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Extender composition, osmolality, cryoprotectant and equilibration time effects on fresh sperm motility of two Characiformes fish: piracanjuba (*Brycon orbignyanus*) and streaked prochilod (*Prochilodus lineatus*)

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Abstract

Studies regarding the effects of extender composition, osmolality, cryoprotectant (CPA) and equilibration time on the induction/suppression of sperm motility are necessary to establish standard activating agents and immobilizing media for improving both artificial fertilization and preservation techniques. Thus, the aim of this study was to evaluate the effects of these factors on fresh sperm motility in piracanjuba (*Brycon orbignyanus*) and streaked prochilod (*Prochilodus lineatus*). Twenty four media, as a combination of six extenders (BTS™ and glucose solutions at 270, 315 and 360 mOsm/kg) with the CPAs DMSO, methanol, methyl glycol (MG) and a control without CPA, were prepared. Immediately after dilution, samples were observed under a light microscope to confirm whether different extender-CPA combinations would suppress the initiation of sperm motility. Motility was then triggered in 92 mOsm/kg NaCl and evaluated immediately after dilution (non-equilibrated samples) and after a 30-min equilibration time at 4°C for motility rate and motility quality score (0 = no movement; 5 = rapidly swimming sperm). In both species, motility was initiated in all samples diluted in BTS-270-control, Glu-270-MG, Glu-270-control and in all combinations containing DMSO. In *B. orbignyanus*, motility rate (77 to 92%) and motility quality score (3.3 to 4.7) of non-equilitrated samples was not significantly affected by any parameter. After 30 min, however, motility quality score decreased in most of the samples, mainly when diluted in BTS™ (3.3 to 4.2). In *P. lineatus*, motility rate was significantly higher in non-equilibrated samples (overall mean = 83%) compared to 30-min equilibrated samples (overall mean = 75%). Motility quality score of non-equilibrated samples was not affected by any parameter (3.3 to 4.2), but samples equilibrated in DMSO yielded the lowest score (3.0). Sperm motility (rate and score) was affected differently in *B. orbignyanus* compared to *P. lineatus*, and this finding should be considered when developing a methodology for sperm cryopreservation.

Keywords: reproduction, semen, sperm quality, teleost.

Introduction

Piracanjuba (*Brycon orbignyanus*) and streaked prochilod (*Prochilodus lineatus*) belong to the order Characiformes and are native to South America. These species have great potential for aquaculture and have been used in restocking programs through artificial propagation (Carolsfeld *et al*., 2003). During the spawning season (October to February), these species migrate to spawning sites. This migratory behavior is known as *piracema* and occurs when the environment is appropriate to stimulate the fish's reproductive biology (Godinho and Godinho, 1994). Changes in the course of rivers, urbanization, pollution, overfishing and hydroelectric dams are some of the reasons why the populations of some migratory fish are declining. The genus *Brycon,* family Characidae, is highly affected by environmental changes, and many species are on the red list of Brazilian threatened fauna, such as *B. orbignyanus*, pirapitinga-do-sul (*B. opalinus*), tiete-tetra (*B. insignis*) and pirapitinga (*B. nattereri*; Rosa and Lima, 2008). *B. orbignyanus* is native to the La Plata River basin and is found in Argentina, Brazil and Uruguay (Lopez *et al*., 1987; Lima, 2003). It is a very tasty, highly priced fish, and its aggressive behavior is appreciated for recreational fishing (Companhia Energética de Minas Gerais - CEMIG and Fundação Centro Tecnológico de Minas Gerais - CETEC, 2000). *P. lineatus* belongs to the family Prochilodontidae and has a large geographical distribution throughout South America, accounting for 50-90% of the total fish biomass in the Paraná River basin. This species is popularly known as curimba, curimbatá or grumatã. Larvae from *P. lineatus* are used as live feed for hatchery-raised endangered species, such as *B. orbignyanus* and jaú (*Zungaro jahu*). Also, this species has been used as a model in a number of studies addressing nutrition, health, genetic diversity and reproduction (Orfão *et al*., 2010).

