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Short-term preservation of *Brycon amazonicus* sperm from a protected area of Brazil

Conservação de curto prazo do esperma de *Brycon amazonicus* de uma área protegida do Brasil

Preservación a corto plazo de espermatozoides de *Brycon amazonicus* de un área protegida de Brasil

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Abstract

The objective of this work was to characterize the in natura semen of the native *Brycon amazonicus* species from refrigeration tests with undiluted semen and diluted in four different solutions. Breeding males from fish farming were used. Milt was frozen and stored in dry shippers immediately after collection. The analyzed parameters were: volume of semen, concentration, pH, motility time and motility rate. The diluents tested were Ringer, Glucose, Sucrose and NaCl. The mean values obtained were: volume = 426.6 µL, concentration = 5.7 x 106spzmL-1, pH = 7.5, motility time = 56.6s and motility rate = 98%. Among the treatments tested, two groups with significant differences were observed: 1) with high motility rate, composed by treatments with undiluted semen and Ringer; 2) with low motility rate and very close to 30%, consisting of NaCl and Glucose treatments. The motility time of *B. amazonicus* pure semen was maintained up to 96 hours after dilution, indicating that this taxon has a good potential for fish farming.

Keywords: Ringer; Motility; Fish; Spermatozoa; *Brycon amazonicus.*

Resumo

Técnicas de conservação seminal têm sido desenvolvidas ultimamente, visando contribuir na operacionalização dos programas de reprodução de espécies cultivadas ou em vias de extinção. Neste trabalho objetivou-se caracterizar o sêmen da espécie nativa *Brycon amazonicus* a partir de refrigeração com o sêmen não-diluído e diluído em quatro soluções diferentes. Foram utilizados reprodutores procedentes de piscicultura de uma zona úmida de interesse internacional dentro do território maranhense (Brasil). Os parâmetros analisados foram: volume, concentração, pH, tempo de motilidade e taxa de motilidade. Os diluentes testados foram Ringer, Glicose, Sacarose e NaCl. Os valores médios obtidos foram: volume = 426,6 µL, concentração = 5,7 x 10^6spzmL^{-1}, pH = 7,5, tempo de motilidade = 56,6s e taxa de motilidade = 98%. Dentre os tratamentos testados, foram observados dois grupos com diferenças significativas: 1) com taxa de motilidade alta, composto pelos tratamentos com sêmen não diluído e com Ringer; 2) com baixa taxa de motilidade e muito próxima dos 30%, composto pelos tratamentos com NaCl e com Glicose. O tempo de motilidade do sêmen puro de *B. amazonicus* foi mantido até 96 horas após a diluição, indicando que o sêmen dos machos reprodutores pode ser mantido in natura e não diluído por um tempo adequado ao trabalho do piscicultor.

Palavras-chave: Ringer; Motilidade; Peixe; Espermatozóides; *Brycon amazonicus.*

Resumen

Actualmente se han desarrollado técnicas de conservación seminales, con el objetivo de contribuir a la operacionalización de programas para la reproducción de especies cultivadas o en peligro de extinción. Este trabajo tuvo como objetivo caracterizar el semen de la especie nativa *Brycon amazonicus* procedente de refrigeración con semen diluido y sin diluir en cuatro soluciones diferentes. Se utilizaron animales de cría de un humedal de interés internacional dentro del territorio de Maranhão (Brasil). Los parámetros analizados fueron: volumen, concentración, pH, tiempo de motilidad y tasa de motilidad. Los diluyentes probados fueron Ringer, Glucosa, Sacarosa y NaCl. Los valores medios obtenidos fueron: volumen = 426,6 µL, concentración = 5,7 x 106 spzmL-1, pH = 7,5, tiempo de motilidad = 56,6 s y tasa de motilidad = 98%. Entre los tratamientos probados, se observaron dos grupos con diferencias significativas: 1) con una alta tasa de motilidad, compuesto por tratamientos con semen sin diluir y con Ringer; 2) con un índice de motilidad bajo y muy cercano al 30%, compuesto por tratamientos con NaCl y Glucosa. El tiempo de motilidad del semen puro de *B. amazonicus* se mantuvo hasta 96 horas después de la dilución, indicando que el semen de los machos reproductores puede mantenerse in natura y sin diluir por un tiempo adecuado al trabajo del piscicultor.

Palabras clave: Ringer; Motilidad; Peces; Espermatozoides; *Brycon amazonicus.*

1. Introduction

The preservation of genetic material, whether frozen or simply cooled, is a technique of great ecological and economic interest, since, by preserving certain genetic variability of several species, it can favor biodiversity conservation and existing breeding programs, bringing greater profitability for producers (Pinheiro et al., 2016; Navarro et al., 2016; Marina et al., 2020).

