Revista Brasileira de Zootecnia
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ISSN 1806-9290
www.sbz.org.br

R. Bras. Zootec., v.40, n.1, p.1-6, 2011

Artificial fertilization of oocytes and sperm activation in pacu: effects of the spermatozoa:oocyte ratio, water volume, and *in natura* semen preservation

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ABSTRACT - The objective of this work was to investigate artificial fertilization and the duration of sperm motility in pacu with different insemination doses, water volume, and *in natura* semen preservation. It was carried out four experimentsfor evaluation of insemination doses (7×10^3, 7×10^4, 7×10^5, 7×10^6, and 7×10^7 spermatozoa oocytes^{-1}) on the artificial fertilization of oocytes; the effect of water volume (0.5, 15.0, 30.0, 45.0, and 60.0 mL water mL^{-1} of oocyte) with insemination doses of 105,481 and 210,963 spermatozoa oocytes^{-1}; the effect of semen dilutions (0.005, 0.05, 0.5, and 5.0 µL semen mL^{-1} of water) on sperm motility duration; and the effect of storage at 15°C for 9h on sperm motility duration and sperm survival ratio. The highest results obtained were: insemination doses from 7×10^3 to 7×10^7 spermatozoa oocytes^{-1}; from 15 to 60mL water mL^{-1} of oocytes; semen dilution of 0.005 µL semen/mL water and 98.65% sperm survival until 2h45min 36s preservation time. Preservation at 15°C for 9h does not influence sperm motility duration. The highest fertilization rates can be observed by using 0.27 to 270 µL semen mL^{-1} of oocytes with 15 at 60 mL water for activation.

Key Words: fish, gametes, insemination doses, Myleinae, reproduction

Fertilização artificial de ovócitos e ativação espermática em pacus: efeito da razão espermatozoide:ovócito, volume de água e preservação do sêmen *in natura*

RESUMO - Objetivou-se foi avaliar a fertilização artificial e a duração da motilidade espermática em pacus com diferentes doses inseminantes, volumes de água e preservação do sêmen *in natura*. Foram realizados quatro experimentos para avaliação do efeito de doses inseminantes (7×10^3, 7×10^4, 7×10^5, 7×10^6 e 7×10^7 espermatozoides ovócito^{-1}) sobre a fertilização artificial dos ovócitos; do efeito do volume de água (0,5; 15,0; 30,0; 45,0 e 60,0 mL de água mL^{-1} de ovócitos) com doses inseminantes de 105.481 e 210.963 espermatozoides ovócito^{-1}; do efeito de diluição do sêmen (0,005; 0,05; 0,5 e 5,0 µL de sêmen mL^{-1} de água) sobre a duração da motilidade espermática; e do efeito do armazenamento a 15 °C por 9 h sobre a duração da motilidade espermática e o índice de sobrevivência espermática. Os maiores resultados obtidos foram: doses inseminantes entre 7×10^3 e 7×10^7 espermatozoides ovócito^{-1}; 15 a 60 mL de água mL^{-1} de ovócitos; diluição de 0.005 µL sêmen mL^{-1} de água e 98,65% de sobrevivência espermática até o tempo de preservação de 2h45min36s. A preservação a 15°C por 9 horas não influencia a duração da motilidade espermática. As maiores taxas de fertilização podem ser observadas no emprego de 0,27 a 270 µL de sêmen mL^{-1} de ovócitos, com 15 a 60 mL de água para ativação.

Palavras-chave: dose inseminante, gametas, Myleinae, peixe, reprodução

Introduction

Pacu, *Piaractus mesopotamicus* is a species in the Characidae family, subfamily Myleinae (Nakatani et al. 2001). It is of great importance in commercial fishing in its original region and there is interest in culturing this species in South American countries along the Paraná and Paraguay Rivers (Urbinati & Gonçalvez, 2005). Because of its importance in Brazilian aquaculture (IBAMA, 2007), pacu has been the focus of research in several areas, including the reproductive management (Urbinati & Gonçalvez, 2005). However, methods of semen manipulation and preservation and their relationship with successful artificial fertilization require further investigation.

In an attempt to optimize reproductive efficiency in fish, many researchers have determined appropriate insemination doses and sperm concentrations for maximal fertilization (Sanches et al., 2009). An appropriate

Received February 26, 2010 and accepted May 6, 2010.
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combination of the number of spermatozoa per oocytes, and the activation solution volume used in artificial fertilization are required, as they significantly affect fertilization rate (Souza, 2007). In addition to improving fertilization ratios, these studies will improve preservation programs of genetic biodiversity in pacu and genetic improvement on fish farms (Denniston et al., 2000), as they allow a rational use of gametes, limiting the number of breeding fish in culture stations, and reducing production costs (Bombardelli et al., 2006).

