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Initiation and suppression of sperm motility is osmolality-dependent in two South American fish species: streaked prochilod (*Prochilodus lineatus*) and piracanjuba (*Brycon orbignyanus*)

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Abstract

Solutions to induce or suppress the initiation of sperm motility in fish have been used to improve reproductive success during artificial fertilization and preservation techniques. The aim of the present study was to evaluate the effects of three solutions (NaCl, glucose, and BTS™) - each prepared with 10 different osmolalities - on the initiation and suppression of fresh sperm motility in *Prochilodus lineatus* and *Brycon orbignyanus*. Sperm was diluted in each of the 30 solutions and immediately observed under a light microscope to determine which solutions triggered or suppressed the initiation of sperm motility. When present, motility rate (% motile sperm) was determined at 0, 30, and 120 s post-activation and the motility quality score ranging from 0 (no movement) to 5 (rapidly swimming sperm) was determined at 0 and 30 s post-activation. Osmolality, but not solution composition, significantly affected both motility rate and quality score. Solutions at osmolalities up to 270 mOsm/kg in *P. lineatus* and up to 180 mOsm/kg in *B. orbignyanus* induced motility in at least 60% of sperm, with a minimum quality score of 3.0, and were therefore classified as activating agents. The greatest motility at 0, 30, and 120 s post-activation was observed with solutions ranging from 135 to 225 mOsm/kg for *P. lineatus* and at 135 mOsm/kg for *B. orbignyanus*. On the other hand, solutions ranging from 360 to 450 mOsm/kg in *P. lineatus* and 270 to 450 mOsm/kg in *B. orbignyanus* suppressed motility in at least 95% of sperm and were classified as immobilizing media. The osmolality of the surrounding medium is the key factor in the initiation or suppression of sperm motility in *P. lineatus* and *B. orbignyanus*.

Keywords: Characiformes, extender, semen, sperm activation.

Introduction

The streaked prochilod (*Prochilodus lineatus*) and the piracanjuba (*Brycon orbignyanus*) are fish from the order Characiformes and are native to South America. During the rainy season (October to February), these species migrate to find clean water and spawn. The detritivorous *P. lineatus* belongs to the Prochilodontidae family and its sediment processing activities are important to the structuring of community dynamics in tropical streams (Flecker, 1996). *P. lineatus* larvae serve as prey for carnivorous fish species and adult fish are used for human consumption, especially in northeastern Brazil (Viveiros *et al*., 2009). As artificial reproduction methods are well established and prolificacy is high, *P. lineatus* has been used as a model species for research in a number of studies addressing nutrition, health, genetic diversity, and reproduction (Viveiros *et al*., 2010). *B. orbignyanus* belongs to the Characidae family and is an omnivorous species that feeds on other fish (in early life stages), fruits, and seeds. Overfishing, the destruction of riparian vegetation, pollution, and hydroelectric dams have led to a decline in populations of *B. orbignyanus,* which is currently classified as an endangered species (Rosa and Lima, 2008). *B. orbignyanus* exhibits fast growth in captivity and has an excellent meat quality, indicating that it can be produced on a commercial scale, which may prevent its extinction (Maria *et al*., 2006a).

Like the majority of teleosts (Morisawa and Suzuki, 1980), the sperm of *P. lineatus* and *B. orbignyanus* are immotile in the male genital tract and motility is triggered when the sperm is released into the water (Maria *et al*., 2006a, b; Viveiros *et al*., 2009). Osmolality, pH, temperature, and ion concentration affect sperm motility (Morisawa and Suzuki, 1980; Alavi and Cosson, 2006). Studies addressing the effects of these factors on the induction of sperm motility are necessary for the establishment of standard activating agents (media that trigger motility) and immobilizing media, which prevent the initiation of sperm motility (also called extenders), for improving artificial fertilization and preservation techniques (Alavi *et al*., 2009b). In 2006, Alavi and Cosson published a review paper addressing the effects of osmolality on fish sperm motility, focusing on species of three orders (Salmoniformes, Cypriniformes, and Acipenseriformes). At that time, no similar study on Characiformes species had been carried out and only four studies had since been conducted to evaluate the effects of osmolality on the induction and suppression of fresh sperm motility in Characiformes. In NaCl solution, sperm motility was reported to be induced at 49 to 147 mOsm/kg and suppressed at 196 to 392 mOsm/kg in *Brycon*