Most fish spermatozoa are immotile in the seminal tract and hyposmotic media initiate sperm motility in freshwater fish (Morisawa and Suzuki, 1980). Besides osmolality, pH, temperature and ion concentration affect sperm motility (Alavi and Cosson, 2006). Studies regarding the effects of these factors on the induction and suppression of sperm motility are necessary to establish standard activating agents (media that trigger motility) and immobilizing media (media that suppress the initiation of sperm motility), also called extenders, for improving both artificial fertilization and preservation techniques (Alavi *et al*., 2009). In Characiformes, there are only a few studies that

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describe the effects of osmolality on fresh sperm motility. In those species, motility was suppressed in NaCl or glucose solutions at a minimum of 360 mOsm/kg in *Prochilodus lineatus* (Gonçalves and Viveiros; unpublished data), 325 mOsm/kg in *B. opalinus* (Orfão *et al*., 2011), ~276 mOsm/kg in *B. orthotaenia* (Melo and Godinho, 2006) and 410 mOsm/kg in *B. insignis* (Shimoda *et al*., 2007). In our previous study (Maria *et al*., 2006b), some media with an osmolality ranging from 240 to 429 mOsm/kg were tested in *B. orbignyanus* sperm. Motility was suppressed in media at 285 mOsm/kg or above. The media tested, however, possessed not only different osmolalities but also different compositions, thus, conclusions regarding osmolality only could not be drawn. All these studies suggest that sperm motility in Characiformes is triggered in a hyposmotic medium, and that the minimum osmolality to suppress the initiation of sperm motility is different among species.

The aim of the present study was to investigate the effects of extender composition, osmolality, cryoprotectant agent and equilibration time on fresh sperm motility of *B. orbignyanus* and *P. lineatus*.

Materials and Methods

Fish handling, sperm collection and initial evaluation

All fish were handled in compliance with published guidelines for animal experimentation (Van Zutphen *et al*., 2001). *B. orbignyanus* (n = 6) and *P. lineatus* (n = 6) males were selected from earthen ponds at the Hydrobiology and Fish Culture Station of Furnas, state of Minas Gerais, Brazil (20°43'07" S; 46°18'50" W) during the spawning season (November and December). All males with detectable running sperm under soft abdominal pressure were given a single intramuscular dose of carp pituitary extract (cPE; Argent Chemical Laboratory, Redmond, Washington, USA) at 3 mg/kg body weight. After 5 h (*B. orbignyanus*) or 8 h (*P. lineatus*) at ~25°C, the urogenital papilla was carefully dried and approximately 5 ml of sperm from each male was hand stripped directly into test tubes. Sperm collection was carried out at room temperature (~22°C). Soon after collection, tubes containing sperm were placed in a polystyrene box containing chemical ice (4 ± 2ºC). Contamination of sperm with water, urine or feces was carefully avoided. Immediately after collection, 5 µl of each sample was placed on a glass slide and observed under a light microscope (model L1000, Bioval, Jiangbei, China) at 400X magnification. As the sperm in the seminal plasma of both species should be immotile, any sperm motility observed was attributed to urine or water contamination and the sample was discarded. All samples were immotile and sperm motility was then triggered in 25 µl of 92 mOsm/kg NaCl (~0.29% NaCl) as an activating agent (Maria *et al*., 2006a). Because the sticking of sperm to a glass slide has not been observed in the Characiformes species, the addition of BSA or any other protein in the activating agent was unnecessary. Immediately after, motility rate was subjectively estimated and expressed as the percentage of motile sperm. All sperm samples possessed at least 80% motile sperm and were used in the subsequent analyses. Motility quality scores were assigned using an arbitrary grading system ranging from 0 (no movement) to 5 (rapidly swimming spermatozoa), as described in Viveiros *et al*. (2011). Sperm concentration (hemacytometer Neubauer chamber, Boeco, Hamburg, Germany) was also determined. Approximately 1.5 ml of each sperm sample was centrifuged (MiniStar, Shanghai, China) at 2000 g for 30 min at room temperature and the seminal plasma osmolality (Semi-Micro Osmometer K-7400, Knauer, Berlin, Germany) was measured.

