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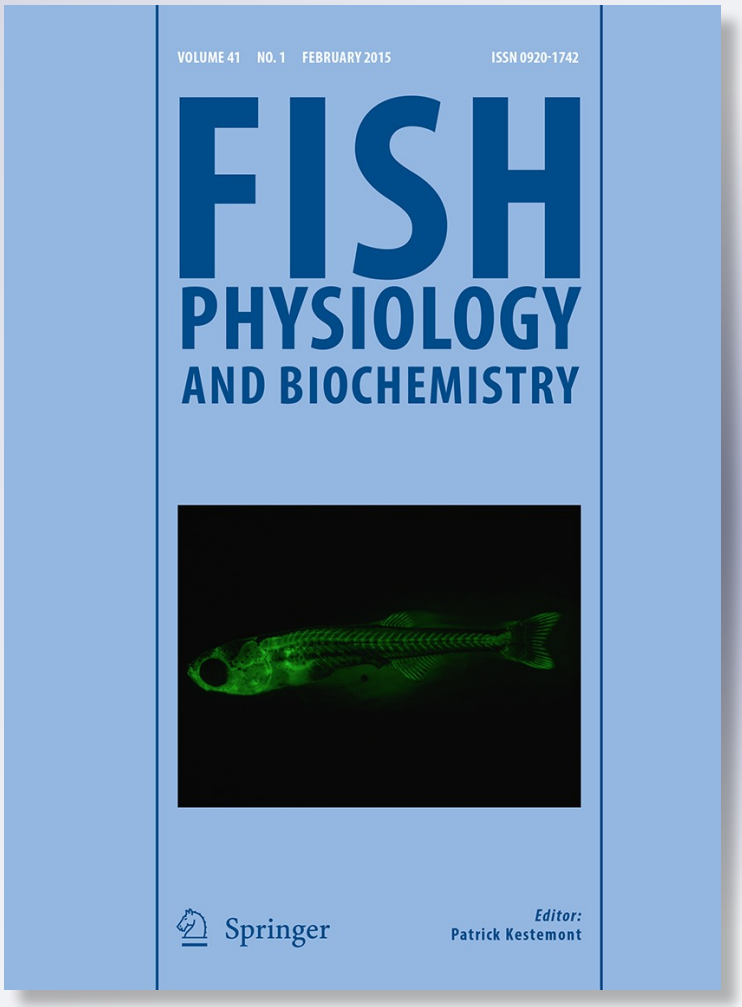

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Methyl glycol, methanol and DMSO effects on post-thaw motility, velocities, membrane integrity and mitochondrial function of *Brycon orbignyanus* and *Prochilodus lineatus* (Characiformes) sperm

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Abstract The aim of this study was to use more accurate techniques to investigate the effects of cryoprotectants (CPAs) and extenders on post-thaw sperm quality of *Brycon orbignyanus* and *Prochilodus lineatus*. Six freezing media comprising the combination of three CPAs (DMSO, methanol and methyl glycol) and two extenders (BTS and glucose) were used. Sperm was diluted in each medium, loaded into 0.5-mL straws, frozen in a nitrogen vapor vessel (dry-shipper), and stored in liquid nitrogen at −196 °C. Post-thaw sperm motility rate and velocities (curvilinear = VCL; straight line = VSL; average path = VAP) were evaluated using a computer-assisted sperm analyzer. Membrane integrity and mitochondrial function were determined using fluorochromes. Post-thaw quality was considered high when samples presented the following minimum values: 60 % motile sperm, 140 μm/s of VCL, 50 % intact sperm membrane and 50 % mitochondrial function integrity. High post-thaw quality was observed in *B. orbignyanus* sperm frozen in BTS–methyl glycol and in *P. lineatus* sperm frozen in BTS–methyl glycol, glucose–methyl glycol and glucose–methanol. All samples frozen in DMSO yielded low quality. The presence of ions in the BTS extender affected post-thaw sperm quality positively in *B. orbignyanus* and negatively in *P. lineatus*. Methyl glycol was the most suitable CPA for both fish species, leading to a good protection of cell membrane, mitochondrial function and motility apparatus during the cryopreservation process. For an improved protection, *B. orbignyanus* sperm should be frozen in an ionic freezing medium.

