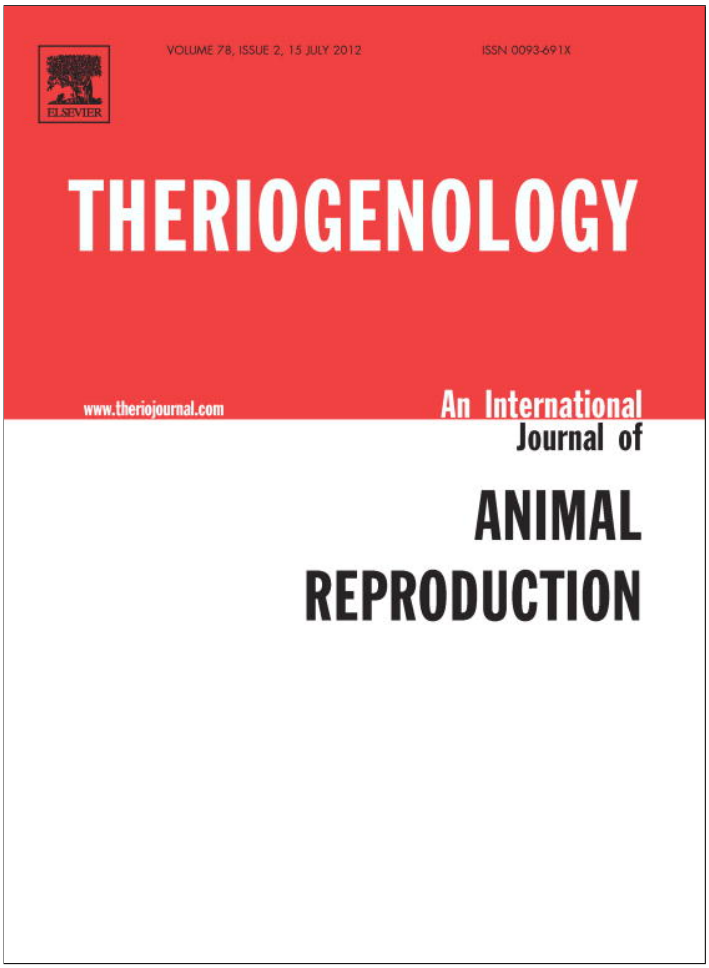

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Effects of extenders, cryoprotectants and freezing methods on sperm quality of the threatened Brazilian freshwater fish pirapitinga-do-sul *Brycon opalinus* (Characiformes)

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Abstract

The objective was to develop a suitable freezing method to cryopreserve *Brycon opalinus* (Characiformes) sperm. Extenders (NaCl and glucose at 325 and 365 mOsm/kg), cryoprotectants (dimethyl sulfoxide = dimethyl sulfoxide (DMSO) and methyl glycol = methyl glycol (MG)), equilibration times (15 and 30 min), thawing temperatures (30 and 60 °C), and straw sizes (0.5 and 4.0 mL) were tested. Sperm were frozen in a liquid nitrogen vapor vessel at −170 °C and subsequently stored in liquid nitrogen. Post-thaw sperm quality was always evaluated in terms of motility (expressed as percentage of motile sperm), duration of motility and vitality (eosin-nigrosin staining, expressed as percentage of intact sperm). The best freezing method was also tested for fertility and hatching (expressed as the percentage of fertilized eggs). Post-thaw sperm quality was highest when sperm were cryopreserved in Glucose 365 mOsm/kg and MG, after a 30-min equilibration and thawed at 60 °C for 8 s, of regardless straw size: 74 ± 7% motile sperm, 47 ± 4 s of motility duration, 69 ± 3% intact sperm, 64 ± 4% fertilization and 63 ± 3% hatching. The freezing method developed in the present study was efficient and can be used to maximize larvae production for both aquaculture purposes and for conservational programs, since *B. opalinus* is a threatened species.

