuetz, 1983, 1985; Pickford and Morris, 1999). We previously determined that cAMP signaling, via activation of Gαi, is involved in P_4 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 GVBD induced by T (Figure 2). Treatment also significantly stimulated T secretion during both seasonal periods, whereas P_4 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 et al., 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 E_2 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, P_4 receptor-dependent decrease in intracellular cAMP caused by AC inhibition (Sadler and 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 and Shuetz, 1985). Specifically, transient incubation of whole *R. arenarum* follicles (obtained during both non-reproductive and reproductive periods) with forskolin or db-cAMP stimulated P_4 and T secretion (Figures 4-5), 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 E_2, which is involved with aromatization and follicle growth in the ovary, whereas during the reproductive period, P_4 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, USA) and testosterone (Sigma, USA) were dissolved in absolute ethanol to obtain a stock solution of 4 mM and 10 µM, respectively. db-CAMP (Sigma, USA) 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 $CaCl_2$/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 three 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 h 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 watchmakers' forceps, and then gently shaking the oocytes with follicular cells for 5 min (100 oscilations per min) (Zelarayán et al., 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, USA) 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 h), 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

Batchs of 20 follicles were incubated in AR solution for 24 h; 1 µM T for 24 h; 1 µM T + 0.5 mM db-cAMP or 10 µM forskolin for 24 h; or 0.5 mM db-cAMP or 10 µM forskolin for

8, 16, or 24 h. At the end of the incubation period, the follicles from the db-cAMP- or forskolin-only batches (8, 16, or 24 h) were washed thoroughly and then transferred to an AR solution for the remaining time (total of 24 h).

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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 h); 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 ECLIA. Meiotic resumption (GVBD) was determined in the follicles at 20 h.

4.5. Extraction and quantification of steroids for ECLIA

Steroids were extracted from the incubation medium, and quantified using ECLIA with the method validated previously by our group (Arias Torres et al., 2016b). In this analytical system, the reporting range for P_4 is 0.095-191 nM, for T is 0.087-52.0 nM, and for E_2 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.

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Legends

Figure 1. Schematic of the oocyte surrounded by follicular cells. In follicular cells, the activation of the Luteinizing hormone receptor (LHR) coupled to Gαs activates AC at the plasma membrane, thus increasing cAMP levels so that steroidogenesis is activated via PKA. Forskolin (Forsk) can also stimulate AC, causing a similar increase in cAMP that activates PKA. MIS synthetized and released by follicular cells interacts with the oocyte, and decreases cAMP levels in the oocyte via a Gαi. Depletion in cAMP decrease triggers GVBD and leads to oocyte maturation.

Figure 2. Effects of cAMP and forskolin on T-induced oocyte maturation. Oocyte samples (20) were preincubated for 1 h 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 h of incubation. Each bar represents the mean ± standard error (n=7), performed in duplicate with different animals. Means with different letters are significantly different ($p < 0.05$).

Figure 3. Seasonal follicular secretion of T, P_4, and E_2 under gonadotropic stimulation. Whole follicles from reproductive- (RP) and non-reproductive (NRP)-period animals were incubated for 6, 9, or 12 h in 1 ml of AR, or AR plus hCG (10 UI/ml). Steroids – (a) T, (b) P_4, and (c) E_2 – 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 h 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 P_4 in the presence of forskolin. Whole follicles from reproductive (RP) and non-reproductive (NRP) period animals were incubated for 6, 9, or 12 h in 1 ml of AR, or AR plus forskolin (10 µM). Steroids – (a) T and (b) P_4 – 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 h 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 P_4 in the presence of cAMP. Whole follicles from reproductive (RP) and non-reproductive (NRP) period animals were incubated for 6, 9, or 12 h in 1 ml of AR, or AR plus db-cAMP (0.5 mM). Steroids – (a) T and (b) P_4 – 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 h 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 ($p < 0.05$).