The objective of this study is to evaluate the effects of spermatozoa per oocyte ratios and the water volume on the artificial fertilization rates. It was also assessed the effect of semen preservation on sperm motility duration and sperm survival in pacu, *P. mesopotamicus*.

Material and Methods

It was used 17 pacu, *P. mesopotamicus* breeders, 10 males and 7 females at 7 years of age, provided by Centro de Pesquisa em Aqüicultura Ambiental - CPAA, Toledo, Paraná, Brazil, in December of 2006.

Broodfish were selected in the experimental culture tank. Males that expelled semen upon light abdominal pressure and females with a rounded abdomen and swollen and reddish urogenital papilla were selected. The percentage of oocytes whose germinal vesicles migrate after immersion in Serra solution (60 mL alcool 90 ºGL; 30 mL formaldeyhd; 10 mL glacial acetic acid) was determined by an ovarian biopsy (Romagosa et al., 1990). All selected females presented more than 80% of oocytes with polar vesicles and with the aforementioned characteristics.

The selected animals were transferred to the laboratory. They were weighed, marked, separated by sex in round 2,000-L tanks with constant water renewal (750 L hour^{-1}). Male and female fish were subjected to hormonal induction by injecting carpa pituitary extract (CPE), intraperitoneal via in the pectoral fin region. It was used a total dose of 2.5 and 5.5 mg kg^{-1} body weight, for males and females respectively (Woynarovish & Horváth, 1983), splited in two doses. The first one corresponded to 10% and the second to 90% of the total. Gametes were collected 240 degree-hours (sum of water temperature over time in hours) after the second hormone injection. Fish were restrained and dried with a tissue, and the abdominal region was massaged in the cephalo-caudal direction.

In experiment 1, six males and three females with an average weight of 3,000 ± 992.47 and 4,110.00 ± 608.28 g, respectively, were used. The total volume of semen stripped from each fish was measured after collection, pooled, and analyzed for sperm concentration by cell count in a Neubauer chamber. A 5-µL aliquot of semen was diluted in 5,000 µL buffered formol-saline, according to Streit Junior et al. (2004a), to obtain a final dilution of 1:1,000.

Female gametes were collected after a preliminary evaluation in a conditioning tank. Breeders that easily expelled oocytes upon light abdomen pressure were used. The two selected females were transferred to a dry place, and the oocytes were obtained by stripping and later they were tested for fertilization. Oocytes were placed on clean, dry Petri dishes. Those gametes contaminated by urine or feces were discarded.

After collecting female gametes, the relative number of oocytes mL^{-1} of stripped material per female was determined by counting three 0.1 mL aliquots of oocytes. After that, 20 aliquots (2.0 mL) of oocytes were separated for fertilization experiments. They were mixed with several volumes of known semen concentrations and activated with a fixed volume of artesian well water (30 mL) (pH = 6.80; oxygen = 4.26 mg L^{-1}; ammonia = 0.126 mg L^{-1}).

A randomized experimental design was used, with five insemination doses and four replicates. The insemination doses were 7×10^3, 7×10^4, 7×10^5, 7×10^6, and 7×10^7 spermatozoa oocytes^{-1}. The eggs were incubated in 20 conical experimental PVC incubators with 2.5 L of water. It was considered an experimental unit one incubator containing 2.0 mL of oocytes (≅ 2,700 oocytes). The fertilization ratio was measured 8h after oocyte hydration by counting three samples with approximately 250 eggs per incubator.

In experiment 2, the effect of water volume on the artificial fertilization trials using two spermatozoa doses (105,481 and 210,963 spermatozoa oocytes^{-1}) was tested. The volume ratios tested were 0.5, 15.0, 30.0, 45.0, and 60.0 mL of water mL^{-1} of oocytes. The insemination doses used in this experiment were based on the results from experiment 1. The fertilization rates were measured to evaluate the effects of the water volume.

In trial 1, three males and three females weighing 2,800.00 ± 400.00 and 4,200.00 ± 1,479.87 g, respectively, were used. In trial 2, it was used only one male and one female weighing 3,200 g and 2,800 g, respectively. In trials 1 and 2, all experimental units contained 2,960 and 2,880 (2.0 mL) oocytes, respectively.

