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The role of dimethyl sulfoxide in the cryopreservation of Curimba (*Prochilodus lineatus)* semen

O papel do dimetilsulfóxido na criopreservação de sêmen de Curimba (*Prochilodus lineatus*)

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Abstract

Cryopreservation of Curimba semen (Prochilodus lineatus) is ecological and commercial importance. The objective of this study was to evaluate the effect of different concentrations (2, 5, 8 and 11%) of dimethyl sulfoxide (DMSO) diluted in Betsville Thawing Solution (BTS) on the quality of post-thaw semen Curimba. We analyzed the rate and period motility, sperm viability, membrane integrity and DNA, mitochondrial functionality, and fertilization and hatching rate. The plasma membrane and DNA integrity of a DMSO concentration of 11% obtained better results than the concentration of 5% (p <0.05). However, treatment of 5% DMSO resulted in a longer latency and a higher fertilization rate and hatching, in other sperm quality equal to that of fresh semen. The results of this study indicate that 5% DMSO is ideal for cryopreservation of semen Curimba.
Key words: Freezing, fertilization, hatching, mitochondria, *Prochilodus lineatus*

Resumo

A criopreservação de sêmen de Curimba (*Prochilodus lineatus*) é de importância ecológica e comercial. O objetivo deste estudo foi avaliar o efeito de diferentes concentrações (2, 5, 8 e 11%) de dimetilsulfóxido (DMSO) diluído em *Betsville Thawing Solution* (BTS) sobre a qualidade do sêmen de Curimba pós-descongelamento. Foram analisadas a taxa e período de motilidade, viabilidade espermática, integridade da membrana plasmática e do DNA, funcionalidade mitocondrial, e taxa de fertilização e eclosão. A membrana plasmática e a integridade do DNA a uma concentração de DMSO de 11% obtiveram melhores resultados que na concentração de 5% (p <0,05). Contudo, o tratamento de 5% de DMSO resultou em um aumento da latência e uma maior taxa de fertilização e de eclosão, com as demais qualidades do esperma sendo semelhante ao de sêmen fresco. Os resultados deste estudo indicam que 5% de DMSO é ideal para a criopreservação do sêmen Curimba.
Palavras-chave: Congelamento, eclosão, fertilização, mitocôndrias, *Prochilodus lineatus*

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Introduction

The Curimba (*Prochilodus lineatus*) is a migratory species native to South American rivers that plays an important ecological role, especially as food source for carnivorous species threatened with extinction, such as the Piracanjuba (*Brycon orbignyanus*) and Jaú (*Zungaro jahu*) (VIVEIROS et al., 2009). Spawning in Curimba can be dramatically affected by anthropogenic factors such as altered river flow and pollution (VIVEIROS et al., 2009). Because of its hardiness and high growth rate, this fish is one important pisciculture specie intended for human consumption (FROESE; PAULY, 2013). Gamete cryopreservation is one of the methods for the preservation of this species.

Cryopreservation is helpful in maintaining its germplasm and thus its genetic diversity. Moreover, freezing of semen improves the competitiveness of this species by enabling long-term storage of semen and enhancing breeding success, thus optimizing reproduction (e.g., increasing the rate of larval production) (VIVEIROS et al., 2009). However, cryopreservation procedures (i.e., freezing and thawing) are stressful to sperm cells because of excessive water loss and the formation of ice crystals and transitions in membrane phospholipids (PARKS; GRAHAM, 1992). To improve the efficiency of this process, a cryoprotectant solution is typically used to provide a nutritionally and osmotically ideal environment for semen.

Successful cryopreservation of semen has been reported in spawning species such as *B. orbignyanus* (MARIA et al., 2006), *P. lineatus* (MURGAS et al., 2007), and pirapitinga (*Brycon nattereri*) (OLIVEIRA et al., 2007) by using Beltsville Thawing Solution® (BTS), which was designed for use in cooling boar semen. Among the components of the BTS is likely that glucose is one of the most responsible for the reported success because this monosaccharide acts as an external cryoprotectant agent preventing sperm damage during freeze and/or thaw (PURDY, 2006).

