Neotropical Ichthyology, 13(1): 157-164, 2015

DOI: 10.1590/1982-0224-20140066

Fresh, equilibrated and post-thaw sperm quality of *Brycon orbignyanus* (Valenciennes, 1850) and *Prochilodus lineatus* (Valenciennes, 1837) treated with either salmon GnRHa and domperidone or pituitary extract

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The effects of reduced doses of Ovaprim™ (GnRHa + domperidone) on sperm release of *Brycon orbignyanus* and *Prochilodus lineatus* were evaluated. Furthermore, sperm quality was compared among fresh, equilibrated and post-thaw samples. Males received a single and reduced dose of Ovaprim™ (0.125 or 0.25 ml/kg); control males received pituitary extract (cPE; 3 mg/kg). Fresh sperm was evaluated for volume, concentration, seminal plasma osmolality and seminal plasma pH. Then sperm was diluted in a freezing medium, equilibrated for 15-20 min and frozen in nitrogen vapor vessel (dry-shipper). Sperm motility was analyzed during 60 s post-activation in fresh, equilibrated and post-thaw samples. Sperm quality of males treated with Ovaprim™ (both doses) were not different from that of cPE-treated males, thus these data were pooled. In *B. orbignyanus*, motility was higher in fresh (99%) than in equilibrated sperm (81%); post-thaw motility dropped to 42%. In *P. lineatus*, motility was similar in fresh (99%) and equilibrated sperm (92%); post-thaw motility was 73%. Motility decreased as a function of time post-activation, and this decrease was significant after 60 s in fresh and equilibrated sperm, and as soon as 30 s in post-thaw sperm, in both species. Ovaprim™ at ¼ of the recommended dose can successfully replace cPE.

O efeito de doses reduzidas de Ovaprim® (GnRHa + domperidona) na liberação do sêmen de *Brycon orbignyanus* e *Prochilodus lineatus* foi avaliado. Além disso, a qualidade do sêmen foi comparada entre as amostras frescas, equilibradas e descongeladas. Os machos receberam dose única e reduzida de Ovaprim® (0,125 ou 0,25 ml/kg); os machos-controle receberam extrato de hipófise (cPE; 3 mg/kg). O sêmen fresco foi avaliado quanto ao volume, concentração, e osmolalidade e pH do plasma seminal. Em seguida, o sêmen foi diluído num meio de congelamento, equilibrado por 15-20 min e congelado em botijão de vapor de nitrogênio (*dry-shipper*). A motilidade espermática foi analisada durante 60 s pós-ativação no sêmen fresco, equilibrado e descongelado. A qualidade do sêmen não diferiu entre os machos tratados com Ovaprim® (ambas as doses) ou cPE, assim foi feito um *pool* desses dados. Em *B. orbignyanus*, a motilidade foi maior no sêmen fresco (99%) do que no equilibrado (81%); a motilidade do sêmen descongelado caiu para 42%. Em *P. lineatus*, a motilidade foi semelhante entre o sêmen fresco (99%) e equilibrado (92%); a motilidade do sêmen descongelado foi 73%. A motilidade caiu em função do tempo pós-ativação, e essa queda foi significante após 60 s no sêmen fresco e equilibrado, e tão precoce quanto 30 s no sêmen descongelado, em ambas as espécies. Ovaprim® a ¼ da dose recomendada pode substituir o cPE com sucesso.

Keywords: CASA, Characiformes, cryopreservation, Ovaprim™, sperm motility.

Introduction

The piracanjuba *Brycon orbignyanus* and the streaked prochilod *Prochilodus lineatus* are migratory Characiformes species native to South America. The *B. orbignyanus* belongs to the family Characidae, is endemic to the Paraná-Paraguay River basin (Godoy, 1975) and its population is declining due mainly to overfishing, destruction of riparian vegetation, pollution and construction of hydroelectric dams (Rosa & Lima, 2008). *Brycon orbignyanus* has a great potential for aquaculture and restocking programs, for recreational fisheries, and artificial propagation; reintroduction have been emphasized for conservation, management and recovery of this species (Maria *et al*., 2006). *Prochilodus lineatus* belongs to the family Prochilodontidae and has a large geographical distribution in the Prata River basin

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and Paraíba River basin (Godoy, 1975). Because *P. lineatus* larvae are used as live food for endangered carnivorous species such as *B. orbignyanus* and *Zungaro jahu* (Ihering, 1898), in addition to serving as a human food source, *P. lineatus* are of great importance to the aquaculture industry (Viveiros *et al*., 2009). As artificial reproduction methods are well established and its ability to produce an offspring with a large number of individuals is high, the *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).

