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Extender composition and osmolality affects post-thaw motility and velocities of piracanjuba *Brycon orbignyanus* (Valenciennes, 1850) (Characiformes) sperm

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Summary

The aim of this study was to evaluate the effects of extender composition and osmolality on post-thaw motility of *Brycon orbignyanus* sperm. Eight extenders comprising combinations of two compositions (NaCl and glucose) and four osmolalities (285, 325, 365 and 405 mOsm kg^{-1}) were tested. Methyl glycol was used as cryoprotectant. Diluted sperm was loaded into 0.25 ml straws, frozen in a nitrogen vapor vessel (*dry shipper*) and stored in a liquid nitrogen vessel. Straws were thawed in a water bath at 60°C for 3 s and sperm was immediately evaluated for motility rate and velocities (curvilinear = VCL; straight line = VSL; average path = VAP). Seminal plasma osmolality was also determined (249 mOsm kg^{-1}). Both extender composition and osmolality affected post-thaw sperm motility. In general, sperm cryopreserved in NaCl was of better motility than that in glucose, and at lower osmolalities better than at higher ones. High post-thaw quality, with motility above 60% and VCL above 140 $\mu m\ s^{-1}$, was observed only in samples frozen in NaCl at 285 mOsm kg^{-1}. Different from most of the sperm from freshwater species that need a cell membrane protector (such as sugars), *B. orbignyanus* sperm should be frozen in an ionic solution for better protection during freezing and thawing processes. Furthermore, this solution should be prepared at an osmolality just above seminal plasma osmolality. Cryopreserved sperm can be used both for aquaculture purposes and for conservational programs, since *B. orbignyanus* is a threatened species.

Introduction

The piracanjuba *Brycon orbignyanus* is a native fish species to South America and belongs to the order Characiformes, family Characidae and subfamily Bryconinae. During the spawning season (November to February), *B. orbignyanus* migrate to spawning sites. This migratory behavior is known as *piracema* and occurs when the environment is appropriate to stimulate fish reproduction (Godinho and Godinho, 1994). Alterations to the river stream, overfishing, urbanization, pollution and hydroelectric dams are some of the reasons why the status of *B. orbignyanus* is currently 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 *B. opalinus*, *B. insignis* and *B. nattereri* (Rosa and Lima, 2008). The *B. orbignyanus* exhibits fast growth in captivity and has an excellent meat quality, indicating that this species can be produced on a commercial scale and thus preventing it from extinction (Maria et al., 2006).

Most fish spermatozoa are immotile in the seminal tract (Morisawa and Suzuki, 1980), and hyposmotic media or water initiate sperm motility in freshwater fish (Alavi and Cosson, 2006). Osmolality, pH, temperature, and ion concentration affect sperm motility (Morisawa and Suzuki, 1980; 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 (also called extenders; media that suppress the initiation of sperm motility), for improving both artificial fertilization and preservation techniques (Alavi et al., 2009). Osmolality, but not the presence of ions, seems to be the key factor for the induction or suppression of fresh sperm motility in Characiformes fish species. Motility was completely suppressed when sperm was diluted in a solution at 270 mOsm kg^{-1}or higher in *B. orbignyanus*, or at 360 mOsm kg^{-1} or higher in *Prochilodus lineatus*, regardless of extender composition (NaCl, glucose or BTS™; Gonçalves et al., 2013). In frozen sperm, however, detailed information on the effects of extender osmolality on post-thaw sperm quality in Characiformes species is not yet available, but the presence of ions affects post-thaw quality, and this occurs differently among fish species. Sperm of *B. nattereri* (Oliveira et al., 2007) can be successfully frozen in ionic solution, while sperm of *B. opalinus* (Viveiros et al., 2012), of *Salminus brasiliensis* (Viveiros et al., 2009a) and of *P. lineatus* (Viveiros et al., 2009b) are better frozen in sugar solutions. However, sperm of *Piaractus mesopotamicus* (Orfão et al., 2010) needs a combination of both ions and sugar (BTS™) for better protection during the freezing and thawing processes.

The aim of this study was to evaluate the effects of extender composition (ionic and sugar extenders) and osmolality on post-thaw motility and velocities of piracanjuba *Brycon orbignyanus* sperm, using a Computer-Assisted Sperm Analyzer (CASA).

Materials and methods

Fish handling and sperm collection

All fish were handled in compliance with the guidelines for animal experimentation described by Van Zutphen et al.

