Different extenders solutions for tambaqui semen cooling

Raycon Roberto Freitas Garcia[1], Ana Carina Nogueira Vasconcelos[1], Jayme Aparecido Povh[2], Eneder Rosana Oberst[1], Lidiane Raquel Eloy[1] and Danilo Pedro Streit Junior[1]

[1]Universidade Federal do Rio Grande do Sul, Faculdade de Agronomia, Departamento de Zootecnia, Rua Bento Gonçalves, nº 7712, CEP 91540-000 Porto Alegre, RS, Brazil. E-mail: raycongarcia@live.com, anacarina.vasconcelos@hotmail.com, oberst@ufrgs.br, lidianeloy@hotmail.com, danilo.streit@ufrgs.br [2]Universidade Federal de Mato Grosso do Sul, Faculdade de Medicina Veterinária e Zootecnia, Avenida Senador Filinto Muller, nº 2443, Vila Ipiranga, CEP 79074-460 Campo Grande, MS, Brazil. E-mail: jayme.peixegen@gmail.com

Abstract – The objective of this work was to evaluate the efficiency of extenders solutions in tambaqui (*Colossoma macropomum*) semen cooling at 6°C. The treatments consisted of semen dilutions with three extenders – BTS, HBSS, and ACP-104 –, and a control with undiluted semen. Cooled semen was evaluated by 24, 48, 72, 96, and 120 hours. There were differences for the duration of motility and fertilization rates among the semen dilutions. Motility duration proved a highly variable evaluation after 72 hours. Dilution testing can be performed by the three products, and cooling storage for 72 hours is most effective with BTS extender.

Index terms: *Colossoma macropomum*, ACP, BTS, HBSS, motility, sperm.

Diferentes soluções diluidoras para o resfriamento do sêmen de tambaqui

Resumo – O objetivo deste trabalho foi avaliar a eficiência de soluções diluidoras no resfriamento do sêmen de tambaqui (*Colossoma macropomum*) a 6°C. Os tratamentos consistiram de diluições de amostras de sêmen com três soluções – BTS, HBSS e ACP-104, e de um controle com sêmen não diluído. O sêmen resfriado foi avaliado por 24, 48, 72, 96 e 120 horas. Houve diferenças quanto à duração da motilidade e taxa de fertilização entre as diluições do sêmen. A duração da motilidade mostrou-se altamente variável depois de 72 horas. Os testes de diluição podem ser realizados pelos três diluidores, e seu armazenamento por 72 horas é mais eficaz com o diluidor BTS.

Termos para indexação: *Colossoma macropomum*, ACP, BTS, HBSS, motilidade, espermatozoide.

Popularly known as tambaqui, *Colossoma macropomum* is a Brazilian fish species widely accepted in the consumer market, and it stands out currently as the most produced fish in the country (Pedroza Filho et al., 2016). However, some difficulties are encountered in the production of fingerlings, and a viable alternative to control reproduction is the storage of semen through cooling, preserving the most of their fertilizing capacity (Zaniboni-Filho & Nuñer, 2004).

The use of the extender BTS – Beltsville thawing solution (Minitüb GmbH, Tiefenbach, Germany) –, which was developed for pigs, has shown satisfactory results for fish semen (Murgas et al., 2004; Viveiros et al., 2014a), and the same success can be observed with HBSS (Hank's balanced salt solution) (Wayman et al., 1998). The extender solution powdered coconut water ACP (ACP Biotecnologia, Fortaleza, CE, Brazil), was developed for several freshwater species; this solution has recently been adapted for semen fish, received the name ACP-104 (Viveiros et al., 2010), and was tested in *Prochilodus lineatus* (Viveiros et al., 2014b).

The use of cooled semen has been addressed as a solution for the problems of reproductive asynchrony between males and females, as it can be used for some hours or even days (Zaniboni-Filho & Nuñer, 2004; Carneiro et al., 2006). Besides being cheap and practical, this technique application can ensure the productivity of fingerling producing stations, and it can utilize semen in several females which may come spawning at different times (Marques & Godinho, 2004; Zaniboni-Filho & Nuñer, 2004).

The objective of this work was to evaluate the efficiency of extenders solutions to maintain the quality of tambaqui semen cooling.