Keywords Cryoprotection · CASA · Semen · Neotropical · Teleost · Fish

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Introduction

The piracanjuba *Brycon orbignyanus* and streaked prochilod *Prochilodus lineatus* are fish species endemic to South America and belonging to the order Characiformes. These species, as many other Brazilian fish species, need to migrate for spawning. However, the reproductive cycle of some migratory species has been disrupted due to dam construction,

water quality deterioration, loss of riparian habitats and overfishing. The genus *Brycon* is highly affected by these environmental changes, and many species are categorized in the red list of Brazilian threatened fauna including *B. orbignyanus* (Rosa and Lima 2008). On the other hand, *P. lineatus* is well adapted to captivity, artificial fertilization methods are well established, and this species has been used as a model in a number of studies addressing nutrition, health, genetic diversity and reproduction. In our laboratory, those two species have been submitted to a number of studies on spawning and spermiation inducers (Viveiros et al. 2013), on initiation and suppression of sperm motility (Gonçalves et al. 2013) and on sperm preservation (Maria et al. 2006a, b; Viveiros et al. 2008, 2009; Lopez-Galvis 2014).

The cryopreservation of fish sperm provides a powerful tool thanks to which reproduction is optimized and larval production is increased, thereby improving breeding and fish conservation programs (Kopeika and Kopeika 2008). Sperm cryopreservation facilitates procedures for artificial reproduction, as having access to viable sperm stored in liquid nitrogen, it is needed to induce spawning and collect gametes only from females. Cryopreserved sperm may be kept in germplasm banks for an indefinite period, which allows the establishment of breeding programs, eliminates the problem of asynchronous reproductive activity between males and females, and enables maintenance of fewer male broodfish individuals (Godinho and Viveiros 2011). However, cryopreservation reduces sperm motility rate and velocity and increases membrane and organelles damages (Godinho and Viveiros 2011; Cabrita et al. 1998; De Baulny et al. 1999; Li et al. 2006). Several cryoprotectant agents (CPA), such as dimethyl sulphoxide (DMSO), methyl glycol and methanol, just to mention a few, have been tested in fish species, so as to reduce these damages. During the past decade, it has been reported that methyl glycol was the most effective CPA for the sperm cryopreservation of several characiforms species, such as *Brycon insignis* (Viveiros et al. 2011), *B. orbignyanus* (Maria et al. 2006a, b), *Colossoma macropomun* (Carneiro et al. 2012), *Piaractus brachypomus* (Nascimento et al. 2010), *Piaractus mesopotamicus* (Orfão et al. 2010) and *P. lineatus* (Viveiros et al. 2008). With few exceptions, post-thaw sperm quality was mostly assessed using simple techniques such as subjective analysis of motility rate and membrane integrity after eosin–nigrosin staining.

Thus, the aim of this study was to use more accurate techniques to investigate the effects of CPAs and extenders on post-thaw sperm quality of *B. orbignyanus* and *P. lineatus*. Motility rate and velocities were evaluated using computer-assisted sperm analyzer (CASA), and membrane integrity as well as mitochondrial function assessment using fluorochromes.

Materials and methods

Fish handling, sperm collection and initial evaluation

All fish were handled in compliance with the guidelines for animal experimentation described in Van Zutphen et al. (2001). *Brycon orbignyanus* ($n = 5$; 1.3 ± 0.5 kg of body weight) and *Prochilodus lineatus* ($n = 5$; 1.4 ± 0.3 kg of body weight) males were selected from earthen ponds at the Hydrobiology and Fish Culture Station of Furnas in the city of São José da Barra (20°43′07″S; 46°18′50″W), state of Minas Gerais, Brazil, during the spawning season (December–February). Males with detectable traces of sperm released under soft abdominal pressure received a single intramuscular dose of carp pituitary extract (cPE; Argent Chemical Laboratory, Redmond, Washington, USA) at 3 mg/kg body weight. After 5 (*B. orbignyanus*) or 8 h (*P. lineatus*) at ~25 °C, the urogenital papilla was carefully dried and 3–5 mL of semen was hand-stripped from each male directly into test tubes. Sperm collection was carried out at room temperature (~25 °C). Right after collection, tubes containing sperm were placed in a cooler (9 ± 11 °C) containing dry ice foam (Polar Technics CRI Ltd, Brazil). Contamination of sperm with water, urine or feces was carefully avoided.

