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Sperm cryopreservation of tiete tetra *Brycon insignis* (Characiformes): effects of cryoprotectants, extenders, thawing temperatures and activating agents on motility features

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Abstract

The aim of this study was to test the effects of cryoprotectants [dimethyl sulphoxide (DMSO) and methylglycol], extenders (0.9% NaCl, 5% glucose, Beltsville Thawing Solution™ and Merck III™), thawing temperatures (30 and 60 °C) and activating agents (0.29% NaCl and 1% $NaHCO_3$) on the cryopreservation process of tiete tetra *Brycon insignis* sperm. Sperm was loaded in 0.5 mL straws, frozen in nitrogen vapour at − 170 °C and stored in liquid nitrogen. Post-thaw sperm quality was evaluated in terms of subjective motility rate, quality motility score (0 = no movement; 5 = rapidly swimming spermatozoa), duration of motility and vitality (eosin–nigrosin staining). Post-thaw sperm motility rate was greater in methylglycol (76–88%), compared with DMSO (23–59%). In general, the highest quality motility scores were observed when sperm was thawed at 30 °C and triggered in 1% $NaHCO_3$ (3.5–4.3). Duration of motility was longer when triggered in 1% $NaHCO_3$ (95–120 s) compared with 0.29% NaCl (69–107 s). Sperm vitality was not affected by any of the parameters tested and varied from 51% to 69% intact sperm. *Brycon insignis* sperm frozen in methylglycol combined with any of the extenders tested and using the methods described above yields motility above 57% and that should last long enough to fertilize oocytes.

Keywords: fish, semen, motility, duration of motility, vitality

Introduction

Many South American fish species migrate upstream to find clean water and spawn. This process is known as *piracema* and occurs when the environment is adequate to stimulate the fish reproductive physiology, such as hormonal stimulus (Godinho & Godinho 1994). Changes in the river stream, overfishing, urbanization, pollution and hydroelectric dams are some of the reasons why the status of many migratory fish species is set as endangered. The genus *Brycon* is highly affected by these environmental changes, and many species are in the red list of Brazilian threatened fauna, such as tiete tetra *Brycon insignis*, pirapitinga *Brycon nattereri*, pirapitinga-do-sul *Brycon opalinus* and piracanjuba *Brycon orbignyanus* (Rosa & Lima 2008).

The *B. insignis* (Steindachner 1877), known as tiete tetra in English and as piabanha in Portuguese, is a teleost fish member of the order Characiformes, family Characidae and subfamily Bryconinae. This is an endemic Brazilian freshwater species, native to the Paraíba do Sul River Basin in the southeastern Brazil (Bizerril 1999). In the past, *B. insignis* was an important fishery resource for both commercial and sportive fishing (Machado & Abreu 1952; Shimoda 2004), but nowadays this species is threatened in nature and there is very little information about its reproductive behaviour. Knowledge of reproductive biology and physiology is critical for conservation and species management, allowing the

prevention of species extinction and the utilization of males in natural and artificial reproductive programmes (Wildt 2005).

The cryopreservation of fish sperm provides a tool by which reproduction is optimized during the reproductive period. Cryopreserved sperm serves as genetic bank or germplasm, which may help to ensure genetic diversity and reproductive success for population management strategies.

