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# Sperm cryopreservation of freshwater fish bocachico (*Prochilodus magdalenae*) in DMSO and glucose and its effects on fertilization and hatching efficiency

**J.G. Martínez, A.M. Tarazona-Morales, S.C. Pardo-Carrasco[1]**

Universidad Nacional de Colombia - Medellín - Facultad de Ciencias Agropecuarias, Departamento de Producción Animal, Grupo de Investigación en Biodiversidad y Genética Molecular BIOGEM, código postal 472, Colombia.

## Abstract

Internal cryoprotectants (dimethylsulfoxide - DMSO), as well as external ones (glucose) have been of great importance for sperm cryopreservation in freshwater fish. The aim of this study was to evaluate both the fertilization and hatching rates of eggs fertilized with bocachico (*Prochilodus magdalenae*) spermatozoa cryopreserved in different combinations of DMSO and glucose. Nine treatments were evaluated by a combination of three concentrations of DMSO: 5% (701 mM), 10% (1402 mM), 15% (v/v; 2103 mM) and three concentrations of glucose: 5.5% (305 mM), 6% (333 mM), 6.5% (w/v; 361 mM). Semen from males obtained by abdominal stripping 6 h after hormonal induction with carp pituitary extract was submitted to each treatment. The semen was frozen in 0.5 ml straws in a nitrogen vapor dry shipper for 30 min and then in liquid nitrogen (-196°C). Five days later they were placed in water with a temperature of 60°C for 8 sec and analyzed. A high total motility (71.0 ± 7.0%) was observed when DMSO concentration was 10% and glucose was 6%, and a high linearity displacement (62.8 ± 6.3%) was observed when DMSO concentration was 5% and glucose was 5.5%. In conclusion, we found that for the purposes of cryopreservation of bocachico spermatozoa, the combinations of 10% DMSO + 5.5 or 6% glucose and 5% DMSO + 5.5 or 6% glucose produced the best results in terms of fertilization and hatching rates. This becomes the first report to successfully demonstrate the fertilizing capacity and larvae obtaining capabilities of cryopreserved bocachico semen.

**Keywords:** computer assisted semen analysis, fertility, freshwater fish, hatchability, sperm cryopreservation.

## Introduction

Bocachico (*Prochilodus magdalenae*) is a South American Characiformes, native to the Magdalena River, of great economic importance for fisheries and aquaculture, for which artificial reproductive technologies and larviculture have been widely investigated and standardized (Atencio-García, 2001; Atencio-García *et al*., 2003). However, reliable semen cryopreservation protocols have not yet been developed for this particular species. Not only has this lack of technology hindered the bocachico from attaining significant scale production, but it has also greatly limited genetic exchange between producers as well as stunted the creation of gene banks. Thus, sperm cryopreservation for this species becomes fundamental regarding the development of new reproductive technologies, particularly when considering the great potential of this biotechnology as an instrument for biodiversity conservation (Wildt and Wemmer, 1999), most specifically when associated with endangered species (Mongkonpunya *et al*., 1995). Other advantages of the furthering of this study include broadening the processes of artificial fertilization in aquaculture (Watson and Holt, 2001) as well as increasing the availability of semen during naturally occurring periods of lesser sperm production in reproductively mature fish.

Dimethylsulfoxide (DMSO) is the most successful cryoprotectant used in seminal cryopreservation for the majority of South American Characiformes (Carolsfeld *et al*., 2003; Viveiros and Godinho, 2009); however, there are no reports that indicate similar results for bocachico semen. Considering that each protocol may potentially vary between species, the effectiveness of DMSO must be evaluated in each particular case and consequently adapted and tested individually among the species. Likewise, exacting immobilization of sperm during the cryopreservation process is absolutely necessary in order to ensure natural motility post-thawing (Yang *et al*., 2006). The activation of motility before or during this process causes technical failure for post-thawing cellular immobilization due to short duration of sperm motility once initiated (Billard and Cosson, 2005).

