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Short-term storage of oocytes from the neotropical teleost fish *Prochilodus marggravii*

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Abstract

The loss of oocyte viability after ovulation is one of the limiting factors in controlled reproduction of several fish species. Experiments were performed with 15 feral *Prochilodus marggravii* female fish induced to spawn with crude carp pituitary extract to evaluate the viability of oocytes retained within the ovarian cavity (in situ storage) and outside of the ovarian cavity (ex situ storage). Because fertility rates rapidly declined after ovulation, simultaneously with an increase in the number of deformed larvae, *P. marggravii* oocytes could only be successfully stored for 1 h ex situ at room temperature (~26 °C). There was a highly negative correlation ($r = -0.82$) between fertilization and deformed larvae during in situ storage at ~26 °C. Ex situ cooling (18 °C) caused a drastic reduction in fertilization rates as compared with storage at ~26 °C. Oocyte structure was preserved during 2 h storage and the cortical reaction was induced before spawning. Since the micropylar apparatus remained open, it was not the primary cause for the loss of oocyte fertility. The cytoskeleton of the oocyte appeared to be affected since ooplasmic segregation was altered after 2 h storage.

Keywords: Fish; Oocyte viability; Oocyte storage; Oocyte surface; *Prochilodus marggravii*

1. Introduction

Oocyte viability refers to the time period during which fertilization of ovulated oocytes remains possible once they have been emitted by the female [1]. Fertility of fish gametes undergoing external fertilization is limited to a few seconds or minutes in water because

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oocytes are activated and undergo cortical reaction leading to micropyle closure [2]. Short-term preservation aimed at increasing postspawning gamete longevity may improve hatchery management, minimizing problems resulting from inbreeding and asynchronous brooder maturation [3]. Ovulated oocytes retained in the ovarian cavity undergo over-ripening due to gradual morphological and biochemical changes that negatively affect fertility and larval development [4,5]. Unlike salmon and trout, oocytes of the few neotropical fishes examined to date lose their viability within a few hours of storage in the ovarian cavity [6–10].

The causes of loss of oocyte viability are not well known. According to Stoss [11], successful short-term oocyte preservation is obtained in species whose postspawning activation can be controlled so that it will only occur after the oocyte is released in the water, as in salmonids. In other fish groups such as *Carassius* and *Cyprinus* auto-activation occurs after ovulation, and also when oocytes are stored in Ringer solution [11]. Thus, precluding mechanical activation of the oocyte appears to be critical for the success of short-term storage [12]. In *Carassius auratus*, the chorion expansion and loss of oocyte viability in water may involve proteolysis, a more complex process than simple mechanical micropyle closure [13]. A group of ovulatory proteins, produced by the trout ovary and secreted in the coelomic fluid during ovulation, acts as protease inhibitors and are responsible for maintaining oocytes in a nonactivated state [14]. The role of the cytoskeleton in fertilization has been experimentally demonstrated. In *Brachydanio rerio*, a 0.2–0.5-μm thick layer of ooplasm, rich in actin filaments and poor in organelles, lies beneath the plasma membrane, increasing in thickness in the micropyle region [2,15]. Oocytes of *Rhodeus ocellatus ocellatus* treated with cytochalasin-B before fertilization showed depolymerization of actin filaments; thus inhibiting spermatozoal movement in the oocyte cortex [16]. In *Silurus glanis*, oocyte ageing phenomenon may result in errors in chromosome distribution during fertilization such as aneuploidy, triploidy and tetraploidy, and several malformations in the larvae as a consequence of the alterations of the cytoskeleton in mitotic divisions [17].

Prochilodus marggravii, a large fish endemic to the São Francisco River basin, Brazil (latitude: 13°21′S and longitude: 36°48′W), reaching over 10 kg body weight, is highly important for commercial and sport fisheries and intensively used in hatcheries for mass juvenile propagation programs. It is a highly fecund migratory species with a relatively short reproductive period within the rainy season (October–March) and whose oocytes are not naturally spawned in captivity [18]. The purpose of this study was to evaluate the effect of in situ and ex situ *P. marggravii* oocyte storage on its fertilization capacity and investigate possible structural changes in the oocyte surface, micropylar apparatus and ooplasm that might hinder fertilization.