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orthotaeniae (Melo and Godinho, 2006), induced at 0 to 274 mOsm/kg and suppressed at 410 to 547 mOsm/kg in *Brycon insignis* (Shimoda *et al*., 2007), and induced at 0 to 270 mOsm/kg and suppressed at 450 mOsm/kg in *Colossoma macropomum* (Carneiro *et al*., 2012). In a glucose solution, sperm motility was reported to be induced at 62 to 310 mOsm/kg and suppressed at 410 to 620 mOsm/kg in *Prochilodus magdalenae* (Martínez *et al*., 2011). In $NaHCO_3$ solution, sperm motility was reported to be induced at 0 to 270 mOsm/kg and suppressed at 450 mOsm/kg in *C. macropomum* (Carneiro *et al*., 2012). In another study involving Characiformes, sperm from *Brycon opalinus* was diluted in NaCl and glucose solutions from 245 to 365 mOsm/kg and motility was completely suppressed only at osmolalities greater than 325 mOsm/kg, regardless of the extender composition (Orfão *et al*., 2011).

The use of 0.29% NaCl as an activating agent and 0.9% NaCl, 5% glucose, and 5% BTS™ (Beltsville Thawing Solution) as an immobilizing medium for sperm from *P. lineatus* and *B. orbignyanus* has been tested at these standard concentrations by our research group (Maria *et al*., 2006a, b; Viveiros *et al*., 2009, 2010; Orfão *et al*., 2010). Solution composition and the osmolality range in which maximum sperm motility is either triggered or suppressed, however, require further investigation. Thus, the aim of the present study was to evaluate the effects of three solutions (NaCl, glucose, and BTS™) prepared at osmolalities ranging from 45 to 450 mOsm/kg on the initiation and suppression of fresh sperm motility in *P. lineatus* and *B. orbignyanus*.

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*. (2001). During the spawning season (December to January), *P. lineatus* 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) and the Fish Culture Station of the Minas Gerais Power Company (CEMIG) in the city of Itutinga (21°17'36'' S; 44°37'02'' W) - both located in the state of Minas Gerais, Brazil. *B. orbignyanus* males were selected only from the Furnas Fish Culture Station. Males with detectable running sperm under soft abdominal pressure received intramuscular doses of carp pituitary extract (cPE; Argent Chemical Laboratory, Redmond, Washington, USA) using the routine method at each fish culture station. At Furnas, all males received a single dose of 3 mg cPE/kg BW and were stripped after 8.5 h (*P. lineatus*; n = 7) and 5 h (*B. orbignyanus*; n = 7) at 25-26°C. At CEMIG, *P. lineatus* males (n = 7) received two doses of cPE (0.25 and 0.75 mg/kg BW) with a 12-h interval between injections and were stripped after 7.5 h at 27-28°C. The urogenital papilla was carefully dried and approximately 5 ml of sperm were hand stripped directly into graduated glass tubes. Sperm collection was carried out at room temperature (22-24°C). Immediately after collection, the tubes containing sperm were maintained in a cooler (9-11°C) containing dry ice foam (Polar Technics CRI Ltd., 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 sperm sample were placed on a glass slide and observed using a light microscope (Model L1000, Bioval, Jiangbei, China) at 400X magnification. Any sperm motility observed was considered premature induction caused by urine or water contamination. All sperm samples were immotile and the motility rate (expressed as percentage of motile sperm) was subjectively estimated following the addition of 50 µl of an activating agent composed of 0.29% NaCl (~98 mOsm/kg) using the method employed in our laboratory for Characiformes species (Viveiros *et al*., 2009, 2010; Orfão *et al*., 2010). As the sticking of sperm to glass slides has not been observed in Characiformes species, the addition of BSA or any other protein in the activating agent is not necessary (Orfão *et al*., 2011). The motility quality score was assigned using an arbitrary grading system ranging from 0 (no movement) to 5 (rapidly swimming sperm), based on Viveiros *et al*. (2011). Sperm concentration was determined using a hemacytometer/Neubauer chamber (Boeco, Hamburg, Germany). The osmolality of the seminal plasma was measured (Semi-Micro Osmometer K-7400, Knauer, Berlin, Germany) after centrifugation of the sperm at 2000 x g for 30 min (MiniStar, Shanghai, China). Sperm characteristics of all samples were evaluated at room temperature (~25°C) by the same well-trained technician.

Solution composition and osmolality

Thirty solutions (NaCl, glucose or BTS™ – each at 10 different osmolalities ranging from 45 to 450 mOsm/kg) were prepared in 100 ml amber glass bottles, stored in a refrigerator at 6-8°C and used within 48 h. BTS™ (Beltsville Thawing Solution, Minitube™, Tiefenbach/Landshut, Germany) is a boar sperm extender composed of 79.9% glucose, 12.7% sodium citrate, 2.7% EDTA, 2.7% $NaHCO_3$, 1.5% KCl and 0.5% gentamycin sulfate.

