

## Role of arachidonic acid cascade in *Rhinella arenarum* oocyte maturation

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### Summary

There are no studies that document the production of prostaglandins (PGs) or their role in *Rhinella arenarum* oocyte maturation. In this study, we analysed the effect of arachidonic acid (AA) and prostaglandins (PGs) on maturation, activation and pronuclear formation in *R. arenarum* oocytes. Our results demonstrated that AA was capable of inducing maturation in time-dependent and dose-dependent manner. Arachidonic acid-induced maturation was inhibited by indomethacin. PGs from AA hydrolysis, such as prostaglandin  $F_{2\alpha}$  ( $PGF_{2\alpha}$ ) and, to a lesser extent,  $PGE_2$ , induced meiosis resumption. Oocyte maturation in response to  $PGF_{2\alpha}$  was similar to that produced by progesterone ( $P_4$ ). Oocyte response to  $PGE_1$  was scarce. *Rhinella arenarum* oocyte  $PGF_{2\alpha}$ -induced maturation showed seasonal variation. From February to June, oocytes presented low sensitivity to  $PGF_{2\alpha}$ . In following periods, this response increased until a maximum was reached during October to January, a close temporal correlation with oocyte response to  $P_4$  being observed. The effect of  $PGF_{2\alpha}$  on maturation was verified by analysing the capacity of oocytes to activate and form pronuclei after being injected with homologous sperm. The cytological analysis of activated oocytes demonstrated the absence of cortical granules in oocytes, suggesting that  $PGF_{2\alpha}$  induces germinal vesicle breakdown (GVBD) and meiosis resumption up to metaphase II. In turn, oocytes matured by the action of  $PGF_{2\alpha}$  were able to form pronuclei after fertilization in a similar way to oocyte matured by  $P_4$ . In microinjection of mature cytoplasm experiments, the transformation of pre-maturation promoting factor (pre-MPF) to MPF was observed when oocytes were treated with  $PGF_{2\alpha}$ . In summary, our results illustrated the participation of the AA cascade and its metabolites in maturation, activation and pronuclei formation in *R. arenarum*.

Keywords: Arachidonic acid cascade, Oocyte maturation, Prostaglandins, *Rhinella arenarum*.

### Introduction

In amphibians, as in almost all vertebrates, oocytes are arrested in meiotic prophase I. Just before ovulation a surge in luteinizing hormone (LH) stimulates ovarian follicles to unleash multiple signals that ultimately

trigger oocytes to re-enter the cell cycle in a process called maturation (Albertini & Carabatsos, 1998). Oocytes progress through meiosis to metaphase II, where they are again arrested until after fertilization, when meiosis is completed. Oocytes arrested at prophase I have a nucleus with an intact nuclear envelope or germinal vesicle (GV). Germinal vesicle breakdown (GVBD) is the first clearly visible marker of meiosis resumption (Brunet & Maro, 2005).

For oocyte maturation to occur in amphibians, a surge in LH secretion must stimulate a follicular production of progesterone ( $P_4$ ). This hormone acts non-transcriptionally to cause GVBD by interaction with a receptor located at the cell membrane, and that was demonstrated in *Xenopus* oocytes (Maller, 2001).  $P_4$  starts a cascade of transmembrane signaling events (Zelarayán *et al.*, 2007; 2012), leading to the

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activation of a key regulator of the G<sub>2</sub>-M phase transition (Liu *et al.*, 2005), the cytoplasmic maturation promoting factor (MPF), a complex of the cyclin-dependent kinase p34<sup>cdc2</sup> and cyclin B that induces GVBD (Sánchez Toranzo *et al.*, 2006). However, the mechanisms involved in this process have not been fully elucidated to date.

Large numbers of signaling mechanisms and second messengers have been implicated in P<sub>4</sub>-induced oocyte maturation. There is a general consensus that a transient decrease in cAMP intracellular levels, resulting at least partly from the inhibition of membrane adenylyl cyclase (AC) activity, is an obligatory step in the mechanism by which P<sub>4</sub> induces oocyte maturation (Kwon *et al.*, 1989; Zelarayán *et al.*, 2012). In addition to cAMP, other second messengers have been studied in the P<sub>4</sub>-induced maturation of *Rhinella arenarum* (ex *Bufo arenarum*) oocytes (Zelarayán *et al.*, 2000). However, the role of arachidonic acid (AA) metabolites in P<sub>4</sub>-induced oocyte maturation in amphibians has not been clarified.

Prostaglandins (PGs), produced in almost all cells, are important mediators of functions in the female reproductive tract. They result from the hydrolysis of polyunsaturated fatty acids (PUFAs), which are natural constituents of cell membranes. They may derive from 8,11,14-eicosatrienoic acid (PGE<sub>1</sub>) or from 5,8,11,14-eicosatetraenoic acid or AA (PGF<sub>2α</sub> and PGE<sub>2</sub>). Cyclo-oxygenases (COX) catalyze their conversion to PGs (Grosser *et al.*, 2002; Sirois *et al.*, 2004), considered to be important physiological regulators of ovarian functions. Other eicosanoids, such as leukotrienes, can be formed from PUFAs by the action of lipoxygenases (LOX).

Although PGs have been associated with ovulation and their relevance has been acknowledged in many vertebrates, studies conducted in zebrafish oocytes have demonstrated their role during maturation (Lister & Van Der Kraak, 2008). However, their participation during meiosis resumption and their role as paracrine regulation agents are not yet definitely known.

Arachidonic acid and its metabolites may induce meiosis resumption in oocytes of some species of starfish (Meijer *et al.*, 1986), fish (Sorbera *et al.*, 1998), mice (Downs & Longo, 1983) and sheep (Murdoch, 1988). Patiño *et al.* (2003) demonstrated that AA metabolites may induce ovulation in ovarian follicles of Atlantic croaker and may have a permissive role in oocyte maturation. In this sense, AA seems to have an important role in signal transduction leading to ovulation but its role regarding meiosis resumption in certain teleosts is unclear.

In amphibians, in *R. arenarum* in particular, the role of fatty acids and their metabolites during oocyte maturation has been scarcely analyzed. In *R. arenarum*

we showed that AA metabolites produced through COX and LOX pathways play an important role in P<sub>4</sub>-induced oocyte maturation. In agreement with this finding, we demonstrated that phospholipase A<sub>2</sub> (PLA<sub>2</sub>) activation and the consequent AA release induced maturation in *R. arenarum* oocytes in a dose-dependent manner, but PLA<sub>2</sub> inactivation did not affect P<sub>4</sub>-induced maturation. It seems probable that other phospholipases are involved in P<sub>4</sub>-induced maturation in this species. Our results suggest that AA metabolites resulting from the COX or LOX pathways would be involved in P<sub>4</sub>-induced *R. arenarum* oocyte maturation (Ortiz *et al.*, 2013).