2. Materials and methods

2.1. Fish

The fish *P. marggravii* (Characiformes, Prochilodontidae) were captured in the São Francisco River and maintained in 0.1 ha earthen ponds of a fishery station in southeastern Brazil (18°12′S, 45°15′W) at a stocking rate of 1 kg of fish per 7 m^2. They were fed on

commercial feed (22% crude protein), 1.5% of body weight per day, 5 days per week. Water was supplied to the ponds from the Três Marias Reservoir in the São Francisco River. Experiments were performed on recently ovulated oocytes of 15 females (~3 years of age; 44.9 ± 6.8 cm, total length; 1.3 ± 0.6 kg, body weight). Fifteen males (~3 years of age; 48.0 ± 4.4 cm, total length; 1.2 ± 0.2 kg, body weight) were used. The experiments were conducted during the peak (January) of the reproductive period in 1998 and 1999.

2.2. Collection of oocytes

Females with a large, flaccid abdomen and reddish urogenital papilla and males that released milt with slight abdominal pressure were considered to be ready for treatment. Females and males selected for use were measured and weighed and transferred to 3.0 m × 1.0 m × 0.8 m brick-lined tanks with running water, where they were kept apart throughout the induction process. The water supplied to the tanks had the following characteristics: temperature between 25.0 and 26.0 °C; dissolved oxygen of 5.5–6.8 mg/l, pH 6.0–6.5; and conductivity of 50–80 μS/cm^2. Fishes were induced to spawn with crude carp pituitary extract (CCPE) according to Sato et al. [19]. The females received two doses containing, respectively, 0.8 ± 0.2 and 6.0 ± 0.6 mg/kg body weight of CCPE, with a 14-h interval between doses, injected in the coelomic cavity. The males received a single dose containing 2.8 ± 0.5 mg/kg body weight of the same extract at the time the second dose was given to the females. The reproductive condition of the females was evaluated at 30-min intervals, starting at 200 h-degrees (sum of the water temperatures taken at each hour), after the second treatment with CCPE, at a water temperature of ~26 °C. Ovulation occurred at ~220 h-degrees and stripping was performed by manual massage of the ventral abdominal wall and the oocytes collected in 1-l plastic bowls.

2.3. Storage of ovulated oocytes

Recently ovulated oocytes were subjected to one of the following storage treatments:

- *Treatment 1* (in situ storage at ~26 °C): five ovulated, not stripped females (i.e. with their oocytes retained in the ovarian cavity) were kept together in one of the brick-lined tanks with continuous water renewal. Water characteristics were maintained as during the process of oocyte obtainment.
- *Treatment 2* (in situ storage at 18 °C): seven ovulated, not stripped females were kept isolated in styrofoam boxes (70 l capacity) containing water cooled at 18 °C.
- *Treatment 3* (ex situ storage at ~26 and 18 °C): 3–5 g aliquots of oocytes from three ovulated females were manually stripped and stored in 350-ml plastic bags inflated with air and kept in a water bath at ~26 °C or cooled to 18 °C.

2.4. Fertilization

Aliquots of oocytes (3–5 g; 960 oocytes/g of ova) of each female were fertilized immediately after ovulation (fertilization at 0 h = controls) and at 30-min intervals until 120 min. Milt of one male was used to fertilize the oocytes of each female.

The oocytes were placed in dry plastic bowls by first gently mixing the oocytes and milt together and then adding water from the experimental tanks to allow fertilization. The fertilized oocytes were rinsed several times with water to remove excess milt and allow the beginning of hydration before transfer to the incubators.

2.5. Incubation

Oocytes were placed in funnel type fiberglass incubators in which the water enters at the incubator vertex and flows upwards [20] with 20 l capacity (treatment 1) or with 2 l capacity (treatments 2 and 3), all under continuous water flow at ~26 °C.