Sperm from each male (14 specimens of *P. lineatus* and 7 specimens of *B. orbignyanus*) was diluted at 1:10 (sperm:total volume) in each of the 30 solutions directly on a glass slide and immediately observed under a light microscope to determine which solutions would trigger

or suppress the initiation of sperm motility. Motility rate, when present, was subjectively determined at 0, 30, and 120 s post-activation and expressed as the percentage of motile sperm. The motility quality score was determined on the same glass slide and evaluated at 0 and 30 s post-activation. Solutions triggering sperm motility in at least 60% of sperm with a quality score of 3 or higher were classified as activating agents, and those for which the initiation of sperm motility was suppressed in at least 95% of sperm were classified as immobilizing media.

Statistical analyses

Data are expressed as mean ± standard deviation (SD). Statistical analyses were carried out using the computational program of R Development Core Team (2010). Data that did not exhibit normal distribution were transformed using the bootstrap method (Efron and Tibshirani, 1993) with 2000 samplings for the analysis of multiple comparisons. Statistical significance was tested using analysis of variance (ANOVA) followed by the Scott-Knott test. The level of significance for all statistical tests was set at 5% ($P < 0.05$).

Results

Fresh sperm features

Table 1 displays the observed values for body weight, characteristics of fresh sperm and osmolality of the seminal plasma evaluated in the present study. For *P. lineatus,* mean sperm motility was similar between fish culture stations (89% motile sperm and a quality score of 4.1-4.4). However, males from the Furnas station had lower sperm concentration (19.2 x 10^9 sperm/ml) and greater seminal plasma osmolality (337 mOsm/kg) in comparison to those from the CEMIG station (26.8 x 10^9 sperm/ml and 286 mOsm/kg). *B. orbignyanus* exhibited 89% motile sperm, a quality score of 4.0, concentration of 6.0 x 10^9 sperm/ml and seminal plasma osmolality of 301 mOsm/kg.

Table 1. Body weight and some fresh sperm features in *Prochilodus lineatus* originated from two different fish culture stations (Furnas and CEMIG) and *Brycon orbignyanus* from the Furnas station after carp pituitary treatment (cPE).

Features	*P. lineatus*				*B. orbignyanus*	
	Furnas (3.0 mg cPE/kg)		CEMIG (0.25 + 0.75 mg cPE/kg)		Furnas (3.0 mg cPE/kg)	
	Mean ± SD	Min-Max	Mean ± SD	Min-Max	Mean ± SD	Min-Max
Body weight (kg)	1.4 ± 0.4[a]	1.0 - 2.0	1.1 ± 0.3[a]	0.7 - 1.4	1.0 ± 0.8	0.6 - 2.3
Motility rate (% motile sperm)	89 ± 7[a]	75 - 95	89 ± 4[a]	85 - 95	89 ± 5	85 - 95
Motility quality score[1] (0 - 5)	4.1 ± 0.4[a]	4.0 - 5.0	4.4 ± 0.5[a]	4.0 - 5.0	4.0 ± 0.5	4.0 - 5.0
Concentration (sperm x 10^9/ml)	19.2 ± 2.9[b]	15.1 - 23.9	26.8 ± 2.7[a]	23.1 - 28.9	6.0 ± 2.4	2.7 - 8.9
Seminal plasma osmolality (mOsm/kg)	337 ± 8[a]	328 - 346	286 ± 7[b]	276 - 291	301 ± 11	289 - 313

[1]The motility quality score was assigned using an arbitrary grading system ranging from 0 (no movement) to 5 (rapidly swimming sperm).
[a,b]Means within the same row for *P. lineatus* followed by different superscript letters are significantly different ($P < 0.05$; Scott-Knott test).

Solution composition and osmolality

Osmolality, but not solution composition, significantly affected sperm motility (both rate and quality score) in both species. In *P. lineatus*, solutions with osmolalities up to 270 mOsm/kg induced motility ranging from 65 to 95% motile sperm and a quality score of 3.9 to 4.9, and were therefore classified as activating agents. The only exception was BTS™ at 270 mOsm/kg, in which motility was triggered in only 54% of sperm in males from the CEMIG station; however this value did not differ significantly from the motility triggered in NaCl or glucose solutions at the same osmolality. Greater ($P < 0.05$) motility rates were observed when sperm was diluted in solutions within 135 and 225 mOsm/kg among individuals from both the Furnas (87-94% motile sperm; quality score of 4.3-4.7) and CEMIG (77-95% motile sperm; quality score of 4.1-4.9) stations, regardless of the solution composition. At 270 and 315 mOsm/kg, considerable variation in motility rate was observed among individuals, as evidenced by the large standard deviations in these samples. Solutions at 360 mOsm/kg or above suppressed motility in at least 95% of sperm and were therefore classified as

immobilizing media for *P. lineatus* (Table 2).