In the past few years, some studies on fish sperm cryopreservation of species of the subfamily Bryconinae, such as matrinxã or yamú *Brycon amazonicus* (Cruz-Casallas, Medina-Robles & Velasco-Santamaria 2006; Ninhaus-Silveira, Veríssimo-Silveira, Senhorini, Alexandre & Chaguri 2006; Velasco-Santamaria, Medina-Robles & Cruz-Casallas 2006), matrinxã *Brycon cephalus* (Ninhaus-Silveira, Foresti, Veríssimo-Silveira & Senhorini 2006), *B. orbignyanus* (Maria, Viveiros, Freitas & Oliveira 2006; Maria, Viveiros, Orfão, Oliveira & Moraes 2006), matrinchã *Brycon orthotaenia* (Melo & Godinho 2006) and *B. nattereri* (Oliveira, Viveiros, Maria, Freitas & Isaú 2007) were published showing effective protocols. In *B. insignis*, only one PhD thesis (Shimoda 2004) and no published article was found after searching in the web (Google, PubMed, Scopus and Web of Science). In general, the typical combination of glucose, egg yolk and dimethyl sulphoxide (DMSO) is used as the freezing medium of Bryconinae sperm (Shimoda 2004; Cruz-Casallas *et al.* 2006; Ninhaus-Silveira, Foresti *et al.* 2006; Ninhaus-Silveira, Veríssimo-Silveira *et al.* 2006; Melo & Godinho 2006; Velasco-Santamaria *et al.* 2006). More recently, the use of a boar sperm extender Beltsville Thawing Solution (BTS™; Minitüb™) combined with methylglycol has yielded greater results (Maria, Viveiros, Freitas *et al.* 2006; Maria, Viveiros, Orfão *et al.* 2006; Oliveira *et al.* 2007). Beltsville Thawing Solution™ and methylglycol have never been tested in *B. insignis*. Bryconinae sperm is mostly frozen in 0.5 mL straws in vapour nitrogen vessel (dry shipper) and thawed in a water bath at 30 to 60 °C. Post-thaw sperm quality is mostly evaluated in terms of progressive motility rate using a light microscope and it ranges from 33% to 39% in *B. amazonicus* (Velasco-Santamaria *et al.* 2006) up to 72% in *B. nattereri* (Oliveira *et al.* 2007). As sperm of fish of the subfamily Bryconinae is immotile in seminal plasma as most of the teleosts, motility is triggered in hyposmotic activating agent, mostly 0.29% NaCl or 1% $NaHCO_3$ (Viveiros & Godinho 2009). Because of the difficulty in obtaining high-quality eggs in this subfamily, only few studies tested post-thaw sperm for fertilization. In *B. insignis*, artificial reproduction using fresh sperm yields hatching rates that varies from 0% to 85% (Andrade-Talmelli, Kavamoto, Narahara & Fenerich-Verani 2002) and is frequently below 30% (D. Caneppele, pers. obs.).

The aim of this study was to test the effects of two cryoprotectant agents (DMSO and methylglycol) combined with four different extenders (0.9% NaCl, 5% glucose, BTS™ and Merck III [M III™]), two thawing temperatures (30 and 60 °C) and two activating agents (0.29% NaCl and 1% $NaHCO_3$) on the cryopreservation process of tiete tetra *B. insignis* sperm. Some fresh sperm features are also presented.

Materials and methods

Fish handling and sperm collection

All fish were handled in compliance with the guidelines for animal experimentation described in Van Zutphen, Baumans and Beynen (1993). Tiete tetra *B. insignis* males of approximately 4–5 years of age (296 ± 28 g) were selected from circular tanks at the Hydrobiology and Aquaculture Station of the Hydroelectric Company of São Paulo (CESP) in Paraibuna city (23°23′10″S; 45°39′44″W), São Paulo state, Brazil, during the spawning season (January and February). Males with detectable running sperm under soft abdominal pressure received a single intramuscular dose of carp pituitary extract (Argent Chemical Laboratory, Redmond, WA, USA) at 3 mg kg^{-1} of body weight. After 8 h at ∼ 25 °C, the urogenital papilla was carefully dried and sperm was hand-stripped directly into test tubes. Sperm collection was carried out at room temperature (23–25 °C), and soon after collection, the tubes containing sperm were placed in a Styrofoam box containing crushed ice (5 ± 2 °C). Contamination of sperm with water, urine or faeces was carefully avoided.

Determination of fresh sperm quality parameters

Immediately after collection, 5 μL of each sample was placed on a slide and observed under a light microscope (model L1000, Bioval, Jiangbei, China) at 400 × magnification. Any sperm motility was considered to be premature induction of motility, resulting from urine or water contamination and the sample was discarded, as fish sperm in seminal plasma should be immotile. In samples thus selected ($n = 18$ males), sperm motility was subjectively estimated following the addition of 25 μL of activating

agent composed of 0.29% NaCl (50 mM), following the methodology used as routine in our laboratory for Characiformes (Maria, Viveiros, Freitas *et al.* 2006; Maria, Viveiros, Orfão *et al.* 2006; Oliveira *et al.* 2007; Viveiros, Orfão, Maria & Allaman 2009; Nascimento, Maria, Pessoa, Carvalho & Viveiros 2010; Viveiros, Nascimento, Orfão & Isaú 2010). Motility rate was scored as the percentage of motile spermatozoa showing progressive motility, in increments of 10%. All sperm samples possessed at least 80% motility and were thus used for subsequent analyses. Qualitative motility scores were assigned ranging from 0 (no movement) to 5 (rapidly swimming spermatozoa), according to Mounib, Hwang and Idler (1968). Sperm volume, concentration (Hemacytometer Neubauer chamber, Boeco, Hamburg, Germany) and osmolality (Semi-Micro Osmometer K-7400, Knauer, Berlin, Germany) were also determined.