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(2001). The *B. orbignyanus* males were selected from earthen ponds at the Fish Culture Station of the Minas Gerais Power Company (CEMIG) in the city of Itutinga (21°17′36″S; 44°37′02″W), State of Minas Gerais, Brazil, during the spawning season (December to February). Males (n = 10, 2.1 kg^{-1} BW) with detectable running sperm under soft abdominal pressure received a single intramuscular dose of carp pituitary extract (cPE; Argent Chemical Laboratories, Redmond, WA; 1 mg kg^{-1} BW). After 8–10 h at 27–28°C, the urogenital papilla was carefully dried, and approximately 10 ml sperm were hand-stripped directly into glass tubes. Sperm collection was carried out at room temperature (25°C). Immediately after collection, the tubes containing sperm were maintained in a cooler (9–11°C) containing dry ice foam (Polar Technics CRI Ltd, São Paulo, Brazil). Contamination of sperm with water, blood, feces or urine was carefully avoided.

Determination of fresh sperm features

Immediately after collection, 5 μl of each sample were placed on a glass slide and observed using a light microscope (Eclipse E200; Nikon, Tokyo, Japan) at 400× magnification. Motility rate (expressed as % of motile sperm) was subjectively estimated following the addition of 50 μl water. Approximately 2 ml of sperm were transported to the Laboratory of Semen Technology at the Federal University of Lavras (UFLA), State of Minas Gerais, Brazil, for further analyses. Sperm concentration was determined using a hemacytometer Neubauer chamber (Boeco, Hamburg, Germany). Osmolality (Semi-Micro Osmometer K-7400, Knauer, Berlin, Germany) and pH (Digimed DM- 22-V1.0, São Paulo, Brazil) of the seminal plasma were measured after semen centrifugation at 2000 ***g*** for 10 min (MiniStar, Shanghai, China).

Extender composition and osmolality

Eight extenders comprising combinations of an ionic (NaCl; Vetec Quimica Fina LTDA, Rio de Janeiro, Brazil) or sugar (glucose; Vetec Quimica Fina LTDA, Rio de Janeiro, Brazil) solution, adjusted to four osmolalities (285, 325, 365 and 405 mOsm kg^{-1}) were prepared in 100-ml amber glass bottles, stored in a refrigerator at 6–8°C and used within 48 h. Methyl glycol (CH_3O $(CH_2)_2OH$; Vetec Química Fina LTDA, Rio de Janeiro, Brazil), was used as cryoprotectant agent, according to our previous results (Maria et al., 2006). Within 9–10 min after collection, sperm was diluted in each freezing media to a ratio of 10% sperm, 10% methyl glycol and 80% extender. Diluted sperm was loaded into 0.25 ml straws (n = 3 replicate straws × 8 extenders × 10 males) and frozen in a nitrogen vapor vessel (dry vapor shipper, Cryoport Systems, Brea, CA) at approximately −170°C after an equilibration period (the period between diluting sperm in the freezing media and freezing) of 15 min. The straws were then transported 50 km from CEMIG to the Laboratory of Semen Technology at UFLA and stored in a liquid nitrogen vessel (MVE XC 34/18, New Prague, MN) within 24 h.

Post-thaw sperm evaluation

After a few weeks, straws were thawed in a waterbath (Water-bath MA 127, Marconi, Brazil) at 60°C for 3 s, and post-thaw sperm quality was immediately estimated using the CASA system, following the methodology described in Viveiros et al. (2015). Briefly, post-thaw sperm was activated in a Makler™ counting chamber (Sefi-Medical Instruments ltd, Haifa, Israel) placed under a phase contrast microscope (Eclipse E200; Nikon, Tokyo, Japan) at 100× magnification with a green filter and pH 1 position. The activating agent used was NaCl at 98 mOsm kg^{-1} at 26–29°C to a final dilution ratio of 1 : 50. The microscope was connected to a video camera (Basler Vision Technologies™ A602FC, Ahrensburg, Germany) generating 100 images s^{-1}; video recording started approximately 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, for brevity, only motility rate, curvilinear velocity (VCL), straight line velocity (VSL) and average path velocity (VAP), were considered for analysis. To determine these parameters, each individual sperm (a mean of 501 spermatozoa per straw) was followed throughout the images, and the sperm trajectory calculated. We considered high post-thaw sperm quality when motility rate was above 60% and VCL was above 140 μm s^{-1}.