In *B. orbignyanus*, solutions at osmolalities up to 180 mOsm/kg induced motility ranging from 71 to 91% motile sperm and a quality score of 4.0 to 5.0, and were therefore classified as activating agents. The greatest motility (89-91% motile sperm; quality score of 4.7-5.0) was found when sperm was diluted at 135 mOsm/kg, regardless of the solution composition. At 225 mOsm/kg, considerable variation in motility rate was observed among individuals, as evidenced by the large standard deviations in these samples. Solutions at 270 mOsm/kg or above suppressed motility in at least 95% of sperm and were therefore classified as immobilizing media for *B. orbignyanus* (Table 2).

Table 2. Motility rate (expressed as percentage of motile sperm) in sperm from *Prochilodus lineatus* (n = 14 males) and *Brycon orbignyanus* (n = 7 males) diluted in three different solution compositions (NaCl, glucose and BTS™) at osmolalities ranging from 45 to 450 mOsm/kg and observed immediately under a light microscope. *P. lineatus* males originated from two different fish culture stations (Furnas and CEMIG) and *B. orbignyanus* males originated only from the Furnas station.

Osmolality	*P. lineatus* (Furnas)				*P. lineatus* (CEMIG)				*B. orbignyanus* (Furnas)			
	NaCl	Glucose	BTS™	Mean	NaCl	Glucose	BTS™	Mean	NaCl	Glucose	BTS™	Mean
45	79 ± 13	83 ± 12	80 ± 9	80 ± 11	74 ± 7	80 ± 8	73 ± 10	76 ± 9	71 ± 6	73 ± 6	71 ± 6	72 ± 5
90	86 ± 9	89 ± 10	86 ± 9	87 ± 9	78 ± 3	81 ± 5	77 ± 6	79 ± 5	78 ± 4	82 ± 6	79 ± 4	80 ± 5
135	88 ± 5	91 ± 6	91 ± 6	90 ± 6	82 ± 4	90 ± 5	82 ± 4	85 ± 6	89 ± 7	91 ± 4	89 ± 6	90 ± 5
180	92 ± 4	94 ± 6	94 ± 6	93 ± 5	89 ± 5	95 ± 3	89 ± 5	91 ± 5	79 ± 6	81 ± 7	80 ± 5	80 ± 6
225	89 ± 6	91 ± 6	87 ± 8	89 ± 6	81 ± 9	90 ± 4	77 ± 10	83 ± 9	46 ± 16	54 ± 16	51 ± 11	50 ± 14
270	79 ± 10	80 ± 14	79 ± 6	79 ± 10	65 ± 16	76 ± 11	54 ± 20	65 ± 18	1 ± 1	2 ± 2	4 ± 4	3 ± 3
315	56 ± 16	44 ± 21	31 ± 17	43 ± 20	22 ± 8	31 ± 12	14 ± 10	22 ± 12	0 ± 0	0 ± 0	0 ± 0	0 ± 0
360	2 ± 4	2 ± 4	0 ± 1	2 ± 3	4 ± 6	1 ± 2	0 ± 1	2 ± 4	0 ± 0	0 ± 0	0 ± 0	0 ± 0
405	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
450	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Within a given osmolality and fish species, solution composition did not affect sperm motility ($P > 0.05$). Beltsville Thawing Solution, Minitube™ (79.9% glucose, 12.7% sodium citrate, 2.7% EDTA, 2.7% $NaHCO_3$, 1.5% KCl and 0.5% gentamycin sulfate).

To track the decrease in sperm motility after activation, the motility rate was evaluated for 120 s and the motility quality score was evaluated for 30 s in each of the solutions classified as activating agents (45 to 270 mOsm/kg for *P. lineatus*; 45 to 180 mOsm/kg for *B. orbignyanus*). As data on *P. lineatus* males from both the Furnas and CEMIG stations and the three solution compositions within a given osmolality were similar, the data were pooled per fish species and osmolality. In *P. lineatus*, motility significantly decreased from a mean of 72-92% to 8-51% motile sperm during the 120 s analysis and the quality score decreased from 4.1-4.7 to 2.5-3.7 during the 30 s analysis. In general, motility (rate and quality score) was greater when sperm was diluted in solutions at 135, 180, and 225 mOsm/kg in comparison to solutions at 45, 90, and 270 mOsm/kg, regardless of the observation time (Fig. 1).