Extender composition, osmolality, cryoprotectant and equilibration time on sperm motility

Six extenders, comprised of the combination of two compositions and three osmolalities, were prepared. The extender compositions were a simple glucose solution and a more complex solution named BTS™ (80% glucose, 12.7% sodium citrate, 2.7% EDTA, 2.7% $NaHCO_3$, 1.5% KCl, 0.5% gentamycin sulfate; Beltsville Thawing Solution Minitüb™, Tiefenbach/Landshut, Germany). Each solution was prepared at three different osmolalities (270, 315 and 360 mOsm/kg) and referred to as Glu-270, Glu-315, Glu-360, BTS-270, BTS-315 and BTS-360. Then, each extender was combined with the following cryoprotectant agents (CPAs): dimethyl sulfoxide (DMSO, $(CH_3)_2SO$); methanol (CH_3OH); methyl glycol (MG, $CH_3O(CH_2)_2OH$) and a control without CPA (Maria *et al*., 2006a, b). All CPAs were purchased from Vetec Química Fina Ltda™, Duque de Caxias, RJ, Brazil. In total, 24 media (6 extenders x 4 CPAs) were tested. Sperm from each male (n = 6 males of each species) was diluted in each medium to a final proportion (v/v) of 10% sperm, 10% CPA and 80% extender. Immediately after dilution, samples were observed under a light microscope to confirm whether all extender-CPA combinations would suppress the initiation of sperm motility. Soon after, and with no equilibration time, diluted sperm was activated and evaluated for motility (rate and quality score) as described for fresh sperm. Because we aimed to test the best extender-CPA combinations as freezing media for cryopreservation, sperm was equilibrated for 30 min at 4 ± 2ºC and evaluated again for motility. The 30-min equilibration time represents the lag period necessary for the permeation of CPA into the cells for protection against cryoinjuries and for sperm manipulation for freezing (dilution, loading, sealing straws, etc). This experiment was carried out with six replicates for each species (1 replicate = 1 male).

Statistical analysis

Values are expressed as mean ± standard deviation (SD). Statistical analyses were conducted with the SISVAR software program (Ferreira, 1999). Sperm motility and motility quality scores were tested for normal distribution using the univariate procedure. When data did not fit the normal distribution, an arcsin transformation was performed. Data were tested for significant differences using ANOVA, followed by the Tukey test, when applicable. The level of significance for all statistical tests was set at 0.05.

Results

Initial sperm evaluation

The following mean sperm values were found for *B. orbignyanus* males (n = 6): 92% motile sperm, motility quality score of 4.5, concentration of 7.1 x 10^9 sperm/ml and seminal plasma osmolality of 300 mOsm/kg; and for *P. lineatus* males (n = 6): 93% motile sperm, motility quality score of 4.3, concentration of 18.6 x 10^9 sperm/ml and seminal plasma osmolality of 306 mOsm/kg (Table 1).

Table 1. Body weight and some fresh sperm features (mean ± SD) of *Brycon orbignyanus* and *Prochilodus lineatus* after carp pituitary extract treatment.

Parameters	*B. orbignyanus*	*P. lineatus*
Number of males	6	6
Body weight (kg)	1.1 ± 0.8	1.4 ± 0.3
Concentration (sperm×10^9/ml)	7.1 ± 5.6	18.6 ± 2.2
Motility rate (% motile sperm)	92 ± 7	93 ± 5
Motility quality score (0-5)†	4.5 ± 0.5	4.3 ± 0.4
Seminal plasma osmolality (mOsm/kg)	300 ± 9	306 ± 10

†Motility quality score was assigned using an arbitrary grading system ranging from 0 (no movement) to 5 (rapidly swimming sperm).

Extender composition, osmolality, cryoprotectant and equilibration time on sperm motility

The initiation of sperm motility (number of samples in which motility was initiated/total number of samples) is presented in Table 2. In both species, motility was initiated in all samples diluted in DMSO (regardless of extender composition or osmolality), BTS-270-control, Glu-270-control and Glu-270-MG. In *B. orbignyanus*, motility was completely suppressed in all samples diluted in BTS-360-control, BTS-360-MG and in all Glu-315 and Glu-360 samples combined with methanol, MG or control. In *P. lineatus*, motility was completely suppressed in all samples diluted in BTS and in glucose at 315 and 360 mOsm/kg combined with methanol, MG or control.

Table 2. Initiation of motility (number of samples where sperm motility was initiated/total number of samples) of *Brycon orbignyanus* (A; n = 6 males) and *Prochilodus lineatus* (B; n = 6 males) sperm diluted in BTS™ and glucose at different osmolalities, combined with cryoprotectants (including a control without a cryoprotectant).