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The study was carried out in the Farm Buriti, in Nova Mutum, MT, and in the Aquaculture Laboratory of the Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.

For this experiment, 32 tambaqui males were used. Breeders were induced hormonally with 2.5 mg kg^{-1} of carp pituitary extract and, after 12 hours, 2 mL semen were collected from each animal, in disposable syringes, by means of a mild massage in the cranio-caudal direction. The motility of sperm cells was evaluated to ensure that there was no early activation from water or urine. After collecting the semen samples, the material was activated in distilled water at 1:50 ratio, then placed between a slide and a cover slip for observations under an optical microscope with a 40X objective. For this evaluation, there were assigned values between 0 and 100% for motility rate. According to the intensity of cellular movement, there were assigned values from 1 to 5 for the spermatic force. At the time of semen dilutions, a chronometer was started, and the time was count in seconds to stop the sperm flagellar beating.

The semen samples were diluted and fixed at 1:1000 saline buffered formaldehyde, and subsequently homogenized and analyzed. Slide preparations were performed by the staining method Rose Bengal (Streit Jr. et al., 2004), and sperm morphology was observed following the recommendations of Miliorini et al. (2011). Cells were placed on a blade, coupled with a dye drop, and a light smeared was performed. Slides were dried at room temperature, the morphological evaluation was performed by counting 300 cells per slide, and the average percentage of normal cells was assigned. For concentration, sperm per milliliter of collected semen of the sample was analyzed by using a Neubauer chamber .

After the seminal analysis, the sample of each animal was equally divided into four subsamples, and the treatments were applied separately on each one, at 1:4 (semen:extender), in 50 mL Falcon tubes. Four treatments were tested: semen diluted in Beltsville thawing solution (BTS); semen diluted in Hank's balanced salt solution (HBSS); semen diluted in powdered coconut water (ACP104); and a control of semen in natura without dilution.

Cooling temperature was set and maintained at 6±2°C, and monitored with an analogue thermometer. Upon dilution, the extender media average temperature was 20±2°C; and six hours after dilution, the cooling temperature was reached. For cooling, the treatments were placed in a cooler, in racks, which were in the interior of a container with water to avoid large variations of temperature. The seminal evaluations were performed over 120 hours (0, 24, 48, 72, 96, and 120 hours).

Six *C. macropomum* females were used to evaluate the efficiency of the diluted semen in the extenders, in comparison to in natura semen as for the oocyte fertilizations. After extrusion, 1 mL oocyte aliquot containing 980±30 cells on average was used for each animal per treatment, and fertilized with 50 μL of each animal semen per treatment. The fertilized oocytes were taken to the experimental incubators and, after eight hours, a sample of each incubator was collected, in order to measure the fertilization rate by counting the viable and nonviable eggs. A hundred of considered viable eggs were again incubated and, after 18 hours, the hatching rates were evaluated.

The experimental design was completely randomized with repeated measurements over time, which were considered in four treatments (BTS, HBSS, ACP, and control), six assessment times (time 1, the hour of collection; time 2, cold for 24 hours; time 3, cold for 48 hours; time 4, cold for 72 hours; time 5, cold for 96 hours; and time 6, cold for 120 hours), and 32 replicates represented by each animal. Not normal variables, even after transformations and were analyzed by Kruskal-Wallis test, at a maximum 5% probability. To perform these analyses, we used the SAS statistical software, version 9.4.

Average sperm concentration in the animals was $14.4\pm10.3x10^9$. Extender positive actions were observed after 24 hours of cooling, since the motility of the control group decreased by more than 50%, in comparison to the various treatments of diluted semen. Diluted semen viability was maintained after 120 hours, and BTS had higher values than ACP (Figure 1 A).

Sperm motility over 120 hours of cooling constantly declined, and can be explained by the reduction of cell activity caused by dilution and semen cooling (Zaniboni-Filho & Nuñer, 2004). Due to the high correlation of this variable with the sperm vigor (Galo et al., 2011), the fall of both occurs concurrently, and losses on the quality of sperm cells range according to native species (Marques & Godinho, 2004). In the present study, sperm motility treatments cooled with

BTS, HBSS, and ACP showed a better motility (50%) after 48 hours of exposure. However, only BTS and ACP extenders maintained motility higher than 30%, after 72 hours of cooling, and HBSS showed no difference.