Although in *R. arenarum* oocytes P<sub>4</sub>-induced maturation takes place throughout the year, oocytes showed a different P<sub>4</sub> response capacity dependent upon the period in which animals were captured. During the reproduction period, *R. arenarum* oocytes reinitiate meiosis with no need of an exogenous hormonal stimulus when they are deprived of their enveloping cells (Zelarayán *et al.*, 1995), a process known as spontaneous maturation. Oocytes that undergo spontaneous maturation are considered to be competent. Coincidentally, during this period, oocytes and follicles exhibit a maximum response to P<sub>4</sub>-induced maturation.

During fertilization, fusion with the fertilizing sperm triggers a series of morphological and biochemical changes in the oocyte that initiate a program that will allow the development of a new individual. This response of the oocyte arrested in metaphase II to make contact with the sperm is known as oocyte activation and includes a series of events such as exocytosis of cortical granules (CG), end of meiosis II, extrusion of the second polar body, pronuclei formation, DNA replication and reorganization of the cytoskeleton. During the process of activation, sperm act as a signal that causes cytoplasmic and nuclear changes in the oocyte. Under experimental conditions, this signal can be replaced by different stimuli (electric shock or puncture with a fine glass needle) that generate the same initial response in the oocyte. However, later in the course of development, the need for genetic information provided by the sperm is required for normal development to occur (Oterino, 2003; Ph.D. Thesis).

The present work aims to investigate the role of AA and PGs during *R. arenarum* oocyte maturation, with special focus on the potential participation of PGF<sub>2α</sub> in oocyte activation and pronuclear formation.

## Materials and methods

### Animals

Adult *R. arenarum* males and females were caught throughout the year in the surrounding areas of San

Miguel de Tucumán (northwestern Argentina). They were kept in captivity at room temperature with natural light–dark cycles until use, up to 15 days after collection.

### Reagents

Prostaglandins (PGE<sub>1</sub>, PGE<sub>2</sub> and PGF<sub>2α</sub>) and AA were tested for their ability to induce maturation. It is important to emphasize that AA is easily oxidized by oxygen, light, and heat; so, a 20 mM stock solution (dissolved in dimethylsulfoxide) was aliquoted in Eppendorf tubes (100 μl), covered with aluminum foil, sealed with parafilm and stored in a N<sub>2</sub> atmosphere at –20°C for almost 1 month. During each experiment one fraction was completely used. Indomethacin was used as a COX inhibitor. Amphibian Ringer solution (AR) (6.60 g NaCl/l, 0.15 g CaCl<sub>2</sub>/l, and 0.15 g KCl/l) with penicillin G-sodium (30 mg/l) and streptomycin sulphate (50 mg/l), pH 7.4, was used as a culture medium in all routine incubations. P<sub>4</sub> (1 μM) was used as a positive control for maturation. All reagents were purchased from Sigma or Merck.

### In vitro cultures

Fully grown follicles (1.6–1.8 mm in diameter) were isolated from ovarian tissues using watchmaker's forceps. Denuded oocytes were obtained by manually pulling off the follicle epithelium and the theca layer with fine forceps. Follicle cells were removed by incubation of defolliculated oocytes in AR for 5 min with gentle shaking (100 oscillations/min) (Zelarayán *et al.*, 1995). The incubations were performed at 26°C in multiwell culture dishes (Costar 3524, Cambridge, MA, USA) with randomized samples of 20 oocytes or follicles distributed into separate wells that contained 2 ml of AR. Two-well duplicates were run in each experimental group.

Dose–response curves of PGs were performed by incubating denuded oocytes or follicles for 22–24 h in the presence of different doses of the compound (0–200 ng/ml). In the case of AA, dose–response curves were carried out in the dark by incubating samples for only 60 min in the presence of different doses of the substance (0–200 μM). Then the samples were transferred to AR and incubated in the dark. Oocyte maturation was assessed 24 h after reagent addition.

Time–response curves of PGF<sub>2α</sub> were conducted with oocytes and follicles using the dose that had induced the best response. Samples were incubated for 0, 3, 6, 9, 12, 15, 18, 21 or 24 h.

Inhibition experiments were performed by pre-incubating the samples for 60 min in the presence of different doses of indomethacin (0–100 μM). Then, AA was added at the dose that had induced the highest maturation response. Both substances were kept

together during an additional 60 min period. Finally, samples were transferred to AR plus indomethacin and incubated overnight in the dark.

### Maturation criteria

After incubation, samples were fixed overnight at room temperature in Ancel & Vintemberger's solution (10% formol, 0.5% acetic acid and 0.5% NaCl). Meiosis resumption was assessed both by the presence of a transient white spot in the animal pole and by GVBD when fixed samples were dissected under a stereoscopic microscope.

### Mature cytoplasm transfers

Cytoplasm from mature oocytes was obtained using a modified Hedeimann's method (Bühler & Petrino, 1983). Briefly, oocytes matured with PGF<sub>2α</sub> (200 ng/ml) were suspended at the interface of 60% Ficoll (v/v) in AR and then centrifuged (10,000 *g*, 20 min) at 4°C (Eppendorf centrifuge 5417C). After this procedure, oocytes displayed four distinct phases: yolk platelets, pigment granules, clear cytoplasm, and a lipid layer. Denuded fully grown immature oocytes were microinjected with 40 nl of cytoplasm obtained from the clear cytoplasm layer, which contains MPF. Microinjection was performed using intracytoplasmic sperm injection (ICSI) micropipettes (Humagen™ Fertility Diagnostics) attached to a micromanipulator (Leitz Wetzlar). After microinjection, oocytes were incubated in AR for 18–20 h, fixed and GVBD was scored. As the negative control, a batch of immature oocytes was microinjected with the same volume of cytoplasm obtained from immature oocytes (immature cytoplasm). Another batch was microinjected with mature cytoplasm obtained from P<sub>4</sub>-treated oocytes (1 μM) as the positive control.

### Oocytes activation techniques

Oocyte activation was carried out in oocytes matured with PGF<sub>2α</sub> (200 ng/ml) or P<sub>4</sub> (1 μM) (control) by using the following techniques:

- Mechanical activation: by puncture in the animal hemisphere with a glass needle.
- Electric activation: by electric shock using an acrylic chamber (25 × 20 × 0.5 mm) with a platinum electrode connected to an electrical stimulator; 20 oocytes were placed in the chamber with 2 ml of AR and stimulated with 10 manual pulses of direct current (DC) (pulse duration: 2 s; intensity: 220 mV) (Oterino 2003; PhD thesis).

The activation parameters used were: disappearance of the white spot, elevation of the vitelline envelope and exocytosis of CG. Twenty minutes after activation,

oocytes were fixed and the presence or absence of CG was analyzed by a histochemical technique (Alcian Blue, pH 2.5).