2.6. Fertility and deformed larvae

To determine the fertility rate, at each storage interval time, triplicate aliquots of oocytes (minimum of 100 oocytes) from each female were collected 8–12 h after fertilization at which time the embryos were past the blastopore closure stage. Upon collection, the oocytes were immediately fixed in 4% neutral formalin solution and examined to obtain the percentage of viable embryos. At least 50 larvae from each sample were analyzed under a stereomicroscope to establish the rate of deformed larvae at each interval time in treatment 1 (in situ storage at ~26 °C).

2.7. Histology and ultrastructure

For each interval time, oocyte samples were fixed in a solution of 3% glutaraldehyde in 0.1 M phosphate buffer at pH 7.3 for 6–8 h at 4 °C. The specimens were embedded in glycol–methacrylate plastic resin, sectioned at 3–5 μm thickness and stained with toluidine blue and basic fucsin. Serial sections of the micropylar apparatus were obtained to evaluate the opening of the micropylar canal in control and 2 h stored oocytes.

Control and 2 h stored oocytes were also fixed in Karnovsky solution (2.5% glutaraldehyde and 2% *para*-formaldehyde) in 0.1 M phosphate buffer at pH 7.3 for 8–12 h at 4 °C for ultrastructural analyses. Postfixation was performed in 1% osmium tetroxide during 2 h at room temperature, in duplicate, intercalated with 1% tannic acid during 20 min. The samples were then dehydrated, dried at a critical point with CO_2, metallized with gold during 1–2 min at 15 mV and examined under a Zeiss DSM-950 scanning electron microscope.

For transmission electron microscopy, the samples were submitted to postfixation in 1% osmium tetroxide with 1.5% potassium ferrocyanide during 2 h at room temperature, dehydrated, and embedded in epon–araldite plastic resin. The ultrathin sections contrasted with uranyl acetate and lead citrate were examined under a Zeiss EM-10 transmission electron microscope.

2.8. Statistical analysis

One-way ANOVA, followed by Tukey's test, was used for comparing the fertilization rates of stored oocytes with controls (treatments 1 and 2). Multiple ANOVA, followed by

Tukey's test, was also used for comparison of ex situ storage at 18 and 26 °C (treatment 3). $P < 0.05$ were considered significant. The fertilization rates were regressed on interval times to obtain a linear equation. Pearson's correlation was used to correlate the fertilization rates and deformed larvae rates.

3. Results

3.1. Fertilization

In general, there was a significant decline ($P < 0.05$) in the rate of fertilization over the period of in situ and ex situ oocyte storage; in some cases, however, the differences were not statistically significant (Fig. 1A–C). Oocytes stored in situ for 30 min, at 26 or 18 °C, showed ~30% decline in the rate of fertilization as compared with controls. At 60 min, the fertilization rate had decreased by over 50%, and at 120 min by over 90% (Fig. 1A and B). In oocytes stored ex situ at 26 °C, the fertilization rates at 30 and 60 min of storage were statistically similar to those of the controls (Fig. 1C). Ex situ storage at 18 °C caused a drastic reduction in fertilization rates as compared with ex situ storage at 26 °C (Fig. 1C). Student's *t*-test revealed no significant differences in fertilization rates in 20 and 2 l incubators.

3.2. Deformed larvae

The rate of deformed larvae, which was $5.3 \pm 2.8\%$ in controls, increased significantly ($P < 0.05$) over the storage period in treatment 1 (Fig. 1A). After 30 min of storage, the rate of deformed larvae was 25%; after 60 min, it was 43% greater than the controls. There was a high negative correlation ($r = -0.82$) between fertilization and deformed larvae rates in the treatment in situ at 26 °C.

3.3. Oocyte histology

Control oocytes showed yolk globules filling most of the ooplasm (Fig. 2A), cortical vesicles in the peripheral ooplasm, and a thick zona radiata with transverse striations (Fig. 2B). Thin basophilic granules, tending to accumulate in the micropylar region, were present among the yolk globules (Fig. 2C). In some oocytes, the perivitelline space was beginning to be formed (Fig. 2A). In the three treatments, oocytes stored in situ or ex situ showed general characteristics histologically similar to the controls during storage (Fig. 2D–F). However, in contrast to controls, the basophilic granules were concentrated among the yolk globules in several oocytes after 2 h storage (Fig. 2E). The micropylar apparatus of stored oocytes was open as in controls (Fig. 2F).