Keywords: Sperm motility; Vitality; Hatching rate; Methyl glycol; DMSO; Cryopreservation

1. Introduction

The *Brycon opalinus* (Cuvier, 1819), known as pirapitinga-do-sul in Brazil, belongs to the order Characiformes, family Characidae and subfamily Bryconinae, and is native to the Paraíba do Sul and Doce River basins in Southeastern Brazil [1]. The genus *Brycon* is highly affected by environmental changes caused by human activities, e.g., construction of hydroelectric dams and overfishing. Many species of this genus are on the list of Brazilian threatened fauna (e.g., *B. opalinus*, *B. orbignyanus*, *B. insignis* and *B. nattererii* [1]). In the Hydrobiology and Aquaculture Station of the Hydroelectric Company of São Paulo (CESP) in the city of Paraibuna, Brazil, many males and females of *B. opalinus* and other native species are maintained as broodfish for restocking programs in the Paraíba do Sul River basin. The success of the artificial reproduction and larvae production of *B. opalinus* carried out at

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CESP, and the fast growth observed, indicates the suitability of this species for aquaculture [2].

The cryopreservation of fish sperm provides a tool by which reproduction is optimized and larval production is increased, thereby improving breeding and fish conservation programs. The first step when developing a cryopreservation method is to choose an appropriate freezing medium composed of an extender (commonly a saline or glucose solution with an appropriate osmolality), and a cryoprotectant agent. Although a cryoprotectant protects cells against freezing damage because of intracellular ice crystals formation and excessive dehydration, it may also cause detrimental effects on sperm physiology, due to osmotic shock or direct biochemical injury, particularly if sperm are exposed to cryoprotectant for too long [3]. Thus, post-thaw sperm quality depends greatly on cryoprotectant concentration and the interval from addition of a cryoprotectant to freezing (equilibration time).

In Characiformes fish species, dimethyl sulfoxide (DMSO), mainly in combination with glucose and egg yolk, has been the cryoprotectant most widely used for sperm cryopreservation [4]. However, during the past few years, our research group reported that methyl glycol (methyl glycol (MG); also known as 2-methoxyethanol, propane-1,2-diol or ethylene glycol monomethyl ether) was better than DMSO for cryopreserving sperm of some Characiformes, including three species of the genus *Brycon*, namely *B. orbignyanus* [5], *B. nattererii* [6] and *B. insignis* [7]. Furthermore, by replacing DMSO with MG, the addition of egg yolk was unnecessary. In that regard, egg yolk impaired visualization of sperm during motility analysis, under both light microscope and computer-aided, more difficult.

In our previous study, the effects of extender composition (glucose and NaCl), extender osmolality (245, 285, 325 and 365 mOsm/kg) and cryoprotectant (DMSO or MG) on the motility of *B. opalinus* were examined [8]. In that study, an extender with a high osmolality was necessary to prevent initiation of sperm motility, regardless of extender composition or cryoprotectant. Furthermore, with sperm diluted in extender at high osmolality, following activation, more than 77% of sperm were motile, which should last long enough to fertilize oocytes. Thus, the aim of this study was to develop a suitable freezing method for *B. opalinus* sperm, testing various combinations of freezing media, as well as equilibration time, thawing temperature and straw volume. Post-thaw sperm quality was evaluated in terms of motility, duration of motility and vitality, as well as fertilization and hatching rates.

2. Materials and methods

2.1. Fish handling and sperm collection

All fish were handled in compliance with guidelines for animal experimentation described in van Zutphen, et al. [9]. *Brycon opalinus* males approximately 7 yrs of age (body weight 321 ± 13 g and total length 30 ± 1 cm) were selected from circular tanks at the Hydrobiology and Aquaculture Station of CESP in the city of Paraibuna (23°232010“S; 45°392044“W), state of São Paulo, Brazil, during the spawning season (October and November). Males with detectable running sperm under soft abdominal pressure were given a single intramuscular treatment (5 mg/kg body weight) of carp pituitary extract (cPE; Argent Chemical Laboratory, Redmond, Washington, USA) to increase semen volume and decrease viscosity, as a routine method for Characiformes [10]. After 8 h at ~25 °C, the urogenital papilla was carefully dried, and sperm were hand-stripped directly into test tubes. Sperm collection was done at room temperature (~22 °C), and soon after collection, tubes containing sperm were placed in a polystyrene box containing crushed ice (4 ± 2 °C). Contamination of sperm with water, urine or feces was carefully avoided.

