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Determination of the ideal volume of activating solution and the optimal ratio of spermatozoa per oocyte for *Prochilodus brevis* fertilization

Mayara Setúbal Oliveira-Araújo[1], Júlia Trugilio Lopes[1], Larissa Teixeira Nunes[1], Priscila Silva de Almeida-Monteiro[1], Renata Vieira do Nascimento[1], Vanessa Alves Pereira[1], Yasmim Maia Ferreira[1], Raphael William Ponte Neres[2], Assis Rubens Montenegro[1], Liliane Veras Leite-Castro[3] and Carminda Sandra Brito Salmito-Vanderley[4]

[1]Postgraduate Programme in Veterinary Science, State University of Ceará, UECE, Fortaleza, CE, Brazil; [2]Faculty of Veterinary Medicine, State University of Ceará, UECE, Fortaleza, CE, Brazil; [3]Dept of Biology, Federal Institute of Education, Science and Technology of Ceará, IFCE, Acopiara, CE, Brazil and [4]Dept of Biology, State University of Ceará, UECE, Fortaleza, CE, Brazil

Research Article

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Author for correspondence:
Mayara Setúbal Oliveira-Araújo, Faculty of Veterinary Medicine, State University of Ceará, UECE, Fortaleza, CE, 60740-000, Brazil.
Tel:/Fax: +85 3101 9851.
E-mail: maysetubal31@gmail.com

Summary

Prochilodus brevis is a rheophilic species with a threatened natural population that promotes studies aimed at optimizing reproduction in captivity. The correct quantity of inseminating dose and activating solution volume significantly improves fertilization rates, thereby increasing productivity in captivity. The objective of this study was to determine the proportion of sperm per oocyte and the ideal volume of activating solution to be used in the assisted fertilization of *P. brevis*. Gametes were collected and fertilization performed in two steps. In step 1, the ideal proportion of spermatozoa was determined based on the fertilization rate:oocyte by testing six doses of semen: D1 = 30×10^3, D2 = 150×10^3, D3 = 300×10^3, D4 = 3×10^6, D5 = 5×10^6, and D6 = 10×10^6. In step 2, the fertilization and hatching rates were evaluated using different volumes of activating solution (V1 – 25 ml, V2 – 50 ml, V3 – 75 ml,V4 – 100 ml, V5 – 125 ml, and V6 – 150 ml). A linear regression equation was estimated from steps 1 and 2. The Student–Newman–Keuls test was used to compare the means. In step 1, the percentage of fertilization increased linearly, reaching a plateau of 51.69%. In step 2, the best fertilization rates were obtained with an estimated ideal volume of 75.64 ml per 2 ml of oocytes. Therefore, the proportion of 928,410.29 sperm:oocyte, associated with the volume of 75.64 ml of water per 2 ml of oocytes, provided the maximum reproductive performance for *P. brevis*.

Introduction

The growing increase in fish demand is intrinsically related to the search for healthier foods. However, to supply the consumer market, a greater fish catch is required, causing reduction of natural populations. Therefore, to reduce the influence on these populations, the search for new reproductive strategies that optimize the reproduction of captive fish has commenced (Fao, 2016).

Brazilian fish farming has grown annually owing to its great potential for aquaculture production (Anuário Brasileiro da Piscicultura Peixe Br, 2018). Among the freshwater species cultivated are species of the genus *Prochilodus*, which are among the most important in continental fisheries, both for commercial and subsistence purposes, in the rivers of South America (Castro, 1991; Lizama, 2000). In Brazil, there are several species of this genus that are widely distributed in the basins (Souza, 2007), among these is the Brazilian bocachico (*Prochilodus brevis* or *P. cearenses*, Steindachner, 1875), which is a species of rheophilic fish native to the northeast, and is endemic to the states of Piauí, Ceará, and Rio Grande do Norte (Dourado, 1981).