Determination of fresh sperm features

In order to evaluate initial sperm quality, 5 μL of each sample was placed on a glass slide and observed under a light microscope (model L1000, Bioval, Jiangbei, China) at 400× magnification. Prior to any dilution, sperm samples were immotile; thereafter, motility rate (expressed as % of motile sperm) was subjectively estimated following the addition of 25 μL of an activating solution composed of 0.29 % NaCl (92 mOsm/kg; Viveiros et al. 2009). Samples

displaying at least 80 % motile sperm were used in the subsequent analyses. Motility velocity score was assigned using a subjective grading scale ranging from 0 (no movement) to 5 (rapidly swimming spermatozoa), as previously described in Viveiros et al. (2011). Sperm concentration was determined using a Neubauer-type hemacytometer chamber (Boeco, Hamburg, Germany). The osmolality of the seminal plasma was measured (Semi-Micro Osmometer K-7400, Knauer, Berlin, Germany) in the supernatant resulting from centrifugation of the sperm at 2,000*g* for 30 min (MiniStar, Shanghai, China). Fresh sperm characteristics of all samples were evaluated in duplicate for each sperm sample, at room temperature (~25 °C) and by a well-trained technician.

Sperm cryopreservation

Six freezing media comprising the combinations of three CPAs and two extenders were prepared. Dimethyl sulfoxide [DMSO; $(CH_3)_2SO$], methanol (CH_3OH) and methyl glycol [$CH_3O(CH_2)_2OH$] were purchased from Vetec Química Fina Ltda™ (Duque de Caxias, RJ, Brazil). Each CPA was combined with one of the two extenders: a simple glucose solution and a more complex solution named BTS (Beltsville Thawing Solution™, Minitüb™, Tiefenbach/Landshut, Germany) composed of 80 % glucose, 12.7 % sodium citrate, 2.7 % EDTA, 2.7 % $NaHCO_3$, 1.5 % KCl and 0.5 % gentamycin sulfate. Both extenders were adjusted to an osmolality value of 315 mOsm/kg (Nascimento et al. 2012). Approximately 10 min after collection, sperm of each male was diluted in each of the six freezing media to a final proportion (v/v) of 10 % sperm, 10 % CPA and 80 % extender (Maria et al. 2006a; Viveiros et al. 2009). Immediately after, without any equilibration time, diluted sperm was aspirated into five 0.5-mL replicate straws and frozen in a nitrogen vapor vessel (Cryoporter™ LN_2 dry vapor shipper, Cryoport Systems, Brea, CA, USA) at approximately −170 °C. In total, 5 replicate straws × 6 media × 5 males for each species were frozen. Within 24 h, straws were transferred to a liquid nitrogen vessel (M.V.E. Millenium, XC 20, Chart, MN, USA) at −196 °C for storage. Within a week, straws were transferred back to the nitrogen vapor vessel and transported from Furnas to the Laboratory of Semen Technology of the Federal University of Lavras (UFLA), city of Lavras, state of Minas Gerais, Brazil (approximately 260 km). Upon arrival, straws were stored in liquid nitrogen vessel (M.V.E. Millenium, XC 20, Chart, MN, USA) until further analysis. For post-thaw evaluation of sperm quality, straws were thawed in a water bath at 60 °C for 8 s and each straw was assayed for motility and velocity (3 replicate straws = 90 straws per species), for membrane integrity (1 replicate straw = 30 straws per species) and for mitochondria function (1 replicate straw = 30 straws per species), as described below.