In *B. orbignyanus*, motility significantly decreased from a mean of 72-90% to 1-32% motile sperm during the 120 s analysis and the quality score decreased from 4.0-4.9 to 2.3-3.3 during the 30 s analysis. In general, motility (rate and quality score) was greater when sperm was diluted in solutions at 135 mOsm/kg in comparison to solutions at 45, 90, and 180 mOsm/kg, regardless of the observation time (Fig. 2).

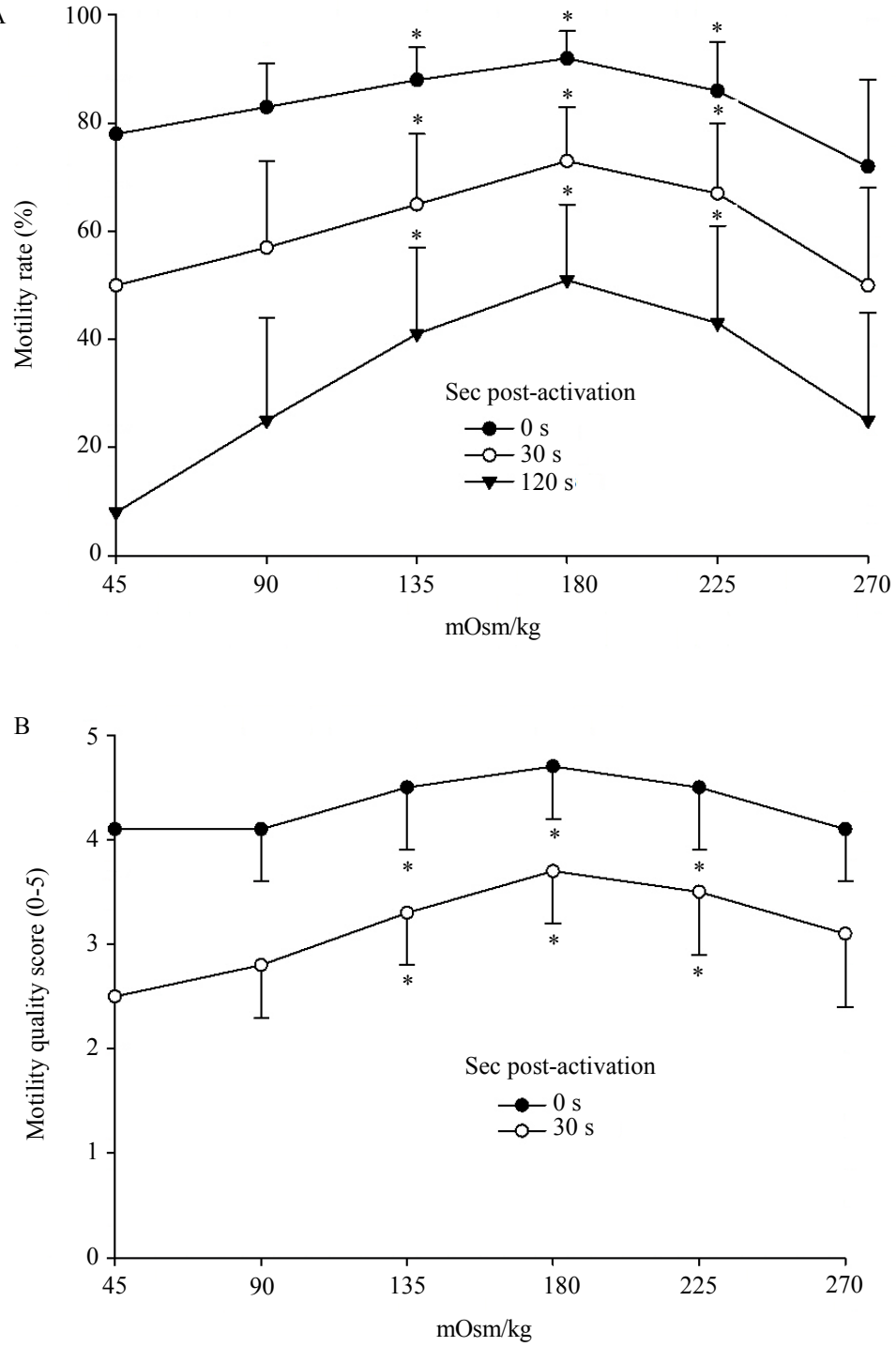

Figure 1. Motility rate (A) and quality score (B) for sperm from *Prochilodus lineatus* (n = 14) diluted in activating agents at osmolalities ranging from 45 to 270 mOsm/kg. Motility rate was determined at 0, 30, and 120 s post-activation, while quality score was determined at 0 and 30 s post-activation. Motility quality score was assigned using an arbitrary grading system ranging from 0 (no movement) to 5 (rapidly swimming sperm). Sperm was diluted in three different solution compositions (NaCl, glucose, and BTS™). Because there was no effect of composition on motility rate or quality score, data were pooled. *Means followed by an asterisk on each line were significantly higher than the others ($P < 0.05$; Scott-Knott test).