### Sperm extract preparation and microinjection

Sperm suspensions were obtained by gently disrupting the testes in 4 ml AR and centrifuging at 1085 *g* for 10 min. The resulting pellet was resuspended in 2 ml of 10% AR and kept at 4°C until use. Sperm concentration was determined by counting in a Neubauer chamber and adjusted to  $2 \times 10^6$  sperm/ml (Bonilla *et al.*, 2008).

Sperm microinjections were performed in denuded oocytes matured with PGF<sub>2α</sub> (200 ng/ml) and P<sub>4</sub> (1 μM) as positive control. Oocytes that showed a white spot were transferred to calcium-free Tris-buffered saline. The sperm suspension was injected into the animal pole of matured oocytes (30–40 nl/oocyte) using ICSI micropipettes (Humagen™ Fertility Diagnostics) attached to a micromanipulator as described above. Buffer microinjection did not result in pronuclei formation in the oocytes (negative control). Two hours after microinjection, the oocytes were fixed overnight in Ancel & Vintemberger's solution, subjected to histological technique (Manes & Nieto, 1983) and stained with Ehrlich's hematoxylin-eosin to visualize pronuclei formation (Bühler *et al.*, 1987).

### Statistical analysis

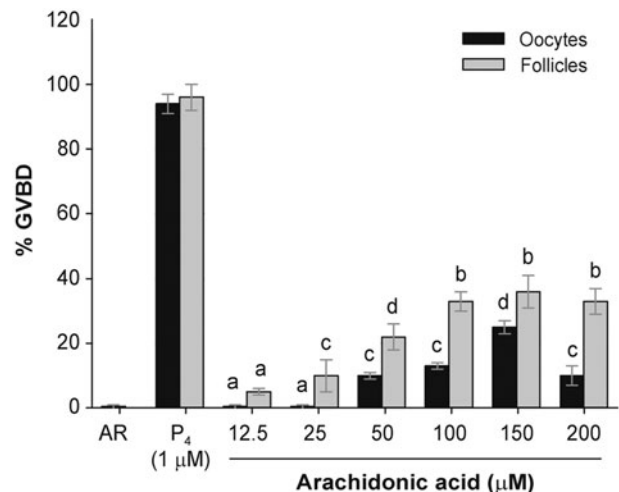
Results are expressed as means ± standard deviation (SD) from experiments carried out in triplicate using different animals in each experiment. Comparisons among different treatments were carried out using one-way analysis of variance (ANOVA) followed by Tukey's honest significant difference (HSD) test. A value of  $P < 0.05$  was considered to be statistically significant.

## Results

### *In vitro* incubation of follicles and oocytes in AA

In a previous work we demonstrated the role of PLA<sub>2</sub> in meiosis resumption in *R. arenarum* oocytes (Ortiz *et al.*, 2013). It is possible that their activation may lead to maturation as a result of PUFAs release, which have been involved in maturation in other species (Sorbera *et al.*, 2001). On the basis of these results, experiments were conducted to analyse the effect of AA on the maturation of oocytes and follicles of *R. arenarum*.

PUFAs such as AA are extremely labile and undergo rapid oxidation by exposure to light, so that their availability as a source of metabolites is short-lived. In preliminary experiments we observed that continuous



**Figure 1** Effect of arachidonic acid (AA) on maturation. Oocytes and follicles were incubated with increasing doses of AA (12.5–200 μM) in the dark. Samples incubated with progesterone (P<sub>4</sub>) (1 μM) were used as positive control. Amphibian Ringer (AR) negative control was performed only with oocytes. Germinal vesicle breakdown (GVBD) was assessed after 18–24 h of incubation. Data represent the mean ± standard deviation (SD) ( $n = 8$ ) of experiments performed in triplicate with different animals. <sup>a–d</sup>Means with different letters are significantly different ( $P < 0.05$ ).

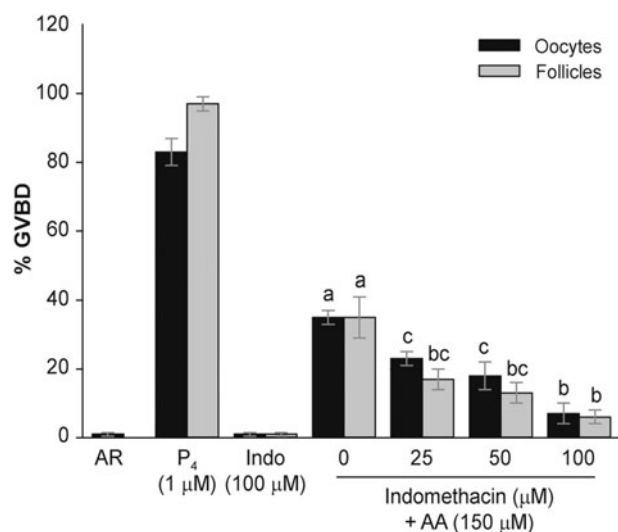
exposure of denuded oocytes and follicles to AA at any of the doses assayed was toxic and caused a gradual lysis or an excessive increase in cell volume during the first few hours of incubation. Considering these unexpected effects, the experiments were conducted as indicated in the Materials and methods section.

The results obtained show that incubation in AA stimulates the maturation of denuded oocytes and follicles in a dose-dependent manner, maximum response being obtained with the dose of 150 μM ( $25 \pm 2\%$  and  $36 \pm 5\%$ , respectively) (Fig. 1). In all cases, the response of the follicles was higher than that obtained in denuded oocytes. Larger doses were less effective.

When AA-induced maturation was studied in comparison with maturation induced by P<sub>4</sub>, a steroid considered the physiological maturation inducer in *R. arenarum* (Zelarayán *et al.*, 1995), the response of both denuded oocytes and follicles to AA was twice as slow as their response to P<sub>4</sub>. After 20 h of incubation in the presence of AA, signs of maturation were observed. In contrast, 50% of the oocytes and follicles treated with P<sub>4</sub> revealed a white spot at 8–10 h after the hormonal stimulus.

### Inhibition of AA-induced maturation with indomethacin

AA is usually metabolized by an oxidation process in which COX and/or LOX participate. In this sense,