Like the vast majority of fish species reared in captivity, *B. orbignyanus* and *P. lineatus* exhibit some form of reproductive dysfunction. In females there is a failure to undergo final oocyte maturation, ovulation and spawning, while in males sperm production is reduced and/or of low quality. Such reproductive dysfunctions are mainly due to the fact that fish in captivity do not experience the natural conditions of the spawning grounds and as a result the pituitary fails to release the maturational gonadotropin, luteinizing hormone (LH), (Zohar & Mylonas, 2001). Therefore, the use of hormone therapy to increase semen volume and quality is a common technique in several species, including the Characiformes.

Despite the great success of pituitary extract (PE) as spermiation inducer of several fish species, its use has been associated with various drawbacks such as the great variability in pituitary luteinizing hormone (LH) content, and the potential for disease transmission from donor fish to recipient broodstock (Zohar & Mylonas, 2001). These concerns led the Brazilian Ministry of Agriculture (MAPA) to prohibit the use of PE in some States, including Minas Gerais. Ovaprim™ is a commercial product that contains a combination of salmon gonadotropin releasing hormone analog (sGnRHa) and a dopamine antagonist, that has been used with success in species of Cypriniformes (Cejko *et al*., 2008, 2012; Jamróz *et al*., 2008; Seifi *et al*., 2011), Osmeriformes (Król *et al*., 2009), Siluriformes (Viveiros *et al*., 2002), among others, at the recommended dose of 0.5 mL/kg BW set by the manufacturer. To the best of our knowledge, the efficiency of Ovaprim™ as spermiation inducer has been reported in only two Characiformes species. *P. lineatus* males treated with a primer dose of *Clarias* PE (0.4 mg/kg) followed by a dose of Ovaprim™ (0.25 mL/kg) produced semen volume, sperm concentration, sperm motility rate, sperm velocities, semen pH and semen osmolality not significantly different from males treated with two doses of *Clarias* PE (0.4 + 4 mg/kg) (Viveiros *et al*., 2013). Similarly, semen volume and concentration of *Colossoma macropomum* (Cuvier, 1816) were not affected by Ovaprim™ (0.5 mL/kg) when compared to cPE (1.5 mg/kg) treatment (Arias Acuña & Hernández Rangel, 2009). There is a need for more research on hormone therapies to replace cPE in Characiformes species, such as the use of other hormones (GnRHa combined with other dopamine antagonist, and human Chorionic Gonadotropin), as well as the test of single and double doses and the least effective concentration.

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 for endangered species such as *B. orbignyanus*, which may help ensure genetic diversity and reproductive success for population management strategies (Viveiros *et al*., 2009). Both *B. orbignyanus* and *P. lineatus* have undergone sperm cryopreservation studies, with promising results (Maria *et al*., 2006; Viveiros *et al*., 2009; among others). However, the cryopreservation process reduces post-thaw sperm motility and velocities, and increases membrane and organelles damages, as cells are exposed to toxic substances (such as cryoprotectants), osmotic shock and intracellular freezing (Li *et al*., 2006). This loss of quality can take place even before freezing, when sperm is diluted and equilibrated in a given freezing medium. These damages can only be detected when not only post-thaw samples but also equilibrated samples are evaluated. However, sperm quality of equilibrated samples is hardly determined nor reported.

Thus, in the present study, we evaluated fresh sperm quality of males treated with single and reduced doses of Ovaprim™ and compared with that of males treated with cPE, in *Prochilodus lineatus* and *Brycon orbignyanus*. Ovaprim™ was tested at ½ and ¼ of the recommended dose. Furthermore, sperm was cryopreserved and the quality was compared among fresh, equilibrated (after dilution and before freezing) and post-thaw sperm.