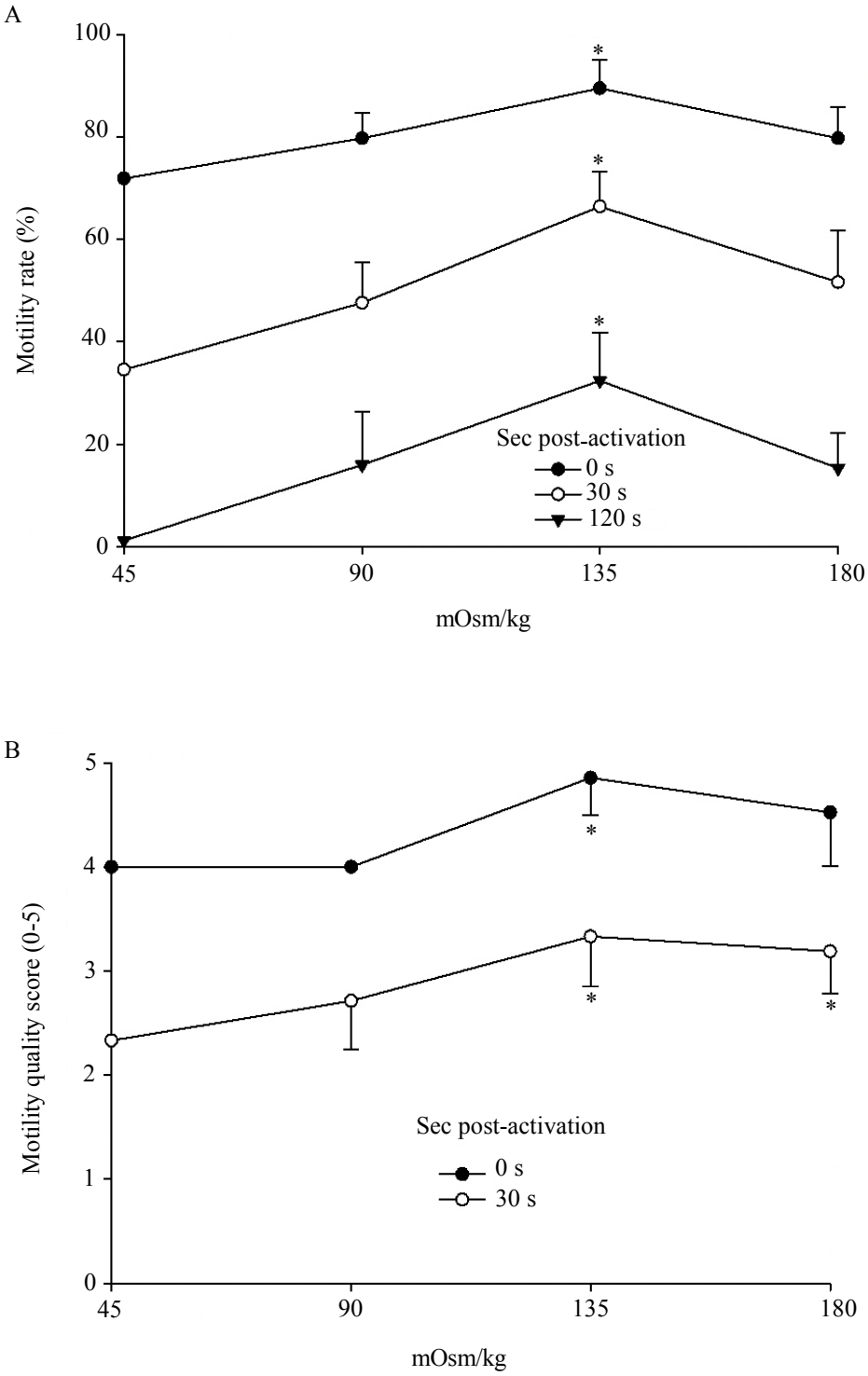

Figure 2. Motility rate (A) and quality score (B) for sperm from *Brycon orbignyanus* (n = 7) diluted in activating agents at osmolalities ranging from 45 to 180 mOsm/kg. Motility rate was determined at 0, 30, and 120 s post-activation, while quality score was determined at 0 and 30 s post-activation. Motility quality score was assigned using an arbitrary grading system ranging from 0 (no movement) to 5 (rapidly swimming sperm). Sperm was diluted in three different solution compositions (NaCl, glucose, and BTS™). Because there was no effect of composition on motility rate or quality score, data were pooled. *Means followed by an asterisk on each line were significantly higher than the others ($P < 0.05$; Scott-Knott test).

