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Time and temperature on the storage of oocytes from jundiá catfish, *Rhamdia quelen*

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ABSTRACT

The objective of this study was to evaluate the effects of three water and storage temperatures on the oocytes of the jundiá catfish, *Rhamdia quelen*. A factorial experimental design over time, with treatments completed in triplicate every 48 h, was used ($5\times3\times3\times3$) to study the exposure of the oocytes to temperatures of 15, 25 and 35 °C and activated with water at 15, 25 and 35 °C each at 0, 45, 90, 135 and 180 minutes post-collection. Linear regression analysis for the response surface model indicated an interaction ($p<0.05$) between time and temperature of exposure with greater values for fertilization, hatching and normal larvae rates at the time of oocyte collection ($70.2\pm8.4\%$ fertilized oocytes, $66.7\pm29.4\%$ hatched eggs and $30.3\pm25.0\%$ normal larvae). According to the statistical model, the water temperature that resulted in the highest fertilization rate was 25.6 °C ($p<0.05$). The rates of fertilization, hatching and normal larvae correlated positively ($p<0.05$) with one another, showing that these parameters can be used in the measurement of oocyte quality. Artificial fertilization of oocytes is recommended immediately after collection; if storage is necessary, it should be carried out at 15 °C.

1. Introduction

The Jundiá catfish, *Rhamdia quelen* is a representative of the Heptapteridae family and the Siluriformes order (Bockmann and Guazzelli, 2003); it originates from the hydrographic basins of Central and South America, which extend from the southwest of Mexico to the center of Argentina (Froese and Pauly, 2009). It is a species of fish that is well adapted to farming and well accepted in the consumer market (Marchioro and Baldisserotto, 1999). In intensive farming systems in Southern Brazil, it has a feed conversion rate of around 1.8, copes well with periodic handling and does not stop growing during the winter (Fracalossi et al., 2004); moreover, it responds positively to the process of hormonal induction (Carneiro and Mikos, 2008; Sampaio and Sato, 2006).

Control of the reproductive process is essential for aquaculture activity that is in full expansion (Scorvo Filho, 2008). The use of artificial propagation or induced reproduction is a key aspect of the intensification of fish production, accompanied by both economic and environmental sustainability (Romagosa, 2006). To this end, quality male and female gametes must be used to promote maximum fertilization and, subsequently, normal development of the embryo (Bobe and Labbé, 2009).

In general, there is little information in the literature about viability *ex situ* of the gametes of the ichthyofauna native to the South American continent. The recommended procedure is the rapid mixing of gametes (artificial fertilization by extrusion) based on the hypothesis that oocytes and semen can lose viability over time. Thus, knowledge about the mechanisms of viability loss and the development of methods for ensuring the longevity of gametes are important to optimize the use of breeders and the techniques of artificial propagation of fish (Babin et al., 2007; Rana, 1995).

According to Suquet et al. (1999), Rizzo et al. (2003) and Babin et al. (2007), gametes have been successfully stored short-term (temperatures near zero) both with and without using diluents, thus ensuring their viability for hours or days. One of the advantages of this technique is that it overcomes some of the difficulties associated with the gametes' breeding asynchrony that occurs when hormone therapy is used (Marques and Godinho, 2004; Rana, 1995). Another advantage is that the procedure is inexpensive, so cryoprotective solutions are not needed; it is possible to achieve cooling at refrigeration temperatures (1–15 °C), which facilitates reproductive management and increases the efficiency of artificial reproduction (Marques and Godinho, 2004).

The reproduction of the jundiá catfish is based on artificial fertilization via the "dry" method, which consists of extrusion and mixing of the gametes in the absence of water and adding water after complete mixing to induce activation of the spermatozoa and fertilization of the oocytes (Sampaio and Sato, 2006). During this procedure, the

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gametes are vulnerable to temperature effects; however, knowledge of these effects is still limited and inexistent in the literature.

This study was conducted with the objective of performing a modeling of the drop on the viability of oocytes over time at a range of storage temperature from 15 to 35 °C. Specifically, we used estimates of the fertilization rates of oocytes, the hatching of eggs and the percentage of normal larvae during 180 minutes post-collection in the jundiá catfish, *Rhamdia quelen*, as indicator of the viability of oocytes.

2. Materials and methods

The experiment was conducted in the Laboratory of Reproductive Technology of Cultivable Aquatic Animals (LATRAAC) at the Universidade Estadual do Oeste do Paraná (UNIOESTE), which is located in the Environmental Aquaculture Research Center (CPAA) in Toledo, Paraná, Brazil.

Thirty-two (32) F1 breeding jundiá catfish (*Rhamdia quelen*) 2 years of age were used from the research station; of these, 17 were female (mean ± standard error — 292.4 ± 14.4 g and 24.4 ± 0.5 cm) and 15 were male (212.7 ± 8.4 g and 22.8 ± 0.3 cm). In February of 2008, the jundiá catfish were selected according to the following external characteristics: females — convex womb and protruding and reddened genital papilla; males — semen flowing under light abdominal compression as described by Bombardelli et al. (2006).

The selected breeders were separated by gender and kept in circular boxes (1500 L) and provided with constant water renewal (750 L h^{-1}) by means of a water pump with independent entrance and exit of water. This system showed water temperature of 25 °C and approximately 13.5 h of daylight and 10.5 h of dark.

The fish were hormonally induced through the application of crude carp pituitary extract (CCPE) as described by Woynarovich and Horvath (1983) and Bombardelli et al. (2006). The females received a total of 5.5 mg CCPE kg^{-1} that was applied in two doses; the first, 10% of the total, was completed 12 h before the second application. The males received a single dose of 2.5 mg CCPE kg^{-1}. The injections were given in the muscle of the dorsal region near the dorsal fin.