A) *B. orbignyanus*

Extender		Cryoprotectant (motility initiated/total samples)			
Composition	mOsm/kg	control	DMSO	methanol	methyl glycol
BTS™	270	6/6	6/6	1/6	3/6
	315	2/6	6/6	1/6	1/6
	360	0/6	6/6	1/6	0/6
Glucose	270	6/6	6/6	2/6	6/6
	315	0/6	6/6	0/6	0/6
	360	0/6	6/6	0/6	0/6

B) *P. lineatus*

Extender		Cryoprotectant (motility initiated/total samples)			
Composition	mOsm/kg	control	DMSO	methanol	methyl glycol
BTS™	270	6/6	6/6	1/6	2/6
	315	0/6	6/6	0/6	0/6
	360	0/6	6/6	0/6	0/6
Glucose	270	6/6	6/6	1/6	6/6
	315	0/6	6/6	0/6	0/6
	360	0/6	6/6	0/6	0/6

BTS™ (Minitüb): 80% glucose, 12.7% sodium citrate, 2.7% EDTA, 2.7% $NaHCO_3$, 1.5% KCl, 0.5% gentamycin sulfate.

Motility rate upon activation was affected differently in *B. orbignyanus* compared to *P. lineatus*. In *B. orbignyanus*, sperm motility was not significantly affected by any of the parameters tested and varied from 77 to 92% motile sperm (Table 3A). In *P. lineatus*, motility rate was significantly higher (overall mean = 83 ± 10%) in non-equilibrated samples compared to 30-min equilibrated samples (overall mean = 75 ± 11%; Table 3B). In non-equilibrated samples, an interaction between osmolality and CPA was observed.

Samples diluted in BTS-DMSO, Glu-DMSO and Glu-methanol produced a higher motility rate at 270 mOsm/kg compared to the same media at 360 mOsm/kg. On the other hand, samples diluted in BTS-methanol yielded a higher motility rate at 360 mOsm/kg (85%) compared to 270 mOsm/kg (75%). In 30-min equilibrated samples, an interaction between extender composition and CPA was observed. Sperm diluted in BTS-control yielded a higher motility rate (79%) compared to samples diluted in BTS-methanol (70%). Sperm equilibrated in Glu-control (80%), Glu-MG (82%) and in Glu-methanol (76%) yielded a higher motility rate than samples equilibrated in Glu-DMSO (66%).

Table 3. Motility rate (mean ± SD) of *Brycon orbignyanus* (A; n = 6 males) and *Prochilodus lineatus* (B; n = 6 males) sperm diluted in BTS™ and glucose at different osmolalities, combined with cryoprotectants (including a control without a cryoprotectant). Motility was evaluated after 0 (non-equilibrated) and 30 min of equilibration time at 4°C and 92 mOsm/kg NaCl was used as activating agent.

A) *B. orbignyanus*

		Cryoprotectant			
Extender	mOsm/kg	control	DMSO	methanol	methyl glycol
		Non-equilibrated samples (% motile sperm)			
BTS™	270	89 ± 2	89 ± 2	90 ± 0	88 ± 7
	315	90 ± 3	88 ± 5	92 ± 3	88 ± 7
	360	92 ± 4	88 ± 3	92 ± 3	88 ± 7
Glucose	270	89 ± 2	85 ± 4	88 ± 3	88 ± 11
	315	88 ± 5	83 ± 5	85 ± 12	87 ± 14
	360	88 ± 3	83 ± 5	87 ± 5	87 ± 14
		30-min equilibrated samples (% motile sperm)			
BTS™	270	84 ± 4	77 ± 5	80 ± 0	78 ± 7
	315	83 ± 4	77 ± 5	80 ± 0	83 ± 5
	360	84 ± 4	77 ± 5	80 ± 0	83 ± 5
Glucose	270	84 ± 2	83 ± 5	83 ± 10	85 ± 4
	315	83 ± 5	83 ± 5	78 ± 14	82 ± 9
	360	85 ± 3	83 ± 5	77 ± 21	80 ± 12