In experiment 3, semen from three males from experiment 2 (trial 1) was used. It was used a randomized experimental design containing four dilutions in three replicates. The dilution rates were the activation of spermatozoa with of 0.005, 0.05, 0.5, and 5.0 µL semen mL^{-1} water (0.5, 5.0, 50.0 and 500.0 µL semen 100 mL^{-1} water). After dilution, 5 µL of

the mixture was used to evaluate the time needed for loss of motility by approximately 50% of spermatozoa assessed under light microscopy (400 X) (Sanches et al., 2009).

In experiment 4, it was used semen from experiment 2 (trial 1). After collection, the semen was transferred to 10.0 mL glass test tubes and placed in boxes with ice. Sperm survival rate and sperm motility duration were evaluated every 30 minutes after collection until 9 hours of preservation.

The sperm motility duration was measured as in experiment 3. The sperm survival rate was determined by the eosin-nigrosin staining method (Blom, 1950), mixing 30 µL semen and 90 µL of each dye and by preparation of a smear. One slide processing was visualized at 400×(light microscopy). Overall, 400 spermatozoa were assessed. Pink cells were dead, whereas unstained cells were live.

Fertilization rates of experiments 1 and 2 were subjected to analysis of variance ($P<0.05$) of one-way ANOVA and after that, Tukey's means test were performed by using software Statistica 7.0®. The presupposed were checked according to Quinn & Keough (2002).

Sperm motility duration, and sperm survival ratio were subjected to regression analysis ($P<0.05$) by using software SAEG 7.0 - Sistema de Análises Estatísticas e Genéticas (UFV, 1997).

Results and Discussion

In experiment 1, the mean of collected semen volume was 13.64 ± 6.82 mL with a concentration of 3.50×10^{10} spermatozoa mL^{-1}. The fertilization rates between insemination doses 7,000 and 7,000,000 sperm oocytes^{-1} remained constant ($P>0.05$). For 70,000,000 spermatozoa oocytes^{-1} there was a decrease on fertilization rates ($P<0.05$) compared to other insemination doses (Figure 1).

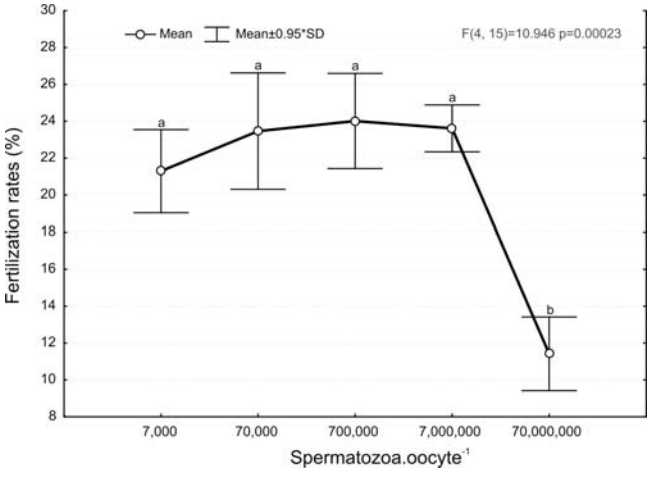

Figure 1 - Fertilization rates of pacu oocytes subjected to various spermatozoa per oocyte ratios in artificial fertilization.

In experiment 2, trial 1 (105,481 spermatozoa oocytes^{-1}), the mean of collected semen volume was 12.03 ± 1.62 mL with a concentration of 5.30×10^{10} spermatozoa mL^{-1}. For trial 2 (210,963 spermatozoa oocytes^{-1}), the collected semen volume was 9.5mL with a concentration of 2.75×10^{10} spermatozoa mL^{-1}.

Fertilization ratio results using 105,481 and 210,963 spermatozoa oocytes^{-1} exhibited similar behavior in relation to the volume of water used in the treatments, with constant values ranging from 15 and 60 mL water mL^{-1} of oocytes ($P>0.05$) (Figure 2). For both insemination doses, the lowest fertilization rates were observed in 0.5 mL water mL^{-1} of oocytes ($P<0.05$) (Figure 2).

In experiment 3, the sperm motility duration was linearl and proportional ($P<0.05$) to the increase in the semen dilution for the water (Figure 3), with greater results (26.34 ± 0.81s) for 0.005 µL semen mL^{-1} water.

In experiment 4, the sperm motility duration was not affected ($P>0.05$) by the semen cooling (Figure 4). However, the survival rate exhibited LRP (Linear Response Plateau) behavior ($P<0.05$) with increasing cooling storage time (Figure 4). The survival rates remained constant up to 2h45min36s for 98.65% of live spermatozoa. More prolonged storage resulted in an inversely proportional relation between the survival rate and the cooling storage time.