Discussion

The present study evaluated the effects of solutions with different compositions and osmolalities on fresh sperm motility and other features in *P. lineatus* and *B. orbignyanus*. The initial sperm quality for the specimens of *P. lineatus* and *B. orbignyanus* analyzed in this study were all within the range previously reported for both species following carp pituitary treatment (Viveiros and Godinho, 2009; Godinho and Viveiros, 2011). Improved knowledge of sperm quality is necessary for the evaluation of sperm quality in commercial hatcheries prior to artificial reproduction as well as in experiments carried out under laboratory conditions (Viveiros and Godinho, 2009; Orfão *et al*., 2011).

The composition of the solution exerted no effect on the initiation or suppression of sperm motility in the *P. lineatus* and *B. orbignyanus* specimens. The media tested herein were a simple ionic solution (NaCl), a simple sugar solution (glucose), and a more complex solution containing both ions and sugar (BTS™). Likewise, solution composition was not found to affect the initiation or suppression of sperm motility in a previous study carried out by our research group using the same fish species (Nascimento *et al*., 2012) or in other studies with Characiformes species such as *B. opalinus* (Orfão *et al*., 2011) and *C. macropomum* (Carneiro *et al*., 2012), and species from other orders, such as *Barbus barbus* (Alavi *et al*., 2009a), *Vimba vimba* (Alavi *et al*., 2010), and *Esox lucius* (Alavi *et al*., 2009b; Table 3).

Table 3. Reported seminal plasma osmolality and solutions considered as sperm activating agents and immobilizing media for some fish species.

Order (Family)	Species	Seminal plasma (mOsm/kg)	Solution composition	Activating agent[1] (mOsm/kg)	Immobilizing medium[2] (mOsm/kg)	Reference
Characiformes	*B. insignis*	--	NaCl	0 - 274	410 - 547	Shimoda *et al.*, 2007
(Characidae)	*B. opalinus*	297 - 340	NaCl	245	325 - 365	Orfão *et al.*, 2011
		297 - 340	Glucose	245	325 - 365	Orfão *et al.*, 2011
	B. orbignyanus	289 - 313	NaCl	45 - 180	270 - 450	Present study
		289 - 313	Glucose	45 - 180	270 - 450	Present study
		289 - 313	BTS™*	45 - 180	270 - 450	Present study
		300	Glucose	--	315 - 360	Nascimento *et al.*, 2012
		300	BTS™*	--	360	Nascimento *et al.*, 2012
	B. orthotaeniae	--	NaCl	~49 - ~147	~196 - ~392	Melo and Godinho, 2006
		--	NaCl	0 - 270	450	Carneiro *et al.*, 2012
	C. macropomum	--	$NaHCO_3$	0 - 270	450	Carneiro *et al.*, 2012
Characiformes	*P. lineatus*	276 - 346	NaCl	45 - 270	360 - 450	Present study
(Prochilodontidae)		276 - 346	Glucose	45 - 270	360 - 450	Present study
		276 - 346	BTS™*	45 - 270	360 - 450	Present study
		306	Glucose	--	315 - 360	Nascimento *et al.*, 2012
		306	BTS™*	--	315 - 360	Nascimento *et al.*, 2012
	P. magdalenae	250 - 300	Glucose	62 - 310[C]	410 - 620[C]	Martínez *et al.*, 2011
Cypriniformes	*B. barbus*	249 - 294	NaCl	3 - 230[V]	350[V]	Alavi *et al.*, 2009b
(Cyprinidae)		249 - 294	KCl	3 - 215[V]	345[V]	Alavi *et al.*, 2009b
		249 - 294	Sucrose	4 - 210[V]	330[V]	Alavi *et al.*, 2009b
	V. vimba	236 - 318	NaCl	30 - 240[V]	285[V]	Alavi *et al.*, 2010
		236 - 318	KCl	50 - 240[V]	290[V]	Alavi *et al.*, 2010
		236 - 318	Sucrose	30 - 210[V]	300[V]	Alavi *et al.*, 2010
Esociformes	*E. lucius*	228 - 350	NaCl	0 - 300[V]	375[V]	Alavi *et al.*, 2009a
(Esocidae)		228 - 350	Sucrose	0 - 235[V]	375[V]	Alavi *et al.*, 2009a
		228 - 350	Mannitol	0 - 235[V]	400[V]	Alavi *et al.*, 2009a
Perciformes	*P. fluviatilis*	298	Glucose	90 - 260[V]	--	Boryshpolets *et al.*, 2009
(Percidae)						

[1]Solutions were classified as activating agents when motility was triggered in at least 60% of sperm. [2]Solutions were classified as immobilizing media when motility was suppressed in at least 95% of sperm. *Beltsville Thawing Solution, Minitube™ (79.9% glucose, 12.7% sodium citrate, 2.7% EDTA, 2.7% $NaHCO_3$, 1.5% KCl, and 0.5% gentamycin sulfate). Motility was always evaluated subjectively unless stated otherwise ([C]computer-assisted sperm analysis, [V] video-recorded frames).