B) *P. lineatus*

		Cryoprotectant			
Extender	mOsm/kg	control	DMSO	methanol	methyl glycol
		Non-equilibrated samples (% motile sperm)			
BTS™	270	88 ± 3	92 ± 4^{A}	75 ± 5^{B}	83 ± 8
	315	91 ± 2	85 ± 6^{AB}	79 ± 11^{AB}	82 ± 7
	360	89 ± 5	77 ± 8^{B}	85 ± 8^{A}	80 ± 14
Glucose	270	88 ± 4	92 ± 4^{A}	85 ± 10^{A}	75 ± 10
	315	91 ± 2	87 ± 8^{AB}	73 ± 12^{B}	77 ± 15
	360	88 ± 4	82 ± 7^{B}	75 ± 8^{A}	78 ± 16
		30-min equilibrated samples (% motile sperm)			
BTS™	270	79 ± 5	75 ± 8	70 ± 11	70 ± 15
	315	78 ± 8	72 ± 10	70 ± 15	80 ± 6
	360	81 ± 5	75 ± 14	69 ± 12	78 ± 10
Mean ± SD		79 ± 6^{a}	74 ± 11^{ab}	70 ± 12^{b}	76 ± 11^{ab}
Glucose	270	78 ± 4	67 ± 12	77 ± 10	80 ± 9
	315	80 ± 6	63 ± 20	77 ± 8	85 ± 8
	360	82 ± 8	68 ± 12	75 ± 10	82 ± 4
Mean ± SD		80 ± 6^{a}	66 ± 14^{b}	76 ± 9^{a}	82 ± 7^{a}

BTS™ (Minitüb): 80% glucose, 12.7% sodium citrate, 2.7% EDTA, 2.7% $NaHCO_3$, 1.5% KCl, 0.5% gentamycin sulfate. $^{a\text{-}b,A\text{-}B}$Means followed by different superscripts (uppercases for columns and lowercase for rows) are significantly different (Tukey; $P < 0.05$).

Motility quality score was affected differently in *B. orbignyanus* compared to *P. lineatus*. In *B. orbignyanus*, the motility quality score of non-equilibrated sperm was high (above 4.0) in all samples, except in BTS-270-control, BTS-DMSO at all osmolalities and Glu-270-control. After 30 min of equilibration, motility quality score decreased in most of the samples, mainly when diluted in BTS™. The highest scores (above 4.0) were observed only in samples equilibrated in BTS-360-control, Glu-315-DMSO, Glu-360-DMSO and in all samples in Glu-methanol and Glu-methyl glycol (Table 4A). In *P. lineatus*, the motility quality score of non-equilibrated sperm was not affected by any parameters evaluated, and varied from 3.3 to 4.2. After 30 min, samples equilibrated in DMSO yielded the lowest score (3.0) compared to the control (3.7), methanol (3.8) and MG (3.9), regardless of extender composition or osmolality (Table 4B).

Table 4. Motility quality score (mean ± SD) of *Brycon orbignyanus* (A; n = 6 males) and *Prochilodus lineatus* (B; n = 6 males) sperm diluted in BTS™ and glucose at different osmolalities combined with cryoprotectants (including a control without a cryoprotectant). Motility was evaluated after 0 (non-equilibrated) and 30 min of equilibration time at 4°C and 92 mOsm/kg NaCl was used as activating agent.

A) *B. orbignyanus*

		Cryoprotectant			
Extender	mOsm/kg	control	DMSO	methanol	methyl glycol
Non-equilibrated samples (score 0-5†)					
BTS™	270	$3.5 \pm 0.8^{B,b}$	3.7 ± 0.5^{b}	$4.0 \pm 0.0^{B,ab}$	4.7 ± 0.5^{a}
	315	$4.2 \pm 0.4^{A,ab}$	3.7 ± 0.5^{b}	$4.3 \pm 0.5^{AB,ab}$	4.7 ± 0.5^{a}
	360	$4.2 \pm 0.8^{A,ab}$	3.7 ± 0.5^{b}	$4.7 \pm 0.5^{A,a}$	4.3 ± 1.0^{ab}
Glucose	270	$3.3 \pm 0.8^{B,b}$	4.7 ± 0.5^{a}	4.0 ± 0.0^{ab}	4.5 ± 0.8^{a}
	315	4.2 ± 0.4^{A}	4.7 ± 0.5	4.7 ± 0.5	4.5 ± 0.8
	360	4.3 ± 0.5^{A}	4.7 ± 0.5	4.7 ± 0.5	4.5 ± 0.8
30-min equilibrated samples (score 0-5†)					
BTS™	270	3.5 ± 0.8	3.3 ± 0.5	4.0 ± 0.0	4.0 ± 0.0
	315	3.8 ± 0.4	3.3 ± 0.5	3.7 ± 0.5	3.7 ± 0.5
	360	4.2 ± 0.4	3.7 ± 0.5	4.0 ± 0.0	3.7 ± 0.5
Mean ± SD		3.9 ± 0.7^{a}	3.4 ± 0.5^{a}	3.9 ± 0.3^{a}	3.8 ± 0.4^{a}
Glucose	270	3.5 ± 0.8	3.7 ± 0.5	4.3 ± 0.5	4.7 ± 0.5
	315	3.8 ± 0.4	4.7 ± 0.5	4.3 ± 1.0	4.3 ± 1.0
	360	4.0 ± 0.6	4.7 ± 0.5	4.7 ± 0.5	4.3 ± 1.0
Mean ± SD		3.8 ± 0.6^{b}	4.3 ± 0.7^{a}	4.4 ± 0.7^{a}	4.4 ± 0.9^{a}

