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Extender composition, osmolality and cryoprotectant effects on the motility of sperm in the Brazilian endangered species *Brycon opalinus* (Characiformes)

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ABSTRACT

Sperm preservation is an important tool for conservation of endangered fish species, such as the *Brycon opalinus* (Characiformes). An optimum medium should prevent the initiation of sperm motility during storage. The aim of this paper was to study the effects of extender composition, osmolality and cryoprotectant agent (CPA) on the motility of *B. opalinus* sperm after a 30-min equilibration time. Eight media were prepared by switching extender compositions (NaCl or glucose) and osmolalities (245, 285, 325 or 365 mOsm/kg). These media were then divided in three aliquots and combined with two CPAs (dimethyl sulfoxide, DMSO, or methylglycol, MG) at 1.4 M; the third aliquot remained without CPA (control). After dilution, all samples were observed under light microscope to confirm whether all extender-CPA combinations actually prevented the initiation of sperm motility. Then, sperm motility was triggered in NaCl 92 mOsm/kg as activating agent after 0- and 30-min equilibration time at 4 °C and the percentage of motile sperm and duration of motility were determined. All combinations of glucose or NaCl media at high osmolalities (325 and 365 mOsm/kg), completely suppressed the initiation of sperm motility. Low extender osmolalities (245 mOsm/kg), however, did not prevent the initiation of sperm motility and more than 50% of sperm diluted in all combinations of glucose media, NaCl-control and in NaCl-DMSO were motile. When motility was triggered after both 0- and 30-min equilibration times, more than 77% motile sperm were observed in all combinations of NaCl and glucose media, except for glucose 245-DMSO and glucose 285-DMSO. The duration of motility in sperm diluted in all media was above 40 s, except for glucose 245-DMSO. Based on these findings, we can conclude that the initiation of sperm motility is triggered by low osmolality rather than the ionic composition of the surrounding medium in *B. opalinus*. Glucose or NaCl solutions at high osmolalities combined with either DMSO or MG prevent the initiation of sperm motility during storage. Sperm diluted in these media yields motility upon activation above 77% and that should last long enough to fertilize oocytes. These media are recommended as freezing media for future essays in cryopreservation of *B. opalinus* sperm.

1. Introduction

The *Brycon opalinus* (Cuvier, 1819), known as pirapitinga-do-sul in Brazil, belongs to the order Characiformes, family Characidae and subfamily Bryconinae, is native to the Paraíba do Sul and Doce River basins in Brazil (Rosa and Lima, 2008). Changes in the river stream, urbanization, pollution and hydroelectric dams are some of the reasons why the status of *B. opalinus* is currently set as endangered. The genus *Brycon* is highly affected by environmental changes, and many species are in the red list of Brazilian threatened fauna, such as piracanjuba *B. orbignyanus*, tiete tetra *B. insignis* and pirapitinga *B. nattereri* (Rosa and Lima, 2008). Because species of the genus *Brycon* can easily adapt to captivity and commercial food, grow fast, in addition to serving as human food source, many species are exploited in commercial fisheries and in fish farming (Narahara et al., 2002; Zaniboni-Filho et al., 2006). Based on this, *B. opalinus* may also become a new species potentially interesting for future human consumption, as what happened with some fish communities, such as red snapper *Lutjanus argentimaculatus* in Southeast Asia and Australia (Vuthiphandchai et al., 2009), eurasian perch *Perca fluviatilis* in European countries (Boryshpolets et al., 2009) and dusky grouper *Epinephelus marginatus* in many areas of the European Atlantic coast and in the Mediterranean Sea (Glamuzina et al., 2000). Furthermore, because *B. opalinus* is endemic of headwater streams of the Paraíba do Sul and Doce River basins, this specie can be

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used as a model to indicate environmental conditions, since the relative health of a fish community is a sensitive indicator of direct and indirect stresses on the entire aquatic ecosystem (Fausch et al., 1990).