The association of external cryoprotectants such as glucose and internal cryoprotectants is able to potentiate cellular protection. In this context, the internal cryoprotectant Dimethyl sulfoxide (DMSO) is a cryoprotectant widely used to freeze fish semen because it reduces the formation of intracellular ice crystals, thus preserving the fertilizing potential of sperm cells (SUQUET et al., 2000) and could be a promising alternative in association with the BTS (MILIORINI et al., 2011). However, the data on the optimal concentration of DMSO are discrepant. While some authors have reported that this cryoprotectant is toxic at concentrations exceeding 5% (BEDORE, 1999), others have tested its effects at 10% concentration (MARTÍNEZ et al., 2012). Furthermore, there are few studies on the effects of cryoprotectants on semen quality parameters other than motility (RANA; MCANDREW, 1989). Therefore, investigations on the optimal concentration of DMSO required to maintain cellular structures and sperm motility are required. The aim of the current study was to evaluate the post-thawing effects of different concentrations of DMSO diluted in BTS on the quality parameters of *P. lineatus* semen.

Materials and Methods

Sperm collection and evaluation

We used *P. lineatus* males from a commercial farm located in Pimenta Bueno, RO, Brazil (11°41′46.95″ S and 61°13′47.50″ W) to provide milt during their reproductive season from December 2010 to February 2011. All fish were kept in fish ponds (800 m^2) and were fed (an amount equivalent to 3% of the total live weight of the fish) thrice weekly with a commercial diet containing 40% crude protein and 2900 kcal/kg metabolizable energy. Males (n = 12, 1.25 ± 0.4 kg BW) with detectable running semen under soft abdominal pressure were administered a single intramuscular dose of carp pituitary extract (1 mg/kg BW). The males two-year-old were maintained in two tanks

with a 0.7m water column (maximum four fish per tank). After 6.5 h, it were removed from the tanks, dried them with a cloth towel, and then cleaned and dried the urogenital papilla with a paper towel. We collected sperm samples in a 15mL conical tube by applying abdominal massage, while avoiding the simultaneous extrusion of feces and urine to prevent milt contamination and sperm activation (STREIT JUNIOR et al., 2006).

To assess the rate of motility firstly was added 1µL of semen on a microscopy slide. Subsequently, the semen was activated by the addition of 100 µL of distilled water at 25 °C on semen for examination. Rate of motility (the percentage of motile sperm visually observed via microscopy) was observed, and the motility period was defined as the interval from activation to the moment the sperm stopped moving. Rate of motility and motility period were then evaluated using phase contrast microscopy (BX 41 Olympus®) at magnification 200 x. The same technician performed all investigations. All sperm samples had at least 80% sperm motility at 10 s after activation (STREIT JUNIOR et al., 2006).

Sperm cryopreservation

Immediately after collection semen from each male was diluted 1: 4 (v / v) in BTS (80% glucose, 12.7% sodium citrate, 2.7% EDTA, 2.7% NaHCO3, 1.5% KCl) (VIVEIROS et al., 2009) and homogenized. After dilution BTS samples of each male was divided into four treatments: 2, 5, 8 and 11% of final concentration of DMSO. After, the different treatments was loaded in 250µL straws and these remained in metal racks by two minutes at room temperature (24 °C) before being placed in dryshipper (Taylor-Wharton®, CP 300), allowing greater contact with the cryoprotectants so that they penetrate the sperm cells. After two minutes, the racks were placed in dryshipper for 12 hours. After 12 hours the straws were stored in liquid nitrogen container at –196 °C at least 15 days until thawed.

Fertilization and post-thawing sperm evaluation

Twelve *P. lineatus* females were used (1.7 ± 0.5 kg), one per male, and maintained them under the same conditions, as described above for males. We induced the females to spawn by injecting them with 5 mg/kg carp pituitary extract diluted in 2 mL of saline (0.9% NaCl) in the dorsal lateral region (CECCARELLI et al., 2000). After 9 h, oocytes were collected in a beaker by applying abdominal massage. Oocytes from each female were divided into 6 aliquots (2 g each), and each aliquot was fertilized with the semen from one straw for each treatment totaling 4 treatments for male (1 aliquot of oocytes = 1 straw) and two for semen *in natura*.