Immediately after collection, 5 μL of each sample were placed on a glass slide and observed under a light microscope (Model L1000, Bioval, Jiangbei, China) at 400 X magnification. As fish sperm in seminal plasma should be immotile, any sperm motility observed was attributed to urine or water contamination and the sample discarded. In immotile samples (n = 20 males), 25 μL activating agent, composed of NaCl 92 mOsm/kg (~50 mM), was placed in the same glass slide at a final dilution ratio of 1:5 (sperm: activating agent; v: v), following the methodology used as routine in our laboratory for Characiformes fish species [5–7,11–13]. Immediately thereafter, motility was subjectively estimated as the percentage of motile sperm. All sperm samples used in this study had at least 80% motile sperm. Duration of motility was assessed on the same conditions: a stopwatch was triggered when activating agent was added and stopped when only 10% of sperm were still moving. To determine whether the subjective evaluation of motility would be accurate and represent the actual percentage of sperm with an intact membrane, motility was compared with an objective test in which vitality was determined following eosin-nigrosin

staining (5% B eosin, 10% nigrosin, Sigma-Aldrich, Steinheim, Germany; pH = 6.9). The concept of using eosin to mark dead cells with damaged membranes which take up eosin, and nigrosin as a background stain, to increase the contrast between faintly stained cells and an otherwise bright background, is well known and has been widely used since 1950 [14]. A total of 300 cells per slide (one slide per male) were counted on a light microscope, at 1000 X magnification. Vitality was calculated as the percentage of unstained or light pink cells, representing sperm with an intact membrane (intact sperm).

Semen volume and concentration (hemocytometer/Neubauer chamber) were also determined. Characteristics of all samples were evaluated by the same technician and at room temperature (~22 °C).

2.2. Experiment 1. Freezing media and equilibration times

All chemicals were obtained from Vetec Química Fina Ltda (Duque de Caxias, RJ, Brazil). Eight freezing media, comprising the combinations of four extenders and two cryoprotectants (DMSO = $(CH_3)_2SO$; and MG = $CH_3O(CH_2)_2OH$) at 1.4 M, were prepared and maintained in crushed ice (4 ± 2 °C) for 30 min. The extenders were prepared as a factorial of two compositions (NaCl and glucose) and two osmolalities (325 and 365 mOsm/kg), and referred to as NaCl-325, NaCl-365, Glucose-325 and Glucose-365. To achieve these osmolalities, the solute concentration was 0.95% NaCl, 1.05% NaCl, 5.85% glucose and 6.55% glucose, respectively. Sperm samples (n = 8 males) were diluted in each freezing medium to a final proportion (v: v) of 10% sperm, 80% extender and 10% cryoprotectant and equilibrated in crushed ice (4 ± 2 °C) for 15 or 30 min. During equilibration, sperm samples were loaded into 0.5-mL straws (n = 3 replicate straws × 8 media × 8 males) and sealed with a metallic sphere, at room temperature. Straws were frozen in a nitrogen vapor vessel (Cryoporter LN_2 dry vapor shipper, Cryoport Systems, Brea, CA, USA) at approximately −170 °C and then transferred to liquid nitrogen (M.V.E. Millenium, XC 20, Chart, MN, USA) at −196 °C within 20 to 24 h for storage. After 7 days, straws were thawed in a water bath at 60 °C for 8 s [5 to 7.11] and post-thaw sperm motility (as percentage of motile sperm), duration of motility and vitality (as percentage of intact sperm) were immediately estimated (as described for fresh sperm).