3.4. Oocyte ultrastructure

The micropylar apparatus was funnel shaped, with a smooth surfaced vestibule and deep micropylar canal (Fig. 3A). In the micropylar region, thin and thick pore-canals were

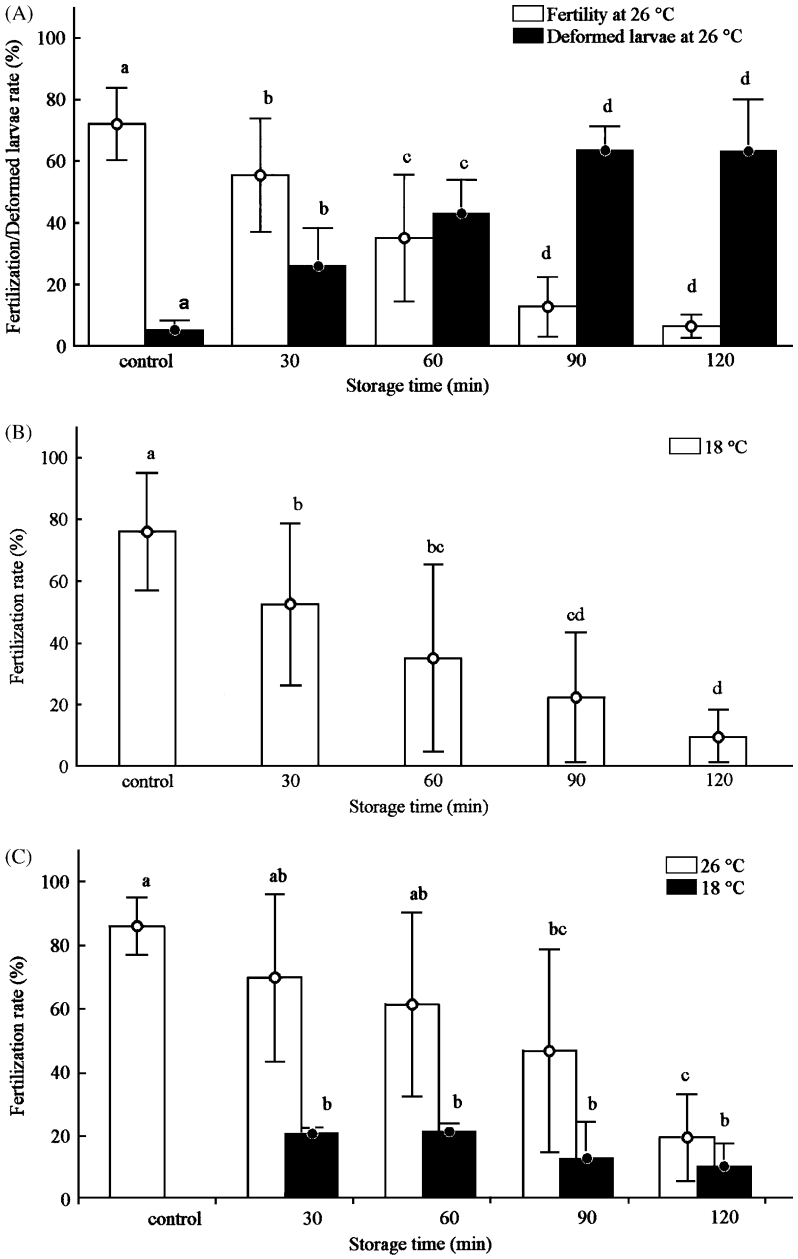

Fig. 1. Mean rates of fertilization (A–C) and deformed larvae (A) of *P. marggravii*. Oocytes were submitted to the following storage treatments: (A) in situ storage at ~26 °C; (B) in situ storage at 18 °C; (C) ex situ storage at ~26 °C (white bar) and 18 °C (black bar). Values with common superscripts indicate that mean rates do not differ significantly ($P > 0.05$). The decline in *P. marggravii* fertilization rates was slower in oocytes stored ex situ at ~26 °C ($Y = 87.82 - 0.52X$; $r^2 = 0.53$) than in situ at ~26 °C ($Y = 71.91 - 0.57X$; $r^2 = 0.94$), in situ at 18 °C ($Y = 71.35 - 0.59X$; $r^2 = 0.81$) and ex situ at 18 °C ($Y = 59.96 - 0.51X$; $r^2 = 0.56$).