For each male, we used four straws (one per treatment) thawed in a water bath at 45 °C for 5 sec. For each aliquot of oocytes was used 150 µL of each sperm sample (n = 4). Concurrently, two other aliquots were fertilized with the same number of sperm per oocyte, with fresh sperm previously collected from the other two males (semen *in natura*) to account for the quality of eggs (all repetitions with fertilization <70% were excluded). Of fresh semen for use in fertilization rate was on average 82.3% and 21.2 motility of sperm motility second time. For fertilization, eggs and sperm were kept in a beaker and were gently homogenized for 5 sec. The mean number of sperm per egg was 7×10^4 sperm per oocyte. We activated the sperm by adding 2 mL of distilled water at 29°C and was homogenized the content again for 5 sec and allowed it to stand for 2 min. Subsequently, we added 20 mL of distilled water at 29°C to each aliquot to hydrate the fertilized eggs. Thereafter, the fertilized eggs were incubated under a gentle, ascending, and constant water flow (150 mL/min) in 2-L conical incubators. The developmental stages was monitored with a stereo microscope (Olympus SZX 7). Fertilization rate was assessed after 8 h (i.e., the number of eggs with cell division per total number of eggs; n = 200). Hatching rate was calculated as the number of hatched embryos from all evaluated embryos (VARELA et al., 2012).

We transported the sperm samples to the ReproPel laboratory for further analyses. Two straws from each treatment were thawed in a water bath at 45°C for 5 sec and re-suspended them in 400 µL of BTS (1:3, v/v) at 22°C in a 1.5mL conical tube to minimize the potential toxic effects of the cryoprotectant.

To evaluate sperm membrane integrity, DNA integrity, and mitochondrial functionality, we counted and analyzed 200 sperm with an epifluorescent microscope at 400× magnification (Olympus® BX 51, América Inc, São Paulo, SP, Brazil).

To check for sperm membrane integrity, was diluted 10 µL sperm sample in a 40 µL working solution of an isotonic saline solution containing 1.7 mM formaldehyde, 20 µM carboxyfluorescein diacetate (CFDA), and 7.3 µM propidium iodide (IP). Sperm that emitted a green fluorescence was classified as viable since their metabolic activity allowed the CFDA to accumulate in their cytoplasm. Sperm that emitted a red or red/green fluorescence were classified as nonviable (VARELA JUNIOR et al., 2012). The percentage of sperm membrane integrity was determined calculating the proportion of sperm emitting green fluorescence to the total number of sperm (green, red, or red/green fluorescence).

We evaluated sperm DNA integrity prior to putting a 45 µL sperm sample in 50 µL of TNE (0.01 M Tris-HCl; 0.15 M NaCl; 0.001 M EDTA; pH 7.2). After 30 sec was added 200 µL of Triton solution (1x); after another 30 sec was added 50 µL of acridine orange (2 mg/mL in deionized H_2O). And a,fter 5 min, we registered the observations without exceeding slide exposure for >1 min. Sperm emitting green fluorescence were considered to have intact DNA, whereas those emitting red or orange fluorescence were considered to have denatured DNA (VARELA JUNIOR et al., 2012). The rate of DNA integrity was determined by the proportion of sperm emitting green fluorescence to the total number of sperm (green, red, or orange fluorescence).

Mitochondrial functionality was analyzed after incubating 10 µL sperm sample with 40 µL rhodamine 123 solution (13 µM), at 20°C for 10 min. Sperm with positive rhodamine staining (i.e., green fluorescence) were considered to have functional mitochondria. On the other hand, nonfunctional mitochondria were characterized by negative rhodamine staining (i.e., sperm with no fluorescence) (VARELA JUNIOR et al., 2012). Rate of mitochondrial functionality was determined by estimating the proportion of sperm emitting green fluorescence to the total sperm count (green or no fluorescence).