Sperm motility and velocity parameters evaluation

Post-thaw sperm motility and velocities were estimated using the CASA system according to the methodology used in our laboratory (Viveiros et al. 2013). Briefly, motility was triggered in 0.29 % NaCl directly in a Makler™ counting chamber (Sefi-Medical Instruments Ltd, Haifa, Israel) placed under a phase contrast microscope (Nikon™ Eclipse E200, Tokyo, Japan), objective magnification of 10×, ocular magnification of 10×, with a green filter and phase 1 position. The microscope was connected to a video camera (Basler Vision Technologies™ A602FC, Ahrensburg, Germany) generating 100 images/s; video recording started 10 s post-activation. Each image was analyzed using the standard settings for fish by Sperm Class Analyzer™ software (SCA™ 2010, Microptics, S.L. Version 5.1, Barcelona, Spain). Although the SCA™ simultaneously assesses more than 15 sperm motility endpoints, only motility rate, curvilinear velocity (VCL), straight-line velocity (VSL) and average path velocity (VAP) were considered for analysis, as similar effects were observed for all parameters. For determination of these parameters corresponding to each individual sperm sample, an average of 340 sperm traces for each straw of *B. orbignyanus* and of 634 sperm traces for each straw of *P. lineatus* was followed throughout the recorded video images from which sperm trajectories were evaluated.

Membrane integrity and mitochondrial function

Membrane integrity, expressed as the percentage of sperm with intact membrane (% intact sperm), was

assessed using the membrane permeant dye SYBR™ 14 which stains DNA in living cells, and propidium iodide (PI) which stains DNA in damaged cells that have lost membrane integrity (LIVE/DEAD™ sperm viability kit; Molecular Probes, Invitrogen, USA). Mitochondrial functionality, expressed as the percentage of cells with functional mitochondria (% mitochondrial function), was assessed using Rhodamine 123 (R 123) and PI. We followed the staining methodology for evaluation of membrane integrity described in Daly and Tiersch (2012) and adapted it to evaluate mitochondria function. Briefly, 10 μL of thawed sperm (previously diluted 1:10) was further diluted to 1:100 (final volume: 100 μL, $\sim 1 \times 10^6$ - cells/mL) in the same extender used during freezing (BTS or glucose). In a 2-mL micro-tube covered with aluminum foil, 100 μL of diluted sperm was mixed in 0.5 μL of SYBR™ 14 (20 μM) or 1.3 μL of R 123 (100 μM) and incubated in the dark for 10 min at room temperature. Then, 0.5 μL of PI (2.4 mM) was added and again incubated for another 10-min period in the dark. A 50 μL drop of stained sperm was placed on a slide under a cover slip and immediately analyzed in an epi-fluorescence microscope (Nikon™ Eclipse E200, Tokyo, Japan) with excitation filter of 546–590 nm for PI, of 450–490 nm for SYBR™ 14 and of 450–490 nm for R 123. By use of a digital camera (Sony DSC-W530 4× Optical Zoom, Sony Corp. China), six pictures were taken from three different fields and a mean of 205 *B. orbignyanus* sperm and of 396 *P. lineatus* sperm was evaluated.

We considered high post-thaw sperm quality when samples presented the following minimum values: 60 % of motile sperm, 140 μm/s of VCL, 50 % of intact sperm and 50 % of mitochondria function.

Statistical analysis

Values are expressed as mean ± standard deviation (SD). Statistical analyses were conducted with the R software program version 2.9.0 (R Development Core Team 2010). Sperm motility and velocity parameters, membrane integrity and mitochondrial functionality were tested for normal distribution using the univariate procedure; all parameters showed a normal distribution. Data were tested for significant differences using ANOVA, followed by the Tukey test, when applicable. The level of significance for all statistical tests was $P < 0.05$.

Results

Fresh sperm features

The following mean values were observed for *B. orbignyanus* males ($n = 5$): motility rate of 89 %, velocity score of 4.0, concentration of 7.4×10^9 sperm/mL and seminal plasma osmolality of 300 mOsm/kg; and for *P. lineatus* males ($n = 5$): motility rate of 96 %, velocity score of 4.6, concentration of 18.4×10^9 sperm/mL and seminal plasma osmolality of 306 mOsm/kg (Table 1). Fresh sperm motility (both rate and velocity score) and seminal plasma osmolality were similar between species, but sperm concentration was higher in *P. lineatus*.