Glucose has commonly been used as an external cryoprotectant for various cell types (Purdy, 2006) but also as a non-ionic immobilizer in freshwater fish, making it the principle additive used for sperm cryopreservation (Horváth *et al*., 2003; Cruz-Casallas *et al*., 2004; Viveiros *et al*., 2009). However, the external concentration of this molecule required to produce cellular immobilization must be determined for bocachico semen during cryopreservation in order to avoid damage resulting from extreme osmotic changes (Cosson *et al*., 1999).

[1]Corresponding author: scpardoc@unal.edu.co
Phone: +57(4)430-9044; Fax: +57(4)430-9025
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Although motility has been considered a good estimator of spermatic quality in fish (Rurangwa *et al*., 2004), fertilization and hatching are becoming relied upon as more dependable indicators for the same purpose. These qualities are considered to be precise variables of the spermatic quality in a more integral manner (Bobe and Labbé, 2010), principally for the evaluation process of cryopreservation (Linhart *et al*., 2000; Kurokura and Oo, 2008).

This study evaluated the effect of the interaction between different concentrations of DMSO and glucose on the post-thawing quality of bocachico sperm, analyzing both fertilization and hatching ability.

## Materials and Methods

*Sperm collection for cryopreservation*

Semen from adult bocachico males was subjected to treatment. All individuals measured uniform length and weight (Table 1). All specimens were part of a group of brood fish adapted and kept in captivity in ponds at the Fish Research Center (CINPIC), University of Córdoba, Colombia. The CINPIC is located in the municipality of Monteria (Córdoba), at geographic coordinates 8°48' North latitude and 75°22' West longitude and an altitude measuring 15 m above sea level. The average annual temperature is 27.5ºC and the relative humidity hovers around 85%. Córdoba receives an average annual rainfall of 1,100 mm, which is distributed asymmetrically during two periods: the rainy season, April through November, during which approximately 85% of the total annual rainfall is received, and the dry season, December through March, during which the remaining 15% falls.

All specimens were selected based on demonstrated physical sexual maturity (Atencio-García, 2001) and were then moved to circular enclosures (6 $m^3$) where they remained for 24 h. The spermiation process began with an intramuscular injection of carp pituitary extract (CPE) equivalent to 4.5 mg/kg body weight (Atencio-García, 2001). After 6 h of hormonal induction, the broodfish were anaesthetized with 2-phenoxyethanol (300 ppm, Sigma Chemical Co., St. Louis, MO, USA; Cruz-Casallas *et al*., 2006) and semen was obtained by stripping in a cephalo-caudal direction, then collected in a 1.5 ml polyethylene vial in order to avoid traces of external contaminants such as water, urine or feces. Seminal evaluation was conducted immediately to determine whether sperm quality was adequate for cryopreservation. The initial variables measured were total and rapid motility (percentage of sperm with speed >100 µm/sec), the values of which exceeded 90 and 75%, respectively.

Table 1. General characteristics of the biological material used for cryopreservation and fertilization trials.

| | n | Body weight (g) | Total length (cm) | Linearity (%) | Total motility (%) | Gamete concentration | Gamete quantity |
|---|---|---|---|---|---|---|---|
| ♂** | 6 | 254 ± 7.5[a] | 28.23 ± 0.3[a] | 42.77 ± 4.6[a] | 99.83 ± 0.2[a] | 17,371.6 ± 434.0 (x$10^6$ SC/ml)[a] | 1.05 ± 0.1 ml[a] |
| ♂*** | 6 | 244 ± 19.4[a] | 28.10 ± 0.5[a] | 44.93 ± 2.4[a] | 98.60 ± 0.8[a] | 17,277.7 ± 415.0 (x$10^6$ SC/ml)[a] | 0.45 ± 0.1 ml[b] |
| ♀*** | 1 | 382 | 32.9 | - | - | 1,750 eggs/g | 82 g |

** Males used for sperm cryopreservation process.
***Specimens used for post-thaw fertilization trials: Control group males, and one female whose eggs were distributed among the treatments to be fertilized. SC: Sperm cells.
Values bearing common letters within the same column are not statistically different (P > 0.05).