Most of fish spermatozoa are immotile in the seminal tract and hyposmotic media initiate sperm motility from freshwater fish species (Morisawa and Suzuki, 1980). Besides osmolality, pH, temperature, and ion concentration affect sperm motility (Alavi and Cosson, 2006; Alavi et al., 2006). Studies regarding the effects of these factors on the induction of sperm motility are necessary to establish standard activating agent (those media that trigger motility) and immobilizing media (those media that prevent the initiation of sperm motility) for improving both artificial fertilization and preservation techniques (Alavi et al., 2009a). In Characiformes species, only three studies describe the effects of osmolality on sperm motility. In *B. orbignyanus*, motility was induced when sperm was diluted in a medium at 240 mOsm/kg but not in media ranging from 285 to 429 mOsm/kg (Maria et al., 2006a). The media tested, however, possessed not only different osmolalities but also different compositions, thus conclusions regarding osmolality only could not be drawn. In matrinchã *Brycon orthotaeniae* sperm was diluted in NaCl solutions ranging from 25 to 200 mM (~46 to 368 mOsm/kg). The initiation of sperm motility was completely suppressed when NaCl at 150 mM (~276 mOsm/kg) or higher was used (Melo and Godinho, 2006). In a similar study, *B. insignis* sperm was diluted in NaCl solutions ranging from 0 to 547 mOsm/kg and the initiation of sperm motility was suppressed when NaCl at 410 mOsm/kg or higher was used (Shimoda et al., 2007). These studies suggest that sperm motility in Characiformes (at least in species of the genus *Brycon*) is triggered in hyposmotic medium, and that the minimum osmolality to suppress the initiation of sperm motility is different among species. A non-ionic medium in different osmolalities, however, has not yet been tested in similar experiments as activating and/or immobilizing medium in species of this order.

The aim of the present paper was to study the effects of extender composition (ionic and non-ionic), osmolality and cryoprotectant agent (CPA) on the motility of *B. opalinus* sperm after a 30-min equilibration time at 4 °C. The extenders were evaluated by switching compositions (NaCl or glucose) and osmolalities (245, 285, 325 or 365 mOsm/kg). Because we plan to test these media in future essays on sperm cryopreservation, the CPAs that are mostly used in Characiformes, dimethyl sulphoxide (DMSO) and methylglycol (MG), were added to each of these media. Furthermore, semen volume, concentration, total number of spermatozoa, pH, motility and duration of motility of fresh sperm, and seminal plasma osmolality were determined and possible correlations among these parameters were evaluated.

2. Material and methods

2.1. Fish handling and sperm collection

All fish were handled in compliance with the guidelines for animal experimentation described by Van Zutphen et al. (1993). *B. opalinus* broodfish were captured from the wild (Paraíba do Sul River basin) 5 to 7 years ago, and maintained in circular tanks at the Hydrobiology and Aquaculture Station of the Hydroelectric Company of São Paulo (CESP) in Paraíbuna (23°23′10″S; 45°39′44″W), São Paulo, Brazil. Males of approximately 7 years of age (body weight: 304 ± 9 g; total length: 30.4 ± 0.4 cm) were selected during the spawning season in October and November. Males with detectable running sperm under soft abdominal pressure received a single intramuscular dose of carp pituitary extract (cPE; Argent Chemical Laboratory, Redmond, Washington, USA) at 5 mg/kg body weight to increase semen volume and decrease viscosity, as a routine method for Characiformes (Viveiros and Godinho, 2009). 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 (~22 °C), and soon after collection, the tubes containing sperm were placed in crushed ice (4 ± 2 ºC).

2.2. Determination of semen characteristics

Immediately after collection, 5 μL of each sample was placed on a glass slide and observed using a light microscope (Model L1000, Bioval, Jiangbei, China) at 400 × magnification. Any sperm motility observed was considered as premature induction of motility caused by urine or water contamination and the sample discharged, assuming that in this species, sperm in seminal plasma should be immotile. In immotile samples ($n = 29$ 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 (volume/volume) of 1:5 (sperm: activating agent), following the methodology used as routine in our laboratory for Characiformes fish species (Maria et al., 2006a,b; Oliveira et al., 2007; Nascimento et al., 2010; Viveiros et al., 2009, 2010). Because sticking of sperm to glass slide has not been observed in Characiformes species, the addition of BSA or any other protein in the activating agent is unnecessary. Immediately after, motility was subjectively scored under light microscope as the percentage of spermatozoa showing progressive motility, in increments of 10%. All sperm samples used in this experiment possessed at least 80% motile sperm upon activation. Duration of motility was assessed on the same conditions: a stopwatch was triggered at the time of activating agent addition and stopped when 10% of the spermatozoa were still moving, and presented as seconds (s). Semen volume, concentration (hemacytometer/Neubauer chamber, Boeco, Hamburg, Germany), total number of spermatozoa (concentration × volume) and pH (pH/mV meter pH 21 Hanna, Hanna Instruments, Rhode Island, United States) were also determined in each semen sample. The osmolality of the seminal plasma was measured cryoscopically (Semi-Micro Osmometer K-7400, Knauer, Berlin, Germany) after centrifugation of sperm at 2000 *g* for 30 min at room temperature. Semen characteristics of all samples were evaluated by the same technician and at room temperature (~22 °C).