Material and methods

Fish handling and sperm collection. All fish were handled following the guidelines for animal experimentation in Van Zutphen *et al*. (2001). During the spawning season (December to February), *B. orbignyanus* and *P. lineatus* males were selected from earthen ponds of the Hydrobiology and Fish Culture Station of Furnas in the city of São José da Barra, Minas Gerais State, Brazil (20°43'07'' S; 46°18'50'' W), where they were hatched and reared; during that time, fish were fed commercial diet containing 32% of crude protein. *B. orbignyanus* males (n = 18) with an average body weight (BW) of 0.9 ± 0.1 kg and *P. lineatus* males (n = 21) with an average BW of 1.1 ± 0.3 kg were divided into three groups. Each group received one single intramuscular dose of: cPE (Argent Chemical Laboratory, Redmond, WA, USA) at 3 mg/kg BW which is the routine method to induce spermiation in this Fish Culture Station; Ovaprim™ at 0.125 mL/kg BW; or Ovaprim™ at 0.25 mL/kg BW. One mL of Ovaprim™ (Syndel Laboratories Ltd., Qualicum Beach, BC, Canada) contains 20 µg of salmon GnRH analogue and 10 mg of domperidone. Water temperature was kept at approximately 27°C. Because the hour-degree from hormone injection to spermiation is different between these two species, *B. orbignyanus* males were hand-stripped after 4 h

and *P. lineatus* after 8 h; the urogenital papilla was carefully dried, sperm was collected into glass tubes and maintained in a cooler (9-11ºC) containing dry ice foam (Polar Technics CRI Ltd., Brazil). Sperm collection was carried out at room temperature (22-24°C), and contamination with water, feces or urine was carefully avoided.

Fresh sperm evaluation. 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 x magnification. Motility rate (expressed as % of motile sperm) was subjectively estimated in intervals of 5% following the addition of 500 µL of an activating agent, composed of a NaCl solution at an osmolality of 120 ± 10 mOsm/kg (60-70 mM NaCl), according to our previous results (Gonçalves *et al*., 2013). Motility velocity score (a subjective analysis of sperm velocity) was determined using an arbitrary grading system ranging from 0 (no movement) to 5 (rapidly swimming sperm), according to Viveiros *et al*. (2011). Because motility analysis was carried out in the field, we could not use our Computer-Assisted Sperm Analyzer (CASA); all we could transport from the lab was the light microscope. To track the decrease of sperm motility after activation, both rate and quality score were evaluated at 0 (~3s), 20, 40 and 60 s post-activation. Sperm concentration (hemacytometer; Neubauer chamber; Boeco, Hamburg, Germany) was also determined. The osmolality (Semi-Micro Osmometer K-7400, Knauer, Berlin, Germany) and pH (pHmeter DM-22, Digimed, São Paulo, Brazil) of the seminal plasma was measured after the centrifugation of sperm at 2000 x g for 30 min (MiniStar, Shanghai, China). Sperm characteristics of all samples were evaluated by the same well-trained technician and at room temperature.

Equilibrated sperm evaluation. Sperm of each male was diluted in a freezing medium containing methyl glycol (Vetec Química Fina Ltda, Duque de Caxias, RJ, Brazil) as cryoprotectant and one extender, according to our previous results. Beltsville Thawing Solution (BTS™) at 342 mOsm/kg (~5%) was used as extender for *B. orbignyanus* sperm (Maria *et al*., 2006) and glucose at 315 mOsm/kg (~5%) for *P. lineatus* sperm (Viveiros *et al*., 2009). BTS™ (Minitub™, Tiefenbach, Landshut, Germany) contains 222.0 mM glucose, 24.4 mM sodium citrate, 4.4 mM EDTA, 15.4 mM $NaHCO_3$, 10.7 mM KCl and 5 g/L gentamycin sulfate. The final dilution ratio was set at 10% sperm, 10% methyl glycol and 80% extender. After 10-15 min of equilibration time in a cooler (9–11ºC) containing dry ice foam, motility rate and motility velocity score were subjectively estimated at 0, 20, 40 and 60 s post-activation, as described for fresh sperm analysis.

Sperm cryopreservation and post-thaw sperm evaluation. During the 15-20 min equilibration time, sperm was loaded into triplicate straws. The total amount of straws was 54 for *B. orbignyanus* (three 0.25-mL straws x 3 hormone treatments x 6 males) and 63 for *P. lineatus* (three 0.5-mL-straws x 3 hormone treatments x 7 males). All straws were frozen in a nitrogen vapor vessel (Cryoporter LN_2 *dry vapor shipper*, Cryoport Systems, Brea, CA, USA) at approximately -170°C, immediately transported from Furnas to the Laboratory of Semen Technology at the Federal University of Lavras (UFLA), Lavras, Minas Gerais, Brazil (approximately 250 km) and then transferred to liquid nitrogen vessel (M.V.E. Millenium, XC 20, Chart, MN, USA) at -196°C within 20 to 24 h for storage.