*Estimation of sperm motility*

In both fresh and cryopreserved semen the following variables were analyzed: rapid motility (%), total motility (%) and linearity of sperm displacement (%; linearity is the ratio between straight line velocity and curvilinear velocity and it allows for the observation of the linearity degree and the importance of directionality for sperm displacement during activation, a good indicator of motion quality during fertilization). This was accomplished with the goals of evaluating the process of cryopreservation as well as determining the relationship between total motility and linearity with fertilization and hatching. To estimate motilities and velocities, 0.25 µl of fresh and cryopreserved semen was placed on a Makler counting chamber (Sefi, Medical Instruments Ltd., Israel). Motility was then activated with 75 µl of distilled water (hypo-osmotic shock activation, 0 mOsm/kg, tested prior to the experiment) to obtain a final dilution of 1:301 (sperm:water; procedure previously standardized with software to capture between 300 and 400 sperm per field). Next, the sample was analyzed employing a contrast optical microscope (Nikon, E50i, Japan)

adapted to a seminal analysis system with a computer-assisted Sperm Class Analyzer (SCA VET 01, Microptic SL, Spain). This analysis obtained the average sample rate, a product of two separate analyses by the software, both recorded within the first 4 sec after activation of sperm motility.

*Sperm concentration*

This variable was estimated in order to verify the concentration of semen that would be cryporeserved as well as the fresh (unfrozen and non mixture with cryoprotectant) semen (control group for fertilization). Additionally, this would allow for the determination of the sperm: egg ratio; in other words, the seminal volume that contains the approximate number of sperm (~100,000 cells) to be added for fertilization of each egg available (Cruz-Casallas and Velasco-Santamaría, 2006). In order to calculate this variable, a 0.5 µl semen sample was taken from each of the six selected males and diluted in distilled water at a ratio of 1:1,500 (semen:distilled water). Once the semen was diluted, 10 µl was taken and placed on the Makler counting chamber where sperm concentration was determined. This process was carried out with Sperm Class Analyzer software and performed three times per sample. This is the second time this software has been used worldwide to estimate sperm quality in fish.

*Semen cryopreservation*

Extenders consisted of: 1) glucose in sterile distilled water at concentrations of 5.5% (305 mM), 6% (333 mM) and 6.5% (361 mM); 2) 5% (701 mM), 10% (1402 mM) and 15% (2103 mM) dimethylsulfoxide (DMSO, Sigma Chemical Co., St. Louis, MO, USA), and 3) 12% (v / v) chicken egg yolk (Cruz-Casallas *et al*., 2006). This way, 9 interactive treatments (extenders) were generated: three concentrations of glucose x three concentrations of cryoprotectant.

The three levels of glucose (5.5, 6 and 6.5%) were chosen according to previous studies in our laboratory based upon activation of sperm motility in this species (Martinez *et al*., 2011). The levels of DMSO and egg yolk were chosen for their success during cryopreservation among some Characiformes (Carolsfeld *et al.,* 2003; Cruz-Casallas *et al.,* 2004).