2.3. Extender composition, osmolality and CPA

All chemicals were obtained from Vetec Química Fina Ltda™ (Duque de Caxias, RJ, Brazil). Eight media comprising combinations of an ionic (NaCl) or non-ionic (glucose) extender, in four osmolalities (245, 285, 325 and 365 mOsm/kg) were prepared. The extender osmolalities were also measured cryoscopically. Then each medium was divided in three and combined with the two CPAs mostly used for Characiformes sperm: DMSO and MG (Viveiros and Godinho, 2009), at 1.4 M. The third aliquot of each media remained without CPA and served as control. Each of the 24 media were placed in 20-mL beakers, and cooled at 4 ± 2 ºC in a styrofoam box containing crushed ice for 30 min to equilibrate.

Sperm from each male was diluted 1:10 (sperm:total volume) in each medium (8 males × 24 media). Immediately after dilution, samples were observed under light microscope to confirm whether all extender-CPA combinations would prevent the initiation of sperm motility. If motility was observed, the percentage of motile sperm was annotated. Then, sperm motility was triggered in NaCl 92 mOsm/kg as activating agent (volume/volume; 1:5), and the percentage of motile sperm and duration of motility were determined (expressed as equilibration time = 0 min). Because we plan to use the best extender-CPA combination as freezing media in future essays on cryopreservation, sperm was stored for 30 min at 4 ± 2 ºC and evaluated again for the percentage of motile sperm and duration of motility upon activation (expressed as equilibration time = 30 min). The 30-min equilibration time represents the lag period necessary for the CPA permeation into the cells for protection against cryoinjuries and for sperm manipulation for freezing (dilution, loading and sealing straws, etc.). This experiment was carried out with eight replicates (1 replicate = 1 male).

2.4. Statistical analyses

Possible relationships among semen volume, concentration, total number of spermatozoa, pH, percentage of motile sperm and duration of

motility of fresh sperm, and seminal plasma osmolality were analyzed by Spearman's rank correlation ($P<0.05$).

Percentage of motile sperm and duration of motility were expressed as mean ± SEM and analyzed by the R computational program (version 2.9.0; R Development Core Team, 2007). A factorial design (2 extender compositions × 4 osmolalities × 3 CPAs) was used to investigate the effects of extender compositions, osmolality and CPA on sperm motility. The premature initiation of sperm motility cause by some extender-CPA combinations (percentage of motile sperm) was analyzed by nonlinear regression, using an exponential model. Motility triggered in activating agent (percentage of motile sperm and duration of motility) were analyzed by linear regression. Because data set showed normal distribution, there was no need for transformation. The level of significance for all statistical tests was set at 0.05.

3. Results

3.1. Semen characteristics and correlations

The observed values for body weight, characteristic of fresh semen and osmolality of seminal plasma evaluated in this study are presented on Table 1. The correlation matrix of these characteristics is depicted on Table 2. The highest correlations were observed between semen volume and concentration ($R=-0.723$; $P<0.05$) and between semen volume and total number of spermatozoa ($R=0.814$; $P<0.05$).

3.2. Extender composition, osmolality and CPA

To confirm whether all of the 24 extender-CPA combinations actually prevented the initiation of motility, diluted sperm was immediately observed under light microscope (Fig. 1). All combinations of glucose or NaCl media at higher osmolalities (325 and 365 mOsm/kg) prevented the initiation of sperm motility. However, extender at low osmolality (245 mOsm/kg) induced motility in 53 to 61% sperm in all combinations of glucose and NaCl, except for NaCl-MG (16%). At 285 mOsm/kg, motility was induced in 34 to 46% sperm diluted in NaCl-control and NaCl-DMSO and in 10 to 16% sperm diluted in NaCl-MG and in all combinations of glucose.