With great economic value in the region (Araújo & Gurgel, 2002), *P. brevis* is one of the species threatened by anthropogenic activities, including overfishing, deforestation of rainforests, urban pollution, silting, building of river dams for hydroelectric use, and indiscriminate use of agrochemicals, which has led to their decline in natural environments (Oliveira-Silva, 2016). Environmental costs and market opportunities (i.e. fish farming) have highlighted the problem to scientific groups and has led to the intensification of studies on their reproductive biology because, after acquiring this knowledge, there has been an increase in the development of conservation and breeding programmes of this species (Denniston *et al.*, 2000).

One of the main aspects for the intensification of fish production, accompanied by economic and environmental sustainability, is the use of artificial propagation or induced reproduction (Romagosa, 2006). To optimize the use of reproductive systems in assisted fertilization, studies

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have been performed that define the ideal proportion of spermatozoa per oocyte (inseminating dose) and the volume of fertilizer solution suitable for fertilization. When these aspects are correctly defined, there is significant improvement in the fertilization rate (Leite *et al.*, 2013; Sanches *et al.*, 2009), which leads to increased productivity, thereby increasing the supply of fish to renew natural stocks and for commercial purposes.

Studies related to the ideal number of sperm per oocyte and/or volume of the activating solution have been applied to several species of freshwater teleosts, such as *Rhamdia quelen* (Bombardelli *et al.*, 2006; Adames *et al.*, 2015), *Rhinelepis aspera* (Sanches *et al.*, 2009), *Brycon orbignyanus* (Felizardo *et al.*, 2010), *Piaractus mesopotamicus* (Sanches *et al.*, 2011), *B. insignis* (Shimoda *et al.*, 2007), *Salminus brasiliensis* and *Colossoma macropomum* (Leite *et al.*, 2013), *P. magdalenae* (García *et al.*, 2015), and *Steindachneridion parahybae* (Sanches *et al.*, 2016). However, for *P. brevis* there are no reports in the literature on such reproductive aspects. Therefore, the objective of the present study was to define the proportion of sperm per oocyte and the ideal volume of activating solution to be used in the assisted fertilization routine of *P. brevis*.

Material and methods

Collection location

The experiment was performed at the Fish Reproduction Biotechnology Laboratory (LBRP), located at the Itaperi campus of the State University of Ceará (UECE; 3°47′36.2″S; 38°33′30.1″W), in Fortaleza, Ceará, Brazil. The experiment was carried out in two steps: (1) to determine the proportion of sperm per oocyte; and (2) to determine the ideal volume of activating solution to be used in the assisted fertilization routine.

Management of animals and gametes

In total, 30 males and six females of *P. brevis*, distributed among steps 1 and 2, belonging to LBRP were used. The animals were maintained in 7100 L fibreglass tanks, with a water recirculation rate of 310 L/min. They were fed daily with commercial feed (28% crude protein) provided at a rate of 3% live weight, divided into two meals per day, according to routine laboratory management procedures. Males with secondary characteristics indicative of reproductive maturity such as urogenital hyperemic papilla and easy release of semen were submitted to mild abdominal pressure. For females, the ones that presented massive abdomen and hyperemic genital papilla were selected.

Males and females were selected and weighed (137 g and 21 cm, and 22 cm and 195 g, respectively) and reproduction was hormone-induced by the application of two doses of carp pituitary extract (0.3 and 3.0 mg.kg^{-1}, 5 and 5.0 mg.kg^{-1}, respectively), with a 12-h interval between applications. After the application of the second dose, the temperature was measured (27 ± 2°C) until the collection hours of the gametes were reached.

For the collection of semen, males were sedated with clove oil solution (Eugenol, Sigma-Aldrich) in a ratio of 1:10:10000 (eugenol:alcohol:water) until the loss of equilibrium was demonstrated. Their eyes were wrapped with a damp cloth and the urogenital papilla was cleaned and dried with paper towel to prevent premature activation of semen by contact with the animal's water, faeces, or urine. Semen was collected with sterile syringes by means of slight abdominal pressure in the anteroposterior direction. The samples were then stored in polyethylene graduated tubes and kept in a thermal box (4°C) until fertilization. In addition, the seminal pH, kinetics, concentration, membrane integrity, and sperm morphology were measured.