In the present study, all solutions at osmolalities within the range of 45 to 270 mOsm/kg and 45 to 180 mOsm/kg for *P. lineatus* and *B. orbignyanus*, respectively, induced motility in at least 60% of sperm and were therefore classified as activating agents. These activating agents were all hyposmotic in comparison to the seminal plasma of the males used in this study (276 to 346 mOsm/kg in *P. lineatus* and 289 to 313 mOsm/kg in *B. orbignyanus*). The results demonstrated that motility is triggered by hyposmotic solutions in both *P. lineatus* and *B. orbignyanus,* regardless of the solution composition. A number of studies have reported the triggering of fresh sperm motility in Characiformes species by solutions at osmolalities up to 270-310 mOsm/kg in species of the Prochilodontidae family and up to 147-274 mOsm/kg in species of the Characidae family (Table 3). In similar studies with fish species of other orders, sperm motility has been triggered in solutions at osmolalities up to 210-240 mOsm/kg in Cypriniformes, up to 235-300 mOsm/kg in Esociformes, and up to 260 mOsm/kg in Perciformes (Table 3). Rapid, acute changes in environmental osmotic pressure may be differentially involved in the activation mechanisms of fish sperm motility. Under natural spawning conditions, the rapid change in the osmolality of the surrounding medium is one of the triggers for the activation fish sperm motility (Bondarenko *et al*., 2013).

To track the decrease in motility following activation, the motility rate was evaluated for 120 s and the motility quality score was evaluated for 30 s. In *P. lineatus*, only samples diluted in solutions within the range of 135 and 225 mOsm/kg exhibited greater than 40% motile sperm after 120 s and had a motility quality score above 3.0 after 30 s. In *B. orbignyanus*, only samples diluted in solutions at 135 mOsm/kg exhibited greater than 30% motile sperm after 120 s and had a motility quality score above 3.0 after 30 s. In both species, motility decreased faster at the lowest osmolality tested (45 mOsm/kg). Similarly, motility was reported to decrease faster when activated in solutions within the range of 0 and 100 mOsm/kg in comparison to the range of 140 to 270 mOsm/k in *C. macropomum* (Carneiro *et al*., 2012), and when activated in distilled water in comparison to solutions within the range of 200 to 230 mOsm/kg in *E. lucius* (Alavi *et al*., 2009b). Moreover, the exposure of sperm to distilled water led to different types of flagellum damage following sperm activation in *E. lucius*, such as cytoplasmic blebs along the length of the flagellum and a curling structure at the tip of the flagellum (Alavi *et al*., 2009b). It has also been reported that the ATP content at the end of motility was very low following carp sperm activation at lower osmolalities in comparison to higher osmolalities (Billard *et al*., 1995). It is possible that the *P. lineatus* and *B. orbignyanus* sperm activated in solutions with lower osmolalities were affected by some kind of flagellum damage induced by a large water influx, similar to that reported for *E. lucius*, and/or a lack of ATP content to promote flagellar movement for longer periods.

In the present study, motility was suppressed in at least 95% of sperm when the osmolality of the surrounding medium ranged from 360 to 450 mOsm/kg and 270 to 450 mOsm/kg for *P. lineatus* and *B. orbignyanus*, respectively. Solutions within these osmolality ranges can therefore be used as immobilizing media (extenders) for the conservation of sperm from these species. A number of studies found that fresh sperm motility was suppressed in solutions at or above 315 mOsm/kg in species of the Prochilodontidae family and at or above ~196 mOsm/kg in species of the Characidae family (Table 3). In similar studies with fish species of other orders, sperm motility was suppressed in solutions at or above 285 mOsm/kg in Cypriniformes and at or above 375 mOsm/kg in Esociformes (Table 3).

In conclusion, the initiation and suppression of sperm motility is osmolality-dependent in *P. lineatus* and *B. orbignyanus*. To trigger motility in the highest number of sperm, activating agents should be within the range of 135 to 225 mOsm/kg for *P. lineatus* and at 135 mOsm/kg for *B. orbignyanus*. Moreover, immobilizing media should be at least 360 mOsm/kg for *P. lineatus* and at least 270 mOsm/kg for *B. orbignyanus* to maintain the sperm during storage.

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