The percentage of motile sperm upon activation was subjectively evaluated after 0- and 30-min equilibration time in each of the 24 media (Fig. 2). Immediately after dilution (0-min equilibration time) in glucose 245 mOsm/kg, motility was lower (21% motile sperm) in DMSO, and higher (83 to 96%) in MG and control. At 285 mOsm/kg, motility was again lower (68%) in DMSO, and higher (95 to 98%) in MG and control. At higher osmolalities, 93 to 99% motile sperm was observed in all combinations of glucose regardless the addition of DMSO or MG. In NaCl media, 80 to 100% motile sperm was observed in all extender osmolalities and regardless the addition of DMSO or MG. After a 30-min equilibration time at 4 °C, again the medium glucose-DMSO yielded the lowest motility at both 245 (38%) and 285 mOsm/kg (51%). When sperm was diluted in glucose-MG and in glucose-control, 82 to 95% motile sperm was observed regardless the extender osmolality. In NaCl media, 70 to 95% motile sperm was observed in all extender osmolalities and regardless the addition of DMSO or MG.

Table 1
Body weight and fresh semen characteristics of *Brycon opalinus*.

Parameters	Number of males	Mean ± SEM	Range
Body weight (g)	29	304 ± 9	270–325
Motile sperm upon activation (%)	29	94 ± 1	80–100
Duration of motility (s)	29	99 ± 2	80–120
Semen volume (mL)	29	7.7 ± 0.8	2–15
Concentration (spermatozoa × 10^9/mL)	29	60.3 ± 3.2	32–96
Total number of spermatozoa (× 10^9)	29	435 ± 40	162–767
Semen pH	25	8.3 ± 0.1	8.0–8.6
Seminal plasma osmolality (mOsm/kg)	18	318 ± 3	297–340

The duration of motility upon activation was evaluated after 0- and 30-min equilibration time in each of the 24 media (Fig. 3). Immediately after dilution (0-min equilibration time) in glucose-DMSO, duration of motility was shorter at 245 mOsm/kg (18 s) compared to higher osmolalities (33 to 49 s). When sperm was diluted in glucose-MG and glucose-control, the duration of sperm motility ranged from 44 to 69 s. In NaCl media, the duration of sperm motility ranged from 40 to 47 s in DMSO, 47 to 60 s in MG and 57 to 74 s in control. After a 30-min equilibration time at 4 °C, again the medium glucose-DMSO yielded the shortest duration of motility at 245 mOsm/kg (20 s), compared to higher osmolalities (41 to 45 s); in glucose-MG and glucose-control, duration of motility was similar and ranged from 46 to 61 s. In NaCl media, the duration of sperm motility ranged from 42 to 62 s in DMSO, 57 to 65 s in MG and 61 to 72 s in control.

4. Discussion

In the present study, 24 media comprising combinations of extender compositions, osmolalities and CPAs were tested in their capacity of storing *B. opalinus* sperm for 30 min at 4 °C without reducing the percentage of motile sperm and duration of motility. Furthermore, we showed that some extender-CPA combinations induced the initiation of sperm motility, while other combinations suppressed motility during storage. This is the first report on the sperm response to ionic and osmolality effects of *B. opalinus.*

4.1. Fresh semen characteristics

Fresh sperm of *B. opalinus* was assessed after hormonal treatment with carp pituitary extract. Semen volume (7.7 mL), sperm pH (8.3) and seminal plasma osmolality (318 mOsm/kg) for the males utilized in this study were all within the range observed for fresh sperm of others species of Characiformes (Viveiros and Godinho, 2009). Sperm concentration (60.3×10^9 spermatozoa/mL), however, was the highest values compared to other Characiformes which ranges from 4.4×10^9 spermatozoa/mL in piaussú *Leporinus macrocephalus* to 37.4×10^9 spermatozoa/mL in pacu *Piaractus mesopotamicus* (Viveiros and Godinho, 2009).

A strong negative correlation was observed between semen volume and sperm concentration. After hormone treatment to induce spermiation, the first observed event is the testicular hydration which leads to an increased volume of extracted semen and a dilution of spermatozoa (Viveiros et al., 2002). This is the reason why reduced semen volume and increased sperm concentration is frequently reported in fish stripped without hormone treatment, compared to fish of the same species stripped after hormone treatment (Viveiros and Godinho, 2009). On the other hand, a strong positive correlation between semen volume and total number of spermatozoa was observed. Increasing semen volume, even at a low sperm concentration, leads to an increase in the total number of spermatozoa of the extracted semen in this fish species.