The collection of female gametes was performed in the same way as that described for males; however, the females were not sedated and the oocytes were collected and analyzed in clean and dry Petri dishes. The weight of the spawning of each female, staining, diameter, and oocyte concentration were measured.

Step 1 – Determination of the proportion of sperm per oocyte

After collection of the gametes, a pool of semen was formed using samples from 15 males and a pool of oocytes from three females. Subsequently, the concentrations of sperm and oocytes were measured in each pool. The oocyte pool was fractionated into 24 equal samples of 3.4 g, which were individually fertilized with different doses of semen to approach the following numbers of sperm per oocyte: $D1 = 30 \times 10^3$, $D2 = 150 \times 10^3$, $D3 = 300 \times 10^3$, $D4 = 3 \times 10^6$, $D5 = 5 \times 10^6$, and $D6 = 10 \times 10^6$. Each dose corresponded to the following volume of semen: D1 = 4.53 µl, D2 = 22.68 µl, D3 = 45.33 µl, D4 = 453.33 µl, D5 = 755.56 µl, and D6 = 1511.11 µl.

The gametes were carefully mixed and the tank water was added as the activating solution, waiting for 5 min for sperm activation and oocyte hydration. The time of water addition was considered the beginning of fertilization (time zero). After hydration, the eggs were transferred to PVC incubators, with a cylindrical format and a useful volume of 1.5 l, with four replicates per dose, resulting in in total, 24 incubators.

Step 2 – Use of different volumes of activating solution in assisted fertilization

Three pools of semen from five males each were formed and the spawning of three females were kept separate to be used individually. In this step, fertilization was performed using the best ratio of sperm:oocyte obtained from step 1, following the same methodology as that described previously.

The spawning of each female was fractionated in six equal samples of 2 ml of unhydrated ova, which were individually fertilized with the semen pools and carefully mixed. Six different volumes of activating solution (water from the incubation system) were added: V 1 – 25 ml, V2 – 50 ml, V3 – 75 ml, V4 – 100 ml, V5 – 125 ml, and V6 – 150 ml. The time of water addition was considered as the beginning of fertilization (time zero), afterwards the eggs were transferred to incubators (3 females × 6 volumes × 2 replicates = 36 incubators) and evaluated for fertilization and hatching rates. The physical and chemical parameters of the water were evaluated.

Seminal evaluation

The semen was evaluated for pH by depositing an aliquot of 10 µl in test strips. Sperm kinetics were analyzed using a Computer Assisted Sperm Analysis system with the aid of a Sperm Class Analyzer (SCA®, version 5.0, Microptic S.L., Barcelona, Spain). Then, 1 µl of semen was activated with 100 µl of water from the incubation system and submitted immediately for analysis. Only samples with sperm motility equal to or greater than 90% were used. The following parameters were analyzed: percentage of moving sperm, curvilinear velocity (VCL, µm s^{-1}), straight line velocity (VSL, µm s^{-1}), and average trajectory velocity (VAP, µm s^{-1}), and at least 2000 sperm were evaluated.

The sperm concentration (sperm ml^{-1}) was measured after the fixation of each semen pool in 4% formulated citrate solution

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(1:4000; semen:fixative). For this, an aliquot of the sample was deposited in a Neubauer chamber, where sperm counts were performed (Nunes *et al.*, 2016).

The integrity of the membrane was determined using the eosin–nigrosin staining method, with a ratio of 5:10:10 (semen:eosin: nigrosin) for the preparation of the smear. In total, 200 sperms were analyzed on a slide under a light microscope (Opton, Uberlândia, Brazil; ×400 magnification). Membrane integrity was expressed as a percentage and sperm stained pink or red were considered as ruptured membranes, and those that colourless had intact membranes (adapted from Oliveira-Silva *et al.*, 2016).