The gametes conservation techniques are aimed at the conservation of genetic variability, the prevention of reproductive asynchrony between males and females (Carolsfeld, 2003; Ribeiro & Godinho, 2003; Suquetet al., 2000) and the maintenance of the population size without the expenses with many breeding (Carneiro, 2007) since the induction and spawning process does not damage the condition factor of some species of fish (Ventura et al., 2020).

Oliveira-Araujoet al. (2017) affirm that the short term preservation of fish semen is indicated to facilitate the management and increase the efficiency of the artificial reproduction

in pisciculture stations. However, conserved semen banks must be implanted before a given species is at risk of extinction, as often under these conditions there will not be sufficient genetic variability for population recomposition (Maria & Carneiro, 2012).

The species commonly known as Matrinchã belongs to the genus Brycon, a group of 43 species with a wide distribution in South America and Central America and currently, there are already studies of semen cooling for some species of this genus Brycon (Oliveira et al., 2007). However, the species *B. amazonicus* is still poorly studied in terms of seminal conservation and semen refrigeration tests with or without diluents are unknown. In Maranhão, the species *B. amazonicus* has been grown on small farms in the Baixada Maranhense Environmental Protection Area, a region of international interest that is part of the Convention on Wetlands of International Importance, known as the Ramsar Convention (Ramsar, 2017). This Ramsar Convention establishes frameworks for national actions and for cooperation between countries with the objective of promoting the conservation and rational use of wetlands in the world, but in the Baixada Maranhense Environmental Protection Area, there is still little research toward conservation of native fish semen (Navarro, 2013). In this scenario, semen conservation techniques are important strategies, requiring knowledge about the use of diluting solutions and the definition of semen storage conditions of species of economic interest (Sanches & Cerqueira, 2010). On the other hand in all applications in fish sperm, cryopreservation thechniques needs to overcome a lack in standardization of methodologies and a correct assay of seminal quality (Cabrita et al., 2010). The objective of this work was to characterize the in natura semen of the native *Brycon amazonicus* species from refrigeration tests with undiluted semen and diluted in four different solutions.

2. Methodology

The breeders (n = 20) used came from a fish farm located in Arari - MA. This region is part of the Baixada Maranhense Environmental Protection Area, which is a humid zone that is part of the Ramsar Convention (Ramsar, 2017).

In this work, the Deductive Method was used (Pereira et al., 2018). Semen collection was performed by means of slight pressures in the abdominal region in the cranial-caudal direction. The parameters determined were: sperm motility rate (percentage of sperm motility), duration of cell movement activity (in seconds) and sperm concentration (number of sperm mL-1 semen) of fresh semen (according to Ribeiro & Godinho, 2003). Then, an sperm

viability evaluation was performed by sperm activation and observation under an optical microscope (CM 505, Solaris Scientific, China) of a given aliquot (2 ul) of the collected material (according to Melo & Godinho, 2006). Sperm activation was performed by addition of 10 μL of distilled water and timed until total stoppage of the spermatozoa (reference). When considered viable (motility <5%), the semen was stored in microcentrifuge tubes (IMEC) with volume up to 2mL, which were arranged in a cryobox type box and the same were packed in a styrofoam ice box at 4° C. The temperature was controlled with a digital thermometer (Incoterm, Hong Kong) equipped with a sensor.

The other seminal physical-chemical characteristics of *B. amazonicus* were: a) Volume (μL), verified using a micropipette of variable volume, which was used in the transfer to microcentrifuge tubes; B) pH of the semen, verified with the use of pH indicative tape (pH indicatorstrips - Merck KGaA, Germany); C) Sperm concentration (number of sperm per mL of semen), where semen samples (n = 20) were diluted in 1 mL of 1% formol saline solution in the proportion of 1:500 (semen: saline formaldehyde solution) and 10 μL aliquot was transferred to the Neubauer Hematimetric Chamber (0.100 mm depth, Precicol HBG, Germany) and focused under the microscope on the 40x objective, remaining at rest for a period of up to five minutes; The sperm count was performed in at least three replicates, reworking them when the difference between them was greater than 10%.