B) *P. lineatus*

		Cryoprotectant			
Extender	mOsm/kg	control	DMSO	methanol	methyl glycol
Non-equilibrated samples (score 0-5†)					
BTS™	270	3.5 ± 0.5	4.2 ± 0.8	3.3 ± 0.5	3.3 ± 0.8
	315	3.8 ± 0.4	4.0 ± 0.6	3.5 ± 0.5	3.7 ± 0.5
	360	4.0 ± 0.6	3.7 ± 0.5	3.8 ± 0.4	3.8 ± 0.8
Glucose	270	3.7 ± 0.8	4.0 ± 0.0	3.8 ± 0.8	3.5 ± 0.5
	315	3.8 ± 0.4	4.0 ± 0.0	3.5 ± 0.5	3.7 ± 0.5
	360	4.0 ± 0.6	3.8 ± 0.4	3.8 ± 0.4	3.5 ± 0.5
30-min equilibrated samples (score 0-5†)					
BTS™	270	3.3 ± 0.5	2.8 ± 0.4	3.8 ± 0.4	3.8 ± 0.4
	315	3.7 ± 0.5	2.8 ± 0.4	3.7 ± 1.0	4.0 ± 0.0
	360	3.8 ± 0.8	3.7 ± 0.5	3.5 ± 0.8	3.8 ± 0.4
Glucose	270	3.7 ± 0.5	3.0 ± 0.6	3.8 ± 0.8	3.8 ± 0.4
	315	3.8 ± 0.4	2.7 ± 0.5	4.2 ± 0.4	4.2 ± 0.4
	360	3.8 ± 0.4	3.3 ± 0.5	4.0 ± 0.6	4.0 ± 0.6
Mean ± SD		3.7 ± 0.5^{a}	3.0 ± 0.6^{b}	3.8 ± 0.7^{a}	3.9 ± 0.4^{a}

BTS™ (Minitüb): 80% glucose, 12.7% sodium citrate, 2.7% EDTA, 2.7% $NaHCO_3$, 1.5% KCl, 0.5% gentamycin sulfate. †Motility quality scores were assigned using an arbitrary grading system ranging from 0 (no movement) to 5 (rapidly swimming spermatozoa). $^{a\text{-}b,A\text{-}B}$Means followed by different superscripts (uppercases for columns and lowercase for rows) are significantly different (Tukey; $P < 0.05$).

Discussion

In the present study, some fresh sperm features and the effects of extender (composition and osmolality), cryoprotectant and equilibration time on fresh sperm motility of *B. orbignyanus* and *P. lineatus* were evaluated. Fresh sperm quality for *B. orbignyanus* and for *P. lineatus* was all within the range previously reported for both species after carp pituitary treatment (Godinho and Viveiros, 2011). A better understanding of the characteristics of fresh sperm before manipulation is necessary to evaluate sperm quality in commercial hatcheries before artificial reproduction and in laboratories before experiments (Orfão *et al*., 2011).

Extender composition affected neither motility rate in *B. orbignyanus* nor motility rate or motility quality score in *P. lineatus*. Similarly, in zebrafish (*Danio rerio*; Wilson-Leedy *et al*., 2009), Northern pike (*Exos lucius L.*; Alavi *et al*., 2009), pirapitinga (*Brycon nattereri*; Oliveira *et al*., 2007) and *B. opalinus* (Orfão *et al*., 2011), no difference was observed in sperm motility after dilution in NaCl or a sugar solution.