The sperm morphology analysis was performed by fixing the sperm of each pool in 4% formulated citrate solution (1:10; semen:fixative) and staining with Rose Bengal (3:20; staining:fixed semen). Two slides were made for each pool and 100 sperm were evaluated per slide. The readings were performed under an optical microscope (Opton, Uberlândia, Brazil; 400X. Sperm pathologies were classified according to Nunes *et al.* (2016).

Analysis of oocytes and embryos

The spawning weight of each female was measured using a digital scale. The evaluation of staining and the uniformity of oocytes was performed by direct observation of a trained evaluator. Measurement of oocyte diameter was performed using photographic records analyzed in ImageJ software. The number of oocytes per female was determined by counting three aliquots of 0.5 g of each sample (Leite *et al.*, 2013). In addition, the spawn index of each female was measured (ID = ova weight × 100/female body weight). This index indicates spawning yield (in percentage) relative to body weight, demonstrating the efficiency of hormone treatment and extrusion.

Embryonic development was monitored, and the phases were recorded using a digital camera coupled in a stereoscopic microscope (Opton, Uberlândia, Brazil; ×400 magnification). The fertilization rate was calculated after the analysis of 300 embryos from each incubator. The eggs were considered fertilized when the embryos reached the gastrula stage (Leite *et al.*, 2013), approximately 6 h (~158 h-degree) after fertilization (Alves *et al.*, 2016). The hatching rate was verified by observing 100 eggs/larvae under a stereoscopic microscope, and the larvae whose fertilization membrane was ruptured were considered to have hatched, allowing free swimming of the larvae.

Evaluation of physical and chemical parameters of the water

The temperature and dissolved oxygen of the water were measured using a meter (Phtek, model DO-100). The pH was measured using a pH meter (KASVI, model K39–0014P) and pH tapes. Commercial analysis (LabconTest) kits were used for ammonia and nitrite parameters.

Statistical analysis

Data were initially submitted to Shapiro–Wilk and Bartlett tests to verify the hypotheses for performing parametric evaluations (normal waste distribution and homoscedasticity). The regression equation for fertilization and hatching rates as a function of the number of sperms per oocyte was estimated using the linear response plateau model of the non-linear regression (NLIN) procedure. From this, the ideal proportion of sperm per oocyte was calculated. A completely randomized design was used to verify the effect of the volume of the activating solution on the fertilization and hatching rates, followed by a linear regression analysis. The Student–Newman–Keuls test was used to compare the means. The analyses were performed in the SAS program (2002) and the level of significance was $P < 0.05$.

Table 1. Characteristics of the semen in nature of *P. brevis*

Parameters semen	Mean ± standard deviation	
	Step 1[a]	Step 2
Membrane integrity (%)	98	100 ± 0.0
Duration of motility (min)	1′13′′	43′′
pH	8	8.3 ± 0.3
Sptz morphologically normal (%)	75.5	69.3 ± 0.4
Concentration (sptz/ml)	37.9×10^9	43.1×10^9
Seminal volume (ml)	0.87 ± 0.4	0.67 ± 0.4
Total motility (%)	97.9	96.37 ± 2.7
VCL (μ/s)	89.2	93 ± 19.7
VSL (μ/s)	46.0	39.9 ± 10.6
VAP (μ/s)	72.5	65.3 ± 17.1

[a]Data referring to a single pool made with the semen of 15 males.

Table 2. Spawning characteristics of *P. brevis*

Parameters oocytes	Step 1	Step 2
Number of oocytes per gram (oocytes/g)	1334 ± 172.3	1052 ± 199.8
Colouring	Greenish	Greenish
Spawning index (%)	17.34	5.33
Oocyte diameter (micrometre)	612.8	612.8
Average female spawn weight (g)	42.80 ± 14.61	14 ± 1

Results

The animals responded positively to the hormonal treatment used in the present study, with the collection of semen and spawning occurring at 134 and 185 h-degree, respectively, after the second dose of induction. The seminal characteristics and oocytes of *P. brevis* are shown in Tables 1 and 2.