Post-thaw sperm quality

In general, post-thaw sperm possessed higher quality in *P. lineatus* (varying from 26 to 81 %, with an

Table 1 Body weight and some fresh sperm features in piracanjuba *Brycon orbignyanus* and streaked prochilod *Prochilodus lineatus* after carp pituitary treatment

Features	*B. orbignyanus*		*P. lineatus*	
	Mean ± SD	Min–max	Mean ± SD	Min–max
Concentration (sperm × 10^9/mL)	7.4 ± 3.1[b]	4.8–10.7	18.4 ± 1.3[a]	16.9–20.1
Motility rate[1] (%)	89 ± 5	80–95	96 ± 5	90–100
Motility velocity score[2] (0–5)	4.2 ± 0.4	4.0–5.0	4.6 ± 0.5	4.0–5.0
Seminal plasma osmolality (mOsm/kg)	300 ± 9	289–313	306 ± 10	290–318

Means within the same row followed by different lowercase letters are significantly different ($P < 0.05$; ANOVA)

[1] Motility rate subjectively evaluated under a light microscope, as described in "Materials and methods"

[2] Motility velocity score was assigned using a subjective grading scale ranging from 0 (no movement) to 5 (rapidly swimming sperm)

Table 2 Post-thaw quality (mean ± SD) of *Brycon orbignyanus* ($n = 5$ males) and *Prochilodus lineatus* ($n = 5$ males) sperm frozen in three cryoprotectants (CPA) combined with two extenders

Extender	CPA (10 %)	Motility (%)	VCL (μm/s)	VSL (μm/s)	VAP (μm/s)	Membrane integrity (%)	Mitoch function (%)
B. orbignyanus							
BTS	DMSO	31 ± 22^{b}	102 ± 23^{b}	58 ± 16^{b}	80 ± 16^{c}	27 ± 7^{b}	20 ± 14^{b}
	Methanol	33 ± 18^{b}	$119 \pm 16^{a,b}$	72 ± 27^{b}	96 ± 29^{c}	30 ± 19^{b}	26 ± 21^{b}
	MG	63 ± 18^{a}	140 ± 24^{a}	90 ± 23^{a}	113 ± 24^{a}	57 ± 11^{a}	61 ± 8^{a}
Glucose	DMSO	16 ± 17^{a}	$90 \pm 38^{a,b}$	49 ± 21^{a}	76 ± 27^{a}	$13 \pm 10^{a,b}$	15 ± 17^{a}
	Methanol	6 ± 20^{b}	63 ± 29^{b}	45 ± 23^{a}	63 ± 25^{a}	4 ± 4^{b}	3 ± 5^{b}
	MG	19 ± 16^{a}	104 ± 23^{a}	52 ± 22^{a}	77 ± 23^{a}	17 ± 8^{a}	20 ± 11^{a}
P. lineatus							
BTS	DMSO	26 ± 11^{c}	69 ± 8^{b}	27 ± 11^{b}	41 ± 14^{b}	50 ± 11^{b}	44 ± 12^{b}
	Methanol	57 ± 16^{b}	144 ± 30^{a}	76 ± 18^{a}	117 ± 29^{a}	60 ± 17^{b}	$57 \pm 4^{a,b}$
	MG	72 ± 12^{a}	152 ± 27^{a}	82 ± 14^{a}	124 ± 28^{a}	68 ± 15^{b}	61 ± 29^{a}
Glucose	DMSO	45 ± 18^{c}	77 ± 7^{c}	41 ± 12^{c}	58 ± 12^{c}	57 ± 13^{b}	52 ± 14^{b}
	Methanol	68 ± 17^{b}	161 ± 38^{b}	93 ± 22^{b}	136 ± 36^{b}	$67 \pm 22^{a,b}$	53 ± 18^{b}
	MG	81 ± 10^{a}	197 ± 31^{a}	110 ± 20^{a}	170 ± 30^{a}	78 ± 14^{a}	80 ± 11^{a}

Motility rate and velocities (VCL = curvilinear, VSL = straight line and VAP = average path) were evaluated using a computer-assisted sperm analyzer (CASA), and membrane integrity and mitochondrial function were assessed using fluorochromes

BTS = Beltsville Thawing Solution™: glucose, sodium citrate, EDTA, NaHCO3, KCl, gentamycin sulfate (see "Materials and methods")

Mean values within the same column and extender composition, for each species, followed by different superscripts are significantly different ($P < 0.05$; Tukey Test)

overall mean value of 56 %) than in *B. orbignyanus* (varying from 6 to 63 %, with an overall mean value of 30 %). Sperm of both species yielded higher quality when frozen in methyl glycol and lower quality when frozen in DMSO. The extender composition, however, affected sperm of *B. orbignyanus* and *P. lineatus* differently.