The number of spermatozoa was expressed in spermatozoa / mL and was defined according to the following formula (Tiba et al., 2018): CE = N x Fc, where CE = Sperm concentration; N = Number of cells counted in the Neubauer Chamber; Fc = Correction factor, which was calculated as: Fc = qx fd / d, where, q = 5, ratio between the total number of squares of the Neubauer Chamber and the number of squares of the counting (25/5) ; Fd = Dilution of the semen aliquot; d = 0.1 mm (depth of the Neubauer chamber).

Four diluents were prepared, which were elaborated one day before semen collection, as follows: a) Glucose (350 mOsm / kg): 6.3 g Glucose + 100 mL distilled water with pH adjusted to 7.9; b) Modified Ringer for fish (300 mOsm / kg): NaCl 6.5 g / L; KCl 3.0 g / L; NaHCO 3 0.2 g / L; 0.3 g / L CaCl2; PH 7.8 (Peleteiro et al., 1996); c) Sucrose (300 mOsm / kg): 10.26 g Sucrose + 100 mL distilled water with pH adjusted to 7.5; d) 1.2% NaCl salt solution (343 mOsm / kg): 1.2 g Sodium Chloride (NaCl) per 100 ml of the aqueous solution with distilled water and pH adjusted to 7.4. The osmolarity of the solutions was checked by means of a digital osmometer (Osmette A, Precision Systems).

The collected and nonactivated semen samples were used to form six pools composed of several volumes of fresh semen of 10 fish, totaling 530 μL of semen per pool. After these

procedures, the pools were maintained in cryobox microfuge tubes and the same in a styrofoam ice box at a temperature of approximately 4°C.

For the refrigeration sperm tests, five treatments (n=20) with six replicates were delineated: the first treatment (TI) with 330 µL of semen in natura in each replicate. The following diluents were added for the other treatments and their respective replicates: Glucose (TII), Sucrose (TIII), Ringer (TIV) and Saline solution NaCl 1.2% (TV). The semen / diluent ratio was 1: 8, consisting of 50 µL of semen to 400 µL of the diluent. Therefore, each tube containing the in natura treatments had a total volume of 330 µL, while the volume of tubes with diluted semen was 450 µL.

The microcentrifuge tubes containing the treatments were stored in cryobox and packed in a Styrofoam thermal box with ice under refrigeration at 4°C. The internal temperature of the thermal box was monitored by means of a digital thermometer equipped with a sensor, proceeding with the replacement of the ice to avoid oscillation in temperature.

The percentage and motility time evaluations of each experimental unit were performed immediately after the dilution in (0h), 6h, 18h, 30h, 40h, 52h, 70h and 96h after cooling at 4ºC. For this purpose, 5 µL of each tube was activated (with 10 µL of distilled water for the TI control treatments and 20 µL of distilled water for the others) under a microscope slide and observed at a 100 × increase in the mentioned time intervals (according to Melo & Godinho, 2006).

Statistical analysis of the data was performed using the free software BIOESTAT version 5.3 (available at www.mamiraua.org.br) at a significance level of 5%. Data were expressed as means ± standard deviation. The means were analyzed by Analysis of Variance (ANOVA), and when significant differences between the means were found the Tukey multiple variation test was applied.

3. Results and Discussion

The volume of semen collected was the parameter that presented the highest variation among the *Brycon amazonicus* specimens analyzed (Table 1). These results are in accordance with the pattern observed for other species, such as *Brycon orthotaenia* (Melo and Godinho, 2003) and Astyanax lacustris (Carneiro-Leite et al., 2020). Ribeiro & Godinho (2003) explains that semen volume varies between fish species and specimens according to age, method and time of samples. In this case, both species (genus *Brycon*) have ecological similarity.

Table 1. Descriptive statistics of the seminal physicochemical characteristics of Matrinchã, *Brycon amazonicus* (n=20).

Parameter	Minimun	Maximun	Mean	Standard deviation	Coefficient of variation (%)
Volume (µL)	220,0	800,0	426,6	155,5	36,5
Concentration (x 10^6spzmL^{-1})	3,0	7,0	5,7	1,6	28,1
pH	6,5	8,0	7,5	0,6	8,0
Motility time (s)	38,0	90,0	56,6	16,1	28,4
Motility rate (%)	90,0	100,0	98,0	4,2	4,2

Parameters measured from the undiluted semen of B. amazonicus at the time of samples. Source: Authors.