2.3. Experiment 2. Freezing media, straw sizes and thawing temperatures

Sperm (n = 7 males) was diluted in four freezing media, comprising combinations of two extenders (NaCl-365 and Glucose-365) and two cryoprotectants (DMSO and MG), and equilibrated for 30 min, based on the results of Experiment 1. Then, diluted sperm were frozen in 0.5- and 4.0-mL straws (n = 6 replicate straws × 4 media × 7 males). Both straw sizes were closed with a metallic sphere in one end; the other end of the 4.0-mL straws was kept open. Three 0.5-mL replicate straws were thawed in a water bath at 60 °C for 8 s and the other three straws at 30 °C for 16 s. Three 4.0-mL replicate straws were thawed in a water bath at 60 °C for 24 s and the other three straws at 30 °C for 48 s [7,15]. During thawing, the open end of the 4.0-mL straws was maintained above water level to avoid water contamination (which would activate sperm motility). Post-thaw sperm motility, duration of motility and vitality were evaluated as described for fresh sperm.

2.4. Experiment 3. Fertilization trial

Sperm samples (n = 4 males) were diluted in glucose-365-MG as freezing medium and equilibrated for 30 min. During equilibration, sperm were loaded into 0.5- and 4.0-mL straws (n = 3 replicate straws × 4 males). A small aliquot of diluted and equilibrated sperm was evaluated for motility, duration of motility and vitality, and served as prefreezing (equilibrated-diluted) control sperm. Straws were then frozen as described in Experiments 1 and 2 and thawed at 60 °C for 8 s (0.5-mL straws) or 24 s (4.0-mL straws). Post-thaw sperm were evaluated for motility, duration of motility and vitality, as described above, as well as for fertility and hatching (see below).

To harvest oocytes, females (n = 4; 400 ± 20-g body weight) received two doses of cPE (1 and 4.5 mg/kg body weight), 12 h apart. Concurrent with the second dose, females were given human chorionic gonadotropin (1450 IU hCG/kg body weight; Pregnyl Schering-Plough, Kenilworth, NJ, USA), and were hand-stripped 5 h later, as a routine method at CESP. All females responded positively to the hormonal treatment, and all were used for the fertilization trial. Oocytes remained at room temperature (~25 °C) for a maximum of 30 min.

Post-thaw sperm of each straw were rediluted in Glucose-365 to a final concentration of 410 × 10^6 sperm/mL, and 100 μL of rediluted sperm were mixed with 0.4 g oocytes (~ 100 oocytes), producing an

approximate and constant ratio of 410 000 sperm: oocyte. Fertilization was initiated by the addition of 5 mL tank water, and mixed for 1 min. Subsequently, 10 mL tank water was added, and samples mixed for another 2 min. Finally, eggs were transferred to a PVC basket, 10 cm in diameter, with a 0.5 mm mesh bottom [16], and incubated in a flow-through system at 22 °C. In total, 48 batches of eggs were fertilized with cryopreserved sperm (n = 3 replicate straws × 4 males × 4 females) from each straw volume. To control oocytes quality, 100 μL of freshly collected sperm of another male, diluted in Glucose-365 to the same concentration and thus the same sperm:oocyte ratio was used to fertilize oocytes from the same females, performing four batches of eggs fertilized with fresh-diluted control sperm.

The number of fertilized eggs, as percentage of total oocytes, was determined 16 h after fertilization at 22 °C (during the gastrula stage) using a stereomicroscope (Carl Zeiss and model Stemi 2000, Munich, Germany). The number of hatched larvae, as the percentage of fertilized eggs, was determined 44 h after fertilization.

2.5. Statistical analysis

Values are expressed as mean ± SEM. Statistical analyses were conducted with the R software program, version 2.9.0 [17]. Sperm motility (rate and duration), vitality, fertility and hatching were tested for normal distribution using the univariate procedure. When data did not fit the normal distribution, an arcsine transformation was performed. In all experiments, data for sperm motility, vitality, fertility and hatching were tested for significant differences using ANOVA, followed by the Duncan's test when necessary (Experiment 3). Associations among sperm motility, duration of motility and vitality were analyzed by Pearson's correlation coefficient. The level of significance for all statistical tests was $P < 0.05$.