B. opalinus fresh sperm possessed 94% motile sperm and 99 s duration of motility upon activation. In other species of the genus *Brycon*, fresh sperm possesses 56 to 100% motile sperm and 33 to 56 s duration of motility (Viveiros and Godinho, 2009). A better knowledge of the characteristics of fresh sperm motility before manipulation 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 initiation of sperm motility due to contamination with water or urine (Viveiros and Godinho, 2009). In some studies with carp *Cyprinus carpio* sperm, it was shown that distilled water or urine contamination lead to decrease of sperm motility parameters (percentage, velocity and beat frequency), a decrease in ATP content of spermatozoa (Perchec-Poupard et al., 1998) and changes in spermatozoa morphology (Perchec-Poupard et al., 1996).

Table 2
Correlation matrix (Spearman's coefficient) of body weight and fresh semen characteristics of *Brycon opalinus* ($n = 18$ males).

	Body weight	Motile sperm	Duration of motility	Semen volume	Concentration	Total spermatozoa	Semen pH	Seminal plasma osmolality
Body weight	1.000							
Motile sperm	0.256	1.000						
Duration of motility	0.173	0.100	1.000					
Semen volume	0.578	0.365	−0.019	1.000				
Concentration	−0.087	−0.553	0.133	−0.723*	1.000			
Total spermatozoa	0.008	0.002	0.036	0.814*	−0.246	1.000		
Semen pH	−0.378	0.120	−0.491	−0.015	−0.196	0.220	1.000	
Seminal plasma osmolality	0.217	0.308	0.034	−0.094	0.190	−0.049	0.179	1.000

* $P<0.05$.

4.2. Initiation of sperm motility after dilution in different extender-CPA combinations

Environmental factors such as ions and osmolality stimulate the initiation of sperm motility by changing the properties of the plasma membrane (Morisawa, 1985; Morisawa et al., 1999; Krasznai et al., 2000). In the present study, however, extender composition (ionic NaCl and non-ionic glucose) did not affect the initiation of sperm motility in *B. opalinus*, as observed in barbel *Barbus barbus* (Alavi et al., 2009b). Osmolality, as expected, had a pronounced effect. Both glucose and NaCl media at 245 and 285 mOsm/kg induced motility. These osmolalities are hyposmotic compared to seminal plasma (318 mOsm/kg). Thus, as observed for *C. carpio* (Cosson et al., 1991) and perch *P. fluviatilis* (Alavi et al., 2007; Lahnsteiner et al., 1995), initiation of sperm motility of *B. opalinus* occurs when osmolality of the surrounding medium is lowered, while hyperosmotic medium prevents the initiation of motility. Therefore, in order to store sperm of *B. opalinus*, we recommend the use of a medium at an osmolality of 325 mOsm/kg or higher.

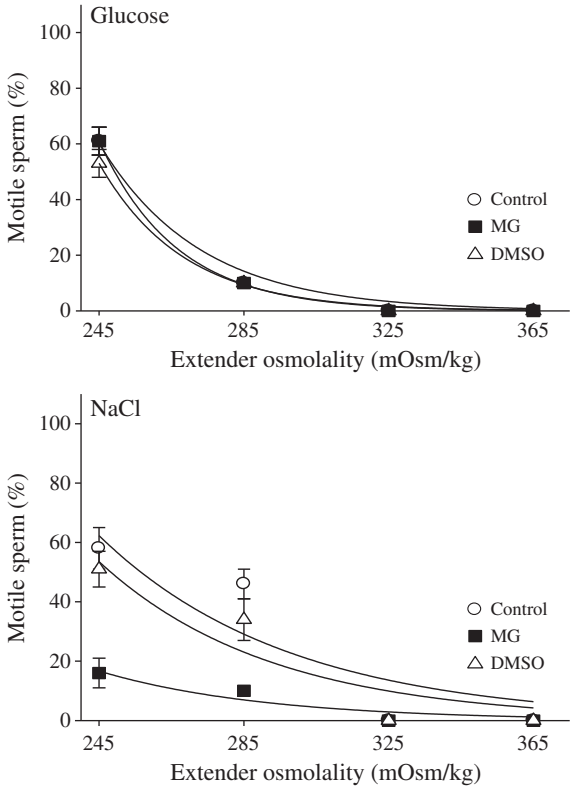

Fig. 1. Effects of extender composition (glucose on the top, NaCl on the bottom), osmolality and cryoprotectant (methylglycol, MG = closed squares, DMSO = open triangles, control without cryoprotectant = open circles) on the initiation of sperm motility (%) of *Brycon opalinus*. Each dot and error bar represents mean ± SEM of eight males and each line represents the predicted value after nonlinear regression analysis ($R^2 = 0.5728$).