The mean water temperature of the culture system was 26.5 ± 3.7° C and the dissolved oxygen concentration was 7.14 ± 0.48 mg L^{-1}. The pH was 10.36 ± 1.56 and the ammonia and nitrite values were 0.58 ± 0.80 and 1.11 ± 1.07 ppm, respectively.

The proportion of sperm:oocyte was obtained by means of the discontinuous regression equation: $y = -0.2778 + 16.08 \times x$ ($P < 0.01$), where, y = fertilization rate and x = number of sperm: oocyte, reaching a high degree of correlation between the variables ($r^2 = 0.90$), as observed in Fig. 1. Therefore, the fertilization rate gradually increased until dose D3 and, when the proportion was between doses, the percentage of fertilized oocytes remained constant. These results suggest that a plateau was reached by stabilizing the proportion of sperm:oocyte, resulting in a fertilization rate of 51.69%. Based on the regression equation obtained, the ideal proportion of 928,410.29 sperm:oocyte was found, which is the starting point of the plateau, representing the minimum proportion required for the maximum fertilization rate to be reached.

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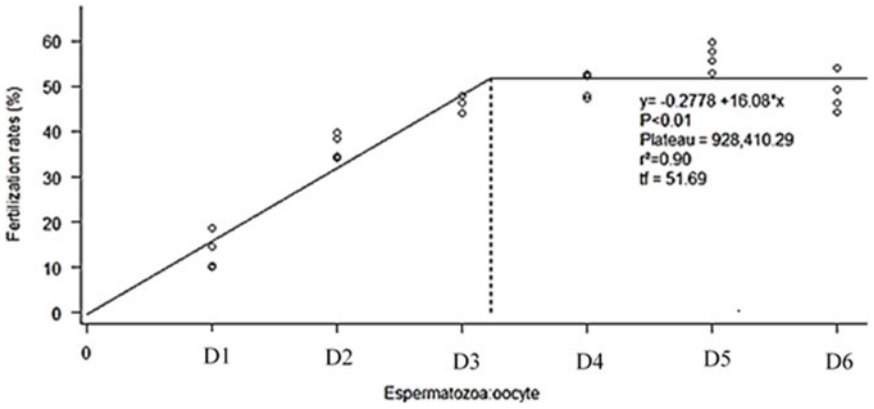

Figure 1. Rates of assisted fertilization of oocytes of common curimatã (*Prochilodus brevis*) artificially fertilized with different doses of sperm:oocyte. D1 = 30×10^3; D2 = 150×10^3; D3 = 300×10^3; D4 = 3×10^6; D5 = 5×10^6; D6 = 10×10^6.

From the ideal proportion of sperm:oocyte obtained in the present study, it was determined that for every gram of ova, 32.7 µl of semen is required. Females produce an average of approximately 28.40 g of roe; therefore, considering the average spawning obtained during the two steps, 0.93 ml of semen is required to fertilize a female of *P. brevis*. Therefore, the semen of two males is required for each female, based on the mean volume obtained for this species in the present study.

In step 2, it was observed that the fertilization rate of *P. brevis* oocytes was affected ($P < 0.05$) by the different volumes of water used during the activation of gametes during fertilization (Table 3). When volumes V1 to V5 were used at the time of fertilization, no significant differences were observed between fertilization rates ($P > 0.05$). However, when volume V6 was used, a sudden decrease ($P < 0.05$) was observed in the fertilization rate. The same results were observed for the hatching rate (Table 3).

To determine the ideal volume of the activating solution, a linear regression analysis was used, where the value of 75.64 ml of water was estimated for 2 ml of oocytes, corresponding to the optimal volume for which a maximum fertilization rate of 58, 67% (Fig. 2) could be obtained.

Discussion

The results obtained in the present study assist researchers to improve the assisted fertilization process for *P. brevis*, because to obtain the best oocyte fertilization rates, it is necessary to define the proportion of sperm:oocyte and the volume of the activating solution (Zaniboni-Filho & Weingartner, 2007).