3. Results

Fresh semen of the 20 males used in this study had an average volume of 8.0 ± 0.1 mL, with 41 billion ± 13 billion sperm/mL, 97 ± 1% motile sperm, with duration of motility 97 ± 2 s, and 96 ± 5% intact sperm. Both fresh and post-thaw sperm motility were positively correlated with vitality ($r = 0.97$; $P < 0.01$), but not with duration of motility ($r = 0.61$; $P > 0.05$).

3.1. Experiment 1. Freezing media and equilibration times

Extender osmolality, but not extender composition, cryoprotectant or equilibration time, significantly affected motility, duration of motility, and vitality. In general, post-thaw sperm quality was the highest when sperm were cryopreserved in NaCl-365-DMSO (77–78% motile sperm, 40–46 s of motility duration and 79–83% intact sperm) and in glucose-365-MG (86–88% motile sperm, 51–52 s of motility duration and 81–84% intact sperm), regardless of equilibration time (Table 1).

3.2. Experiment 2. Freezing media, straw sizes and thawing temperatures

Extender composition and thawing temperature, but not cryoprotectant or straw size, significantly affected motility, duration of motility and vitality. There was a significant interaction between extender composition and cryoprotectant; DMSO was a better cryoprotectant than MG when combined with NaCl, whereas MG was a better cryoprotectant than DMSO when combined with glucose. In general, post-thaw sperm quality was highest when sperm were cryopreserved in glucose-365-MG and thawed at 60 °C (80–82% motile sperm, 48–53 s of motility duration and 80–82% intact sperm), regardless of straw size (Table 2).

3.3. Experiment 3. Fertilization trial

Sperm quality (motility, duration of motility and vitality) was not different ($P > 0.05$) between fresh-diluted control and prefreezing (equilibrated-diluted) control sperm. Both control samples consistently were of better quality than frozen sperm ($P < 0.05$; Table 3). Pooled values for both straw sizes were 74 ± 7% motile sperm, 47 ± 4 s of motility duration and 69 ± 3% intact sperm. When frozen sperm were tested for their ability to fertilize oocytes, fertilization rates were higher for fresh-diluted sperm (78%) and 0.5-mL straw frozen sperm (69%) compared to 4.0-mL straw frozen sperm (42%; $P < 0.05$). Hatching rates, however, were higher for fresh sperm (73%) and 4.0-mL straw frozen sperm (77%), compared to 0.5-mL straw frozen sperm (61%, $P < 0.05$; Table 4).

Egg quality was different among females ($P < 0.05$; Table 4). Using fresh sperm as a reference, fertilization and hatching rates were significantly higher for Female Number 1 (89% fertility and 85% hatching) compared to Female Number 2 (65% fertility and 68% hatching).

Table 1
Extender (composition and osmolality), cryoprotectant (DMSO and MG) and equilibration time on post-thaw motility, duration of motility and vitality (mean ± SEM; n = 8 males × 3 replicate straws) of *Brycon opalinus* sperm (Experiment 1).

Extender		Equilibration time*			
Composition*	mOsm/kg	15 min		30 min	
		DMSO	MG	DMSO	MG
		Motility (% motile sperm)			
NaCl	325	49 ± 5†	21 ± 5	45 ± 3†	25 ± 3
NaCl	365	78 ± 3†‡	68 ± 6‡	77 ± 3‡	70 ± 3‡
Glucose	325	28 ± 2†	18 ± 3	28 ± 6	42 ± 5†
Glucose	365	61 ± 4‡	86 ± 2†‡	54 ± 3‡	88 ± 1†‡
		Duration of motility (s)			
NaCl	325	36 ± 4	30 ± 4	36 ± 3	30 ± 5
NaCl	365	40 ± 2	46 ± 5‡	46 ± 4‡	49 ± 4‡
Glucose	325	13 ± 5	10 ± 3	17 ± 3	28 ± 4†
Glucose	365	42 ± 4‡	51 ± 3‡	37 ± 3‡	52 ± 4†‡
		Vitality (% intact sperm)			
NaCl	325	42 ± 5†	23 ± 1	43 ± 5†	28 ± 6
NaCl	365	83 ± 1†‡	65 ± 8‡	79 ± 2†‡	68 ± 9‡
Glucose	325	30 ± 6	24 ± 3	34 ± 3	47 ± 6†
Glucose	365	57 ± 3‡	81 ± 3†‡	52 ± 10‡	84 ± 4†‡