Sperm viability was examined through microscopic observations of the differential penetration ability of the dyes (e.g., eosin/nigrosin) by adapting Morisson et al. (1997) protocol. 1 µL sperm sample was homogenized in 10 µL staining solution (containing 5 g of eosin Y and 10 g of nigrosin). After 1 min, we prepared a sperm smear and left it to dry. After counting 200 sperm cells using a microscope with an oil immersion objective (100×). Sperm were considered intact when they remained unstained, whereas those having either pink or red staining were not intact.

Statistical analyses

All variables were normally distributed according to the Shapiro-Wilk test. The effect of each concentration on the sperm cells was tested by ANOVA, with a comparison of means conducted by Tukey's test. All analyses were performed by using Statistix®.

Results and Discussion

No statistical differences ($p > 0.05$) were observed in sperm motility, sperm viability, and mitochondrial functionality among the different treatments with DMSO (Table 1).

A comparison of all treatment concentrations showed a decrease in membrane and DNA integrity when the DMSO concentration was 5%. We found significant differences in motility period in the 5% DMSO treatment and that in the 11% DMSO treatment (Table 1).

Table 1. Mean ± SEM post-thaw motility, sperm viability, mitochondrial functionality, membrane integrity, motility period and DNA integrity period for all for *P. lineatus* sperm (n=12).

Seminal Analyses	Concentrations of Dimethylsulfoxide (DMSO)			
	2 %	**5%**	**8%**	**11%**
Sperm Motility (%)	18.0 ± 16.4	22.0 ± 11.0	16.0 ± 5.4	12.0 ± 11.0
Sperm Viability (%)	43.2 ± 22.6	57.6 ± 4.7	51.8 ± 10.9	47.6 ± 10.2
Mitochondrial functionality (%)	37.6 ± 4.0	39.6 ± 13.1	58.0 ± 15.0	46.6 ± 29.4
Membrane integrity (%)	31.2 ± 20.0^{a}	11.4 ± 3.6^{b}	30.0 ± 3.9^{a}	40.6 ± 19.9^{a}
Motility period (s)	16.8 ± 15.3^{ab}	23.4 ± 11.8^{a}	17.4 ±6.7^{ab}	9.6 ± 8.8^{b}
DNA integrity (%)	52.4 ± 26.6^{a}	52.2 ± 8.4^{b}	71.0 ± 18.8^{a}	65.8 ± 8.8^{a}

$^{a-b}$ Within a column, means without a common superscript differ (P < 0.001).

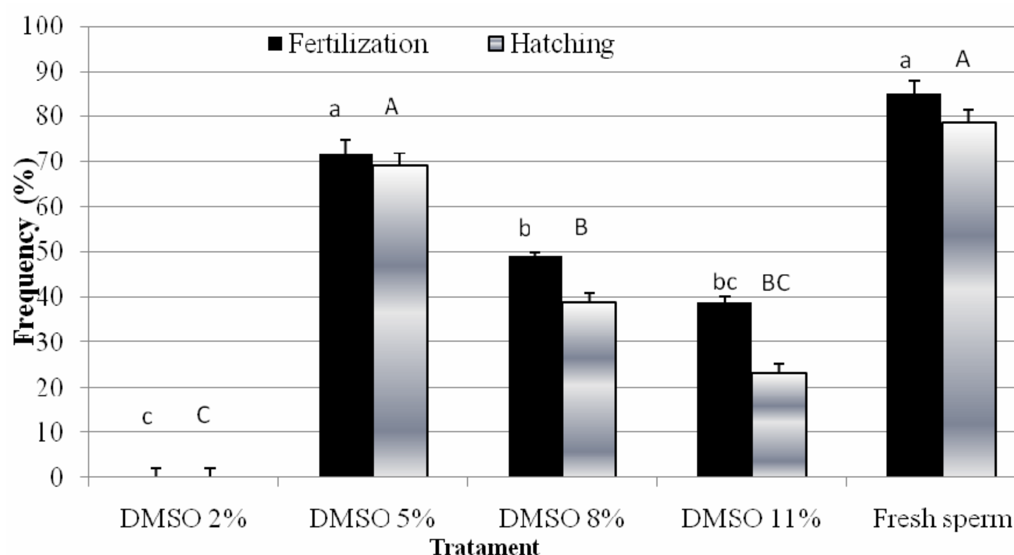
The best fertilization and hatching rates after the longest post-thawing motility period was achieved with 5% DMSO; these rates did not differ significantly ($p > 0.001$) from those observed with fresh semen (Figure 1). There were no significant differences ($p > 0.05$) in sperm quality parameters; however, the fertilization rate in the 2% DMSO treatment was zero. Therefore, the sperm lost their ability to fertilize after thawing (Figure 1).