In the present study, the osmolality of 270 mOsm/kg in samples diluted in BTS-control and in Glu-control did not prevent the initiation of sperm motility for *B. orbignyanus* or *P. lineatus*. Because the seminal plasma osmolality of both species is 300 mOsm/kg or higher, the initiation of sperm motility in a medium of 270 mOsm/kg could be expected. Environmental factors, such as ions and osmolality, stimulate the initiation of sperm motility by changing the properties of the plasma membrane (Morisawa *et al*., 1999; Krasznai *et al*., 2000). For fresh sperm, there are some studies in Characiformes showing that the initiation of sperm motility was completely suppressed in glucose or NaCl at 325 mOsm/kg or higher in *B. opalinus* (Orfão *et al*., 2011), in NaCl at ~276 mOsm/kg or higher in *B. orthotaenia* (Melo and Godinho, 2006), in NaCl at 410 mOsm/kg or higher in *B. insignis* (Shimoda *et al*., 2007) and in glucose solution at 410 mOsm/kg or higher in *Prochilodus magdalenae* (Martínez *et al*., 2011). It is noteworthy that in the present study, although samples diluted in media at 270 mOsm/kg showed some degree of motility, sperm motility in these samples could be triggered after 30 min of equilibration. Possibly, *B. orbignyanus* and *P. lineatus* sperm have the ability of reactivation, as has been reported for *C. carpio* sperm (Perchec *et al*., 1995), rainbow trout (*Oncorhynchus mykiss*) sperm (Christen *et al*., 1987) and *B. opalinus* sperm (Orfão *et al*., 2011). A transient lack of energy and its recovery is one possible explanation, but osmotic reequilibration could occur in sperm during this 30-min equilibration period, reestablishing an internal ionic concentration compatible with a correct motility activation rate. Although the osmolality did not affect sperm motility after 30 min of equilibration compared to non-equilibrated samples, just to be on the safe side, we recommend that the sperm of *B. orbignyanus* and *P. lineatus* should be stored in a medium at 315 mOsm/kg or higher.

In this study, sperm motility was initiated in all sperm samples diluted in DMSO, regardless of extender composition or osmolality. When a CPA is added to an extender, the global osmolality of the surrounding medium is increased. The initiation of sperm motility, however, was not suppressed by such an increase in global osmolality. It has been shown that the addition of DMSO activates striped bass (*Morone saxatilis*; He and Woods, 2003) and *B. opalinus* (Orfão *et al*., 2011) sperm kept quiescent in extenders. In *C. carpio* sperm, a swelling following the addition of DMSO at 1 to 20% (approximately 400 to 3200 mOsm/kg) has been observed, possibly caused by an influx of water (Perchec-Poupard *et al*., 1997). In the present study, DMSO was used at 10% of the total solution, which is within the range of 1 to 20% observed for carp sperm. It is possible that a similar water influx after the addition of DMSO had occurred and triggered sperm motility, despite an increase in global osmolality. Thus, we recommend the use of methyl glycol or methanol instead. However, if DMSO is to be used, then it should be added to the sperm just before freezing to prevent the initiation of sperm motility.

Equilibration time did not affected motility rate in *B. orbignyanus* sperm, but the motility quality score decreased in most of the samples after 30-min equilibration. In *P. lineatus*, motility rate was higher in non-equilibrated samples compared to 30-min equilibrated samples. The motility quality score of *P. lineatus* sperm, however, was not affected by equilibration time, except when DMSO was used as a CPA. Some studies suggest that equilibration time is not necessary (Aral *et al*., 2009), and that excessive contact of spermatozoa with the cryoprotectant before cryopreservation can lead to higher toxicity effects of these cryoprotectants. Equilibration times between 10 and 20 min are the most commonly used for fish sperm (Billard and Zhang, 2001). Since a decrease in sperm motility (rate and/or quality score) was observed after 30 min of exposure to a CPA in both species, we suggest that freezing should occur as soon as the straws are loaded.

In conclusion, the initiation of sperm motility is triggered in a hyposmotic medium (270 mOsm/kg) or when DMSO is added to the medium. Although motility was initiated in samples diluted in these media, motility could still be triggered after a 30-min equilibration time. *B. orbignyanus* sperm should be tested for cryopreservation diluted in glucose at 315 mOsm/kg or higher and combined with MG or

methanol, while *P. lineatus* sperm should be cryopreserved in BTS™ or glucose at 315 mOsm/kg or higher and MG as a CPA. In both species, freezing should occur as soon as the straws are loaded.

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