Table 1 Chemical composition of the media tested as sperm extenders of tiete tetra *Brycon insignis*

	Extenders			
Chemicals (g)*	0.9% NaCl	5% Glucose	BTS™†	M III™†
NaCl	0.9	–	–	–
Glucose	–	5.0	4.00	5.34
$NaHCO_3$	–	–	0.13	0.25
KCl	–	–	0.08	–
Sodium citrate	–	–	0.63	0.18
EDTA	–	–	0.13	0.18
Gentamycin sulphate	–	–	0.02	0.03
Osmolality (mOsmol kg^{-1})	285	308	356	367

*All chemicals were diluted in deionized water to a final volume of 100 mL.
†Donated by Minitüb™.
BTS™, Beltsville Thawing Solution™; M III™, Merck III™.

Experiment 1 – cryoprotectants and extenders

Eight freezing media, comprising combinations of two cryoprotectants and four extenders were selected from our previous studies with other species of the subfamily Bryconinae (Maria, Viveiros, Freitas *et al.* 2006; Maria, Viveiros, Orfão *et al.* 2006; Oliveira *et al.* 2007). The cryoprotectants tested were DMSO and methylglycol, both obtained from Vetec Química Fina™ (Duque de Caxias, RJ, Brazil). The extenders 0.9% NaCl, 5% glucose, 5% BTS™ and 6% M III™ were prepared using deionized water, adjusted to a pH of 7.6 (Viveiros, Isaú, Figueiredo, Leite & Maria 2010) and maintained in crushed ice (~ 5 °C). Beltsville Thawing Solution™ and M III™ are commercial extenders for boar sperm (Minitüb™, Tiefenbach/Landshut, Germany). For extender composition, please refer to Table 1. Sperm ($n = 10$ males) was diluted in each freezing media to a final proportion (v/v) of 10% sperm, 10% cryoprotectant and 80% extender, and loaded into 0.5 mL straws ($n = 3$ replicate straws). Straws were sealed with plastic sphere and immediately frozen in a nitrogen vapour vessel (dry vapour shipper; YDH-8, Chengdu Golden Phoenix Liquid Nitrogen Container, Chengdu, Sichuan, China) at approximately − 170 °C. Using this methodology, straws reach − 170 °C after ~ 329 s in a freezing rate of approximately − 35.6 °C min^{-1} between + 21 and − 170 °C (Maria, Viveiros, Freitas *et al.* 2006). Then, straws were transferred to liquid nitrogen (cryogenic tank YDS-20; Chengdu Golden Phoenix Liquid Nitrogen Container, Chengdu, Sichuan, China) at − 196 °C within 20–24 h for storage.

After a few days, straws were thawed in a 60 °C water bath for 8 s (Maria, Viveiros, Freitas *et al.* 2006; Maria, Viveiros, Orfão *et al.* 2006; Oliveira *et al.* 2007), and post-thaw sperm motility rate was immediately estimated as described for fresh sperm. To trigger motility, 0.29% NaCl was used as an activating agent.

Experiment 2 – extenders, thawing temperatures and activating agents

Sperm ($n = 8$ males) was diluted in methylglycol combined with the same four extenders (0.9% NaCl, 5% glucose, 5% BTS™ and 6% M III™) and frozen following the same methodology described in Experiment 1. After a few days at − 196 °C, straws were thawed in a water bath at two different temperatures: 60 °C for 8 s and 30 °C for 16 s. Post-thaw sperm was evaluated for motility rate, quality motility score, duration of motility and vitality. To evaluate motility features, two activating agents were tested: 0.29% NaCl (50 mM; 92 mOsmol kg^{-1}) and 1% $NaHCO_3$ (119 mM; 184 mOsmol kg^{-1}). The duration of sperm motility was assessed on the same glass slide as the motility rate and quality motility score, using a stopwatch that was started when the activating agent was mixed with the sperm sample and stopped when only 10% of the spermatozoa were still moving.