When CPAs were added to extenders at low osmolalities (245 and 285 mOsm/kg), the global osmolality of the surrounding medium was increased. Although we could not measure this increase because our osmometer measures osmolality by freezing point depression of the solution and this is not possible when CPAs are added to the solution, we calculated the global osmolality. Osmolality increased from 245 to 1525 and to 1559 mOsm/kg, and from 285 to 1565 and to 1599 mOsm/kg, after the addition of DMSO and MG, respectively. The initiation of sperm motility, however, was not suppressed by such increase of global osmolality. It has been shown that the addition of DMSO activates striped bass *Morone saxatilis* sperm kept quiescent in extenders (He and Woods, 2003). In *C. carpio* sperm, a swelling following the addition of DMSO at 1 to 20% (approximately 400 to 3200 mOsm/kg) has been observed possibly caused by an influx of water (Perchec-Poupard et al., 1997). In the present study, DMSO was used at 1.4 M (1280 mOsm/kg), which represents approximately 10% in weight/volume of the total solution. This value is within the range of 1 to 20% observed for carp sperm. It is possible that a similar water influx after DMSO addition has occurred and triggered sperm motility, despite the increase of the global osmolality. No similar study has been carried out using MG as CPA; however, due to such similarity of the results, it is possible that the same mechanism of water influx occurred.

4.3. Motility of diluted sperm upon activation

There was no effect of extender composition (ionic NaCl and non-ionic glucose) on the percentage of motile sperm upon activation after 0- and 30-min equilibration time at 4 °C. Sperm diluted in a non-ionic (glucose-control) or in an ionic (NaCl-control) media yielded similar motility which ranged from 82 to 100%. These results are consistent with those observed in zebrafish *Danio rerio* (Wilson-Leedy et al., 2009) and in Northern pike *Exos lucius* L. (Alavi et al., 2009a), for which no difference on sperm motility was observed after dilution in NaCl or sucrose solution.

When CPAs were added, the percentage of motile sperm upon activation was significantly affected by the combination of low osmolality glucose media and DMSO. At both 0- and 30-min equilibration time, the lowest percentage of motile sperm (21 to 38%) was achieved when sperm was diluted in glucose 245-DMSO medium. At the same low osmolality (245 mOsm/kg), sperm diluted in glucose-MG, NaCl-MG or NaCl-DMSO yielded motility above 70%. After 30-min equilibration time at higher osmolalities (325 and 365 mOsm/kg), motility of sperm diluted in glucose-DMSO increased to ~77%, but these values were still lower than that observed for sperm diluted in glucose-control (85 and 93%) and in glucose-MG (91 to 93%). It is interesting to note that glucose is added to an aqueous solution not only to increase the osmolality of the surrounding medium, but also because of its stabilizing effects on the spermatozoa liposomal membrane during storage (Quinn, 1985). Glucose is commonly used at the concentration of 5% (which is ~277 mOsm/kg) and in combination with DMSO, as freezing medium for sperm of many neotropical fish species (Viveiros and Godinho, 2009). According to our results, motility triggered 30 min after dilution (which means just before freezing) would be 40 to 50%;