*Packaging and freezing*

Semen from each male was divided into 9 equal subsamples. Each subsample and extender were mixed manually in 1.5 ml sterile vials at room temperature (28.0 ± 1.0°C) at a 1:4 dilution (dilution tested successfully for bocachico by our group in previous experiments using cryopreservation). Mixture was achieved by adding 100 µl of semen + 300 µl of extender-cryoprotectant, for a total of 400 µl. Immediately following, the mixture was packed in 0.5 ml straws (Minitüb, Abfül - und Labortechnik GmbH & Co. KG) which were sealed at both ends with polyvinyl alcohol and water. For the freezing process, the straws were placed upright in an aluminum cane designed to accommodate the straws in the freezing thermos in order to improve simultaneous handling of straws and homogenize their distribution inside the thermos. Once the straws were placed in the stand, they were completely introduced into a liquid nitrogen vapor dry shipper (MVE 4/2V, AL, USA) for 30 min (Medina-Robles *et al*., 2007). After 30 min, the straws were quickly transferred (time <2 sec) to a thermal storage unit (MVE 24/2V, AL, USA) and immediately plunged into liquid nitrogen (-196°C).

*Semen thawing process*

After 5 days the semen was thawed for analysis. The thawing was performed by immersing the straws into a serological bath (Memmert ® WNB7, GmbH Co. KG, Germany) at 60°C for 8 sec at a velocity applied with success for curimbatá (*Prochilodus lineatus*) by Viveiros *et al*. (2009) and characterized and described graphically for yamú (*Brycon amazonicus*) in 0.5 ml straws (Medina-Robles *et al*., 2007). The maximum time between thermal extraction and water immersion was 3 sec. After thawing, straws were opened on one end and the semen was poured into an equal number of 1.5 ml sterile vials. The samples were then subjected to motility analysis (total motility and linearity) as well as fertilization and hatching tests.

*Assessment of fertilization and hatching rates*

Oocytes from one adult female (Table 1) in her reproductive period (Atencio-García, 2001) were used for testing fertilization with cryopreserved and fresh sperm (control group; from different males). To obtain the eggs, the female was hormonally induced for oocyte final maturation and ovulation at a dosage of 5 mg of CPE/kg body weight in two doses (Atencio-García, 2001). Oocytes were obtained 5 h after administration of the last dose (water temperature 27.9 ± 0.5°C) through abdominal stripping and were weighed and distributed into 20 ml plastic polyethylene containers. Once thawed, both samples of frozen semen and fresh semen were distributed among groups with equal numbers of oocytes, each group composed of 2 g (ca. 1,750 eggs/g) with six replicates per treatment group. Fresh sperm for fertilization testing was collected from six males (n = 6) in the same manner as the semen used for cryopreservation. Within each group of oocytes, 100,000 sperm per egg were deposited, mixed and activated with 1 ml of distilled water. Each sample was shaken for 50 sec and the eggs hydrated for 1 min. Afterwards, they were transferred to 2 l upstream flow

incubators within a closed recirculation water system (activated carbon filter and ultraviolet light, water temperature 27.0 ± 0.7°C, pH: 8.0 ± 0.4).

Fertilization rate (%) was estimated 5 h after fertilization (HAF) (blastopore closing), establishing the proportion of viable fertilized eggs, spherical and translucent, within the number of total eggs observed. Estimation was performed three times (100 to 150 eggs per sample).

Hatching rate, determined 10 h after fertilization, established the proportion of viable larvae, motile and formed, within the total sample observed. Estimation was performed three times (100 to 120 units per sample).

*Statistical analyses*

To establish a post-thaw behavior and interdependence between variables of motility and fertilization capacity or hatching, we estimated the linear correlation coefficient using Pearson's test. All data for total motility, linearity, fertilization and hatching rate were tested using Brown and Forsythe's Test (homogeneity of variance) and were tested for normal distribution using the Kolmogorov-Smirnov's test. If data did not fit the normal distribution, an arcsine transformation was applied. Data were tested for significant differences by two-way ANOVA (GLM procedure), followed by Tukey's studentized range test. Differences among treatments were considered when $P < 0.05$. All data are expressed as mean ± standard deviation (SD). Statistical analyses were carried out using SAS version 9.0 (SAS Institute Inc., Cary, NC, USA).