DMSO, dimethyl sulfoxide; MG, methyl glycol.
* Extender composition and equilibration time did not affect ($P > 0.05$) motility, duration of motility, or vitality.
† Differences ($P < 0.05$) between cryoprotectants for equal equilibration times.
‡ Differences ($P < 0.05$) between extender osmolalities for equal extender compositions.

4. Discussion

In the present study, eight freezing media, comprising various combinations of extender compositions, osmolalities and CPAs, two equilibration times, two straw sizes and two thawing temperatures, were tested regarding their capacity for protecting *B. opalinus*

Table 2
Extender composition, cryoprotectant, straw size and thawing temperature on post-thaw motility, duration of motility and vitality (mean ± SEM; n = 7 males × 3 replicate straws) of *Brycon opalinus* (Experiment 2).

Extender Composition mOsm/kg	Cryoprotectant	Thawing temperature			
		30 °C		60 °C	
		0.5-mL straw	4.0-mL straw	0.5-mL straw	4.0-mL straw
		Motility (% motile sperm)*			
NaCl-365	DMSO	52 ± 8‡	64 ± 4†‡	72 ± 4‡	72 ± 3‡
NaCl-365	MG	26 ± 5	46 ± 6†	51 ± 4	51 ± 3
Glucose-365	DMSO	69 ± 5†	58 ± 9	67 ± 3	64 ± 3
Glucose-365	MG	75 ± 2‡	69 ± 3‡	82 ± 2‡	80 ± 3‡
		Duration of motility (s)*			
NaCl-365	DMSO	35 ± 6‡	37 ± 5	41 ± 5	41 ± 3
NaCl-365	MG	17 ± 5	38 ± 6†	40 ± 4	45 ± 5
Glucose-365	DMSO	36 ± 4	26 ± 6	45 ± 3	41 ± 4
Glucose-365	MG	50 ± 6‡	41 ± 6‡	48 ± 3	53 ± 2‡
		Vitality (% intact sperm)*			
NaCl-365	DMSO	47 ± 11‡	57 ± 8†	68 ± 9‡	71 ± 4‡
NaCl-365	MG	24 ± 3	43 ± 2†	47 ± 6	48 ± 5
Glucose-365	DMSO	68 ± 1†	57 ± 4	65 ± 2	65 ± 2
Glucose-365	MG	78 ± 2‡	72 ± 9‡	82 ± 1‡	80 ± 1‡

DMSO, dimethyl sulfoxide; MG, methyl glycol.
* Motility, duration of motility and vitality were higher ($P < 0.05$) when straws were frozen in glucose compared to NaCl, and thawed in a water bath at 60 versus 30°C.
† Differences ($P < 0.05$) between straw sizes for equal thawing temperatures.
‡ Differences ($P < 0.05$) between cryoprotectants for equal extender compositions.

Table 3
Prefreezing (equilibrated-diluted) and post-thaw motility, duration of motility and vitality (mean ± SEM; n = 4 males × 3 replicate straws) of *Brycon opalinus* sperm cryopreserved in Glucose 365 mOsm/kg, methyl glycol and two straw sizes. As control, fresh sperm of another male diluted in Glucose 365 mOsm/kg was used (Experiment 3).