To ensure greater reliability in determining sperm motility values, it is necessary to evaluate other sperm quality parameters (AMANN; HAMMERSTEDT, 2002; LOVE, 2011). According to Marques (2001), even if the sperm exhibit motility, structural damage can be caused by the freezing/thawing process, which may prevent fertilization. Thus, it is necessary to consider the additional variables analyzed in our study: membrane integrity, sperm viability, mitochondrial functionality, DNA integrity and motility period.

For DNA integrity only 5% DMSO concentration presented lower mean, however this result demonstrated to be sufficient to obtain about 70% of larvae hatching rate, higher than the other treatments and similar to fresh semen (Figure 1) showing that approximately 50% DNA integrity was able to ensure the fundamental function of DNA, which according (HAINES et al., 2001) is related to embryonic development.

We found that mitochondrial functionality was maintained at all tested concentrations of DMSO, thus ensuring essential energy production in sperm cells and thus, any treatment of DMSO was able to maintain the mitochondrial functionality that promotes the production of ATP for cellular movement (YAO et al., 2000). In agreement with this result it was found that all tested DMSO concentrations did not differ in sperm viability test that checks if the membranes are intact, because eosin is a supravitally dye that does not penetrate into cells with intact plasma membrane, staining stain of pink only the injured membranes (ARRUDA et al., 2010).

Figure 1. Fertilization and hatching rates for all concentrations with frozen and fresh *P. lineatus* sperm. DMSO: Dimethylsulfoxide; [a-c; A-C] (Mean ± SEM) Within a column, means without a common superscript differ (P < 0.001).



Together, the maintenance of mitochondrial functionality and sperm viability independent DMSO concentration were probably responsible for the motility rates do not differ among treatments because the motility that is the percentage of motile cells is dependent on maintaining the selectively permeable membrane (evaluated by sperm viability test) and mitochondrial functionality proporiona ATP production (evaluated by mitochondria functionality test) (ARRUDA et al., 2010; YAO et al,. 2000).

It is observed that as the plasma membrane integrity was different between the concentrations of DMSO, and the concentration of 5% had a low integrity and concentration of 11% showed the highest rate of integrity. This result is curious, given that one of the dyes used for the membrane integrity test, propidium iodide (PI) second Pintado et al. (2000) has positive correlation with dyes such as eosin / nigrosin (r = 0,69; p < 0,01) used in sperm viability test that showed no statistical difference between the different DMSO concentrations in this study with *P. lineatus*. Thus, further studies are needed to elucidate the causes for these different results between dyes with similar performances.

Considering the fertilization and hatching rates were the highest in the 5% DMSO treatment, although these rates were not significantly different from those observed with fresh semen samples. The average motility period for post-thawing using 11% DMSO was significantly shorter than that observed with 5% DMSO, which may explain the lower fertilization ability. When sperm motility is quickly achieved, the number of sperm that are available for fertilization can reduce. This is noteworthy because the rate of sperm motility using 11% DMSO was nearly half of that observed in 5% DMSO, although there was no significant difference among the treatments. Godinho et al. (2003) reported that different concentrations of DMSO in tilapia semen affected sperm motility period, revealing the influence of the cryoprotectant. Furthermore, fertilization and hatching rates were significantly higher at 5% DMSO concentration, thus reinforcing the correlation between motility period and fertilization ability.