Table 1. Effect of a transient increase in cAMP levels on T-induced follicle maturation

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

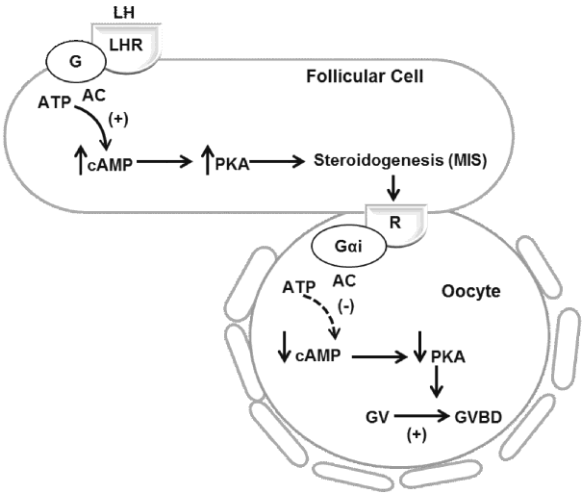

Figure 1

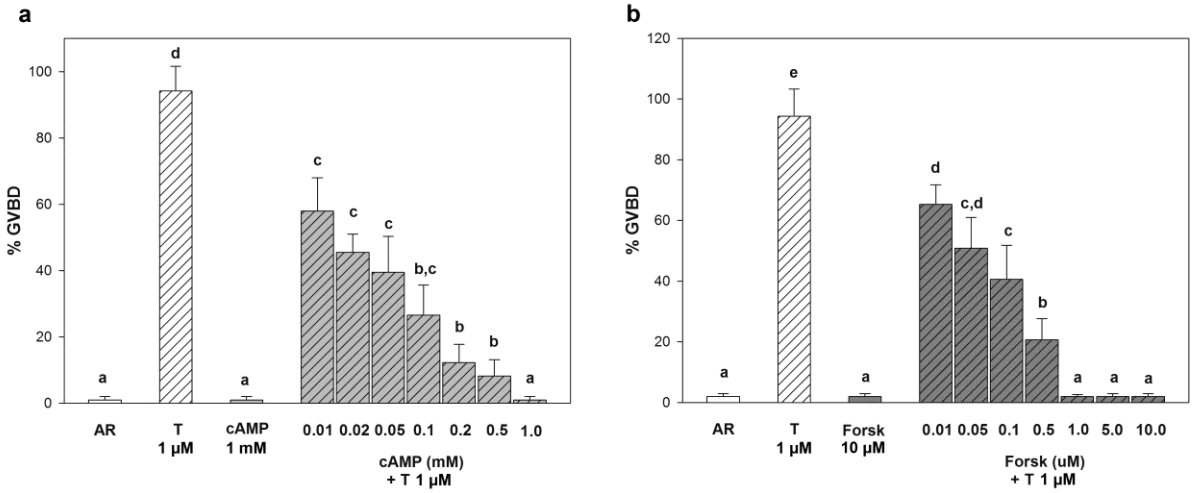

Figure 2

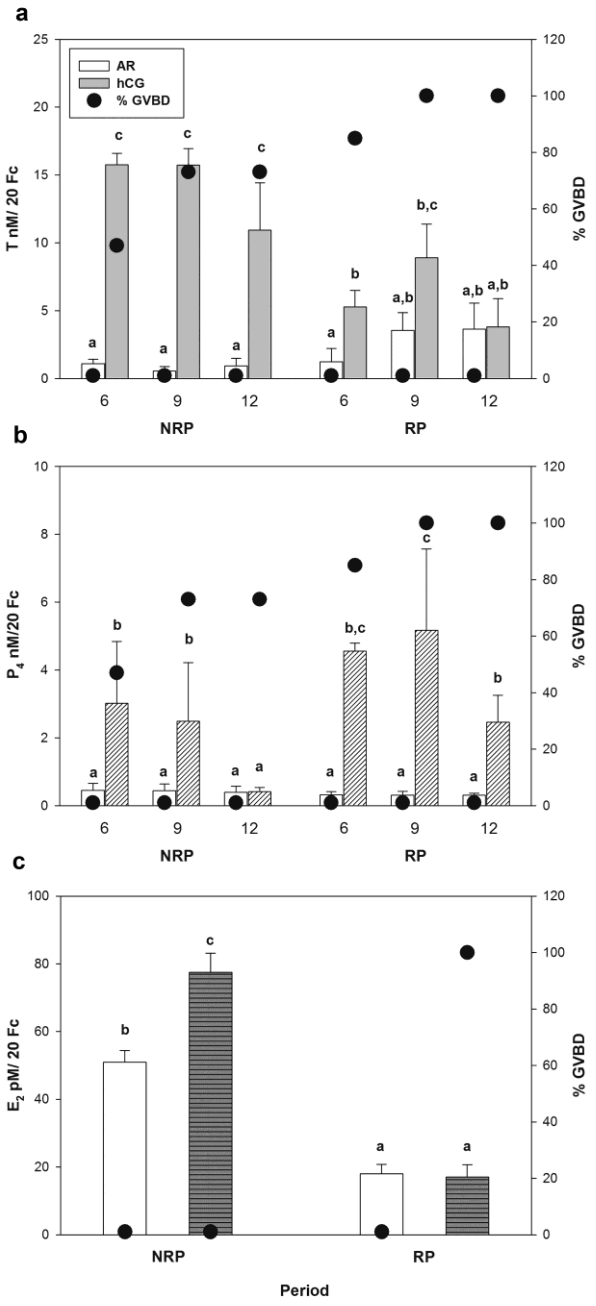

Figure 3

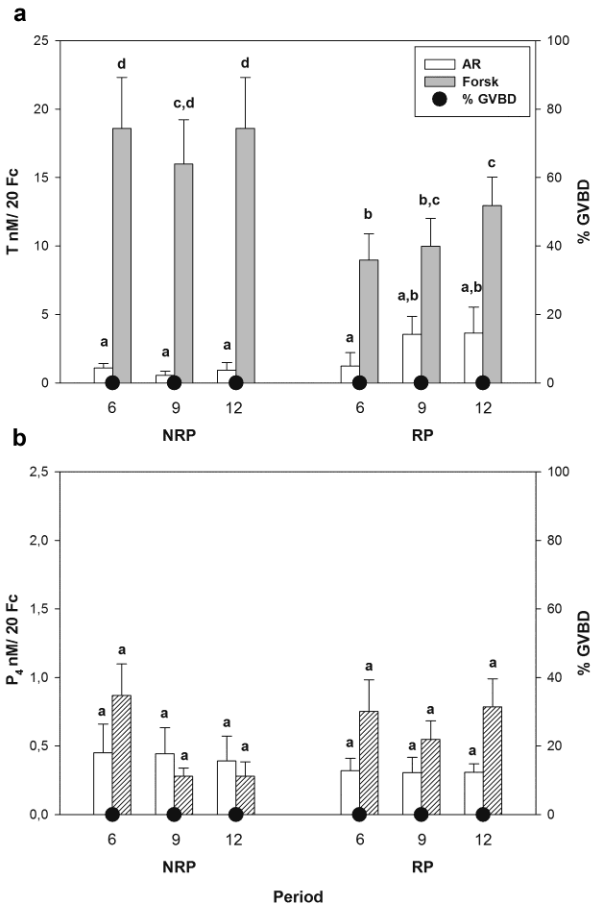

Figure 4

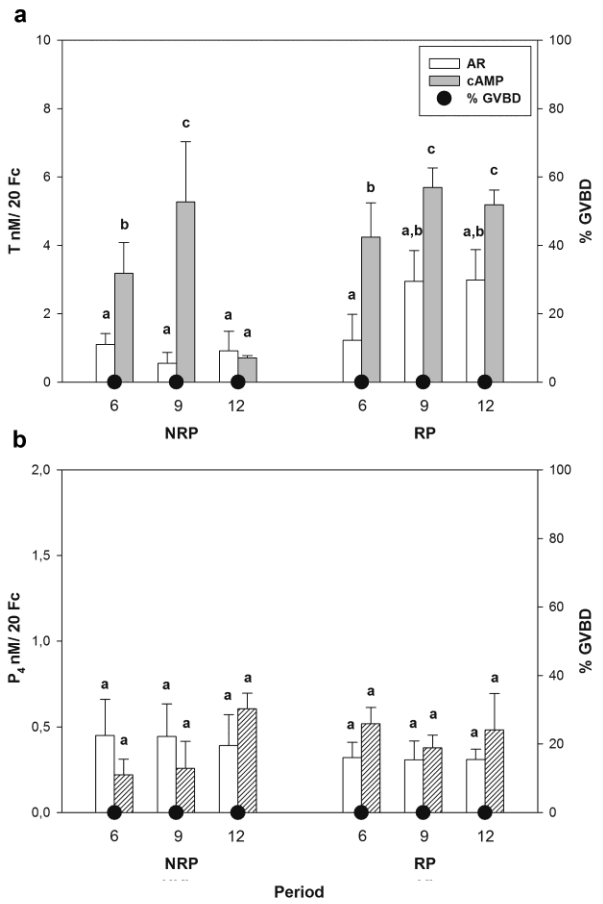

Figure 5