## Results

The total motility of fresh semen used for cryopreservation exceeded, on average, 90% (Table 1). Likewise, rapid motility exceeded 75% in the same semen samples (78.7 ± 2.5%), which generally fulfilled these two selection criteria before subsequent treatment.

*Total motility and linearity*

The best total motility results were observed in treatments where the concentration of DMSO was 10 and 5% in all glucose concentrations, varying from 51.1 ± 6.8% to 71.0 ± 7.0%, without statistical differences between treatments ($P > 0.05$; Table 2).

On the other hand, it was detected that the total sperm motility post-thaw was substantially decreased in all cases where DMSO concentration reached 15% under any glucose concentration (Table 2).

The variable linearity presented a similar, but not equal, behavior (Table 2).

Table 2. Total motility and linearity of frozen-thawed bocachico semen (n = 6) based upon interactions and treatments between DMSO and glucose.

| DMSO concentration (%) | Glucose concentration (%) | Total motility (%) | Linearity (%) |
|---|---|---|---|
| 5 | 5.5 | 51.13 ± 6.76$^{abc}$ | 62.81 ± 6.26$^{a}$ |
| | 6 | 57.66 ± 1.60$^{ab}$ | 46.33 ± 3.87$^{ab}$ |
| | 6.5 | 57.13 ± 5.51$^{ab}$ | 48.31 ± 5.03$^{a}$ |
| 10 | 5.5 | 54.36 ± 4.55$^{ab}$ | 55.28 ± 3.79$^{a}$ |
| | 6 | 71.00 ± 7.02$^{a}$ | 58.75 ± 7.33$^{a}$ |
| | 6.5 | 51.86 ± 6.85$^{abc}$ | 62.59 ± 6.51$^{a}$ |
| 15 | 5.5 | 20.13 ± 2.03$^{c}$ | 19.90 ± 3.07$^{c}$ |
| | 6 | 35.76 ± 3.51$^{bc}$ | 28.42 ± 3.2$^{bc}$ |
| | 6.5 | 34.23 ± 4.15$^{bc}$ | 22.96 ± 0.93$^{c}$ |

Values bearing common letters within the same column are not statistically different ($P > 0.05$).

*Fertilization and hatching rate*

Fresh sperm had a fertilization rate of 69.3 ± 5.2%, differing from frozen-thawed semen ($P < 0.05$). A lower fertilization rate ($P < 0.05$) was recorded for sperm from treatments with 15% DMSO concentration, with values of 0.99 ± 0.13%, 3.63 ± 0.57% and 0.61 ± 0.21%, when interacted with glucose at concentrations of 5.5, 6 and 6.5%, respectively, with no difference between them ($P > 0.05$; Fig. 1).

There was no difference in fertilization rate when frozen-thawed semen was subjected to treatments where glucose concentrations (5.5 and 6%) interacted with 5% DMSO (27.6 ± 2.4% and 23.4 ± 1.8%) or 10% DMSO (25.7 ± 0.3% and 26.8 ± 3.1%; $P > 0.05$), making these the treatments with the highest fertilization rates (Fig. 1).

Whenever glucose concentration reached a maximum of 6.5%, within each group of treatments with 5 or 10% DMSO concentrations, the fertilization rate was lower (13.0 ± 1.2% and 7.2 ± 1.2%) compared with that of glucose at concentrations of 5.5 and 6% ($P < 0.05$; Fig. 1).