Viveiros et al. (2010) compared the efficiency of coconut water- or glucose-based extenders in preserving semen quality parameters in thawed

Curimba semen, and although of the lower motility in glucose-based extenders than in coconut water, the fertilization rates in both treatments were similar indicating that glucose is crucial not only in maintaining sperm structure but also in preserving the fertilization potential.

The glucose present in the BTS probably served as an external cryoprotectant for the spermatozoal cytoplasmic membrane (STREIT JUNIOR et al., 2006), thus preserving cell structure. Miliorini et al. (2011) had also examined the cryopreservation of curimba semen in BTS and found that a DMSO concentration of 7.5% showed a higher potential for maintaining fertilization than 5%, 10%, and 12.5%. Moreover, according to them, DMSO was able to preserve the sperm tail, indicating that the interaction between glucose and DMSO exerts a protective effect on the sperm structure.

Only glucose would not be sufficient to confer protection sperm. Our data suggest that the association of glucose with a specific concentration of DMSO is required for high rates of fertilization and hatching to occur. Our results show that although 11% DMSO led to an increase in membrane integrity, 5% DMSO resulted in relatively higher fertilization and hatching rates, and 2% DMSO showed relatively lower fertilization and hatching rates. The concentration of glucose was the same at all DMSO concentrations; thus, the changes in rate of fertilization can be attributed to changes in the concentrations of DMSO.

The combination of internal and external cryoprotectants in the composition of the extender can justify the difference in results between the different studies on *P. lineatus*. Thus, there is a need for more in-depth research on post-cryopreservation spermatozoa. Miliorini (2006) recorded average fertilization rates of 22.3%, 17.1%, 15.7%, and 7.1% in 5%, 7.5%, 10%, and 12.5% DMSO concentrations, respectively. Moreover, the fertilization rates reported by Miliorini (2006) did not exceed 22.3% (DMSO, 5%), while in the current study, the average fertilization rate achieved was >70% (DMSO, 5%). Despite the fact that in both studies, the best fertilization rate was achieved using 5% DMSO, it should be noted that the cryoprotectant solution used in the current study contained 2.16-fold lower glucose levels than that in the study by Miliorini (2006).

DMSO is a sulfoxide with advantageous properties for use in cryopreservation, as demonstrated in by Carolsfeld et al. (2003). Nevertheless, some studies have shown that other internal cryoprotectants, such as methylglycol, are superior to DMSO (VIVEIROS et al., 2009). Viveiros et al. (2009) found that 5% methylglycol (a derivative of methanol) and glucose produced greater post-thawing motility rates than 5% DMSO combined with glucose (independent of the activator used). In the current study, the highest rate of motility was achieved numerically at 5% DMSO, but this finding was not statistically significant (Table 1). Thus, our results, in addition to the observations by Miliorini et al. (2011), indicate that DMSO is suitable for the cryopreservation of Curimba semen.

The ability of a cryoprotectant to penetrate a cell influences its internal cryoprotection capacity (MILIORINI, 2006). However, an excess amount of the cryoprotectant penetrating the cell can lead to sperm toxicity. These toxic effects on fish semen have been previously observed, especially when cryoprotectants are used in high concentrations (LEUNG, 1991). Thus, it can be assumed that 5% DMSO is less toxic than 11% DMSO. The latter caused mortality and damage to more sensitive cells, impairing their fertilization potential. Another reason for the improved fertilization and hatching rates at 5% DMSO is that this merger would have provided greater mean post-thawing sperm latency times. Therefore, higher fertilization rates are probably due to the prolonged handling capacity of sperm cells, with a greater likelihood of penetrating the still-open micropyle. According to Miliorini et al. (2011), several studies have assessed the effects of cryoprotectants such as DMSO across a range of

concentrations (typically from 5% to 15%) without accurately determining an optimal concentration that would enable greater protection of sperm cells. The current study, however, illustrates an optimal DMSO concentration for the cryopreservation of curimba semen. Furthermore, our study evaluated various aspects of gamete quality. In conclusion, 5% DMSO in combination with BTS is a sufficient and effective treatment for the cryopreservation of *P. lineatus* spermatozoa, with sperm quality similar to that of fresh semen.

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