Statistical analyses

Data are expressed as mean ± standard deviation (SD). Statistical analyses were conducted with the SISVAR software program (Ferreira, 1999). Data were tested for normal distribution using test Shapiro Wilk and for significant differences using ANOVA, followed by the Scott-Knott test, when applicable. The level of significance for all statistical tests was set to 5% ($P < 0.05$).

Results

Fresh sperm features

Fresh sperm of the 10 males used in this study had a mean of 91% motility and 2.7×10^9 spermatozoa ml^{-1}. Seminal plasma was 249 mOsm kg^{-1} with a pH of 8.06 (Table 1).

Extender composition and osmolality

Both extender osmolality and composition affected ($P < 0.05$) post-thaw sperm quality. In general, samples frozen in NaCl yielded higher quality than that in glucose, and at 285 and 325 mOsm kg^{-1} higher than that at 365 and 405 mOsm kg^{-1}. The only exception was VCL, which was not affected ($P > 0.05$) by the osmolality of the glucose solution (Fig. 1).

High post-thaw quality, with motility above 60% and VCL above 140 μm s^{-1}, was observed only in samples frozen in NaCl at 285 mOsm kg^{-1} (Table 2).

Table 1
Body weight and some fresh sperm features of *Brycon orbignyanus* (n = 10 males; mean ± SD; minimum-maximum values) after carp pituitary treatment

Feature	Mean ± SD	Min-max
Body weight (kg)	2.1 ± 0.4	1.5–2.9
Subjective motility rate (%)	91 ± 3	85–95
Concentration (spermatozoa × 10^9 ml^{-1})	2.7 ± 1.3	1.1–5.3
Seminal plasma osmolality (mOsm kg^{-1})	249 ± 17	216–275
Seminal plasma pH	8.06 ± 0.11	7.88–8.21

Discussion

In the present study, eight extenders comprising the combination of two compositions and four osmolalities were tested. Post-thaw sperm motility was evaluated in terms of motility rate and velocities using the CASA system. This is the first detailed report on the evaluation of extender osmolality on post-thaw sperm motility of a Characiformes fish species.

Fresh sperm quality of *B. orbignyanus* was assessed after hormonal treatment with carp pituitary extract. The values observed for sperm quality were all within the range observed for fresh sperm of this species (Nascimento et al., 2012; Gonçalves et al., 2013; Viveiros et al., 2015). It is interesting to observe that the sperm of this species is characterized by a large volume, frequently above 10 ml, and a low concentration, mostly below 10 × 10^9 sperm ml^{-1} (Viveiros and Godinho, 2009). 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).

The extenders herein tested were simple solutions, either ionic (NaCl) or non-ionic (glucose). The presence of ions was beneficial during freezing and thawing of *B. orbignyanus* sperm, as NaCl-frozen sperm yielded higher motility and velocities compared to glucose-frozen sperm. Similarly, post-thaw motility of *B. nattereri* sperm was higher in NaCl- than in glucose-frozen samples (Oliveira et al., 2007). To the contrary, in other Characiformes the sperm of *B. opalinus* (Viveiros et al., 2012), *P. lineatus* (Viveiros et al., 2009b) and *S. brasiliensis* (Viveiros et al., 2009a) frozen in glucose yielded higher motility than that in NaCl, and sperm of *B. insignis* (Viveiros et al., 2011) frozen in ionic or sugar solution yielded similar motility (Table 2). The addition of ions in a freezing medium aims the control of osmotic pressure during cell dehydration, while sugars are cell membrane protectors (Kopeika and Kopeika, 2008) and are present in most of the extenders for fish sperm (Viveiros and Godinho, 2009).

Extender osmolality also affected post-thaw sperm motility of *B. orbignyanus*. Highest motility rate and velocities were observed when sperm was frozen in extenders at 285 and 325 mOsm kg^{-1}, compared to that at 365 and 405 mOsm kg^{-1}. It is important to note that all osmolalities tested here were higher than that of seminal plasma (216–275 mOsm kg^{-1}).