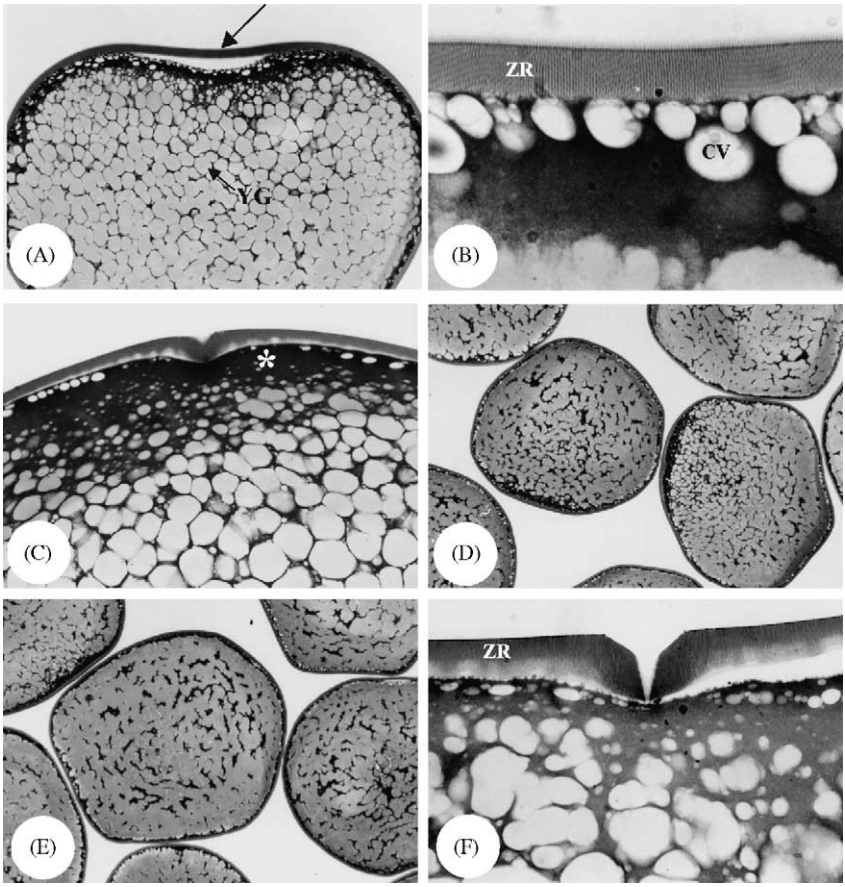

Fig. 2. Histological sections of *P. marggravii* oocytes stained with toluidine blue and basic fucsin: control (A–C), after 2 h of in situ storage at 18 °C or at ~26 °C (D–F). (A) Yolk globules (YG), without liquefaction or fusion of contents; initial formation of the perivitelline space (arrow), 100×. (B) Large cortical vesicles (CV) and thick zona radiata (ZR) with striations, 960×. (C) Concentration of basophilic granules (✻) at the micropylar region, 160×. (D) Intact oocytes after in situ storage at 18 °C for 2 h, 40×. (E) Basophilic granules concentrated among yolk globules after storage at ~26 °C, 48×. (F) Opened micropylar apparatus after 2 h of storage to 18 °C, 380×.

partially covered by a thin fibrillar net (Fig. 3B) which gradually increased in density toward the vegetal pole (Fig. 3C and D). The surface of oocytes stored in situ and ex situ for 2 h showed similar characteristics to those of the controls; ruptures were not seen in the zona radiata and the micropylar apparatus showed no morphological alterations.