Semen production and sperm concentration of *P. mesopotamicus* brood fish varied among experiments. Viveiros & Godinho (2009), observed, in their review, variations in semen of this same species of pacu, with collected volume of 5.02 ± 2.84 up to 12.1 mL and semen concentrations of $13.9 \pm 1.3\times10^{9}$ up to $37.4 \pm 8.0\times10^{9}$ spermatozoa mL^{-1}. These differences are common, inasmuch

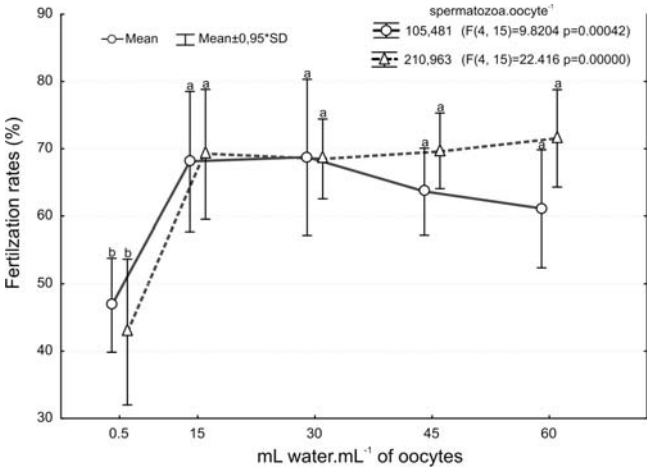

Figure 2 - Fertilization rates of pacu oocytes submitted to two artificial fertilization protocol with various water volumes in 105,481 and 210,963 spermatozoa oocyte^{-1}.

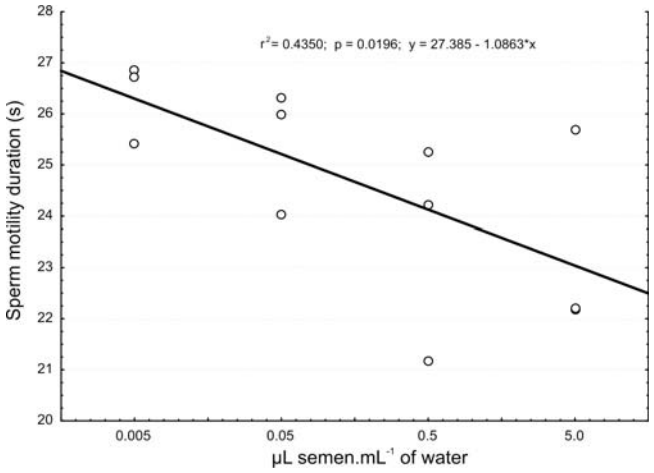

Figure 3 - Sperm motility duration of pacu semen exposed to different semen:water volume ratios of during sperm activation.

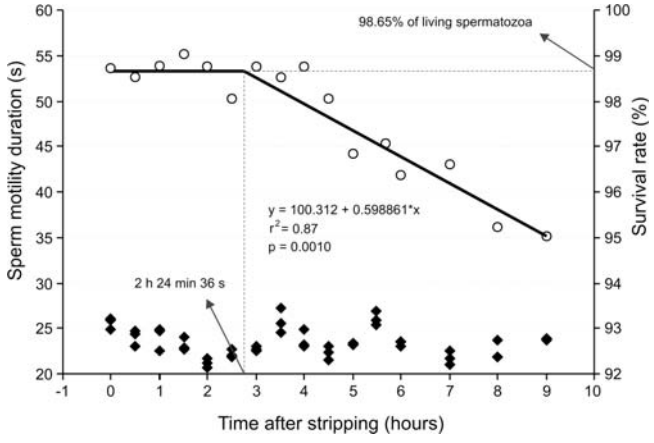

Figure 4 - Effect of *in natura* and under cooling pacu semen storage on the sperm survival rate and sperm motility duration.

as individual size, time of the year, and the sampling method directly affect these variables (Luz et al., 2001). Furthermore, volume of semen cannot be considered as the total volume of semen collected by the stripping method since it does not guarantee the complete stripping of the semen present in the gonads (Ferreira et al., 2001).

Several factors also determine sperm concentration in fish semen, including the species (Godinho, 2007), the age of the breeder (Bastardo et al., 2004), the use of hormone induction (Zaniboni Filho & Weingartner, 2007), the hormones used in hormonal induction (Streit Junior et al., 2004b), and the collection time (Borges et al., 2005).