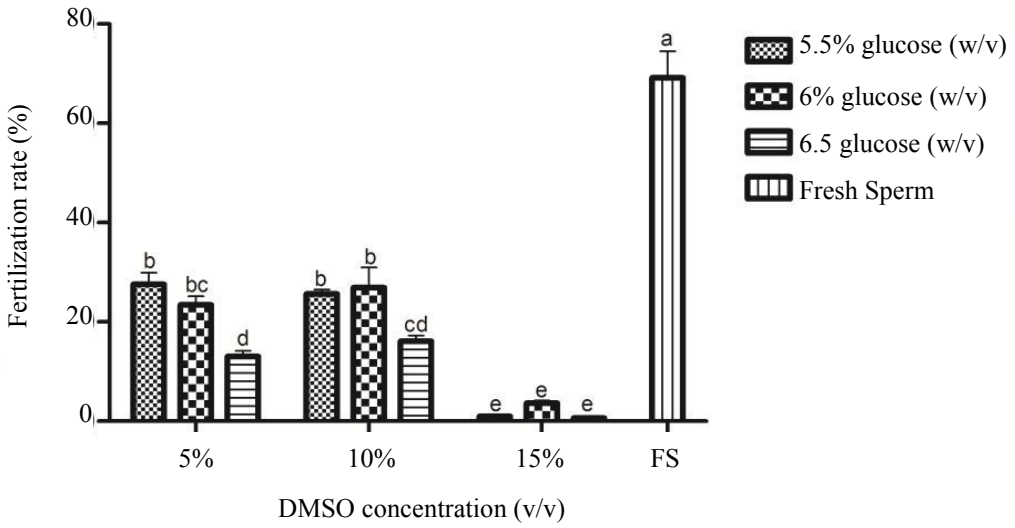


Figure 1. Effect of the interaction between DMSO (5, 10, 15% v/v) and glucose (5.5, 6, 10% w/v) concentrations during bocachico (*Prochilodus magdalenae*) sperm cryopreservation on fertilizing ability after thawing. Fresh semen was not mixed with DMSO or glucose and non-frozen. Fertilization was done with eggs from a single female (about 2 g of eggs per treatment replicate, 1,750 eggs/g, n = 6). Sperm:egg ratio was 100,000:1. Bars bearing common letters are not statistically different (P > 0.05).

The hatching rate for fresh semen (45.8 ± 2.4%) was significantly higher than with cryopreserved semen from any treatment interaction between glucose and DMSO (P < 0.05; Fig. 2).

There was no difference in hatching rate between treatments where bocachico semen was cryopreserved with 5.5 to 6% glucose under both 5 and 10% DMSO concentrations (P > 0.05; Fig. 2). However, these hatching values decreased significantly when any concentration of glucose (5.5, 6 and 6.5%) interacted with DMSO at a concentration of 15% (0.3 ± 0.2%, 1.1 ± 0.9 and 0.6 ± 0.2; P < 0.05).

