



A protocol for cryopreservation of spermatozoa of the fish *Brycon orthotaenia*

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Abstract

The teleost matrinxã *Brycon orthotaenia*, native to the São Francisco River in Brazil, is in the vulnerable category of species in threat of extinction in the State of Minas Gerais, Brazil. We developed a protocol to freeze its spermatozoa to be used in artificial breeding programs aimed at preserving this species. Hand-stripped milt measured 2.0 ± 0.8 ml with a sperm concentration of 14.4×10^9 spermatozoa/ml, and a spermatocrit of $40.2 \pm 9.6\%$. We used post-thaw sperm motility to evaluate the effects of the freezing protocol on the spermatozoa. To measure sperm motility of fresh samples, we used a two-step dilution procedure. Fresh spermatozoa remained immotile when the milt was diluted in a 150- or 200-mM NaCl solution, but motility rates rose when the milt was diluted in NaCl solutions of lower osmolality. Peak motility occurred in a 25- and 50-mM NaCl solutions (88% motile spermatozoa and >50 s duration of motility). Concentrations of 5%, 10%, and 15% of DMSO, ethylene glycol, and propanediol did not significantly induce changes in motility rate of fresh spermatozoa. However, exposure to methanol and dimethyl acetamid in the same concentrations resulted in significantly lower motility rates. We performed cryopreservation assays in a dry shipper cryogenic container. Milt was frozen in extender solutions (1:5 dilution, v/v) containing 10% DMSO, 10% propanediol, or 10% ethylene glycol plus 5% glucose, 10% hen egg yolk, and 75% distilled water. We obtained the highest post-thaw motility rate and duration of sperm motility ($70.5 \pm 11.4\%$ and 62.2 ± 8.5 s respectively) when DMSO was the cryoprotectant and the thawed spermatozoa were activated with 119 mM NaHCO_3 . Thus, the cryopreservation protocol developed in this work successfully preserved spermatozoa of matrinxã.

Keywords: sperm cryopreservation, *Brycon orthotaenia*, teleost.

Introduction

The neotropical genus *Brycon* includes at least 41 species, some of which are important in commercial and sport fisheries in several neotropical river basins. The dependence of these fish on allochthonous food items such as fallen fruits, seeds, and insects (Lima, 2003) makes them highly vulnerable to anthropogenic interventions that affect riparian ecotone zones. As a consequence, six *Brycon* species are considered in threat

of extinction in southeast and southern Brazil (Brazil, 2004).

The São Francisco Basin in southeastern Brazil has experienced a drastic reduction of its native *Brycon orthotaenia* (formerly *B. lundii*) stocks. This condition has placed this fish (popularly known as matrinxã) in the vulnerable category of species in threat of extinction in the State of Minas Gerais (Lins *et al.*, 1997). Matrinxã is a migrant fish (Sato *et al.*, 1997; Sato and Godinho, 2003) that reaches > 30 cm in length (Lima, 2003) and is also important in sport and commercial fisheries (Sato and Godinho, 2003). A conservation program based on restocking using matrinxã juveniles from a governmental hatchery has been conducted in the area of Três Marias, Minas Gerais. Matrinxã, which was practically extinct above Três Marias Dam, has been tenuously reestablished after reintroductions began in 1988 (Sato and Godinho, 2003).

An important issue in fish restocking programs is the genetic quality and variability of the offspring when a low effective breeding number is used in the process (Waples and Do, 1994). In Brazil, the wide use of migratory species in such programs is critical because they are highly fecund (Sato *et al.*, 2003). Thus, a gene bank composed of frozen sperm collections would be an appropriate tool for ensuring genetic diversity in selective breeding programs. Frozen sperm banks would ensure the availability of a large and diverse spectrum of samples. It also would provide a hatchery with the practical advantages of reducing the dependence on synchronous maturation between sexes. However, the scope of sperm banks and their applications to both conservation programs and aquaculture require careful ethical consideration (Carolsfeld *et al.*, 2003).

The main objective in this study was to develop a protocol for freezing matrinxã spermatozoa in a practical, inexpensive manner and in field conditions. We focused on determining the efficiency of selected cryoprotectants and on the feasibility of using a dry shipper as a portable cryogenic device.