In *B. orbignyanus*, samples frozen in BTS solution were of better quality ($P < 0.05$) than those frozen in glucose. High post-thaw sperm quality (according to our criteria) was observed only in samples frozen in BTS–methyl glycol. All samples frozen in glucose possessed low quality with motility below 20 %, whatever the CPA used (Table 2).

In *P. lineatus*, all samples frozen in glucose solution were of better quality ($P < 0.05$) than those frozen in BTS. High post-thaw sperm quality (according to our criteria) was observed in samples frozen in glucose–methyl glycol, glucose–methanol and BTS–methyl glycol (Table 2).

There was a positive and a significant correlation ($P < 0.01$) between sperm motility, velocities, intact sperm and mitochondrial function in both species (Table 3). The strongest correlation was observed between sperm motility and membrane integrity ($R = 0.929$).

Discussion

In the present study, the effects of CPAs and extenders on post-thaw sperm quality were assessed using CASA system and fluorescence dyes. This is the first report where such accurate techniques were used as a better tool to detect differences due to freezing media on *B. orbignyanus* and *P. lineatus* cryopreserved sperm.

Fresh sperm quality

Fresh sperm quality of *B. orbignyanus* and *P. lineatus* was evaluated in the present study, and the observed values were all within the range previously reported for *B. orbignyanus* and *P. lineatus* species after carp pituitary treatment (Godinho and Viveiros 2011). It is interesting to observe the higher sperm concentration

Table 3 Correlations among quality features of *Brycon orbignyanus* ($n = 5$ males) and *Prochilodus lineatus* ($n = 5$ males) cryopreserved sperm

	Motility	VCL	VSL	VAP	Membrane integrity	Mitoch function
Motility	1.000					
VCL	0.864	1.000				
VSL	0.821	0.973	1.000			
VAP	0.847	0.994	0.980	1.000		
Membrane integrity	0.929	0.670	0.580	0.638	1.000	
Mitoch function	0.716	0.451	0.457	0.487	0.849	1.000

Motility rate and velocities (VCL = curvilinear, VSL = straight line and VAP = average path) were evaluated using a computer-assisted sperm analyzer (CASA), and membrane integrity as well as mitochondrial function assessment using fluorochromes

All correlations were significant ($P < 0.01$)

in *P. lineatus* compared with *B. orbignyanus*. It has been reported that *P. lineatus* produces a high concentration and a low volume of sperm (frequently below 5 mL; Viveiros et al. 2013), while *B. orbignyanus* produces a low concentration and a high volume of sperm (frequently above 10 mL; Maria et al. 2006b). A better knowledge of fresh sperm characteristics is necessary to evaluate sperm quality before experiments in order to guarantee high initial quality of sperm parameters.

Extenders

In the present study, BTS and glucose solutions were tested as extender and affected sperm of *B. orbignyanus* and *P. lineatus* differently. Several solutions have been used as fish sperm extenders; some are simple saline (NaCl) or sugar (glucose) solutions, while others have a more complex formulae with a combination of salts and sugars, such as BTS, Powdered Coconut Water (ACP^{TM}) and Hanks Balanced Salt Solution (HBSS), among others.

In *B. orbignyanus*, samples frozen in BTS solution were of better quality ($P < 0.05$) than those frozen in glucose. Despite the high concentration of glucose (almost 80 %) present in BTS, some other component (sodium citrate, EDTA, $NaHCO_3$, KCl) produced a positive effect during the freezing and thawing processes of *B. orbignyanus* sperm. Furthermore, in our previous study (Lopez-Galvis 2014), *Brycon orbignyanus* sperm frozen in ionic solutions (NaCl in several osmolalities) yielded better quality than that frozen in ion-free solution (glucose). The addition of ions in a freezing medium aims to control osmotic pressure during cell dehydration (Kopeika and Kopeika 2008).

In contrast with *B. orbignyanus*, *P. lineatus* sperm was better preserved in an ion-free medium, as all samples frozen in glucose solution were of better quality ($P < 0.05$) than those frozen in BTS. In our previous study (Viveiros et al. 2009), *P. lineatus* sperm frozen in ionic solution (NaCl) yielded very low motility rate (below 20 %), while those samples frozen in glucose solution yielded motility above 90 %. The addition of sugars in a freezing medium aims the protection of cell membrane (Kopeika and Kopeika 2008), and it is present in most of the extenders for fish sperm (Godinho and Viveiros 2011). In *P. lineatus,* the presence of glucose is mandatory for the success of the cryopreservation process, while the presence of ions decreases post-thaw sperm quality. See Table 4 for more details.