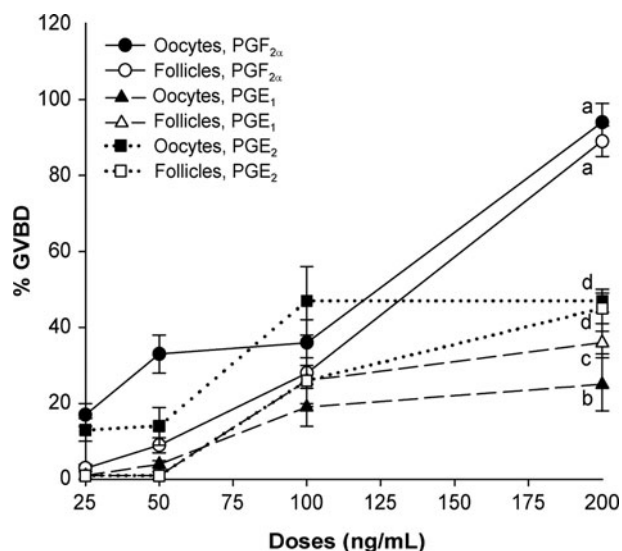
**Figure 2** Effect of indomethacin on arachidonic acid (AA)-induced maturation. Oocytes and follicles were preincubated for 1 h with different indomethacin doses (0–100  $\mu\text{M}$ ). Maturation was induced with AA (150  $\mu\text{M}$ ). Incubations were performed in triplicate in the dark. Samples incubated with progesterone ( $\text{P}_4$ ) (1  $\mu\text{M}$ ) were used as positive control. Amphibian Ringer (AR) negative control was performed only with oocytes. Germinal vesicle breakdown (GVBD) was assessed after 24 h of incubation. Values represent the mean  $\pm$  standard deviation (SD) ( $n = 6$ ) of experiments performed on different animals. <sup>a-c</sup>Means with different letters are significantly different ( $P < 0.05$ ).

in a previous work we demonstrated that  $\text{P}_4$ -induced maturation in *R. arenarum* can be partially inhibited when oocytes are treated with indomethacin (Ortiz *et al.*, 2013). This finding suggests that COX would participate in meiosis reinitiation in *R. arenarum* oocytes. In order to verify if the AA metabolites resulting from the action of COX were involved in AA-induced maturation in *R. arenarum* oocytes, we examined the effect of indomethacin, an inhibitor of the enzyme.

The effect of indomethacin on AA-induced maturation was effective as preincubation of follicles and oocytes with indomethacin (0–100  $\mu\text{M}$ ) inhibited AA-induced maturation in a dose-dependent manner (Fig. 2). Doses of 100  $\mu\text{M}$  of indomethacin inhibited AA-induced maturation in both oocytes and follicles ( $7 \pm 3\%$  and  $6 \pm 2\%$  GVBD, respectively). These results agree with those obtained previously with  $\text{P}_4$  in this species (Ortiz *et al.*, 2013).

### Involvement of PGs in oocyte maturation

The participation of PGs in the maturation process was studied in denuded oocytes and follicles incubated with  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_1$  and  $\text{PGE}_2$  (25–200 ng/ml). The results indicated that these PUFA metabolites induce



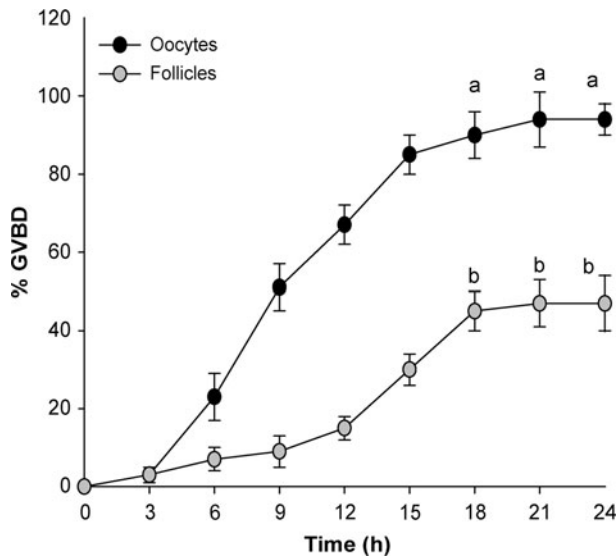
**Figure 3** Dose-response curves of oocytes and follicles treated with prostaglandin  $\text{F}_{2\alpha}$  ( $\text{PGF}_{2\alpha}$ ),  $\text{PGE}_1$  and  $\text{PGE}_2$ . Oocytes and follicles were incubated in the presence of increasing doses of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_1$  and  $\text{PGE}_2$  (25–200 ng/ml). The incubations were performed in triplicate. Germinal vesicle breakdown (GVBD) was assessed after 18–24 h of incubation. Values represent the mean  $\pm$  standard deviation (SD) ( $n = 20$ ) of experiments performed on different animals. <sup>a-d</sup>Means with different letters are significantly different ( $P < 0.05$ ).

maturation in both oocytes and follicles and this response is dose dependent. However,  $\text{PGF}_{2\alpha}$  is more potent than  $\text{PGE}_1$  and  $\text{PGE}_2$  (Fig. 3) and produces results similar to those obtained when maturation is induced by  $\text{P}_4$ . With doses of 200 ng/ml of  $\text{PGF}_{2\alpha}$ , maximum maturation response was obtained in both oocytes and follicles ( $94 \pm 5\%$  and  $89 \pm 4\%$ , respectively). Higher doses of  $\text{PGF}_{2\alpha}$  failed to induce a greater response (data not shown). As  $\text{PGF}_{2\alpha}$  is the strongest prostaglandin to induce meiosis resumption in oocytes or follicles of *R. arenarum* and it is also an AA metabolite, the following experiments were designed only in the presence of this prostaglandin.

$\text{PGF}_{2\alpha}$  induces maturation in *R. arenarum* oocytes and follicles in a time-dependent manner, maximum GVBD percentages being reached at 18 h of incubation ( $90 \pm 6\%$  and  $45 \pm 5\%$  GVBD, respectively) with the dose of 200 ng/ml (Fig. 4). The onset of the white spot as an external sign of maturation was slower with respect to  $\text{P}_4$ -induced maturation.

### Annual variation of oocyte response to $\text{P}_4$ and $\text{PGF}_{2\alpha}$ action

In amphibians, as in other vertebrates, the physiology of the ovary is subject to seasonal variations in the environment. The ability of denuded oocytes

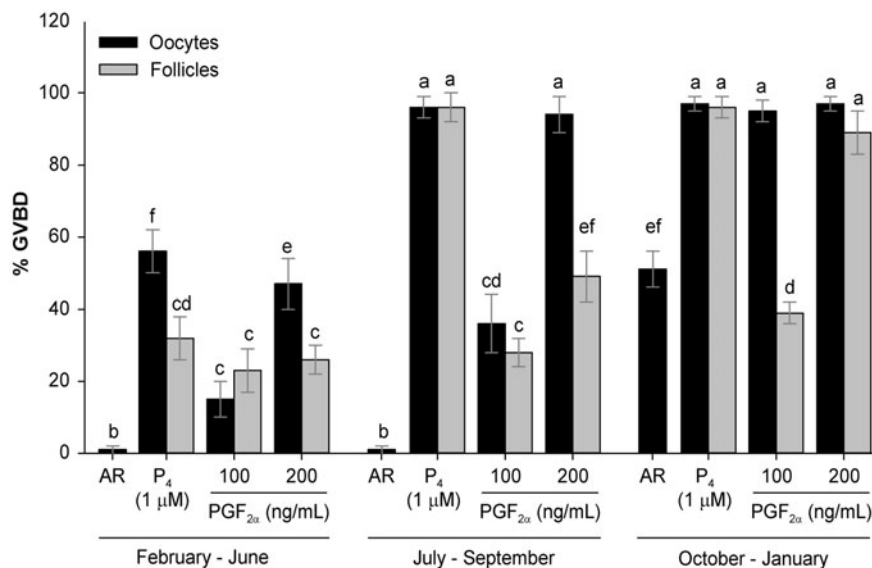


**Figure 4** Time course of maturation induced by prostaglandin  $F_{2\alpha}$  ( $PGF_{2\alpha}$ ) on oocytes and follicles. Maturation was induced with  $PGF_{2\alpha}$  (200 ng/ml) in oocytes and follicles. Samples were fixed at 3, 6, 9, 12, 15, 18, 21 and 24 h of incubation and germinal vesicle breakdown (GVBD) was assessed. Data are represented as the mean  $\pm$  standard deviation (SD) ( $n = 9$ ) of experiments performed in triplicate on different animals. <sup>a,b</sup>Means with different letters are significantly different ( $P < 0.05$ ).