The insemination doses between 7,000 and 7,000,000 spermatozoa oocytes^{-1} did not influence the rates of fertilization by only reducing them down to the dose of 70,000,000 spermatozoa oocytes^{-1} (Figure 1). These results are fundamental for the development of production technology of *P. mesopotamicus* because of the importance of this species in aquaculture in Latin America, allowing optimization of breeding through the rational use of gametes.

The trend of fertilization results obtained in this experiment (Figure 1) was different from that found for "dourado", *Salminus brasiliensis* by Sanches et al. (2009), even though the ideal dose found was 30,722 spermatozoa oocytes^{-1}. The appropriate spermatozoa:oocyte ratios vary among species, por example, those found by Bombardelli et al. (2006) and Shimoda et al. (2007) for " jundiá-cinza," *Rhamdia quelen* and "piabanha," *Brycon insignis*, respectively, were considerably different from those of *P. mesopotamicus*. Those authors observed that the fertilization rates reached a plateau above the ideal insemination dose and remained constant over 89,497 and 314,418 spermatozoa oocytes^{-1}, respectively. According to Rurangwa et al. (1998) and Sanches et al. (2009), fertilization ratios may be affected by low and high spermatozoa:oocyte ratios, and these intervals constrain the fertilization ratio (Tvedt et al., 2001). Besides the spermatozoa per oocyte ratio, other factors can influence the fertilization rates, such as gamete contact time and the fertilization protocol used (Rurangwa et al., 2004). Furthermore, some characteristics, such as oocyte size, spermatozoa swimming distance, sperm motility duration, and micropyle closing time are determinants in the application of insemination doses (Suquet et al., 1995; Chereguini et al., 1999), so as spermatic quality that can be masked with higher inseminations doses (Maria et al., 2004). These characteristics can sharply differ for teleost fish species (Godinho, 2007).

The fertilization rates of oocytes in experiment 2 for 105,481 and 210,963 spermatozoa oocytes^{-1} had a significantly different effect on the treatment (Figure 2). The artificial fertilization rate of oocytes increased up to 15.00 mL water mL^{-1} of oocytes. Above that, the fertilization rates remained constant for increasing amounts of water (Figure 2).

Based on the fertilization rates of experiment 2, the use of large volumes of water as an activating solution, from 15.00 to 60.00 mL water mL^{-1} of oocytes, afforded maximum performance in terms of oocyte fertilization rates. However, it is recommended to use low volumes of water in this range for greater ease in artificial fertilization processing and oocyte hydration. Nevertheless, the low fertilization rates observed in treatments with low water volumes were reached by a possible influence of the reduced medium dilution. This low dilution may have resulted in an inefficient reduction of the osmolarity of the fertilization medium (Alavi et al., 2007), which affected spermatozoa activation, or did not provide

the adequate medium for successful fertilization (Chereguini et al., 1999).

Sperm motility duration of experiment 3 (Figure 3) corroborate the fertilization rates (Figure 1). It was observed that the semen dilution directly affected sperm motility duration and those larger dilutions afforded longer sperm activation times. The fertilization ratios decreased after 7,000,000 spermatozoa oocyte^{-1}, which may be related to the semen dilution. Because the water volume was constant for the treatments, the increase in the spermatozoa per oocyte ratio must have resulted in an inefficient reduction or dilution of the fertilization medium. Sanches et al. (2009) reported similar results for dourado, *Salminus brasiliensis* semen.

In experiment 4 (Figure 4), the *in natura* cooling storage of semen was conducted up to 2h45min36s after stripping without any negative effect on spermatozoa, with survival ratios falling sharply thereafter. The preservation of semen at 15°C for over this time in these conditions is recommended only if the insemination dose is considered as a function of the reduction of the sperm survival ratio.

Souza (2007) observed that the appropriate use of spermatozoa and water volume ratios in artificial fertilization protocols increases the oocyte fertilization ratios and, consequently, the successful artificial reproduction of curimbatá, *Prochilodus lineatus*.

Conclusion

The high fertilization rates of pacu oocytes are observed in the applications of 0,27 at 270 µL semen and 15 at 60 mL of water mL^{-1} of oocytes. Semen of pacu can be stored in ice box (15 ºC) for 2h45min36s without damaging its quality.

Acknowledgements

The authors thank to Universidade Estadual do Oeste do Paraná (UNIOESTE), Centro de Pesquisa em Aquicultura Ambiental (CPAA) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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