The mean number of sperm recorded in the present study was considered low (Table 1) when compared to the values already registered for other species. Cruz-Casallas et al. (2006), analyzing fresh semen of *B. amazonicus* in Colombia, found values of 7.6 ± 1.3 x 10^9 spermatozoa mL-1. Other species of the same order (Characiformes) also presented higher concentrations (Melo-Maciel et al., 2012), such as *Brycon orbignyanus* (7.1 ± 5.6 x 10^9 mL spermatozoa mL-1) and *Prochilodus lineatus* (18.6 ± 2.2 x 10^9 mL spermatozoa mL-1) (Nascimento et al., 2012). The low concentration recorded in *B. amazonicus* does not invalidate efforts for cryopreservation of the semen of the species, as there are studies that show that the concentration of spermatozoa necessary to successfully inseminate lots of oocytes with cryopreserved and fresh semen can be up to 1.6x10^5 mL-1 spermatozoa (Tiba et al., 2018). The average value of the pH parameter found in the fish semen used in this study (Table 1) is in agreement with the acceptable variation (6.5 to 8.5) proposed by Tabares et al. (2005) for semen of freshwater fish. The second largest variation among the parameters evaluated in the *B. amazonicus* semen is the motility time at the time of sample (Table 1). And with the lowest variation, the mean motility rate (Table 1) that is the percentage of motile spermatozoa. According to Melo & Godinho (2006), normally the spermatozoa of teleosts remain mobile for a short period of time after the activation, being less than one minute. It is worth mentioning that sperm motility time in freshwater fish may vary between species (Murgaset al., 2011) and among specimens according to intrinsic and extrinsic factors. It was also observed that no significant differences were observed in the mean of the motility rate (%) between the time of collection and the post-dilution moment of the spermatozoa of the

specimens analyzed in this study (Table 2). For the conservation of semen, it is recommended to use samples with motility above 90% (Cosson et al., 1999), attested by an optical microscope.

Table 2. Motility rate (% mean ± standard deviation) of Matrinchã semen, *Brycon amazonicus*, diluted in different solutions and stored at 4 ° C for up to 96 hours.

Intervalos de observação	Sêmen não diluído	Diluições		
		Ringer	NaCl	Glicose
0	94,2±0,8a	83,3±1,0a	35,0±2,5b	31,7±14,5b
6	93,3±1,0a	70,0±5,1b	29,2±2,7c	-
18	89,2±3,2a	55,8±4,1b	15,8±1,5c	-
30	88,3±3,5a	52,5±2,5b	-	-
40	69,2±3,7a	38,3±1,0b	-	-
52	35,8±2,0a	10,8±0,8b	-	-
70	20,8±1,5	-	-	-
96	17,5±2,1	-	-	-

Values with different letters on the same row differ significantly ($p < 0.05$). Source: authors,

For the purpose of seminal characterization, at the moment of collection, it is common to proceed with the activation of a certain aliquot of the semen to verify the viability of a conservation and for that reason activating solutions are used. In the present study, the activating solution was distilled water, which presented a satisfactory result, similar to that observed in the literature for *Prochilodus lineatus* (Murgaset et al., 2007), *Leporinus macrocephalus* (Ribeiro & Godinho, 2003) and *Prochilodus mesopotamicus* (Streit Jr et al., 2006).

Sperm motility inhibitors, whose physico-chemical compositions resemble that of semen plasma, are used in the dilution of semen in the refrigeration process, in order to increase the spermatozoid's useful life without significantly altering seminal quality (Peñarandaet al., 2010). And as diluents with good results for one species may be unsuitable for others, testing with various diluents is necessary to define the most satisfactory. The ratio (1: 8) of fresh semen to diluent solution, i.e., 50 µl of matrinchã semen for each 400 µl of diluent was considered adequate for carrying out the present work. These proportions are among those considered appropriate to the methodology, since, according to Peleteiro et al. (1996) and Suquet et al. (2000) they may range from 1: 1 to 1:20, although, for most species, dilution ratios of 1: 3 to 1: 6 are most commonly used.

The motile time under refrigeration of the matrinchã in natura semen analyzed in this study reached up to 96 hours (Table 3) after dilution. Among the diluted semen samples, the superiority of the TIV treatment (Ringer) under the effects of refrigeration was clearly observed, as it reached motility up to 52 hours after dilution (Table 3). On the other hand, the TV treatment (NaCl) showed only motility up to 18 hours and the TII treatment (containing Glucose) only at the time of dilution. Also in terms of performance in the post-dilution motility duration, it was observed that the TII (Glucose) treatment presented the highest mean value at time zero, but did not show motility in subsequent observations. These motile duration data are of great importance in practice, since they aim at programming in in vitro fertilization works for this species (Murgas et al., 2007).

Table 3. Motility time (s) (mean ± standard deviation) of Matrinchã semen, *Brycon amazonicus*, diluted in different solutions and stored at 4 ° C for up to 96 hours.