Gamete collection was completed after a 240 degree-hour period (product of water temperature and time), monitored from the second hormonal application (Bombardelli et al., 2006). The criteria used to facilitate handling for gamete collection among males and females are described below.

Males were individually contained and externally dried (using cloths and paper towel), then massaged in the abdominal region in the cephalic–caudal direction. The first drop of semen was not used to prevent possible contamination with urine; the remaining semen was collected (graduated test tube, ± 0.1 mL) to measure the total volume of semen released, then stored under refrigeration (13 °C) as described by Sanches et al. (2009). If bleeding occurred while semen was being collected, collection was stopped, thus preventing any possible effects on the evaluation of sperm quality.

The semen was pooled from five males for use in each experimental protocol; then sperm concentration and sperm viability duration of sperm motility were analyzed.

To measure sperm concentration, a 5 μL sample of semen was diluted in 5000 μL of formaldehyde buffered saline (Streit Jr et al., 2004), resulting in a 1:1000 dilution. Counting the sperm cells was realized from one sample and a Neubauer hemocytometer chamber was used (spermatozoa.mL^{-1}) as described by Wirtz and Steinmann (2006).

The evaluation of the sperm viability was through of the survival rate by eosine–nigrosine staining method (Blom, 1950), using 30 μL of semen and 90 μL of each dye. The slides were analyzed under a light microscope (40× objective), and a total of 400 spermatozoa were counted.

Sperm motility duration was measured according to Sanches et al. (2009) using 5 μL of semen diluted in 200 μL of activating solution (water used in fertilization an incubation procedure), resulting in a semen:water dilution of 0.025:1. After dilution, 5 μL (semen + water) were add in slide and covered for a coverslide and conducted at light microscope (40× objective) to measure the time in that approximately 50% of the spermatozoa lost their movement. The time was measured with a digital chronometer, which was initiated simultaneously with the beginning of sperm activation and stopped when half of the sperm stopped the motility (Hilbig et al., 2008). These analyses were realized in triplicate.

The female released oocytes after light pressure was applied to the abdomen in the cephalo-caudal direction; the oocytes were transferred to a dry place and later underwent the process of artificial fertilization. One pool of oocytes homogeneous for size and color was designated for the fertilization protocols. To estimate the number of oocytes used in each experimental unit, three samples of oocytes were collected (0.1 mL) and counted, and the total number of oocytes was determined. To estimate the total number of oocytes removed from all females, the remaining oocytes that were not used in the experimental protocols were later estimated through three 3.0 mL samples.

A factorial 5 × 3 × 3 × 3 experimental design over time was used. The treatments consisted of exposure of the oocytes after collection to temperatures of 15, 25 and 35 °C and activated with water at 15, 25 and 35 °C for time periods of 0 (control), 45, 90, 135 and 180 minutes post-extrusion. The oocytes were placed in petri dishes and exposed to warm or cooled environments equipped with a thermostat (± 1 °C). The treatments were distributed across 39 experimental units and completed in triplicate. Each replicate conducted in sequential protocols every 48 h, for this, was considering the time as the block factor (Table 1). Each protocol was composed of oocyte fertilization (from one pool of oocytes from a minimum of at least three females) subjected to the different storage temperatures *by* the activation solution temperatures (water) *by* the time post-collection (Table 1). A conical incubator manufactured from PVC (2.5 L) containing 2.0 mL of oocytes (approximately 2.589 ± 185 oocytes — esteemed through of three sample of 0,1 mL of each oocyte pool) was considered an experimental unit. The insemination dose was around 1,000,000 spermatozoa $oocyte^{-1}$ (adapted from Bombardelli et al., 2006).

The semen used in each replicate in the different treatments was the same and was drawn from one pool of semen from five males, maintained under refrigeration at 13 °C (Sanches et al. 2009). For these, the sperm viability and the sperm motility duration were measured (0, 45, 90, 135 and 180 minutes post-collection) over the 180 min of exposure to observe possible effects from the loss of sperm viability over time.

The activation of gametes was performed in plastic containers (150 mL) using 25 mL of water from the artesian well at the research station with a pH of 6.8, 4.26 mg L^{-1} dissolved oxygen, 0.29 mg L^{-1} nitrate, 0.0038 mg L^{-1} nitrite, 0.126 mg L^{-1} ammonia, 43.96 mg de CaCO3 L^{-1} hardness and 49.815 mg de CaCO3 L^{-1} alkalinity.

The effect of the treatments was observed based on the estimates of the rates of fertilization of oocytes, hatching of eggs and normal larvae percentage rates. The fertilization rates were measured after the closing of

Table 1
Illustration of the layout of the experimental units of a replicate across experimental factors (temperature of exposure of the oocytes, temperature of the water and time post-collection for the artificial fertilization of jundiá catfish oocytes, *Rhamdia quelen*).

Fertilization protocol TEO (°C)	15			25			35		
TEO (min.)/AST (°C)	15	25	35	15	25	35	15	25	35
0	–	–	–	x	x	x	–	–	–
45	x	x	x	x	x	x	x	x	x
90	x	x	x	x	x	x	x	x	x
135	x	x	x	x	x	x	x	x	x
180	x	x	x	x	x	x	x	x	x

TEO — temperature of exposure of the oocytes (°C); TEO — time of exposure of the oocytes; AST — activation solution temperature (°C); x — combination of the experimental factors used in each experimental unit.