Materials and Methods

Fish and milt collection

The adult fish, captured in the São Francisco River, were kept in a 200-m² earthen pond belonging to a private hatchery. They were fed 6 d/wk with commercial fish food. Under these conditions, they

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reached gonadal maturation within the normal reproductive period (November - January; Sato *et al.*, 1997). Milt donors were selected from the pond based on: 1) the presence of anal fin spicules that become rugged to hand inspection during the reproductive season and 2) how easily they expressed milt with gentle coelomic wall massage. To collect the milt, the genital papilla of each male was dried with paper towels to avoid contamination with water, urine, and feces. Milt was collected in clean, graduated, plastic tubes, and its volume was measured. Then, body weight and total length were recorded (ranging from 500 - 950 g and 37 - 42.5 cm, respectively), and the fish were returned to the pond.

Experiment 1: Spermatocrit and sperm concentration

Spermatocrit values were obtained from 16 fish by using two fresh milt samples from each one and then centrifuging the sample at 14,000 xg for 25 min. The percentage of packed cell volume to plasma volume was obtained for each sample, and the mean was used to calculate the spermatocrit of each fish. Sperm concentration (number of spermatozoa/ml) was determined for 5 fish in a hematocrit chamber (Taitson and Godinho, 2003) using post-thawed cryopreserved sperm samples after correcting for dilutions made during the cryopreservation process. Six replicates were conducted for each fish, and the mean sperm concentration was used in the calculations.

Experiment 2: Activation of motility of fresh spermatozoa exposed to NaCl solutions

Sodium chloride solutions at the concentrations of 25, 50, 75, 100, 150, or 200 mM in distilled water were used to determine which osmolality would better activate sperm motility. The following parameters were evaluated for six fish: 1) sperm motility (percentage of motile spermatozoa immediately following activation; qualitatively evaluated); 2) latency (time between sperm activation and the observation of motile spermatozoa); and 3) duration of motility (time in seconds from activation and initiation of motility until the time the percentage of motile spermatozoa dropped to ~20%).

In this experiment, we used a modified two-step dilution protocol designed to induce motility in all spermatozoa present in the sample at once (Billard and Cosson, 1992). The first dilution step consisted of a 1:100 milt pre-dilution in a 200-mM NaCl solution that was previously established in this study as non-activator for matrinxã spermatozoa. The second step was performed using a light microscope set at 400x and used a dilution rate of 1:10 with activating solutions of different osmolality that resulted in a final dilution of 1:1000. Duration of motility was recorded from the moment that the second dilution was made to when ~20% of spermatozoa were still motile.

Experiment 3: Activation of motility of fresh spermatozoa exposed to cryoprotectant solutions

The next step was to determine the effect of various cryoprotectants on fresh spermatozoa. Therefore, we evaluated the motility rate, latency, and duration of sperm motility of 6 fish exposed to the following cryoprotectants: dimethyl sulphoxide (DMSO), propanediol, ethylene glycol, methanol, and dimethyl acetamid. For each, we used 5%, 10%, or 15% (v:v) solutions in distilled water. The protocols used for sperm dilution and determination of sperm motility were the same used in Experiment 2. In this experiment however, we replaced the activating solutions used in the second-step dilution with the cryoprotectants and distilled water solutions.

Experiment 4: Sperm cryopreservation and post-thaw sperm motility

The cryoprotectants and respective concentrations used in Experiment 3 which allowed higher sperm motility were selected for the experiment. Thus, aliquots of fresh milt from 10 fish were added to extenders containing 10% DMSO, 10% propanediol, or 10% ethylene glycol plus 5% glucose, 10% hen egg yolk, and 75% distilled water (Carolsfeld *et al.*, 2003); this corresponded to a final cryoprotectant concentration of 8.3%. The ratio of milt to extender (v/v) was 1:5.

Milt-extender mixtures were immediately aspirated by mouth into labeled, 0.5-ml straws (IMV, L'Aigle, France). The external surface of each straw was cleaned, and straws were placed into labeled goblets in bundles of four. The goblets were placed in either the upper or lower holding position of tin canes (Gencor, U.S.A.) and lowered quickly into a fully charged Taylor-Wharton, CP 65, dry shipper (Harsco Corp., Theodore, AL, U.S.A.) that was previously empty (Carolsfeld *et al.*, 2003). The dry shipper was used simultaneously for both freezing and storage. Later, the goblets containing the frozen straws were transferred from the dry shipper to a liquid-nitrogen storage container where they remained until the thawing step.