Cryoprotectants

In the present study, high post-thaw sperm quality (minimum values: 60 % motility, 140 μm/s of VCL, 50 % intact sperm and 50 % mitochondria function) was observed when methyl glycol was used as CPA for both fish species, and methanol for *P. lineatus*.

The methyl glycol and DMSO have similar molar concentrations (approximately 78 and 76 g/mol, respectively), while the methanol has less than half of it (32 g/mol). In the present study, we could not measure the final osmolality of each freezing medium because our osmometer measures osmolality by freezing point depression and this is not possible when CPAs are added to the solution. But another study reported the final osmolality (measured in a vapor pressure osmometer) of the freezing medium using an extender at 300 mOsm/kg (similar to ours)

Table 4 Some cryoprotectant agents (CPA) and extenders tested on the sperm cryopreservation of species of the genus *Brycon* and *Prochilodus*

Species	CPA (10 %)	Extender (mOsmol)[a]	Post-thaw sperm quality	Reference
B. insignis	DMSO	356 BTS	23 % motility[S]	Viveiros et al. (2011)
	DMSO	308 glucose	45 % motility[S]	
	DMSO	285 NaCl	46 % motility[S]	
	Methyl glycol	356 BTS	77 % motility[S], 62 % intact sperm[EN]	
	Methyl glycol	308 glucose	77 % motility[S], 69 % intact sperm[EN]	
	Methyl glycol	285 NaCl	82 % motility[S], 61 % intact sperm[EN]	
B. orbignyanus	DMSO	318 BTS	11 % motility[S]	Maria et al. (2006a)
	Methanol	318 BTS	21 % motility[S]	
	Methyl glycol	318 BTS	68 % motility[S]; 66 % intact sperm[EN]	
	DMSO	285 NaCl + yolk	8 % motility[S]	Maria et al. (2006b)
	Methanol	285 NaCl + yolk	7 % motility[S]	
	Methyl glycol	285 NaCl + yolk	66 % motility[S]	
	Methyl glycol	285 NaCl	63 % motility[C]; VCL of 150 μm/s	Lopez-Galvis (2014)
		285 glucose	40 % motility[C]; VCL of 96 μm/s	
	DMSO	315 BTS	31 % motility[C]; VCL of 102 μm/s; 27 % intact sperm[PI]	Present study
	Methanol	315 BTS	33 % motility[C]; VCL of 119 μm/s; 30 % intact sperm[PI]	
	Methyl glycol	315 BTS	63 % motility[C]; VCL of 140 μm/s; 57 % intact sperm[PI]	
	DMSO	315 glucose	16 % motility[C]; VCL of 90 μm/s; 13 % intact sperm[PI]	
	Methanol	315 glucose	6 % motility[C]; VCL of 63 μm/s; 4 % intact sperm[PI]	
	Methyl glycol	315 glucose	19 % motility[C]; VCL of 104 μm/s; 17 % intact sperm[PI]	
P. lineatus	DMSO	318 BTS	87 % motility[S]	Viveiros et al. (2009)
	DMSO	277 glucose	36 % motility[S]	
	DMSO	285 NaCl	3 % motility[S]	
	Methyl glycol	318 BTS	95 % motility[S]	
	Methyl glycol	277 glucose	95 % motility[S]	
	Methyl glycol	285 NaCl	18 % motility[S]	
	DMSO	300 ACP	8 % motility[S]	Viveiros et al. (2008)
	Methyl glycol	300 ACP	76 % motility[S]	
	DMSO	315 BTS	26 % motility[C]; VCL of 69 μm/s; 50 % intact sperm[PI]	Present study
	Methanol	315 BTS	57 % motility; VCL of 144 μm/s; 60 % intact sperm[PI]	
	Methyl glycol	315 BTS	72 % motility[C]; VCL of 152 μm/s; 68 % intact sperm[PI]	
	DMSO	315 glucose	45 % motility[C]; VCL of 77 μm/s; 57 % intact sperm[PI]	
	Methanol	315 glucose	68 % motility[C]; VCL of 161 μm/s; 67 % intact sperm[PI]	
	Methyl glycol	315 glucose	81 % motility[C]; VCL of 197 μm/s; 78 % intact sperm[PI]	