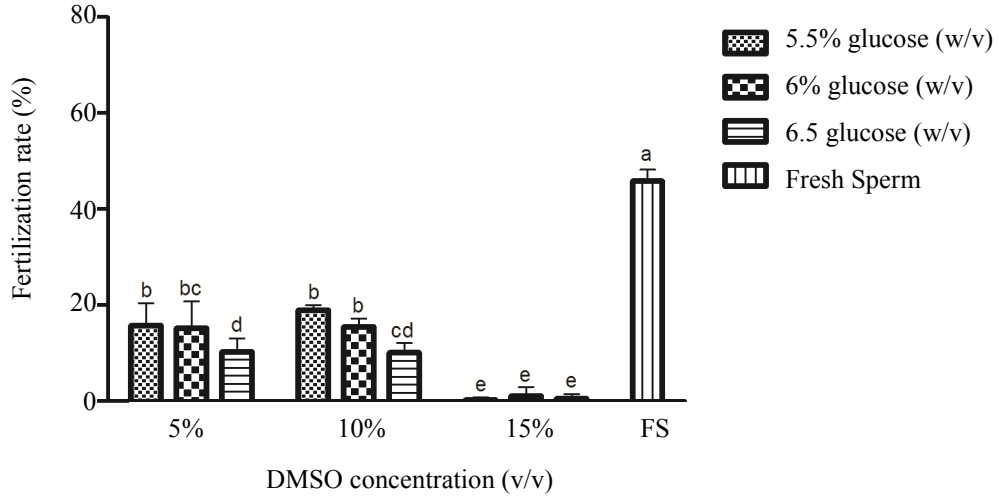


Figure 2. Effect of the interaction between DMSO (5, 10, 15% v/v) and glucose (5.5, 6, 10% w/v) concentrations during bocachico (*Prochilodus magdalenae*) sperm cryopreservation on hatching rate. Fresh semen was not mixed with DMSO or glucose and non-frozen, and was derived from different males than those that provided the frozen sperm. Fertilization was done with eggs from a single female (about 2 g of eggs per treatment replicate, 1,750 eggs/g, n = 6). Sperm:egg ratio was 100,000:1. Bars bearing common letters are not statistically different (P > 0.05).

Hatching rates generated by sperm concentrations at 5.5 (15.8 ± 2.3%) and 6.5% (10.3 ± 1.5%) glucose did not differ when interacting with 5% DMSO ($P > 0.05$). However, when DMSO concentration increased to 10%, glucose at 5.5% generated a hatching rate significantly greater (18.9 ± 1.0%) than at 6.5% (10.1 ± 1.6%; $P < 0.05$), but continued statistically unchanged for the treatment with 6% glucose ($P > 0.05$; Fig. 2).

*Correlation between motility, fertilization and hatching rate*

Fertilization and hatching rates using frozen-thawed sperm presented corollary relationships for both total motility and linearity; however, this relation was highly significant only for total motility (Table 3).

Table 3. Relationship between estimated mobility variables and fertilization and hatching rates for frozen-thawed bocachico semen.

| Correlated variables | r | P | N |
|---|---|---|---|
| TM with FR | 0.7688 | <0.0001 | 27 |
| TM with HR | 0.6956 | <0.0001 | 27 |
| LIN with FR | 0.6050 | <0.05 | 27 |
| LIN with HR | 0.5648 | <0.05 | 27 |

TM: total motility, LIN: linearity, FR: fertilization rate, HR: hatching rate, r: Pearson correlation coefficient.

## Discussion

In this experiment, fertilization rate always decreased when glucose concentration was 6.5%, but remained greater when glucose concentration was 5.5 or 6%. This may be attributable to the external osmotic pressure to which the cell was submitted under these three glucose concentrations, considering the impermeability of this molecule due to its size. Accordingly, it is possible that 6.5% glucose generated a high concentration gradient that in turn displaced a large amount of water outside the cell (potentially intolerable), generating excessive dehydration, which in turn prevents the recovery of water lost during thawing (Kopeika and Kopeika, 2008) and ultimately affects the ultrastructure of the cell (i.e. plasma membrane; Ogier De Baulny *et al*., 1997), an organelle closely related to fusion and fertilization events (Fritz and Baren, 2000; Yu *et al*., 2002).

DMSO has proved to be one of the most suitable cryoprotectants for both freshwater (Viveiros and Godinho, 2009) and saltwater (Suquet *et al*., 2000) species. However, too much of this substance during cryopreservation is always toxic to sperm (Leung, 1987; Gwo, 1993), generating injuries to the cell membrane and decreasing energetic molecules (He and Woods, 2004). This became evident when a significant decrease in sperm fertilization rate occurred when DMSO concentration was 15%, showing the negative effect of a high concentration of this cryoprotectant.

In this study, fertilization rate was positively correlated with sperm motility. This was noted when the highest fertility and motility values were obtained under 5 and 10% DMSO concentrations, while the poorest values were obtained under 15% DMSO. Distinct differing results occurred during the cryopreservation of yamú (*Brycon amazonicus*) sperm (Cruz-Casallas *et al*., 2004), where despite finding that 5% DMSO and 10% DMSO yielded the highest motility values (34.6 and 68.8% on average) and both were statistically different from that of 15% DMSO, they did not reflect similar fertilization rates.