Approximately two months later, straws were thawed in a 60°C water bath (Water-bath MA 127, Marconi, Brazil) for 3 s (0.25 mL straws) or for 8 s (0.5 mL straws), and post-thaw sperm quality was estimated using the CASA system (Computer-Assisted Sperm Analyzer), following the methodology described in Viveiros *et al*. (2013). Briefly, post-thaw sperm was activated directly in a Makler™ counting chamber (Sefi-Medical Instruments ltd, Haifa, Israel) to a final dilution ratio of 1:50 (*B. orbignyanus*) or 1:500 (*P. lineatus*). The Makler™ chamber was placed under a phase-contrast microscope (Nikon Eclipse E200, Tokyo, Japan) at 100 x magnification with a green filter and pH 1 position. The microscope was connected to a video camera (Basler Vision Technologies™ A602FC, Ahrensburg, Germany) generating 100 images/s at 10, 30, 50 and 70 s post-activation (1 s of video at each evaluation time). We could not analyze motility at 0 s post-activation, as we did for fresh and equilibrated sperm, because it takes approximately 10 s to cover the Makler™ chamber, focus and find a good field for motility analysis. Each image was analyzed using the adjusted 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 731 *B. orbignyanus* sperm per field and 383 *P. lineatus* sperm per field) were followed throughout the images and sperm trajectory was calculated.

Statistical analysis. The values are expressed as mean ± standard deviation (SD). Statistical analyses were conducted using the R Development Core Team Computational Program (2010). Data were evaluated for normal distribution using the Shapiro-Wilk test. Data on subjective motility, quality motility score, seminal plasma osmolality and VCL did not fit the normal distribution and thus arcsin transformation was performed. Statistical significance was tested using analysis of variance (ANOVA) followed by Scott-Knott test when applicable. The level of significance for all statistical tests was set to 5% ($P < 0.05$).

Results

Fresh sperm motility. All males responded to the Ovaprim™ and cPE treatments and were thus stripped of sperm. Fresh sperm quality parameters of ovaprim-treated males (both doses) were not different (P > 0.05) from cPE-treated males (Table 1). All together, the 18 *B. orbignyanus* males (pooled data) possessed a mean semen volume of 8.8 mL, concentration of 12.4 x 10^9 spermatozoa/mL, motility rate of 99%, motility velocity score of 4.9, seminal plasma osmolality of 300 mOsm/kg and pH of 7.7. The 21 *P. lineatus* males (pooled data) possessed a mean semen volume of 3.1 mL, concentration of 27.3 x 10^9 spermatozoa/mL, motility rate of 99%, motility velocity score of 4.9, seminal plasma osmolality of 271 mOsm/kg and pH of 7.8.

In order to track the decrease of fresh sperm motility after activation, both rate and velocity score were evaluated during 60 s. As these results were similar between ovaprim- and cPE-treated males, the data were pooled per fish species (Table 2). Motility decreased slowly as a function of time and was significantly lower when evaluated after 60 s compared to that at 0 s. Motility of *B. orbignyanus* fresh sperm decreased from a rate of 99 to 88% and from a velocity score of 4.9 to 3.7, and of *P. lineatus* sperm decreased from a rate of 99 to 84% and from a quality score of 4.9 to 3.6 after 60 s of analysis.

Equilibrated sperm motility. Motility evaluated after a 10-15 min of sperm contact with the freezing medium (equilibrated samples) and for a period of 60 s post-activation was also not affected by Ovaprim™ treatment. Thus, to facilitate the description of these results the data were pooled per fish species (Table 2). Motility decreased slowly as a function of time and was significantly lower when evaluated after 60 s compared to that at 0 s. Motility of *B. orbignyanus* equilibrated sperm decreased from a rate of 81 to 66% and from a quality score of 3.9 to 2.6, and of *P. lineatus* sperm decreased from a rate of 92 to 73% and from a quality score of 4.3 to 2.9 after 60 s of analysis.