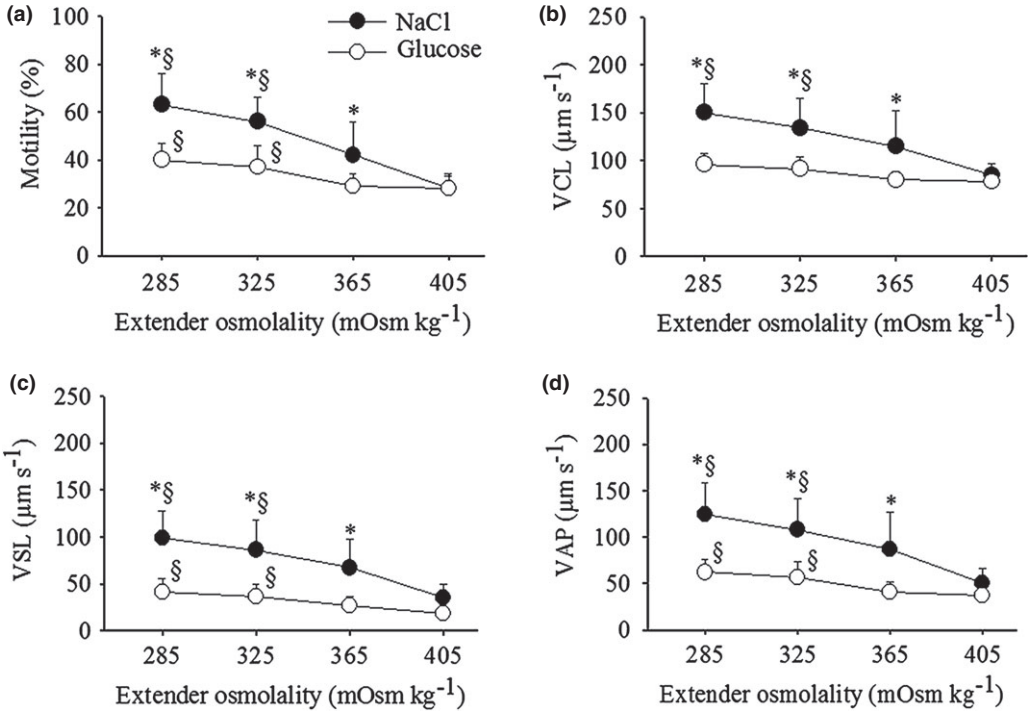

Fig. 1. Motility rate (a), curvilinear (VCL; b), straight-line (VSL; c) and average path (VAP; d) velocities of *Brycon orbignyanus* sperm cryopreserved in eight extenders composed of 2 (NaCl = closed circles; glucose = open circles) × 4 osmolalities. Each dot and error bar represents mean ± SD (n = 3 replicate straws × 10 males). *Differences between extender compositions ($P < 0.05$; ANOVA); § Differences among extender osmolalities ($P < 0.05$; Scott-Knott test); values followed by a symbol are significantly higher

Table 2
Composition and osmolality of some extenders tested during cryopreservation of fish sperm, genus *Brycon*

Species	Extender (mOsm kg^{-1})	Post-thaw sperm quality	Reference
B. insignis	285 NaCl	70% motility[a]; duration of motility 86 s; 64% intact sperm[c]	Viveiros et al., 2011;
	308 Glucose	68% motility[a]; duration of motility 107 s; 51% intact sperm[c]	
	356 BTS	62% motility[a]; duration of motility 85 s; 62% intact sperm[c]	
B. nattereri	~285 NaCl	68% motility[a]	Oliveira et al., 2007;
	~405 NaCl	57% motility[a]	
	~429 NaCl	45% motility[a]	
	~318 BTS	72% motility[a]	
	~277 Glucose	49% motility[a]	
B. opalinus	325 NaCl	25% motility[a]; duration of motility 30 s; 28% intact sperm[c]	Viveiros et al., 2012;
	365 NaCl	70% motility[a]; duration of motility 49 s; 68% intact sperm[c]	
	325 Glucose	42% motility[a]; duration of motility 28 s; 47% intact sperm[c]	
	365 Glucose	88% motility[a]; duration of motility 52 s; 84% intact sperm[c]	
B. orbignyanus	285 NaCl	63% motility[b]; VCL of 150 $\mu m\ s^{-1}$; VSL of 99 $\mu m\ s^{-1}$; VAP of 124 $\mu m\ s^{-1}$	Present study
	325 NaCl	56% motility[b]; VCL of 134 $\mu m\ s^{-1}$; VSL of 86 $\mu m\ s^{-1}$; VAP of 108 $\mu m\ s^{-1}$	
	365 NaCl	42% motility[b]; VCL of 115 $\mu m\ s^{-1}$; VSL of 67 $\mu m\ s^{-1}$; VAP of 87 $\mu m\ s^{-1}$	
	405 NaCl	28% motility[b]; VCL of 85 $\mu m\ s^{-1}$; VSL of 35 $\mu m\ s^{-1}$; VAP of 50 $\mu m\ s^{-1}$	
	285 Glucose	40% motility[b]; VCL of 96 $\mu m\ s^{-1}$; VSL of 41 $\mu m\ s^{-1}$; VAP of 61 $\mu m\ s^{-1}$	
	325 Glucose	37% motility[b]; VCL of 91 $\mu m\ s^{-1}$; VSL of 35 $\mu m\ s^{-1}$; VAP of 56 $\mu m\ s^{-1}$	
	365 Glucose	29% motility[b]; VCL of 80 $\mu m\ s^{-1}$; VSL of 23 $\mu m\ s^{-1}$; VAP of 42 $\mu m\ s^{-1}$	
	405 Glucose	28% motility[b]; VCL of 78 $\mu m\ s^{-1}$; VSL of 18 $\mu m\ s^{-1}$; VAP of 37 $\mu m\ s^{-1}$	
	285 NaCl + yolk	66% motility[a]	Maria et al., 2006;
	404 NaCl + yolk	44% motility[a]	
	429 NaCl + yolk	26% motility[a]	