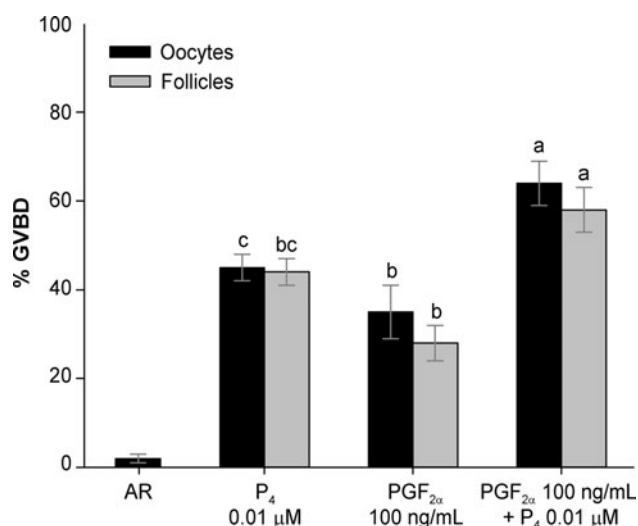
of *R. arenarum* to resume meiosis under the action of  $P_4$  varies throughout the year. Thus, previous observations showed that the oocytes of this species

present a higher response to the action of the hormone during the reproductive period (Ortiz *et al.*, 2013), coincidentally with their ability for spontaneous maturation (Zelarayán *et al.*, 1995). Our experience during the last 4 years allows us to infer that the response of *R. arenarum* denuded oocytes and follicles to  $P_4$  varies seasonally and agrees with the reports of Valdéz Toledo & Pisanó (1980) and Medina *et al.* (2004), which strengthen this idea. Our results *in vitro* could be grouped into three periods according to the seasonal variations of the ovary (Fig. 5).

From February to June, which represents a recovery stage after the summer, non-competent oocytes and follicles exhibit a low ability to respond to  $P_4$  *in vitro* ( $56 \pm 6\%$  and  $32 \pm 6\%$  of maturation, respectively), a gradual increase in this response being observed during subsequent periods. During the July to September period, oocytes respond to the hormonal stimulus but do not mature spontaneously (incompetent oocytes). During the October to January period, animals ovulate in response to the photoperiod and to the increase in gonadotropins. During this period, oocytes can mature spontaneously *in vitro* if they are deprived of their enveloping follicle cells (competent oocytes) ( $51 \pm 5\%$ ) and reach maximum GVBD values in response to  $P_4$  ( $97 \pm 3\%$ ). Similarly, during this period, follicles also reach their maximum response to the hormone ( $96 \pm 4\%$ ) (Fig. 5). The ovary shows its highest activity (ovulation and oviposition). This time is a period of high  $P_4$  response capacity.



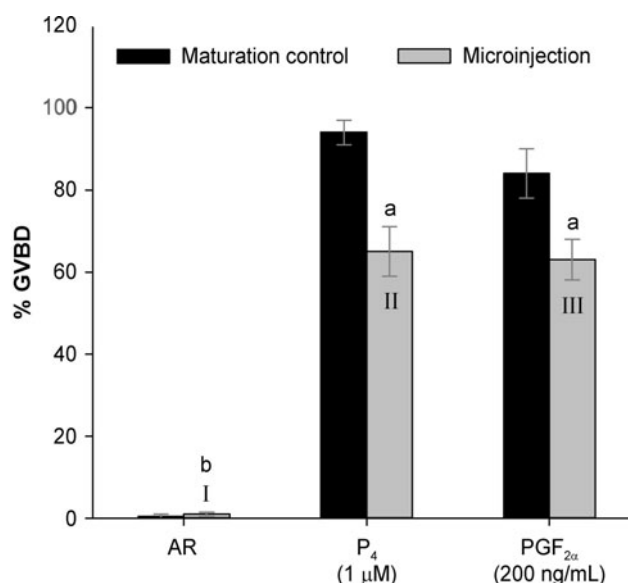
**Figure 5** Seasonal response of oocytes and follicles to progesterone ( $P_4$ ) and prostaglandin  $F_{2\alpha}$  ( $PGF_{2\alpha}$ ). Oocytes and follicles obtained during February to June, July to September and October to January were incubated in the presence of  $P_4$  (1  $\mu$ M) or  $PGF_{2\alpha}$  (100 and 200 ng/ml). Values represent the mean  $\pm$  standard deviation (SD) ( $n = 9$ ) of experiments in triplicate with different animals. Amphibian Ringer (AR) control was performed only with oocytes. <sup>a-f</sup>Means with different letters are significantly different ( $P < 0.05$ ).



**Figure 6** Effect of the combined action of prostaglandin F<sub>2α</sub> (PGF<sub>2α</sub>) and progesterone (P<sub>4</sub>) on meiosis resumption. Oocytes and follicles from animals captured during the July to September period were incubated in the presence of P<sub>4</sub> (0.01 μM) and of PGF<sub>2α</sub> (100 ng/ml). Values represent the mean ± standard deviation (SD) ( $n = 8$ ) of experiments performed in triplicate with different animals. Germinal vesicle breakdown (GVBD) was assessed after 24 h of incubation. <sup>a-c</sup>Means with different letters are significantly different ( $P < 0.05$ ).

Based on the seasonal variation of the oocyte response to P<sub>4</sub> *in vitro*, we investigated the effect of PGF<sub>2α</sub> on maturation in the three periods in the year. The results obtained in the February to June period indicate that oocytes present low sensitivity to PGF<sub>2α</sub>, reaching a maturation percentage of  $15 \pm 5\%$  and  $47 \pm 7\%$  for doses of 100 and 200 ng/ml, respectively (Fig. 5). In contrast, during the October–January period, maturation indices were highest, no significant differences being observed between the doses used ( $95 \pm 3\%$  and  $97 \pm 6\%$ ). In the July–September period, the response to PGF<sub>2α</sub> presented differences according to the dose used ( $36 \pm 8\%$  and  $94 \pm 6\%$  for 100 and 200 ng/ml, respectively). Follicles also exhibited a seasonal response to PGF<sub>2α</sub>. These results indicate that oocytes and follicles of *R. arenarum* mature under the action of this prostaglandin, showing a correlation with the response to P<sub>4</sub> throughout the year.