The mean volume of semen obtained from the breeders used in the present study was higher than that reported by Nunes *et al.* (2016) and Nascimento *et al.* (2017), who presented a mean of 0.56 ± 0.24 and 0.54 ± 0.23 ml, respectively. A similar result was observed with sperm concentration, with the values obtained in the present study being higher than those reported by these authors. These differences are influenced by the nutritional and health status of the animal, period of the year, age of the breeder, and collection methodology, among other factors (Bombardelli *et al.*, 2006; Borges *et al.*, 2005; Sanches *et al.*, 2009).

For pH, membrane integrity, and sperm morphology parameters, the values were similar to those reported in the literature (8.50 ± 0.21, 96.76 ± 1.46%, and 74.18 ± 10.63%, respectively, in Nunes *et al.*, 2016, and 8.39 ± 0, 12; 95.29 ± 5.97%, and 77.21 ± 12.29%, respectively, in Nascimento *et al.*, 2017), as well as for the spermatic kinetics parameter. The mean duration of motility reported in the present study for *P. brevis* semen was 53 s, which was higher than that recorded for other fish species (Murgas *et al.*, 2007; Streit Jr *et al.*, 2004). However, these evaluations present some divergence because there is no standardization among the methods of analysis employed among the different research teams (Martínez-Páramo *et al.*, 2017).

The dry fertilization technique is most commonly used in captivity (Woynarovich & Horváth, 1983), therefore a long duration of sperm motility is not necessary to locate the micropyle, i.e. the opening located in the oocyte where the spermatozoon will fertilize. In this technique, male and female gametes are initially mixed and, only after the addition of water, is sperm activation and oocyte hydration observed (Leite *et al.*, 2013). Therefore, fertilization is optimized by the prior approximation of the gametes (Carneiro, 2007). There are some factors that influence the duration of sperm motility, such as the protocol used, species studied, volume, pH, temperature, and composition of the activating solution, with these factors varying from species to species (Billard *et al.*, 1995).

The numbers of oocytes per gram of ova in the present study were similar to those obtained for tambaqui, a species belonging to the order Characiformes, in an average of 1500 oocytes/g (Leite *et al.*, 2013). The staining of the oocyte, even though it is greenish soon after collection, becomes transparent when it is hydrated. The spawning index observed in the present study was 17.34% during step 1 and 5.33% during step 2, with this analysis being an alternative to evaluation by the gonadosomatic index because that requires the animal to be sacrificed (Vazzoler, 1996). To the best of our knowledge, this is the first study to characterize the spawning of *P. brevis* females.

In the present study, the plateau for the fertilization rate was reached in the proportion of 928,410.29 sperm:oocyte. This value was similar to that found by Souza (2007) for *P. lineatus*, whose ideal proportion was 1,007,660 sperm:oocyte. The variation was due to the size and quality of the oocyte, the sperm motility time, and the distance travelled by the sperm, which varies among species (Lahnsteiner, 2000).

The diameter found for the species studied was 612.8 µm. The diameter directly influences the ideal proportion of sperm:oocyte because the distance to be covered by the sperm influences the fertilization capacity. Shimoda *et al.* (2007) and Suquet *et al.* (1995) reported that the larger the diameter, the more sperm cells are needed to fertilize the oocytes, and the smaller the diameter of the oocyte, the greater the likelihood of spermatozoa reaching the micropyle and fertilizing it.

Recent studies have shown that the sperm dilution rate influences fertilization because it causes changes in sperm motility

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Table 3. Rate of fertilization and hatching of *P. brevis* embryos submitted to different volumes of activating solution in the assisted fertilization routine

	V1	V2	V3	V4	V5	V6
Parameters	25 ml	50 ml	75 ml	100 ml	125 ml	150 ml
Fertilization rate (%)	48.12 ± 4.56^{a}	50.66 ± 2.4^{a}	58.88 ± 2.37^{a}	57.87 ± 6.03^{a}	51.88 ± 3.65^{a}	26.66 ± 0.09^{b}
Hatch rate (%)	41.25 ± 2.6^{a}	47 ± 3.5^{a}	51.66 ± 4.14^{a}	55.75 ± 7.69^{a}	51 ± 2.02	23.83 ± 1.48^{b}

^{a,b}Lowercase letters between columns indicate statistical difference.
$P < 0.05$.