To determine whether the subjective motility test would be accurate and represent the actual percentage of spermatozoa showing intact membrane (so-called live sperm) in a sample, motility rate was evaluated as described in Experiment 1 and

compared with an objective test in that vitality was determined following eosin–nigrosin staining (5% B eosin, 10% nigrosin, pH = 6.9; Sigma-Aldrich, Steinheim, Germany). 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 widely used since Blom (1950). A total of 100 spermatozoa per slide (1 slide = 1 straw) were counted on a light microscope (model L1000; Bioval, Jiangbei, China) at a magnification of × 1000. Vitality was calculated as the percentage of unstained or light pink cells, representing spermatozoa with an intact membrane.

Statistical analysis

Values are reported as means ± standard deviation (SD). Statistical analyses were conducted with the software R, version 2.7.1 (R Development Core Team 2008). In all experiments, data were tested for normal distribution using the univariate procedure. If data did not fit the normal distribution, a Box and Cox (1964) transformation was performed. Data were tested for significant differences by analysis of variance (ANOVA), followed by the Scott–Knott test when applicable. The level of significance for all statistical tests was set at 0.05.

Results

Fresh sperm of the 18 males used in this study possessed a mean of 98% motility rate, score 4.7 of motility quality, volume of 3.9 mL, 17 × 10^9 spermatozoa mL^{-1} and 350 mOsmol kg^{-1} (Table 2).

Experiment 1 – cryoprotectants and extenders

Post-thaw motility rate of sperm cryopreserved in eight freezing media is depicted on Table 3. There was a significant effect of cryoprotectant on motility rate. All sperm samples cryopreserved in methylglycol yielded greater motility rates (77–82%) compared with DMSO (23–46%), regardless of the extender.

Experiment 2 – extenders, thawing temperatures and activating agents

Sperm quality after cryopreservation in four extenders, thawing at 30 or 60 °C and activated in 0.29% NaCl or 1% $NaHCO_3$ is depicted on motility rate, quality motility score and duration of motility (Table 4) and vitality (Table 5).

Post-thaw sperm motility rate was not affected by extenders, thawing temperatures or activation media, and ranged from 57% to 74%.

Post-thaw quality motility score was not affected by extenders and ranged from 2.3 to 4.3. In general, motility scores were higher when straws were thawed at 30 °C and motility was triggered in 1% $NaHCO_3$.

Post-thaw duration of motility was not affected by extenders or thawing temperatures. The activating agent, however, affected the duration of motility. All sperm samples triggered in 1% $NaHCO_3$ were motile for a longer period (95–120 s) compared with samples triggered in 0.29% NaCl (69–107 s).

Post-thaw vitality was not affected by extenders or thawing temperatures, and ranged from 51% to 69% intact sperm. Sperm thawed at 60 °C, however,

Table 2 Body weight and fresh sperm features of tiete tetra *Brycon insignis* after carp pituitary treatment

Features	Number of males	Mean ± SD	Minimum–maximum
Body weight (g)	18	296 ± 28	230–305
Sperm motility rate (%)	18	98 ± 5	80–100
Quality motility score (0–5)*	8	4.7 ± 0.5	4–5
Volume (mL)	8	3.9 ± 0.7	2.5–5.0
Spermatozoa × 10^9 mL^{-1}	8	17 ± 7	6–34
Osmolality (mOsmoL kg^{-1})	8	350 ± 13	332–375

*Qualitative motility scores were assigned ranging from 0 (no movement) to 5 (rapidly swimming spermatozoa).