In both the control and experimental oocytes, the pore-canals in the zona radiata were open and the plasma membrane was not ruptured (Fig. 4A–C). Partial release of cortical alveoli contents into the perivitelline space was occasionally observed in stored as well as in control oocytes (Fig. 4A). Yolk globules, mitochondria, endoplasmic reticulum, annulated lamellae, and ribosomes showed no structural alterations after 2 h of storage

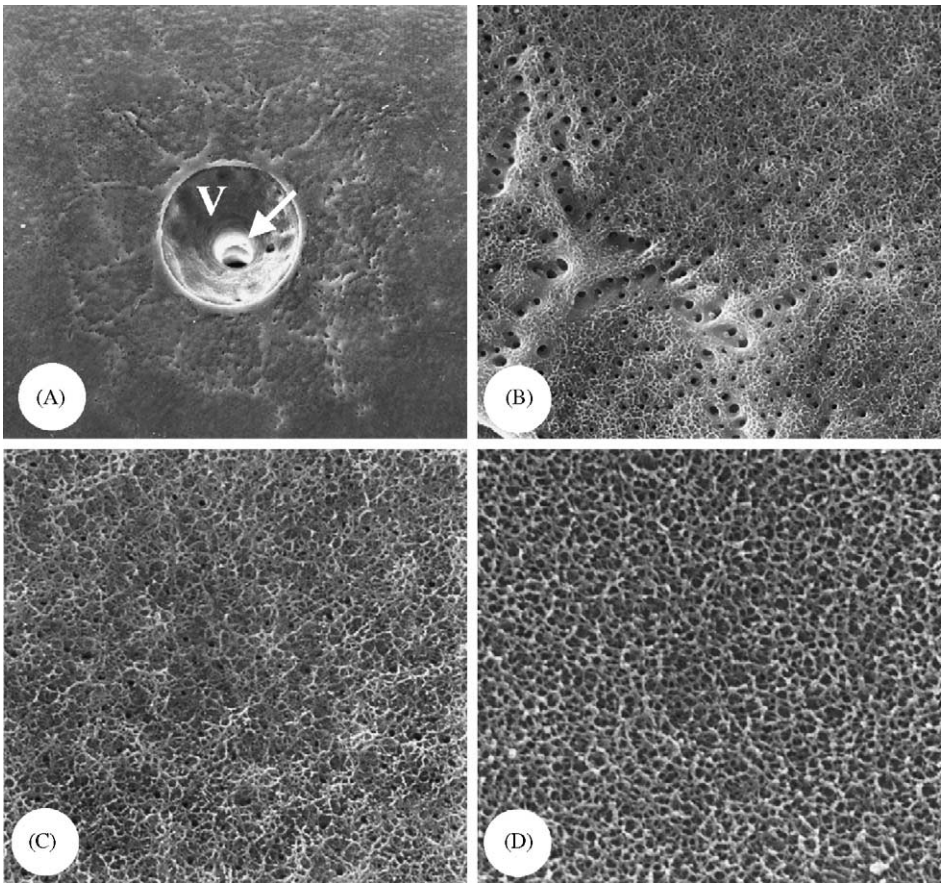

Fig. 3. Scanning electron micrographs of *P. marggravii* oocyte surface after 2 h of in situ (A) or ex situ (B–D) storage. (A) Funnel shaped micropyle with vestibule (V) and open micropylar canal (arrow), 980×; (B) peculiar arrangement of thin and thick pore-canals of the zona radiata in the micropylar region, 4250×; (C) scarce fibrilar net on the zona radiata at the animal pole, 6380×; (D) dense fibrilar net at the vegetative pole surface, 8500×.

(Fig. 4D–G). Swollen mitochondria, dispersed annulated lamellae, and aggregated organelles were found in a small number of control and treated oocytes.

4. Discussion

In this work, the viability of *P. marggravii* oocytes was assessed through fertilization rates evaluated after in situ and ex situ storage. Fertility decreased rapidly as storage time increased and it was recorded close to 0 a few hours after in situ or ex situ storage, similar to reports in several teleost species [6–10,21,22]. Thus, in our experiments, *P. marggravii* oocytes could only be successfully stored for 1 h in ex situ treatment at ∼26 °C. The highest fertility/hatching rate and lowest percentage of deformed larvae were obtained