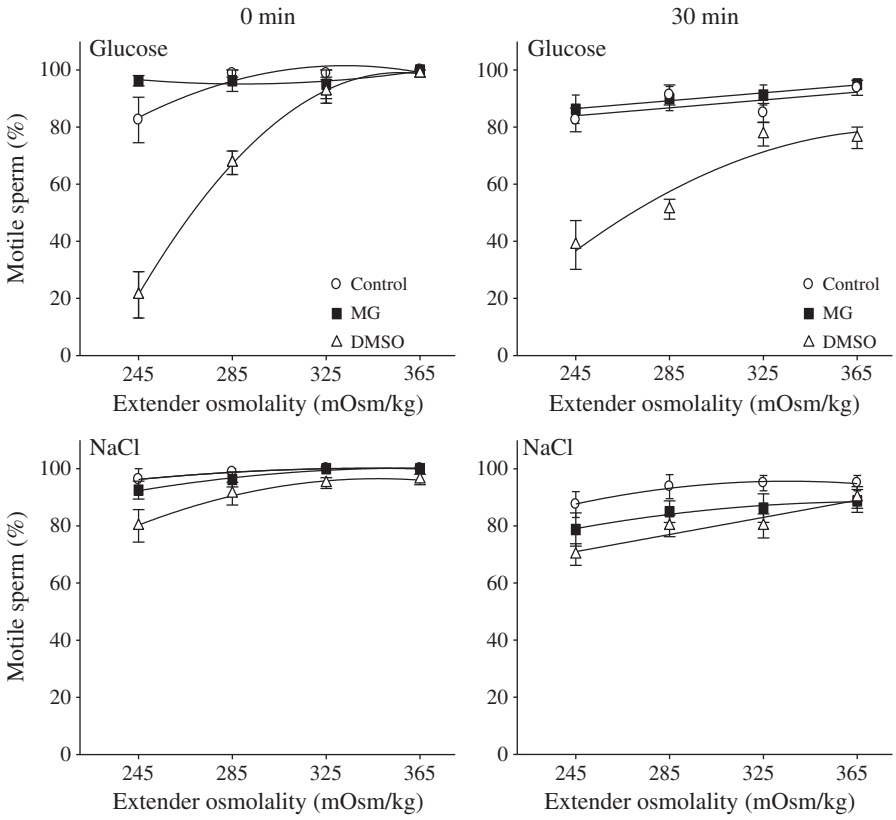

Fig. 2. Effects of extender composition (glucose at the top, NaCl at the bottom), osmolality and cryoprotectant (methylglycol, MG = closed squares, DMSO = open triangles, control without cryoprotectant = open circles) on the percentage of motile sperm upon activation of *Brycon opalinus*. Motility was triggered after 0- and 30-min equilibration times at 4 °C in each media. Each dot and error bar represents mean ± SEM of eight males and each line represents the predicted value in regression analysis ($R^2 = 0.9396$).