As an extender of cooling technique, for semen cryopreservation of Neotropical species, BTS is associated with its ability to keep sperm with good motility capacity. Studies with *Brycon orbignyanus* showed 65% motility until the sixth day (Murgas et al., 2004). This work is a pioneer in testing HBSS for semen cooling of Neotropical fish, with no comparisons in the literature until present.

When compared to the control group, treatments with BTS, HBSS, and ACP showed differences only in the assessments carried out after 24 and 48 hours of cooling. Up to 120 hours under cooling effects, only BTS and ACP showed samples with apparent vigor level (Figure 1 B). The motility reduction rate may be related to gas exchange means between the extender and sperm cells during the days of conservation (Viveiros et al., 2014a).

In general, semen dilution used in the treatments enabled a greater duration of motility over 120 hours (Figure 1 C). Although the energy resources of fish sperm cells are limited, the chemical composition of the extender means may interfere with the motility over time (Alavi & Cosson, 2005). This fact can be observed in the present study, since at the time of dilution, sperm in control group showed a motility time of 157 s, while the term in the treatments of semen diluted with the extenders BTS, HBSS, and ACP was 323, 267, and 190 s respectively. Even after cooling for 72 hours, sperm in the treatments with BTS, HBSS, and

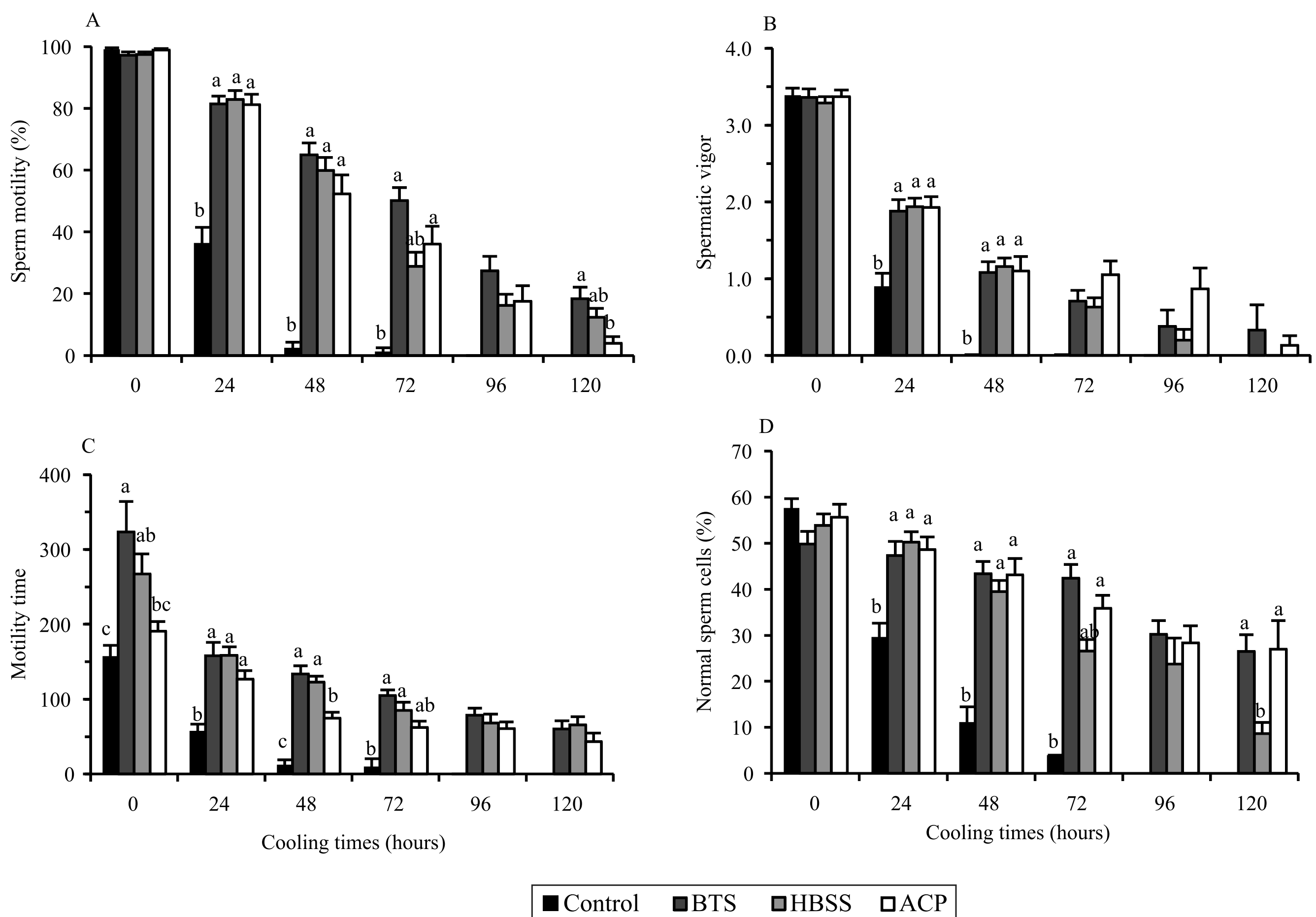

Figure 1. Percentage of sperm motility (A), spermatic vigor (B), motility time (C), and percentage of normal sperm cells (D), for *Colossoma macropomum*, in treatments over 120 hours of cooling at 6°C. Extenders: BTS (Beltsville thawing solution), HBSS (Hank's balanced salt solution), and ACP-104 (powdered coconut water-104).

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ACP moved on average 104, 85, and 62 s respectively, while in control group, motility was only 10 s. These values were similar to those found in seminal cooling of *P. lineatus* with BTS, in which motility found after 72 hours of cooling was 79.1 s (Franciscatto et al., 2002).