Additionally, during the July–September period, experiments were conducted with PGF<sub>2α</sub> (100 ng/ml) in combination with a suboptimal dose of P<sub>4</sub> (0.01 μM) in order to analyze if synergistic effects appeared. Incompetent oocytes and whole follicles were incubated in the presence of both substances for 24 h. Results are shown in Fig. 6. The maturation induced by PGF<sub>2α</sub> (100 ng/ml) ( $35 \pm 6\%$  in oocytes and  $28 \pm 4\%$  in follicles) was potentiated by the



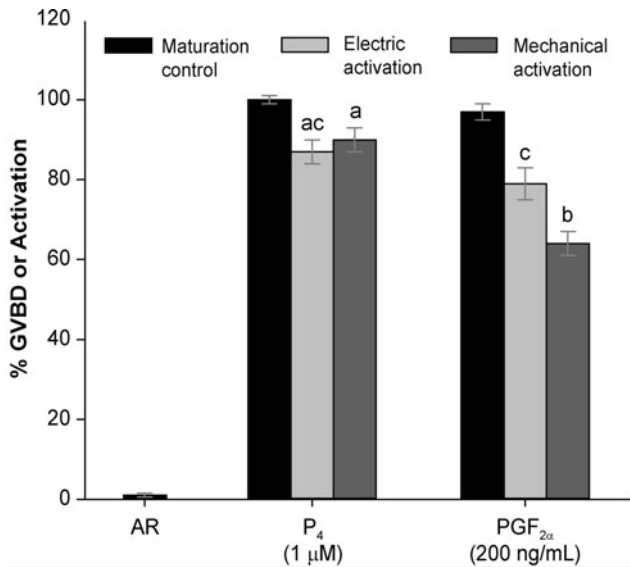
**Figure 7** MPF amplification in denuded oocytes. Immature incompetent oocytes were microinjected with 40 nl of (I) immature cytoplasm, (II) cytoplasm matured with progesterone (P<sub>4</sub>) (1 μM) and (III) cytoplasm matured with prostaglandin F<sub>2α</sub> (PGF<sub>2α</sub>) (200 ng/ml). After microinjection, oocytes were incubated in Amphibian Ringer (AR) solution for 20 h and germinal vesicle breakdown (GVBD) was assessed. Each bar represents the mean ± standard deviation (SD) ( $n = 4$ ) of experiments performed in duplicate on different animals. <sup>a,b</sup>Means with different letters are significantly different ( $P < 0.05$ ).

addition of a suboptimal concentration of P<sub>4</sub> (0.01 μM), so that oocytes and follicles reached significantly greater percentages of GVBD ( $64 \pm 5\%$  and  $58 \pm 5\%$ , respectively) ( $P < 0.05$ ). The treatment of denuded oocytes with PGF<sub>2α</sub> in combination with a suboptimal dose of P<sub>4</sub> showed a synergistic effect of both substances on the maturation process.

#### PGF<sub>2α</sub>-induced maturation and its relationship with MPF activation

In a previous study, our research group demonstrated that microinjection of mature cytoplasm from P<sub>4</sub>-treated oocytes into *R. arenarum* immature oocytes (intact GV) is sufficient to activate an amplification loop that induces the conversion of pre-MPF into active MPF, leading to meiosis resumption (Sánchez Toranzo *et al.*, 2006). In order to analyze in this species if PGF<sub>2α</sub>-induced maturation activates MPF, its amplification was analyzed with a microinjection of cytoplasm from mature oocytes.

When immature oocytes were microinjected with cytoplasm from oocytes treated with PGF<sub>2α</sub> (200 ng/ml), they matured ( $63 \pm 5\%$  of GVBD) after 20 h of incubation (Fig. 7 III). Cytoplasm transfer from



**Figure 8** Activation capacity of oocytes matured with prostaglandin F<sub>2α</sub> (PGF<sub>2α</sub>). Maturation was induced with PGF<sub>2α</sub> (200 ng/ml) or progesterone (P<sub>4</sub>) (1 µM) (control). Oocytes were stimulated mechanically or with electric pulses. After 20 min external signs of activation were assessed. The samples were subjected to conventional histological technique and staining with Alcian Blue dye, pH 2.5, to assess cortical granules (CG) exocytosis. Values represent the mean ± standard deviation (SD) ( $n = 4$ ) of experiments performed in triplicate on different animals. <sup>a-c</sup>Means with different letters are significantly different ( $P < 0.05$ ).

PGF<sub>2α</sub>-matured oocytes produced results statistically similar to those obtained with microinjection of cytoplasm from P<sub>4</sub>-treated oocytes ( $65 \pm 6\%$ ) ( $P < 0.05$ ) (Fig. 7 II). The oocytes microinjected with immature cytoplasm did not resume meiosis (negative control) (Fig. 7 I). Donor oocytes (those from which cytoplasm was obtained) reached  $94 \pm 3\%$  and  $84 \pm 6\%$  GVBD when they were treated with P<sub>4</sub> (1 µM) and PGF<sub>2α</sub> (200 ng/ml), respectively.

#### Activation and formation of pronuclei in PGF<sub>2α</sub>-matured oocytes

To further the study of the role of PGs in meiosis resumption, we analyzed if PGF<sub>2α</sub>-induced maturation is a physiological phenomenon that allows the later activation of *R. arenarum* oocytes. For that purpose, the maturation of denuded oocytes was induced with PGF<sub>2α</sub> (200 ng/ml) or P<sub>4</sub> (1 µM) as control. After 20–22 h of incubation, the oocytes were stimulated mechanically or with electric pulses. The data obtained indicate that at 20 min post-activation  $79 \pm 4\%$  of the oocytes stimulated with electric pulses and  $64 \pm 3\%$  of those stimulated mechanically showed signs of activation (Fig. 8). Higher results were observed

in oocytes treated with P<sub>4</sub> ( $87 \pm 3\%$  and  $90 \pm 3\%$  of activation, respectively). The oocytes showed the external signs characteristic of activation: flattening of the animal pole and disappearance of the white spot (Fig. 9A). Conventional histological technique and staining with Alcian Blue, pH 2.5, verified the complete exocytosis of CG (Fig. 9B). Non-activated oocyte is shown in Fig. 9C.