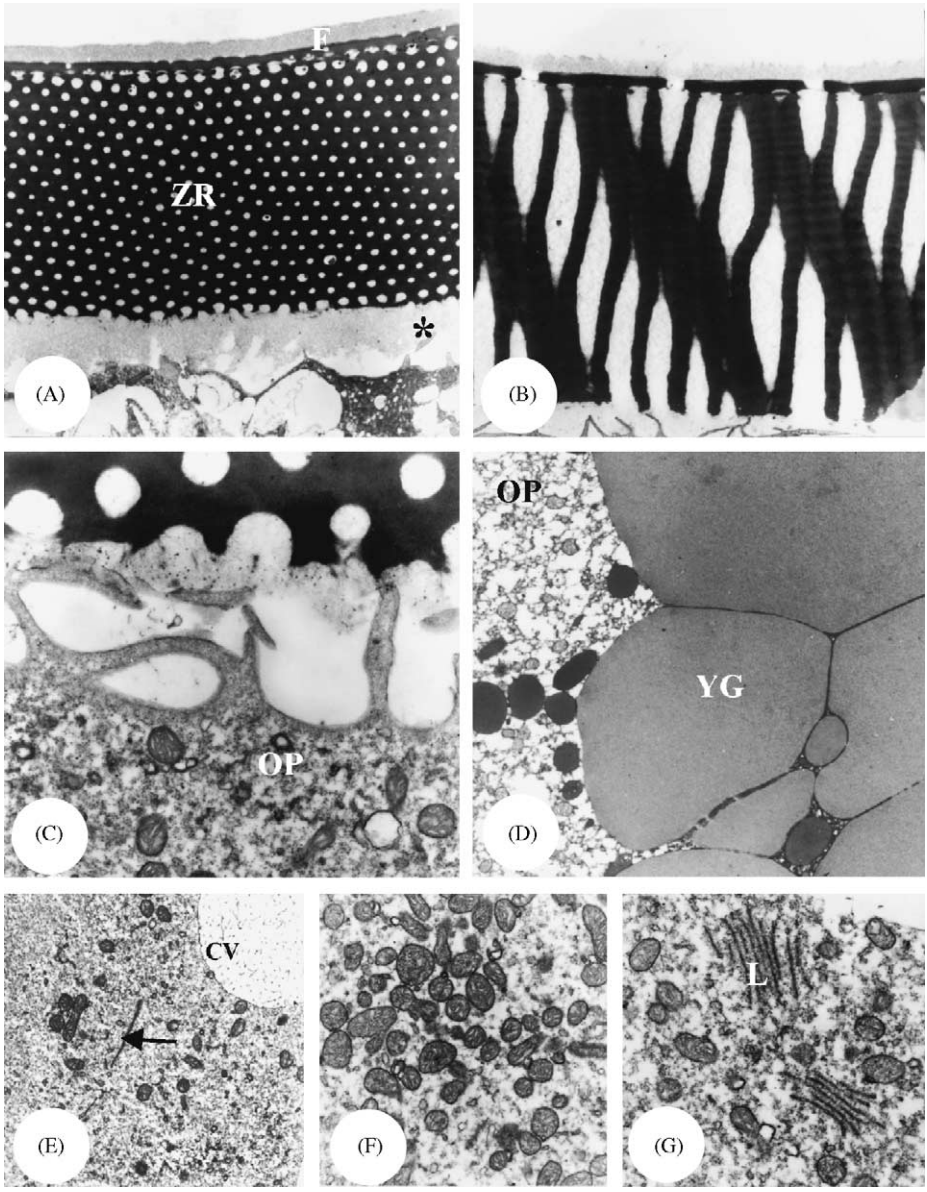

Fig. 4. Transmission electron micrographs of *P. marggravii* oocytes after 2 h of storage: in situ at ~26 °C (A, C, G), ex situ at ~26 °C (B, D, E) and ex situ at 18 °C (F). (A) Intact zona radiata (ZR) and fibrilar net (F); perivitteline space (*) with material of cortical vesicle exocitose, 3140×. (B) Enlarged pore-canals of the zona radiata, 6110×. (C) Oocyte plasma membrane with microvilli, without ruptures; ooplasm (OP) is unaltered, 15,750×. (D) Isolated, nonfused, yolk globules (YG); flocculent ooplasm (OP), 6250×. (E) Unaltered organelles: cortical vesicles—CV, mitochondria, ribosomes and endoplasmic reticulum—arrow, 6810×. (F) Aggregation of mitochondria, 10,380×. (G) Annulate lamellas (L), 12,950×.