One straw from each of the 10 fish in the experimental treatments was thawed by rapidly swirling it in a water bath at 35°C for 7–10 s. The plugged end of the straw was then quickly cut with scissors, and the partially-thawed milt-extender mixture was allowed to run out onto a Petri dish. We tested 25-mM NaCl (which gave the best result in Experiment 2), 119-mM NaHCO₃ (Carolsfeld *et al.*, 2003), and distilled water as post-thaw sperm motility activating solutions. One microliter of the thawed milt-extender mixture was placed on a slide that was placed on the stage of a light microscope previously focused at 400x. The mixture was then immediately activated by adding 10µl of activator and gently stirred. Sperm motility rate,



duration of motility, and latency were recorded as described in Experiment 2.

Statistical analyses

We used the Kruskal-Wallis non-parametric test to evaluate median differences recorded in experiments related to 1) activation of motility of fresh spermatozoa exposed to NaCl solutions of different osmolality or exposed to cryoprotectant solutions of different compositions, and 2) post-thaw motility of spermatozoa submitted to freezing with different cryoprotectants. Differences were considered significant at $P < 0.05$.

Results

Experiment 1: Spermocrit and sperm concentration

The hand-stripped milt (volume = 2.0 ± 0.8 ml)

had a spermocrit of 40.2 ± 9.6 % packed cells and a sperm concentration of $14.4 \pm 2.9 \times 10^9$ spermatozoa/ml.

Experiment 2: Activation of motility of fresh spermatozoa exposed to NaCl solutions

Matrinxã spermatozoa were non-motile when in the fish seminal plasma. Table 1 presents data on matrinxã sperm motility rate, latency, and duration of motility. No motility was observed when the spermatozoa were mixed in 150- or 200-mM NaCl solutions. However, a significant increase in sperm motility was recorded when spermatozoa were mixed in solutions of lower osmolality. The 25- and 50-mM NaCl solutions induced the highest rates of sperm motility, which were comparable to that of distilled water. Latency only occurred when 75- and 100-mM NaCl solutions were used. Spermatozoa were motile for longer periods of time (51- 59 s) when activated with 25 and 75 mM NaCl solutions.

Table 1. Motility rate, duration of motility, and latency of fresh matrinxã (*Brycon orthotaenia*) spermatozoa activated with NaCl solutions of different concentrations (n = 6 fish; mean \pm standard deviation).

Activator	Motility rate (%)	Latency (s)	Duration of motility (s)
NaCl (mM)			
200	0	-	-
150	0	-	-
100	2.8 ± 4.0^a	9.2 ± 3.3^b	24.0 ± 24.2^a
75	75.8 ± 16.2^b	0.5 ± 1.2^a	59.8 ± 7.5^c
50	88.3 ± 10.8^{bc}	0^a	51.2 ± 24.5^c
25	89.2 ± 10.2^c	0^a	50.5 ± 8.0^{bc}
Distilled water	90.8 ± 10.2^c	0^a	39.2 ± 6.0^{ab}

^a Different letters in columns indicate means are different ($P < 0.05$); “-“ = not observed.

Experiment 3: Activation of motility of fresh spermatozoa exposed to cryoprotectant solutions

Table 2 presents the data of evaluation of the effects of different cryoprotectant solutions on sperm activation. Different concentrations of DMSO, propanediol, and ethylene glycol did not interfere with the motility of fresh matrinxã spermatozoa. This was also the case when 5 and 10% concentrations of methanol and dimethyl acetamid were used, but when used at 15%, the sperm motility rate declined significantly. Latency did not occur when dimethyl acetamid was used as the cryoprotectant. For the other cryoprotectants, latency varied from 2-24 s and was longer at higher cryoprotectant concentrations. In general, latency was lowest at the 5% concentration. Motility lasted for 41-60 s in all cryoprotectant solutions, except in dimethyl acetamid in which the

duration was slightly shorter. Cryoprotectant concentrations significantly influenced the duration of motility only in the case of 10 and 15% methanol.

Experiment 4: Sperm cryopreservation and post-thaw motility

Table 3 shows the motility rates and duration of motility of post-thaw sperm. Post-thaw sperm motility rates were significantly different among experiments that used different extender solutions and activators. Independent of the activator used, extenders containing propanediol or ethylene glycol produced lower post-thaw motility rates than those that contained DMSO. Duration of motility of post-thaw spermatozoa activated in 25-mM NaCl and distilled water was significantly lower than in the 119-mM NaHCO_3 solution.

Table 2. Motility rate, latency, and duration of motility of fresh matrinxã (*Brycon orthotaenia*) spermatozoa activated in cryoprotectant solutions of different concentrations (n = 6 fish; mean ± standard deviation).