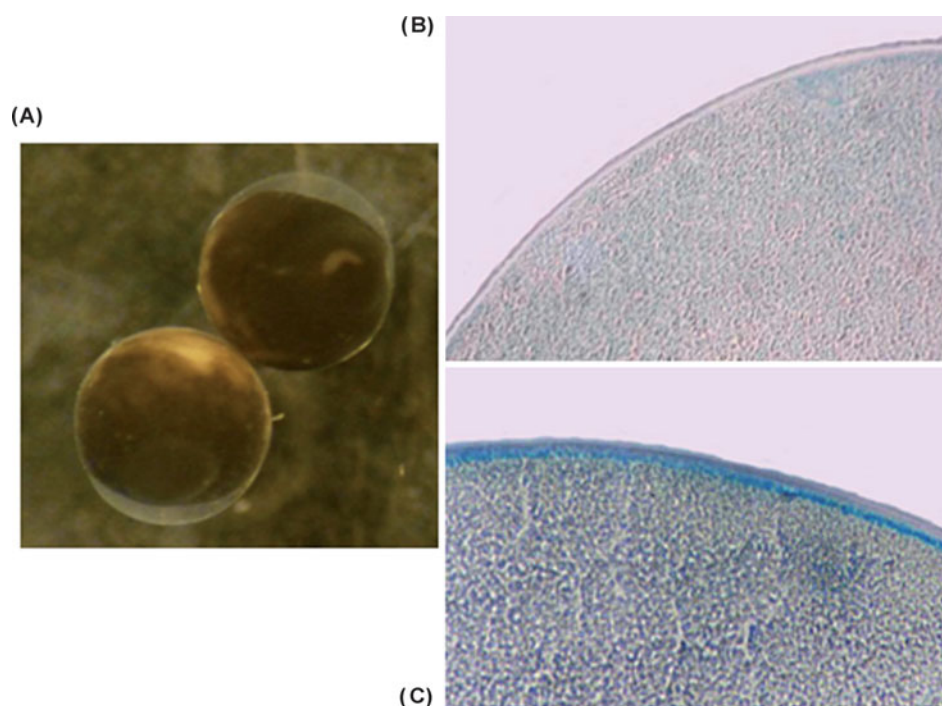
In order to verify if PGF<sub>2α</sub>-induced maturation capacitates *R. arenarum* oocytes to form pronuclei, homologous sperm were microinjected into denuded oocytes previously matured *in vitro* with PGF<sub>2α</sub> (200 ng/ml) or P<sub>4</sub> (1 µM) (control). PGF<sub>2α</sub>-matured oocytes were able to form pronuclei in similar time periods (2 h) to control treated with P<sub>4</sub> (1 µM). The cytological analysis at 120 min of the microinjected and fixed, oocytes revealed the presence of female and male pronuclei (Fig. 10A and 10B, respectively). In the sperm microinjection experiments an incubation time of 2 h is required to visualize the pronuclei formation. We did not perform assays for longer periods of time as the development of the embryos was abortive after 3–4 h of incubation when oocyte maturation was induced by P<sub>4</sub> (Oterino et al, 1997).

## Discussion

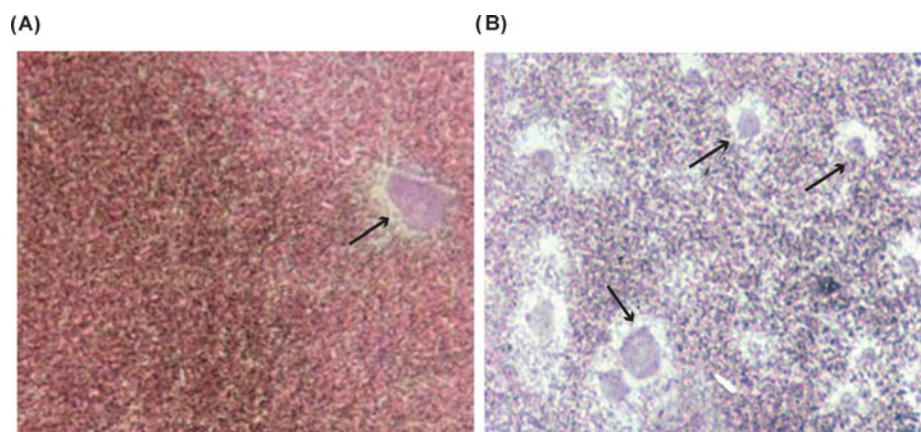
The results of this work constitute the first study that examines the effect of AA and its metabolites, PGs, on *R. arenarum* oocyte maturation, their potential role in the regulation of P<sub>4</sub>-induced maturation and their effect on oocyte activation and pronuclei formation.

In this study, induction of *in vitro* oocyte maturation by AA treatment was observed. Although AA induces maturation of oocytes and follicles in a dose- and time-dependent manner, the maturation rate is lower than in the presence of P<sub>4</sub>. AA-induced meiosis resumption is a slower process. These results agree with the ones obtained in starfish where AA and eicosapentaenoic acid were found to induce maturation in the same way as the natural hormone, 1-MeAde (Meijer et al., 1984). In fish oocytes, AA mobilization and PGs formation are required to induce GVBD (Sorbera et al., 2001; Lister & Van Der Kraak, 2008). However, when oocytes and follicles of *R. arenarum* were exposed to AA in doses higher than 150 µM, the maturation values reached were lower. Our results are similar to those obtained in fish oocytes, in which the low response could be attributed to different causes, such as the rapid oxidation of high AA doses, AA transformation into other compounds (Mercure & Van Der Kraak, 1995) or its low availability to be transformed into PGs. In this paper we only analysed the role of AA in oocyte maturation. However, other PUFAs have been





**Figure 9** Activation of oocytes matured with prostaglandin  $F_{2\alpha}$  ( $PGF_{2\alpha}$ ). (A) External signs of activation can be observed in the oocytes: flattening of the animal pole, separation of the vitelline envelope and disappearance of the white spot. Stereoscopic microscope observations,  $\times 30$  magnification. (B) Cortical granule (CG) exocytosis. Activated oocyte stained with Alcian Blue dye. Absence of CG can be observed,  $\times 400$  magnification. (C) Non-activated oocyte stained with Alcian Blue dye. The presence of stained CG can be observed,  $\times 400$  magnification.



**Figure 10** Fully grown denuded oocytes matured with  $PGF_{2\alpha}$  were injected with a homologous sperm suspension and fixed 2 h later. Photographs show: (A) female pronucleus (arrow); (B) numerous male pronuclei (arrows)  $\times 600$  magnification.

investigated. In fish, their importance in ovulation has been demonstrated when specimens were treated with diets enriched with PUFAs. In that case, the effect of high AA doses ( $300 \mu\text{M}$ ) was analysed during oocyte maturation (Sorbera *et al.*, 2001). In our experiments, lower doses were used. Our results indicate that AA-induced maturation can be inhibited by indomethacin, a COX inhibitor, suggesting that PGs could be implicated in meiosis resumption in *R. arenarum*. In a similar way to the results found by

Van Der Kraak & Chang (1990) for goldfish oocytes, the effect of  $PGF_{2\alpha}$  was blocked by drugs that inhibit COX activation, preventing the formation of PGs from AA. In the ovary of gold fish, the mechanism by which AA affects oocyte maturation is still poorly understood. In other tissues, many experiments indicate that calcium is a mediator of AA action (Törnquist *et al.*, 1994). We may tentatively assume that AA causes the mobilization of  $\text{Ca}^{2+}$  and such mobilization would be related to the maturation of *R. arenarum* oocytes.