Sperm	Motility (% motile sperm)	Duration of motility (s)	Vitality (% intact sperm)
Fresh-diluted control	100[a]	120[a]	95[a]
Prefreezing (equilibrated-diluted) control	95 ± 6[a]	117 ± 9[a]	92 ± 5[a]
Post-thaw 0.5-mL straw	75 ± 3[b]	47 ± 3[b]	70 ± 3[b]
Post-thaw 4.0-mL straw	73 ± 5[b]	47 ± 3[b]	68 ± 3[b]

[ab] within a row, means without a common superscript differed ($P < 0.05$).

sperm during the freezing and thawing processes with regards to effects on motility, duration of motility and vitality. Furthermore, sperm frozen and thawed using the best freezing method developed here, produced hatching rates similar to fresh control sperm. This was apparently the first report on cryopreservation of *B. opalinus* sperm.

4.1. Fresh semen characteristics and evaluation methods

Semen characteristics, such as sperm motility, volume and concentration, are important for assessment of sperm production capacity of a species or individual. In the present study, mean motility (97% motile sperm with 97 s of duration), semen volume (8 mL) and concentration (41×10^9 sperm/mL) determined in *B. opalinus* after treatment with pituitary extract, were within the range previously reported for this species [8].

Fish sperm quality has usually been based on evaluation of motility by observation under light a microscope. Although, this method of evaluation is fast and practical, it may not represent actual sperm quality. Thus, to have a better overview of sperm quality of *B. opalinus*, not only motility (as the percentage of motile sperm), but also vitality (percentage of sperm with an intact membrane) were evaluated. Sperm motility was always positively correlated with vitality, not only in fresh sperm, but also in frozen-thawed sperm. Thus, the percentage of motile sperm actually represented the percentage of intact/live sperm. A similar correlation between sperm motility and vitality was reported in *B. orbignyanus* [5] and *B. insignis* [7].

Duration of motility is another important measure of sperm quality, as the oocyte micropyle remains opened for only few seconds to 1 min after reaching the environment in which fertilization occurs. In the present study, fresh-diluted control sperm were motile for 120 s, prefreezing (equilibrated-diluted) control for 117 s, and post-thaw sperm for 47 s. Although the duration of sperm motility significantly decreased after thawing, post-thaw sperm were able to find and enter the micropyle and fertilize the oocyte during the 47 s of motility. In our previous study with another Bryconinae, *B. insignis*, several sperm were observed on the opening of the micropyle and more than one inside the micropylar canal 20 s after water was added [18]. Therefore, we inferred that sperm of Bryconinae species need only few seconds (perhaps <20 s) of motility to fertilize an oocyte.

4.2. Extenders

In the present study, glucose and NaCl solutions at both 325 and 365 mOsm/kg were tested as extender of *B. opalinus* sperm. Sperm frozen in both solutions at 365 mOsm/kg were consistently of better quality than

Table 4
Fertilization (expressed as the percentage of total oocytes; mean ± SEM) and hatching rate (expressed as the percentage of fertilized eggs; mean ± SEM) of *Brycon opalinus* sperm (n = 3 replicate straws × 4 males × 4 females), cryopreserved in Glucose 365 mOsm/kg, methyl glycol and 0.5- or 4.0-mL straws. As control, fresh sperm of another male diluted in Glucose 365 mOsm/kg was used (Experiment 3).

Female ID	Fertilization rate (%)			Hatching rate (%)		
	Fresh sperm	0.5-mL straw	4.0-mL straw	Fresh sperm	0.5-mL straw	4.0-mL straw
1	89[A]	86 ± 1[A]	75 ± 2[A]	85[A]	77 ± 3[A]	59 ± 3[C]
2	65[B]	53 ± 5[C]	41 ± 1[B]	68[B]	47 ± 4[C]	85 ± 2[A]
3	86[A]	71 ± 4[B]	31 ± 7[B]	66[B]	54 ± 5[B]	90 ± 5[A]
4	71[B]	68 ± 5[B]	32 ± 8[B]	75[B]	65 ± 8[B]	72 ± 8[B]
Mean	78 ± 6[a]	69 ± 4[a]	42 ± 7[b]	73 ± 4[a]	61 ± 6[b]	77 ± 7[a]