Cryoprotectant conc. (%)	Cryoprotectant solution				
	DMSO	Propanediol	Ethylene glycol	Methanol	Dimethyl acetamid
Motility rate (%)					
5	88.3 ± 10.8 ^a	82.5 ± 17.5 ^a	89.2 ± 10.2 ^a	78.3 ± 4.1 ^a	85.0 ± 17.6 ^a
10	86.7 ± 14.4 ^a	80.0 ± 13.8 ^a	81.7 ± 14.0 ^a	65.0 ± 22.4 ^a	84.2 ± 13.2 ^a
15	83.3 ± 19.1 ^a	71.7 ± 22.3 ^a	76.7 ± 12.5 ^a	17.0 ± 31.0 ^b	65.0 ± 28.1 ^b
Latency (s)					
5	3.7 ± 4.1 ^a	1.8 ± 3 ^a	8.2 ± 4.8 ^a	0 ^a	0 ^a
10	8.3 ± 4.5 ^b	9.2 ± 3.5 ^b	16.7 ± 3.9 ^b	2.7 ± 3.0 ^a	0 ^a
15	13.5 ± 8.1 ^b	12.2 ± 5.6 ^b	24.2 ± 2.6 ^b	10.2 ± 9.3 ^b	0 ^a
Duration of motility (s)					
5	41.0 ± 5.2 ^a	41.8 ± 4.9 ^a	52.5 ± 7.4 ^a	41.7 ± 9.2 ^a	33.0 ± 5.1 ^a
10	48.3 ± 9.6 ^a	48.2 ± 4.9 ^a	52.5 ± 7.8 ^a	50.0 ± 8.5 ^b	31.0 ± 2.5 ^a
15	59.0 ± 7.4 ^b	49.8 ± 6.4 ^a	60.5 ± 5.3 ^b	50.5 ± 16.4 ^b	28.5 ± 3.9 ^a

^a Different letters in columns indicate means are different (P < 0.05).

Table 3. Post-thaw motility rate and duration of motility of frozen matrinxã (*Brycon orthotaenia*) spermatozoa in different extender solutions and activated in 25 mM NaCl, 119-mM NaHCO₃, or distilled water (n = 10 fish, mean ± standard deviation).

Activator	Extender solution		
	DMSO	Propanediol	Ethylene glycol
Post-thaw sperm motility rate (%)			
25-mM NaCl	26.0 ± 14.5 ^a	1.1 ± 1.4 ^a	1.2 ± 1.0 ^a
119-mM NaHCO ₃	70.5 ± 11.4 ^{bA}	23.5 ± 16.3 ^{bB}	39.5 ± 16.1 ^{bC}
Distilled water	24.0 ± 13.3 ^a	1.4 ± 1.0 ^a	0.1 ± 0.3 ^c
Post-thaw sperm duration of motility (s)			
25-mM NaCl	30.5 ± 6.2 ^a	20.1 ± 14.5 ^a	20.3 ± 11.6 ^a
119-mM NaHCO ₃	62.2 ± 8.5 ^{bA}	49.7 ± 8.8 ^{bB}	59.8 ± 18.7 ^{bAB}
Distilled water	22.7 ± 4.1 ^c	21.7 ± 3.9 ^a	1.8 ± 5.7 ^c

^a Different letters in columns indicate means are different (P < 0.05).

^A Different letters in rows indicate means are different (P < 0.05)

Discussion

The amount of milt produced by hatchery fish during the reproductive season varies according to the species, the individuals, and the method of collection, among other variables. Treatments given some hours prior to collection are commonly used to increase hand-stripped milt volume (e.g., hCG: Silveira *et al.*, 1985; LHRHa: Ciereszko and Dabrowski, 1993; Ribeiro and Godinho, 2003; steroidal pheromone: Zheng and Stacey, 1996; crude carp pituitary extract (CCPE): Sato *et al.*, 2003). Short-term treatment with hypothalamic or pituitary hormones induces a testicular hydration response that causes milt volume to increase and sperm concentration to decrease, facilitating the recovery of spermatozoa already present in the testis (Billard *et al.*, 1987; Viveiros *et al.*, 2002). Before milt collection, *Brycon* males are routinely treated with either CCPE

(Bedore, 1999; Ninhaus-Silveira *et al.*, 2006; Oliveira, 2006) or hCG (Andrade-Talmelli *et al.*, 2001). However, only Bedore (1999) presented data on the effects of treatment on milt volume. She showed a significant increase in hand-stripped milt volume following CCPE treatment in *Brycon orbignyanus*. The milt volume of the untreated matrinxã used in our work (2.0 ± 0.8 ml) was lower than that recorded for other *Brycon* species (4-15 ml: Bedore, 1999; Ninhaus-Silveira *et al.*, 2006).