In this respect, our research group has previously demonstrated the participation of this cation in spontaneous and  $P_4$ -induced maturation (Zelarayán *et al.*, 2004). However, AA-induced oocyte maturation requires further experiments to clarify it.

In *Rana pipiens* oocytes the participation of fatty acids and different types of 1,2-diacylglycerol (1,2-DAG) has been demonstrated in  $P_4$ -induced maturation. In the oocytes of this species the increase of DAGs in their plasmatic membranes is sufficient to trigger meiosis. In fact,  $P_4$  acts on cell surface receptors to initiate the sequential release of fatty acids from the different isoforms of DAGs, among them AA (Morrill *et al.*, 2000). These authors propose that this mobilization could be associated with the maturation process. In this respect, Buschiazzo & Alonso (2005) demonstrated that the reinitiation of meiosis induced by  $P_4$  in *R. arenarum* is accompanied by an increase in PUFAs such as AA with no significant variations in DAGs in the vitelline platelets. In this sense, it is possible that during *R. arenarum* oocyte maturation a fatty acids mobilization occurs, among which AA would be preponderant, in a similar way to teleosts and starfish. In our species, such mobilization could result from the activation of  $PLA_2$ , since its inhibition with quinacrine prevents meiosis resumption (Ortiz *et al.*, 2013).

To date, there are no studies that document the production of PGs in the ovary of *R. arenarum*. However, our *in vitro* studies demonstrated the role of AA and its metabolites in the maturation process. In this work we demonstrated that PGs from the hydrolysis of AA such as  $PGF_{2\alpha}$ , and to a lesser extent  $PGE_2$ , induce meiosis resumption in *R. arenarum* oocytes. In this species, oocyte response to  $PGE_1$  is scarce, in a similar way to the response observed in fish oocytes (Sorbera *et al.*, 2001). In agreement with this situation, previous studies have demonstrated the participation of PGs in the maturation of *Xenopus* oocytes, in which a gradual increase in the levels of  $PGF_{2\alpha}$  was observed when follicles were treated with hCG, this effect being lower for  $PGE_2$  (Sena & Liu, 2008). In *R. arenarum*, the effects of  $PGF_{2\alpha}$  on the maturation of oocytes and follicles are very similar to those produced by  $P_4$ , its natural inducer. This fact would allow us to suggest that  $PGF_{2\alpha}$  is a metabolite of AA that would mediate its stimulatory effect on meiosis resumption in this species.

The interaction of  $PGF_{2\alpha}$  with its receptor is not well defined. In hepatocytes it has been suggested that this interaction could be coupled to a  $G_i$  protein as the activation of mitogen activated protein (MAP) kinase by  $PGF_{2\alpha}$  can be inhibited by pertussis toxin (Melien *et al.*, 1998). In a previous study, our research group proposed the participation of a  $G_i$  protein in  $P_4$ -induced maturation in *R. arenarum* oocytes (Zelarayán

*et al.*, 2012). In this species, Toranzo *et al.* (2011) demonstrated the role of MAP kinases in oocyte maturation.

In amphibians, ovarian response is influenced by seasonal changes, *R. arenarum* being no exception, as shown by our *in vitro* studies with recently captured animals. In fact, the maturation of oocytes and follicles shows a seasonal response to  $PGF_{2\alpha}$  in a similar way to  $P_4$ , its natural inducer. Thus, during the reproductive period, oocytes and fully grown follicles of *R. arenarum* respond to favourable environmental conditions and to  $PGF_{2\alpha}$ , showing elevated maturation percentages. In denuded oocytes, AA metabolites could promote maturation by a direct effect, potentiating their competence to mature spontaneously when they are deprived of their follicle cells during the October to January period. At this time, the oocytes surrounded by their enveloping follicle cells do not mature spontaneously, but do so under the influence of  $PGF_{2\alpha}$  and  $PGE_2$ . In the case of our species, it is possible that AA metabolites could promote follicle maturation in an indirect, synergistic way, as observed in the follicles of teleost fish where PGs have been associated with ovarian steroidogenesis (Van Der Kraak & Chang, 1990). Additionally, treatment of *R. arenarum* denuded oocytes with  $PGF_{2\alpha}$  and low doses of  $P_4$  showed a synergistic effect on the maturation process.

Taking into account that there are no previous studies on the role of AA metabolites in the maturation of *R. arenarum* oocytes, the effect of  $PGF_{2\alpha}$  on maturation was confirmed by analysing the capacity of the treated oocytes to activate and form pronuclei after being injected with homologous sperm. It is probable that the action of  $PGF_{2\alpha}$  only leads to the dissolution of the nuclear envelope due to an unknown cause and not to physiological maturation. The cytological analysis of the oocytes activated with mechanical or electric stimuli allowed us to rule out that possibility and demonstrate the absence of CG in the oocyte cortex, suggesting that  $PGF_{2\alpha}$  induces GVBD and the continuation of meiosis until metaphase II. In turn, oocytes matured by the action of  $PGF_{2\alpha}$  are capable of forming pronuclei after fertilization in a similar way to  $P_4$  for this species (Oterino *et al.*, 1997).

The existence of pre-MPF in immature oocytes of *R. arenarum* was demonstrated by the microinjection of mature cytoplasm assays. Small amounts of cytoplasm from oocytes matured under the action of  $P_4$  are sufficient to induce maturation in immature oocytes (Sánchez Toranzo *et al.*, 2006). In a similar way, our MPF amplification experiments with oocytes treated with  $PGF_{2\alpha}$  led to the transformation of pre-MPF into active MPF. These data suggest that injection of a small amount of mature cytoplasm was able to induce MPF amplification in immature oocytes and promote its maturation.

This work focuses on the role of AA and PGs, especially PGF<sub>2α</sub>, during the process of *R. arenarum* oocytes maturation. Our results show that AA and its metabolite PGF<sub>2α</sub> induced oocyte maturation and increased maturation induced by low doses of P<sub>4</sub> *in vitro*. Once maturation was reached, the oocytes treated with PGF<sub>2α</sub> were competent to activate and form pronuclei. This fact demonstrates the capacity of PGF<sub>2α</sub> to induce physiological maturation and represents a novel contribution to the scarce data on the participation of the AA cascade in the process of amphibian oocyte maturation. Although the mechanisms involved in PGF<sub>2α</sub>-induced maturation leading to MPF activation in amphibian oocytes have not yet been clarified, the results of this work represent an original contribution to the study of the maturation process. Further studies would be necessary to elucidate the cellular and molecular mechanisms of AA metabolites involved in meiosis resumption.

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