[A-C;ab] within a row (lowercase) or column (uppercase), means without a common superscript differed ($P < 0.05$).

at 325 mOsm/kg. In our previous study, seminal plasma osmolality of *B. opalinus* ranged from 297 to 340 mOsm/kg and initiation of fresh sperm motility was suppressed when the surrounding medium was ≥ 325 mOsm/kg [8]. To confer protection during freezing and thawing, perhaps, extenders should be at a higher osmolality, e.g., 365 mOsm/kg. In most studies carried out with Characiformes sperm, 5% glucose (~277 mOsm/kg) and 0.9% NaCl (~285 mOsm/kg) are commonly used as extenders, not only because of their simple compositions, but also because they are commercially available [10]. However, according to our results, the osmolalities of these commercial extenders may not be adequate to protect sperm from injuries induced by freezing and thawing.

4.3. Cryoprotectants

Cryoprotectants did not affect post-thaw sperm quality of *B. opalinus*, but there was a strong interaction between extenders and cryoprotectants after both 15 and 30 min of equilibration. Although DMSO was a better cryoprotectant than MG when combined with NaCl, MG was a better cryoprotectant than DMSO when combined with glucose. Since DMSO and MG have similar molar concentrations (approximately 78 and 76 g/mol, respectively), this extender-cryoprotectant interaction was probably not related to cellular permeation. However, it is unclear whether cryoprotectant has to permeate a cell to be protective [19]. Although some studies suggested that equilibration is not necessary [20], sperm of some Characiformes species cryopreserved immediately after dilution (without equilibration time) yielded very low post-thaw motility (unpublished data), possibly because this fast exposure was not long enough for the cryoprotectant to permeate and protect these cells. Conversely, excessive contact of sperm with cryoprotectant before cryopreservation can be toxic. Typically, 10 to 20 min of equilibration times is used for fish sperm [21].

4.4. Cryopreservation methods

In this study, two straw sizes and two thawing temperatures were tested. Sperm thawed in a water bath at 60 °C had higher quality compared to sperm thawed at 30 °C. Although the temperature of 60 °C seemed too high to thaw 0.5-mL straws, this thawing method has been used in our laboratory for several Characiformes species with great success [7,12,15].

Post-thaw sperm motility, duration of motility or vitality of *B. opalinus* were not affected by the use of 0.5- or 4-mL straws. When frozen sperm were tested for fertilization ability, there was no significant difference in fertilization rates between fresh-diluted sperm (78%) and 0.5-mL straw frozen sperm (69%), indicating that post-thaw sperm was able to fertilize as many oocytes as fresh sperm. Hatching rates relative to the number of fertilized eggs were similar between fresh-diluted sperm (73%) and 4.0-mL straw frozen sperm (77%), suggesting that, although fertilization rate was lower for frozen sperm, those eggs that actually were fertilized, developed normally up to hatching. Similarly, no difference on sperm motility was reported for sperm cryopreserved in 0.5- and 4.0-mL straws of *B. amazonicus* [22], *B. nattererii* [15], and *P. lineatus* [12]. The 0.5-mL straws are extensively used in fish sperm cryopreservation experiments due to their low cost, as many replicates are needed, and sometimes because sperm volume is low [12]. In practice, to fertilize oocytes of only one female, many 0.5-mL straws are needed. Thus, the use of larger 4.0-mL straws enables fertilization of more oocytes with fewer straws.

5. Conclusions

An effective method for sperm cryopreservation of *B. opalinus* was achieved. Based on the present study, sperm should be frozen in Glucose 365 mOsm/kg and methyl glycol, after a 15 or 30-min equilibration time, in 0.5- or 4-mL straws, and thawed at 60 °C for 8 s. This method can be used to maximize larvae production for both aquaculture and conservational, since *B. opalinus* is a threatened species.

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