Table 3 Motility rate (mean ± SD; $n = 3$ replicate straws × 10 males) of tiete tetra *Brycon insignis* sperm cryopreserved in eight freezing media, comprising combinations of two cryoprotectants (DMSO and methylglycol) and four extenders

	Sperm motility rate (%)	
Extender	DMSO	Methylglycol
0.9% NaCl	46 ± 17^{Ab}	82 ± 15^{Aa}
5% Glucose	45 ± 23^{Ab}	77 ± 17^{Aa}
BTS™	23 ± 17^{Bb}	77 ± 9^{Aa}
M III™	43 ± 21^{Ab}	77 ± 14^{Aa}

Motility was triggered in 0.29% NaCl.
For extender formulae, please refer to Table 1.
Means followed by different superscripts (lowercase for lines and uppercase for columns) are different (Scott–Knott test; $P < 0.05$).
BTS™, Beltsville Thawing Solution™; M III™, Merck III™; DMSO, dimethyl sulphoxide.

Table 4 Motility rate, quality motility score and duration of motility (mean ± SD; $n = 3$ replicate straws × 8 males) of tiete tetra *Brycon insignis* sperm cryopreserved in methylglycol combined with four different extenders and thawed at 30 or 60 °C

		Activating agent	
Extender	**Thawing**	**0.29% NaCl**	**1% $NaHCO_3$**
Sperm motility rate (%)			
0.9% NaCl	30 °C for 16 s	70 ± 19	74 ± 13
5% Glucose		63 ± 23	70 ± 21
BTS™		57 ± 27	67 ± 20
M III™		61 ± 17	66 ± 21
0.9% NaCl	60 °C for 8 s	61 ± 24	59 ± 27
5% Glucose		68 ± 19	71 ± 14
BTS™		62 ± 24	59 ± 25
M III™		60 ± 28	66 ± 20
Quality motility score (0–5)*			
0.9% NaCl	30 °C for 16 s	3.2 ± 1.0^{Aa}	3.8 ± 0.6^{Aa}
5% Glucose		3.3 ± 1.3^{Ab}	4.3 ± 0.9^{Aa}
BTS™		2.3 ± 1.0^{Bb}	3.7 ± 0.9^{Aa}
M III™		2.5 ± 1.1^{Bb}	3.5 ± 0.8^{Aa}
0.9% NaCl	60 °C for 8 s	2.6 ± 0.6^{Bb}	4.0 ± 0.7^{Aa}
5% Glucose		3.3 ± 0.8^{Aa}	2.7 ± 1.1^{Ba}
BTS™		2.6 ± 0.6^{Ba}	3.0 ± 1.0^{Ba}
M III™		2.5 ± 0.7^{Bb}	3.1 ± 1.1^{Ba}
Duration of motility (s)			
0.9% NaCl	30 °C for 16 s	86 ± 24^{Bb}	116 ± 13^{Aa}
5% Glucose		107 ± 23^{Ab}	120 ± 0^{Aa}
BTS™		85 ± 37^{Bb}	113 ± 17^{Aa}
M III™		84 ± 24^{Bb}	113 ± 20^{Aa}
0.9% NaCl	60 °C for 8 s	69 ± 26^{Cb}	95 ± 36^{Ba}
5% Glucose		83 ± 23^{Bb}	114 ± 15^{Aa}
BTS™		79 ± 25^{Bb}	107 ± 26^{Aa}
M III™		87 ± 31^{Bb}	109 ± 18^{Aa}

*Qualitative motility scores were assigned ranging from 0 (no movement) to 5 (rapidly swimming spermatozoa).
For extender formulae, please refer to Table 1.
Means followed by different superscripts (lowercase for lines and uppercase for columns within the same sperm feature) are different (Scott–Knott test; $P < 0.05$).
Motility was triggered in 0.29% NaCl or 1% $NaHCO_3$.

possessed a more homogeneous data as observed by the lower SD, compared with samples thawed at 30 °C.

There was no difference ($P > 0.05$) on data obtained from the subjective motility test (57–74% motile sperm) when compared with the objective vitality test (51–69% intact sperm).

Discussion

This is the first published study on the cryopreservation process of *B. insignis* sperm. Post-thaw sperm quality was initially evaluated on the basis of sperm motility and then confirmed with the analysis of quality motility score, duration of motility and vitality.

Table 5 Vitality (mean ± SD; $n = 3$ replicate straws × 8 males) of tiete tetra *Brycon insignis* sperm cryopreserved in methylglycol combined with four different extenders, and thawed at 30 or 60 °C

	Vitality (% intact sperm)	
Extender	**30 °C for 16 s**	**60 °C for 8 s**
0.9% NaCl	64 ± 23	61 ± 13
5% Glucose	51 ± 33	69 ± 12
BTS™	57 ± 23	62 ± 12
M III™	54 ± 26	63 ± 11

For extender formulae, please refer to Table 1.
There was no significant difference of extender or thawing temperature on sperm vitality ($P > 0.05$); vitality was evaluated after eosin–nigrosin staining, and was expressed as percentage of spermatozoa with intact membrane (unstained cells).