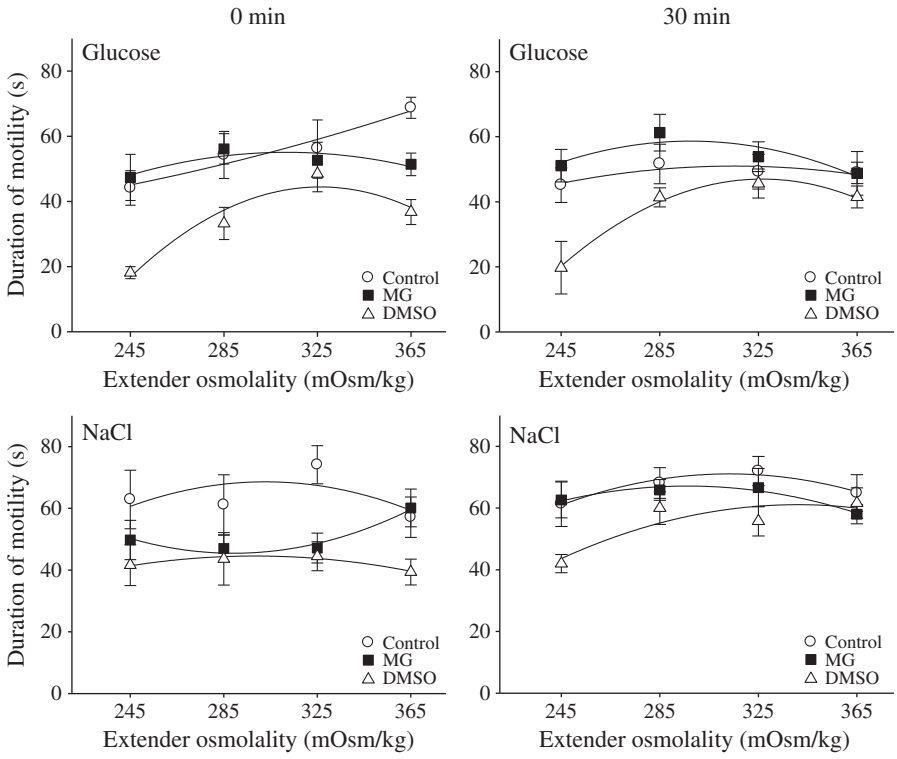

Fig. 3. Effects of extender composition (glucose at the top, NaCl at the bottom), osmolality and cryoprotectant (methylglycol, MG = closed squares, DMSO = open triangles, control without cryoprotectant = open circles) on the duration of sperm motility upon activation of *Brycon opalinus*. Motility was triggered after 0- and 30-min equilibration times at 4 °C in each media. Each dot and error bar represent mean ± SEM obtained with eight males and each line represents predicted value in regression analysis ($R^2 = 0.3968$).

after thawing, even lower percentage of motile sperm would be expected. Our research group compared the use of glucose 277 mOsm/kg combined with DMSO or MG as freezing medium of *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 MG compared to DMSO. It is possible, however, that DMSO combined with glucose at higher osmolalities (such as those tested in the present study) would provide better results.

Another point that merits attention is the fact that, although all extenders at 245 mOsm/kg induced motility in some spermatozoa (Fig. 1), motility could still be triggered upon activation 30 min later. It would be expected that motile sperm (10 to 61%) would run out of energy (ATP) within few minutes and sperm would be permanently immotile. But, in the contrary, motility (39 to 95%) could still be triggered in all samples 30 min after the first induction. It is possible that *B. opalinus* spermatozoa have the ability of reactivation as it has been reported for *C. carpio* (Perchec et al., 1995) and trout *Oncorhynchus mykiss* sperm (Christen et al., 1987). A transient lack of energy and its recovery is one possible explanation, but osmotic reequilibration could occur in *B. opalinus* sperm during this 30-min equilibration period. This could reestablish an internal ionic concentration compatible with a correct motility activation rate. A similar situation was observed for *C. carpio* sperm (Redondo et al., 1991). More experiments specially designed for studies on reactivation of *B. opalinus* sperm are however necessary.

4.4. Duration of sperm motility upon activation

There was no effect of extender compositions (ionic NaCl and non-ionic glucose) on the duration of sperm motility after 0- and 30-min equilibration time at 4 °C. Duration of motility ranged from 44 to 69 s in glucose-control medium, and from 61 to 74 s in NaCl-control medium.

When CPAs were added, duration of sperm motility was affected by DMSO mainly in combination with glucose at low osmolality. The shortest duration of motility (18 and 20 s) was obtained when glucose 245-DMSO was used as medium, compared to glucose-DMSO at other osmolalities and glucose-MG at all osmolalities, after both 0- and 30-min equilibration times. When NaCl was tested, the shortest duration of sperm motility (42 s) was also obtained when the combination of low osmolality and DMSO was used, but only after 30-min equilibration. In *B. orthotaenia*, fresh sperm was diluted in NaCl 200 mM (~368 mOsm/kg) combined with DMSO; the duration of motility was shorter when DMSO was used at 5 and 10% (41 and 48 s) compared to 15% (59 s; Melo and Godinho, 2006). Comparison of duration of sperm motility among different studies is difficult because there is a lack of standardization regarding when the endpoint of motility duration is. In some cases, duration of motility is scored until 20% of spermatozoa are still moving (Melo and Godinho, 2006), others until 10% are still moving (the present study), and yet when all spermatozoa stopped moving (Groison et al., 2010). Nevertheless, after 30-min equilibration, sperm diluted in glucose or NaCl at high osmolalities, combined with either DMSO or MG, was motile for longer than 40 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 canal micropylar 20 s after the addition of water (Maria, 2008). Based on this finding, we can hypothesize that, 40 s is long enough for spermatozoa to find and enter the micropyle and fertilize an oocyte in *B. opalinus*.

Based on these findings, we can conclude that the initiation of sperm motility is triggered by low osmolality rather than the ionic composition of the surrounding medium in *B. opalinus*. Glucose or NaCl solutions at high osmolalities combined with either DMSO or MG prevent the initiation of sperm motility during storage. Sperm diluted in these media yields motility upon activation above 77% and that should last long enough to fertilize oocytes. These media are recommended as freezing media for future essays in cryopreservation of *B. opalinus* sperm.

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