Motility rate evaluated subjectively under a light microscope ([a]) or under a Computer-Assisted Sperm Analyzer ([b]).
[c]Percentage of intact sperm after eosin–nigrosin staining.
BTS™(Beltsville Thawing Solution, Minitub): glucose, sodium citrate, EDTA, $NaHCO_3$, KCl and gentamycin sulfate.

In other studies using species of the genus *Brycon*, sperm frozen in extender at ~285 mOsm kg^{-1} yielded higher post-thaw motility when compared to that at ~404 and at ~429 mOsm kg^{-1}, in both *B. orbignyanus* (Maria et al., 2006) and in *B. nattereri* (Oliveira et al., 2007). It has been stated that since sperm of freshwater fish are very sensitive to osmotic changes, the extender (before the addition of a cryoprotectant) should be iso-osmotic or slightly hypertonic compared to seminal plasma, as the osmotic pressure of the freezing medium is increased substantially by the addition of a cryoprotectant (Kopeika and Kopeika, 2008).

The use of a CASA system has been developed to reduce the amount of time spent in sperm observation and the intra-observer differences, and to improve the accuracy of final results (Rurangwa et al., 2001). This method is objective, accurate, and enables quantification of physical components of sperm movement (Liu et al., 2007). Furthermore, the use of CASA for the evaluation of sperm motility is important because the motility rate and VCL are associated with the reproductive success in fishes (Rurangwa et al., 2001; Viveiros et al., 2010). However, even when analyzing sperm using an objective method, differences among studies are frequent. Among characiforms, *B. orbignyanus* sperm frozen in NaCl and methyl glycol yielded motility of 63% and VCL of 150 $\mu m\ s^{-1}$ (present study), while sperm frozen in BTS™ and methyl glycol yielded lower motility (42%) and similar VCL (160 $\mu m\ s^{-1}$; Viveiros et al., 2015). *P. lineatus* sperm frozen in glucose and methyl glycol yielded motility of 75% and VCL of only 49 $\mu m\ s^{-1}$ in Viveiros et al. (2010) and motility of 73% and VCL as high as 277 $\mu m\ s^{-1}$ in Viveiros et al. (2015). These differences are mostly related to rearing conditions in each fish farm, spermiation inducing methodology, freezing medium, activating agent and differences among individuals. Sperm velocity also depends on several other parameters such as head dimension (diameter of head), beat frequency, length of flagellum, and physical parameters of wave propagation (Alavi et al., 2009) as well as the activating agent (osmolality, composition and viscosity).

Thus, different than most of the sperm from freshwater species that need a cell membrane protector (such as sugars), *B. orbignyanus* sperm should be frozen in an ionic solution for better protection during the freezing and thawing processes. Furthermore, this solution should be prepared at an osmolality just above seminal plasma osmolality. Sperm cryopreserved in this medium should yield a high post-thaw quality with motility above 60% and VCL above 140 $\mu m\ s^{-1}$ and could be used for both aquaculture purposes and conservational programs, since *B. orbignyanus* is a threatened species.

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