Fresh sperm features

Fresh sperm of *B. insignis* was assessed after hormonal treatment with carp pituitary extract. Sperm motility rate (98%), quality motility score (4.7), volume (3.9 mL), concentration (17×10^9 spermatozoa mL^{-1}) and osmolality (350 mOsmol kg^{-1}) for the males utilized in this study were all within the range observed for fresh sperm of other species of the subfamily Bryconinae (Viveiros & Godinho 2009). A better knowledge of the characteristics of fresh sperm motility is necessary to evaluate sperm quality in commercial hatcheries before artificial reproduction and in laboratories before experiments. Preliminary examination of fresh sperm is often carried out in order to discard contaminated samples exhibiting premature induction of motility due to contamination with water or urine (Viveiros & Godinho 2009).

Motility and vitality

The percentage of motile sperm subjectively evaluated under light microscope and the percentage of sperm showing intact membrane after eosin–nigrosin staining were not affected by extender composition, thawing temperatures or activating agents (Experiment 2). Furthermore, post-thaw sperm yielded 57–74% motile sperm, which was similar ($P > 0.05$) to 51–69% intact sperm. The subjective

evaluation of sperm motility is very practical and can be conducted by well-trained personnel in commercial fish farms as an acceptable evaluation of sperm quality, because motility rates evaluated here actually represented the percentage of sperm showing an intact membrane. Similar results were obtained in another Bryconinae, *B. orbignyanus*, when sperm motility and vitality were compared (Maria, Viveiros, Freitas *et al.* 2006).

Cryoprotectants

The two cryoprotectants tested here had a significant effect on the post-thaw sperm motility. All samples cryopreserved in methylglycol yielded greater motility rate (77–82%) compared with DMSO (23–46%). It is interesting to point out that the combination glucose–DMSO supplemented with 5–10% egg yolk has been extensively used as a freezing medium of characiforms sperm (Viveiros & Godinho 2009). However, the addition of egg yolk to the medium interferes with the good visualization of spermatozoa during the motility rate analysis. With this in mind, we have been testing several extender–cryoprotectant combinations without the addition of egg yolk that preserve sperm during storage and yet allow good visualization during motility analysis. Our research group compared the use of glucose combined with DMSO or methylglycol as freezing medium of some characiforms: *B. insignis* (this study), *B. nattereri* (Oliveira *et al.* 2007), streaked prochilod *Prochilodus lineatus* (Viveiros *et al.* 2009) and pirapitinga *Piaractus brachypomus* (Nascimento *et al.* 2010). In all studies, post-thaw sperm quality was significantly higher when glucose was combined with methylglycol compared with DMSO. Based on these studies, it appears that DMSO–egg yolk can be successfully replaced by the use of methylglycol. Methylglycol, also known as 2-methoxyethanol or ethylene glycol monomethyl ether, is derived from methanol (CH_3OH) and ethene oxide (CH_2OCH_2). Methylglycol is relatively nontoxic and was initially used as cryoprotectant for bovine embryos (Takagi, Boediono, Saha & Suzuki 1993).

Extenders

The four extenders tested here (0.9% NaCl, 5% glucose, BTS™ and M III™) did not affect post-thaw motility rate, quality motility score, duration of motility or vitality. Glucose is commonly used as fish sperm extender, both in simple formulae (5% glucose) and in more complex formulae (combined with egg yolk or with ions and antibiotics such as BTS™ and M III™). The addition of energetic substrates, such as glucose or lactate, to the African catfish *Clarias gariepinus* sperm was found to prevent the decrease in intracellular ATP concentration during storage (Zietara, Slominska, Rurangwa, Ollevier, Swierczynski & Skorkowski 2004). On the other hand, a recent review states that spermatozoa from species with external fertilization, such as the *B. insignis*, cannot rely on the medium to provide their energy supply (Bobe & Labbé 2009). Glucose is also added to extenders due to its stabilization effects on the spermatozoa liposomal membrane (Quinn 1985). In the present study, *B. insignis* sperm frozen in glucose-based extenders yielded post-thaw sperm quality similar to sperm frozen in glucose-free extender (0.9% NaCl). It seems that *B. insignis* sperm can cope with the freezing and thawing processes without the need for external glucose stabilizing effects. Similarly, *B. nattereri* sperm frozen in 0.9% NaCl–methylglycol yielded post-thaw motility rate (68%) comparable to sperm frozen in BTS™–methylglycol (72%; Oliveira *et al.* 2007). All of the four extenders tested here are commercially available either as sterile solution (0.9% NaCl and 5% glucose) or as powder (BTS™ and M III™) and can be used in on-farm conditions without the need for laboratory equipment.