when stripping and fertilization were performed immediately after ovulation, as in the neotropicals *Rhamdia sapo* and *Prochilodus platensis* [6,7]. Oocyte quality of the Asian catfish *Pangasius hypophthalmus* starts to decline after 2 h of storage, and the percentage of deformed larvae increases significantly after 3 h of storage [9]. In the South American catfish *R. sapo*, oocyte viability starts decreasing after 9 h at 20 °C or after 5 h at 24 °C, and viable oocytes are totally absent after 15 h at 20 °C or after 8 h at 24 °C [6]. In tilapia, the fertility rate also declines rapidly after 1.5 h of storage in the ovarian cavity, at temperatures below 18–20 °C, but no increases in the percentage of deformed larvae are recorded [8]. Apparently, *P. marggravii* oocytes were very sensitive to temperature reduction, as their viability was greatly reduced when cooled ex situ from ~26 to 18 °C.

Oocyte overripening may be evaluated on the basis of the decline in fertilization and hatching rates [5]. Since fertility and deformed larvae rate are strongly correlated, the fertilization rate alone may be sufficient to indicate the subsequent performance of embryos and larvae [23]. The rate of deformed larvae in *P. marggravii* oocytes stored in situ at ~26 °C (present paper) was negatively correlated ($r = -0.82$) with fertilization rate, similar to findings in the catfish *R. sapo* [6]. Morphological and biochemical alterations during oocyte overripening may result from breakdown of yolk proteins, loss of small organic molecules through oocyte membranes, and dephosphorylation of proteins and lipids [4]. In carps, a disturbance in the aerobic respiration process occurs that leads to the production of lactic acid, which accumulates in the ovarian fluid and reduces its pH, with ultimate loss of oocyte membrane integrity [24]. Although the fertilization in *P. marggravii* was very low after 2 h storage, there were no ruptures at the plasma membrane.

In general, loss of fertility in water or saline solution is believed to result from sealing of the micropylar canal, as a consequence of oocyte activation following cortical reaction. Trypsin treatment of nonfertilized *Orizyas latipes* oocytes for 5 min produced total occlusion of the lower third of the micropylar canal and glycoprotein digestion in the mucous area of the micropylar region, even in the absence of cortical alveoli exocytosis [25]. In our experiments, however, integrity of the zona radiata, yolk globules and most cortical vesicles was recorded. The micropyle was not occluded after 2 h during in situ or ex situ storage; thus, it could not be the primary cause of the loss of fertility in our experiments.

A wave of free cytosolic calcium traverses zebrafish oocytes upon activation; this calcium wave is initiated before fertilization in the region of the micropyle, where an extensive array of endoplasmic reticulum (probably calcium storage) is located beneath the plasma membrane [26]. The calcium wave leads to contraction of the oocyte surface and initial separation of the zona radiata of the plasma membrane as seen in control and experimental oocytes in the present study. Thus, the cortical reaction might have been induced in the ovarian cavity of *P. marggravii* by the release of intracellular calcium reserves.

Distribution of basophilic granules concentrated among yolk globules in the ooplasm was recorded in our experiments. This suggests that the process of organelle migration may have been affected during oocyte storage, since normal segregation of the ooplasm for blastodisc formation depends on the microfilaments of the oocyte cortex [27]. Thus, the spatial organization of the filaments of the cytoskeleton may be important in the preservation of oocyte viability during short-term storage.

In conclusion, gradual loss of viability of *P. marggravii* oocytes occured immediately after ovulation, resulting in a corresponding increase in deformed larvae. Oocyte structures were not altered within 2 h of storage, except for the cytoskeleton. Since the micropylar apparatus remained open, it was not responsible for the loss of oocyte fertility during storage. The increase in deformed larvae due to oocyte overripening renders the timing of manual stripping a critical step in hatchery management of *P. marggravii*.

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