Fish sperm concentration, which is an important parameter in hatchery reproduction management, is highly variable and depends on species, individuals, fish size, and season (Glogowski *et al.*, 1999). Although a Makler chamber, which was used in our study to record sperm concentration, is less frequently used than other chambers (e.g., Neubauer: Andrade-Talmelli *et al.*, 2001), it is reliable, simple to



use, and offers the possibility of estimating motility duration and spermatozoa counting in a single preparation (Taitson and Godinho, 2003). The matrinxã sperm concentration recorded in our study ($14.4 \pm 2.9 \times 10^9$ spermatozoa/ml) falls in the middle range of those of the Brazilian fresh-water fish. For some *Brycon* species, sperm concentration can reach up to 30×10^9 spermatozoa/ml of milt (*Brycon nattereri*: Oliveira, 2006).

Sperm motility in fresh water fish is initiated when spermatozoa come in contact with the aquatic environment and are exposed to an osmolality that is much lower than that of the seminal plasma (Billard and Cosson, 1992; Yang *et al.*, 2006). Thus, motility of matrinxã fresh sperm was best activated only in solutions of low osmolality (< 50 mM), as also seen in *Oreochromis niloticus* (Godinho *et al.*, 2003) and in some Characiformes (Marques and Godinho, 2004). In our experiments, variation in the duration of sperm motility may be ascribed to individual cells' capacity for motility or to a broad range of start times that in turn affect end time of motility, especially when latency was observed. Experiments using computer-assisted sperm analysis (CASA) would be useful to address this issue. The short motility duration (< 1 min) of matrinxã fresh spermatozoa was similar to that of most teleosts (Leung and Jamieson, 1991).

The large number of potential cryoprotectants and the different concentrations in which they can be used make the development of a suitable extender solution a complex task. In addition, depending on the concentration used, some cryoprotectants may be toxic (Leung and Jamieson, 1991). Thus the effects of cryoprotectants on sperm viability should be evaluated when they are intended to be used for a species not previously tested. Because our work was the first to deal with *Brycon orthotaenia* sperm cryopreservation, we tested cryoprotectants commonly used in fish sperm cryopreservation. Our data regarding sperm motility activation after exposure to DMSO, propanediol, or ethylene glycol (Exp. 3) indicated that these chemicals were good candidates for cryopreservation experiments. DMSO has been used successfully in neotropical species (Carolsfeld *et al.*, 2003), including *Brycon* species (*B. orbignyanus*: Bedore, 1999; Maria *et al.*, 2006; *B. cephalus*: Ninhaus-Silveira *et al.*, 2006; *B. insignis*: Shimoda, 2004; *B. nattereri*: Oliveira, 2006). Regarding propanediol and ethylene glycol, no published data are available on the use of these chemicals in *Brycon* sperm cryopreservation, but they have been used in experimental sperm cryopreservation from fish of temperate regions (Lahnsteiner *et al.*, 1996; Billard *et al.*, 2004).

Freezing and thawing rates are critical variables in fish sperm cryopreservation, and they can not be generalized because different extender, straw sizes, and temperatures are used in different studies (Viveiros *et al.*, 2000). The dry shipper used in our study was also successfully used for sperm cryopreservation from various neotropical fish

(Carolsfeld *et al.*, 2003). The cooling rate of $30^\circ\text{C}/\text{min}$ (Carolsfeld *et al.*, 2003) that the 0.5-ml straws of our study were exposed to in the dry shipper is considered adequate to freeze fish spermatozoa (Harvey and Carolsfeld, 1993). Distilled water (Linhart *et al.*, 1993), tap water (Otémé *et al.*, 1996), NaCl solutions (Bedore, 1999; Maria *et al.*, 2006), and in particular 119-mM NaHCO_3 (Stoss and Holtz, 1981; Lahnsteiner *et al.*, 1997; Yao *et al.*, 2000; Carolsfeld *et al.*, 2003; Ribeiro and Godinho, 2003) have been used to induce post-thaw sperm motility. Our results indicated that 119-mM NaHCO_3 was the post-thaw sperm activator of choice for matrinxã spermatozoa. Thus, the combination of a simple extender solution (composed of 10% DMSO, 5% glucose, 10% hen yolk egg, and 75% distilled water), a portable dry shipper (as the cryogenic device), and 119-mM NaHCO_3 (as the post-thaw sperm motility activator) resulted in a good post-thaw motility rate and duration of motility of matrinxã spermatozoa.

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