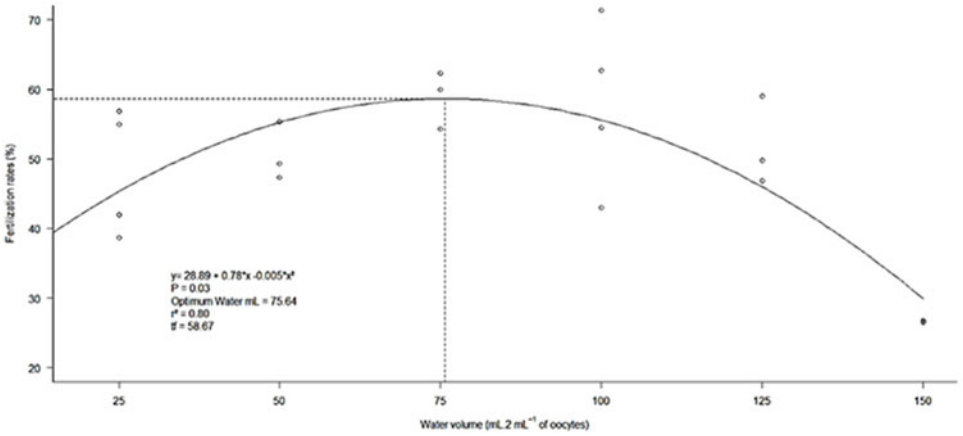

Figure 2. Effects of the addition of different volumes of activating solution for the fertilization of 2 ml samples of *P. brevis* unhydrated oocytes.

parameters, such as speed, percentage of sperm motile, and sperm motility duration (Alavi *et al.*, 2007). Therefore, the volume of activating solution used for sperm activation is as important as the proportion of sperm:oocyte in the routine of captive fertilization. The ideal volume observed in the present study was 75.64 ml of activating solution per 2 ml of oocyte, a result different from that found by Souza (2007) for *P. lineatus*, which was 105.5 ml of water per 2 ml of oocyte. When the ideal value was exceeded, a negative effect on the fertilization and hatching rates found in step 2 of the present study was observed.

The inclusion of large volumes of water as an activating solution leads to a decrease in fertilization rates, possibly due to dilution of the semen and, consequently, to the reduction of the possibility of the spermatozoon and micropole meeting (Bombardelli *et al.*, 2006). However, high dilution rates of semen with the activating solution provide a longer duration of sperm activation (Baggio *et al.*, 2007). Insufficient amounts of activating solution can cause micropyle obstruction by ovary mucus or by contact with another oocyte (Zaniboni-Filho & Weingartner, 2007) and can reduce sperm activation times (Baggio *et al.*, 2007).

These data can also be used to work with cryopreserved semen. As cryopreserved semen of this species is generally diluted 1:9 (semen:diluent, Nunes *et al.*, 2016; Nascimento *et al.*, 2017), it is recommended to use 0.25 ml straws of cryopreserved semen (motile > 80%) to 0.76 g ova or 37.4 straws per female (spawn weight ~28.40 g). In the case of cryopreserved semen with motility close to 50%, it is recommended to use twice the dose mentioned above. In the case of a 1:6 dilution, 0.25 ml straws are recommended for 1.09 g of ova or 26 straws of semen for each female (spawn weight ~28.40 g).

It is important to undertake further studies to define these values to contribute to improving the assisted fertilization of endangered species and for those that present economic value for aquaculture. The inseminating dose that meets the ratio of 928,410.29 sperm: oocytes for the artificial fertilization of *P. brevis* oocytes associated with a volume of 75.64 ml of water per 2 ml of oocytes provides the best reproductive performance in relation to oocyte fertilization rates.

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Conflict of interest. The authors declare that there are no conflicts of interest.

Ethical standards. All procedures were undertaken according to the Ethics Committee for the Use of Animals of UECE (protocol number: 2397704-2016).

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