Navarro *et al*. (2004) found that the best fertilization rates for cryopreserved sperm from cachama (*Piaractus brachypomus*), as with bocachico (both Characiformes), were found in treatments with 5 and 10% DMSO interacting with glucose at a concentration of 5.5% in both cases (25 and 36% on average, respectively), while the lowest fertilization rate with cryopreserved sperm was obtained from the treatment with 15% DMSO + 5.5% glucose, in which sperm motility was observed at only 2% on average, reaching a fertilization rate, in this case, near 0%.

This difference between results for fertilization rates between species, despite similar behavior for motility, could be due to other variables not measured in the study: DNA damage, membrane damage, enzymes or other variables not observable through motility; which are closely correlated with fertilizing ability (Lahnsteiner *et al*., 1996; Yu *et al*., 2002; Gwo *et al*., 2003).

In a similar fashion, this case is considered unique in that although the treatment with 5% DMSO + 5.5% glucose presented a statistically equal rate of total motility and linearity to that of the 15% DMSO + 6% glucose treatment (resulting in the lowest fertilization rates), the results reflect a statistically greater fertilization rate (among the most successful), even when compared to the treatments with 15% DMSO. This phenomenon also occurs in other species, such as Murray cod (*Machulochella pelli pelli*; Daly *et al*., 2008). Similarly, in previous studies by Glogowski *et al*. (2002), it was found that despite the similarities in *post-thawing* motility between treatments with cryopreserved Siberian sturgeon (*Acipenser baeri*) sperm, the hatching rates varied significantly. Similarly, Horváth *et al*. (2005) found that in some North-American Sturgeon species, 5% DMSO produced the

best *post-thawing* motility, but 5% methanol had the best hatching rates.

These conditions indicate that fertilization rate does not depend upon any variable, such as motility, but rather integrates multiple variables and components of sperm quality in order to provide the most successful fertilization rates (Bobe and Labbé, 2010).

The decrease in fertilization rate in this experiment was not attributable to the quality of eggs due to the fact that eggs from only one female were used in all treatments; however, it demonstrated that the variation of fertilization rate mainly depends on treatment, total motility and, in small part, upon the linearity of the sperm.

Despite the evident sperm motile capacity in some treatments, in addition to uniform egg quality, there was a low fertility for cryopreserved sperm treatments compared with the control group. This event may be due to an incorrect sperm: egg ratio for this species when employing frozen semen. Cruz-Casallas and Velasco-Santamaría (2006) achieved the highest fertilization rates in *B. amazonicus* (between 46 and 64%) with cryopreserved semen (10% DMSO + glucose) at a sperm:egg ratio of 75,000:1 and 150,000:1 ($P > 0.05$). In the case of *P. magdalenae*, the maximum fertility value reached under this concentration of DMSO was of 26.9 ± 3.1%. In curimbatá (*Prochilodus lineatus*), the sperm:egg ratio used to fertilize eggs with cryopreserved sperm (DMSO 10% + glucose) is 500,000:1, with an average fertilization rate of 65% (Viveiros *et al*., 2009). Lahnsteiner *et al*. (2004) found that despite obtaining percentages of acceptable motility and velocity after sperm cryopreservation of Starlet (*Acipenser ruthenus* L.), they do not achieve fertilization rates that reflect either the semen from treatments (10% DMSO + NaCl) or with fresh semen (6.9 and 33.9%, respectively). This was attributed to ignorance of the correct sperm: egg proportion, which they, in suitable amounts, can get to compensate for the low motility generated by cryopreservation.

We can conclude that variation and interaction of different DMSO and glucose concentrations significantly influences sperm's capacity to successfully participate in fertilization, which consequently affects hatching. The most efficient treatments found were as follows: 10% DMSO + 5.5% glucose, 6 or 5% DMSO + 5.5 or 6% glucose. Additionally, it remains necessary to maintain feasible variables such as total motility during cryopreservation, considering its significant impact on fertilization rate, which in turn influences hatching rate behavior.

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