The increased number of morphological changes during cooling can be observed with a reduction in the percentage of normal cells (Figure 1 D). Before cooling, normal sperm was over 50% in all treatments and, after 72 hours, normal sperm cells in the BTS, HBSS, and ACP treatments were of 42, 26, and 35%, respectively. In control, only 4% of normal cells were recorded.

Although there is a pattern of morphological changes to mammals (Colégio Brasileiro de Reprodução Animal, 2013), the accepted rates for cellular abnormalities in fish have not yet been defined, making it difficult to compare seminal assessments (Miliorini et al., 2011). The morphological changes can occur due to the reduction of cellular functions, caused by temperature variations of the medium for sperm preservation (Alavi & Cosson, 2005), and these changes are associated with the reduced motility and fertilization capacity (Galo et al., 2011).

The fertilization rate was not different between treatments and the control group at the time of semen dilution (Figure 2). There is probably a higher motility than 50 s, after 120 hours of cooling, and the treatments BTS, HBSS, and ACP were sufficient to fertilize the oocyte. Even when the fertilization rate was low, in the present experiment, it showed no difference between treatments; therefore, this drop can be related to the oocyte quality. This parameter is influenced by preovulatory chronic stress and by the period of spawning and extrusion of oocytes (Donaldson et al., 2000), and may result in lower fertilization rates. Also, the hatching rates did not differ between treatments, and resulted in near 70% hatching of fertilized eggs.

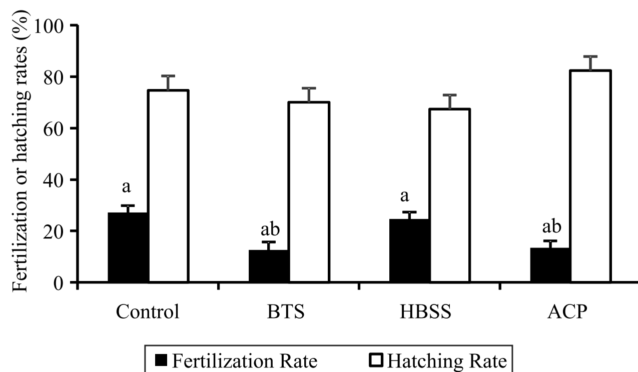

Figure 2. Fertilization and hatching rates of *Colossoma macropomum* using cold semen at 6°C diluted with BTS (Beltsville thawing solution), HBSS (Hank's balanced salt solution) and ACP (powdered coconut water-104).

The use of extenders for the semen cooling of *C. macropomum* may be the alternative for semen dilution and seminal storage in the short term. The tested thinners, mainly the BTS, kept the *C. macropomum* sperm in good conditions under cooling at 6°C for up to 72 hours.

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