Thawing temperatures

The thawing temperatures tested (30 and 60 °C) did not affect post-thaw motility rate, quality motility score, duration of motility or vitality. In general, the quality motility scores were of higher values when straws were thawed at 30 °C. On the other hand, data on vitality were more homogeneous (lower SD) when straws were thawed at 60 °C. Similarly, thawing temperatures did not affect post-thaw sperm motility of *B. amazonicus*; fertilization rate, however, was higher when 0.5 mL straws were thawed at 35 °C (54% fertility) compared with 80 °C (∼ 20% fertility; Velasco-Santamaria *et al.* 2006).

Activating agents

The two activating agents (0.29% NaCl and 1% $NaHCO_3$) tested did not affect post-thaw motility rates or quality motility score, although, in general, higher scores were observed when 1% $NaHCO_3$ was used. Duration of motility, however, was significantly higher

when 1% $NaHCO_3$ was used as an activating agent. Similar results were observed in another Bryconinae. *Brycon orthotaenia* sperm frozen in glucose, egg yolk and DMSO, yielded shorter duration of motility when triggered in 0.15% NaCl (30 s) compared with 1% $NaHCO_3$ (62 s; Melo & Godinho 2006). As observed for most of the freshwater species, sperm motility activation occurs when osmolality of the surrounding medium is lowered (Cosson 2004; Alavi & Cosson 2006). In Northern pike *Esox lucius*, sperm motility was triggered in media ranging from 0 to 400 mOsmol kg^{-1}. In general, the greatest percentage of motile spermatozoa and highest spermatozoa velocity were observed between 125 and 235 mOsmol kg^{-1}. When spermatozoa were exposed to distilled water (0 mOsmol kg^{-1}), an hypo-osmotic shock was observed leading to flagellar damages which in turn limited the duration of motility (Alavi, Rodina, Viveiros, Cosson, Gela, Boryshpolets & Linhart 2009). In the present study, the osmolality of the 1% $NaHCO_3$ medium was higher (184 mOsmol kg^{-1}) than the osmolality of the 0.29% NaCl medium (92 mOsmol kg^{-1}). It is possible that *B. insignis* spermatozoa suffered some sort of flagellar damage at lower osmolality, as observed for *E. lucius*, which limited flagellar movement some time after activation. Experiments specially designed to test the effects of osmolality of the activating agent on sperm motility of *B. insignis* are still needed.

Nevertheless, after activation in both 0.29% NaCl and 1% $NaHCO_3$ media, post-thaw sperm of *B. insignis* was motile for longer than 68 s. When studying the fertilization events of another Bryconinae, *B. nattereri*, we observed several spermatozoa on the opening of the micropyle and more than one inside the micropylar canal 20 s after the addition of water (Maria 2008). Based on this finding, we can hypothesize that 68 s is long enough for spermatozoa to find and enter the micropyle and fertilize oocytes in *B. insignis*.

Conclusions

Brycon insignis sperm frozen in methylglycol combined with any of the four extenders tested and using the methods described above yields very acceptable post-thaw sperm quality, with motility above 57% and lasting long enough to fertilize oocytes. The subjective evaluation of sperm motility is very practical and can be conducted by well-trained personnel in commercial fish farms as an acceptable evaluation of sperm quality, because motility rates evaluated here actually represented the percentage of sperm showing intact membrane. Fertilization trials should be carried out with cryopreserved *B. insignis* sperm so that the effectiveness of the cryopreservation process and the methods of sperm quality evaluation can be determined.

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