BTS = Beltsville Thawing Solution[TM]: glucose, sodium citrate, EDTA, NaHCO3, KCl and gentamycin sulfate

ACP = powdered coconut water[TM]: glucose, proteins, phosphorus, potassium, calcium, magnesium, sodium, iron and among other components

[a] When authors presented extender as % or mM, we estimated the osmolality in order to facilitate comparisons

[S] Motility rate subjectively evaluated under a light microscope

[C] Motility rate evaluated under a computer-assisted sperm analyzer

[EN] Eosin–Nigrosin staining

[PI] SYBR–PI staining

combined with 10 % methanol or methyl glycol as being close to 300 mOsm/kg and with 10 % DMSO as being above 2,000 mOsm/kg (Cuevas-Uribe et al. 2011).

The final osmolality of about 300 mOsm/kg is close to seminal plasma osmolality and corroborates with the high quality of methyl glycol-frozen samples of both species and of methanol-frozen samples of *P.*

lineatus, but not with the low quality of methanol-frozen samples of *B. orbignyanus*. In this species, we have observed that these three CPAs, after 30 min of incubation, do not reduce sperm motility rate (Nascimento et al. 2012) and thus are not toxic to fresh sperm. Possibly, the reduced post-thaw quality of methanol-frozen samples may be related to the inefficiency of this CPA (at least at this concentration) to protect *B. orbignyanus* sperm during dehydration and rehydration events that occur during the freezing and thawing processes.

The huge increase of final osmolality of the freezing medium after addition of DMSO, on the other hand, could explain why DMSO-frozen sperm yielded low quality. Even considering that DMSO penetrates through the cell membrane, the final osmolality (above 2,000 mOsm/kg) may have caused transient osmotic stress causing elevated intracellular ion concentration deleterious to cell membrane and/or some organelles during the freezing or the thawing steps, leading to reduction of all sperm quality parameters, as observed in both *B. orbignyanus* and *P. lineatus* sperm. Similarly, the reduced cryoprotective effects of DMSO in comparison with methyl glycol in terms of motility rate have been reported for sperm of *B. insignis* (Viveiros et al. 2011), *B. orbignyanus* (Maria et al. 2006a, b), *Colossoma macropomum* (Carneiro et al. 2012), *P. mesopotamicus* (Orfão et al. 2010), *P. brachypomus* (Nascimento et al. 2010) and *P. lineatus* (Viveiros et al. 2008). See Table 4 for more details.

Conclusions

Taking all the results together and comparing the fish species, it can be observed that *B. orbignyanus* sperm was more sensitive to the cryopreservation process (overall motility rate of 30 %), compared with *P. lineatus* (overall motility of 56 %). Only one freezing medium (BTS–methyl glycol) protected *B. orbignyanus* sperm during freezing and thawing processes effectively, with post-thaw motility above 50 %. In *P. lineatus*, however, sperm frozen in BTS or glucose combined with methyl glycol or methanol yielded motility rate above 50 %. Either *P. lineatus* is more resistant to dehydration and rehydration events than *B. orbignyanus* sperm, or those media tested in the present study are not ideal to cryopreserve *B. orbignyanus* sperm. We recommend that other ionic solutions, such as KCl, KI or citrate, should be tested as extender for *B. orbignyanus* sperm.

The use of CASA and fluorochromes to assess post-thaw sperm quality improves the analysis accuracy of sperm motility and velocities, as well as membrane integrity and mitochondrial function. Methyl glycol was an efficient CPA and provided efficient cell protection (both functional and structural) during the cryopreservation process, while DMSO caused a decrease of all post-thaw sperm quality parameters and thus should be avoided on sperm cryopreservation of both *B. orbignyanus* and *P. lineatus*. *B. orbignyanus* sperm is more sensitive to the cryopreservation process and should be frozen in an ionic medium, while *P. lineatus* sperm is more resistant and should be frozen in an ion-free medium.

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