Intervalos de observação	Sêmen não diluído	Diluições		
		Ringer	NaCl	Glicose
0	134,4±13,9a	83±6,43b	131±6,21a	200,0±49,9c
6	161,4±21,3a	72,8±13,1b	112,8±15ab	-
18	88,4±8,7a	107,4±32,5a	60,2±5,8a	-
30	97,6±8,5a	64,4±4,9b	-	-
40	72,2±4,9a	55,8±1,9b	-	-
52	58,0±1,9a	42,4±1,2b	-	-
70	56,2±2,1	-	-	-
96	59,6±0,7	-	-	-

Values with different letters on the same line differ significantly ($p < 0.05$). Source: Authors.

Comparing the four treatments at the time of dilution performed with Matrinchã, two groups with significant differences were observed: one with a high average motility rate, composed of treatments with undiluted semen and Ringer's and another with a low motility rate and very close to 30%, where the treatments with NaCl and with Glucose. As defined by Salmito-Vanderley et al. (2012), in terms of practical application, samples of semen kept cold with motility of at least 30% could be used in procedures of induced spawning in the laboratory. Therefore, when considering the results of the treatments in the present study, it can be affirmed that the approximate maximum period (post-dilution) to be viable for semen use would be: (a) for undiluted semen, up to 52 hours; (B) Ringer's, up to 40 hours; (C) NaCl salt solution, until dilution; (D) Glucose, up to dilution. This result can be explained by the characteristics of the diluent in association with the intrinsic conditions of the semen itself.

Minimum conditions are required for a diluent to be suitable for a particular species of fish. Among these conditions Legendre & Billard (1980) emphasize: Isotonicity, so that there is no previous activation of sperm motility, since it can deplete the energy reserve necessary for fertilization; Stability, since its physico-chemical characteristics should not be altered during contact with the semen; High thermal conductivity, allowing the rapid transfer of temperature from the external medium to the spermatozoa; Sterility, that is, they should not link potentially harmful microorganisms to sperm cells; Provide nutrients as energy sources and increase the volume of semen. Waymanet al. (1997), when working with *Pogonias chromis* semen storage, found that the semen motility of undiluted semen was significantly lower than in pre-diluted samples at different concentrations of artificial saline or HBSS (Hanks' balanced saline solution). On the other hand, Harvey & Carolsfeld (1993) affirm that the best strategy is to store the semen fresh and undiluted.

For the species *B. amazonicus* and according to the methodology adopted in this work, the in natura semen preservation without dilution showed results more adequate motility under refrigeration. As regards the simple cooling of the semen with the objective of preservation, Salmito-Vanderley et al. (2012) defines it as a technique that consists in the maintenance of its viability for hours or days in cooling temperatures without reaching the freezing and, therefore, does not require the use of cryoprotectant substances. Another factor that should be considered is the collection of semen, since during collection there is the possibility of contamination by urine, feces, mucus or water, and according to Maria and Carneiro (2012), these contaminants can activate sperm motility making material unsuitable for use in storage (refrigeration or freezing).

In practical terms, the results obtained in the present research with the native species *B. amazonicus* indicate that this taxon has a high potential for fish farming, since the semen of the breeding males can be kept in natura and without dilution. In financial terms this represents more profit for the fish farmer, since it spares the expense of obtaining thinner substances and the maintenance of breeding matrices. In this sense, *B. amazonicus* represents an economically viable and environmentally adequate option to reduce the extractive pressure on fish stocks of protected areas in Brazil.

Future studies with new tests with other diluents are essential to find one that is more efficient than semen in natura (and Ringer) for *B. amazonicus* analyzed here, since the literature reports that the long-term success of cryopreservation is related to several factors , including the composition of the diluent and the intracellular and / or extracellular cryoprotectant used (Pinheiro et al., 2016). In addition, each animal group has a specificity, it

being necessary that the composition of the diluent is adjusted for the seminal characteristics of each species and each seminal technology used (Salmito-Vanderley et al., 2012).

4. Conclusions

The semen of the native species *Brycon amazonicus* analyzed in this work presented high motility rate only in treatments with undiluted semen and with Ringer. The motility time of *B. amazonicus* pure semen was maintained up to 96 hours after dilution, indicating that the semen of the breeding males can be kept in natura and undiluted for one appropriate time to the work of the pisciculturist.

The semen of the native species *B. amazonicus* has a high potential for fish farming. Future research with breeding male semen can be carried out by testing other diluents and cryoprotectant substances.

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