Fresh x equilibrated sperm motility. Because fresh ad equilibrated samples were both analyzed for subjective motility in the field, we could carry out some statistical analysis among these data (Table 2). In *B. orbignyanus*, motility rate and velocity score were always higher (P<0.05) in fresh sperm (99-88%; score 4.9-3.7) compared to sperm equilibrated in the freezing medium (81-66%; score 3.9-2.6), at all observation times. In *P. lineatus*, motility rate was similar between fresh (99-84%) and equilibrated sperm (92-73%), while motility velocity score was always higher (P<0.05) in fresh (4.9-3.6) compared to equilibrated sperm (4.3-2.9), at all observation times.

Table 1. Male body weight and fresh sperm quality (mean ± SD) of *Brycon orbignyanus* and *Prochilodus lineatus* males after treatment with a single dose of carp pituitary extract (cPE) or Ovaprim™.

Features	Hormonal treatment (/kg BW)			Overall mean
	cPE (3 mg)	Ovaprim™ (0.125 mL)	Ovaprim™ (0.25 mL)	
B. orbignyanus				
Spermiated/treated males	6/6	6/6	6/6	18/18
Body weight (kg)	0.9 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	0.9 ± 0.1
Semen volume (mL)	10.8 ± 2.8	6.4 ± 1.9	9.1 ± 5.3	8.8 ± 3.9
Concentration (sperm x 10^9/mL)	11.9 ± 0.4	12.1 ± 1.8	13.4 ± 2.1	12.4 ± 1.7
Subjective motility rate (%)	98 ± 4	98 ± 4	100 ± 0	99 ± 3
Motility velocity score[1] (0 - 5)	5.0 ± 0.0	4.8 ± 0.4	5.0 ± 0.0	4.9 ± 0.2
Seminal plasma mOsm/kg	303 ± 15	302 ± 13	294 ± 10	300 ± 13
Seminal plasma pH	7.8 ± 0.1	7.6 ± 0.3	7.8 ± 0.2	7.7 ± 0.2
P. lineatus				
Spermiated/treated males	7/7	7/7	7/7	21/21
Body weight (kg)	1.1 ± 0.2	1.1 ± 0.3	1.1 ± 0.4	1.1 ± 0.3
Semen volume (mL)	2.9 ± 1.4	3.0 ± 1.5	3.4 ± 1.4	3.1 ± 1.4
Concentration (sperm x 10^9/mL)	28.5 ± 2.0	25.3 ± 3.4	28.1 ± 3.0	27.3 ± 3.1
Subjective motility rate (%)	100 ± 0	99 ± 4	97 ± 3	99 ± 3
Motility velocity score[1] (0 - 5)	5.0 ± 0.0	4.9 ± 0.4	4.7 ± 0.5	4.9 ± 0.4
Seminal plasma mOsm/kg	258 ± 28	274 ± 8	281 ± 8	271 ± 20
Seminal plasma pH	7.6 ± 0.2	7.8 ± 0.2	7.9 ± 0.2	7.8 ± 0.3

[1]A motility velocity score was assigned using an arbitrary grading system from 0 (no movement) to 5 (rapidly swimming sperm).
There was no effect (P > 0.05) of hormonal treatment on fresh sperm quality.

Table 2. Motility rate and motility velocity score (mean ± SD) of fresh and equilibrated sperm of *Brycon orbignyanus* (*n* = 18 males) and *Prochilodus lineatus* (*n* = 21 males) evaluated after 0, 20, 40 and 60s of activation.

S post-activation	Motility rate (%)		Velocity score (0-5) [1]	
B. orbignyanus	Fresh	Equilibrated [2]	Fresh	Equilibrated [2]
0	99 ± 3 [a]	81 ± 10 [b]	4.9 ± 0.2 [a]	3.9 ± 0.3 [b]
20	96 ± 6 [a]	77 ± 11 [b]	4.7 ± 0.5 [a]	3.6 ± 0.5 [b]
40	94 ± 7 [a]	71 ± 11 [b]	4.1 ± 0.6 [a]	2.9 ± 0.4 [b]
60	88 ± 9 [a *]	66 ± 12 [b *]	3.7 ± 0.5 [a *]	2.6 ± 0.5 [b *]
P. lineatus	Fresh	Equilibrated [2]	Fresh	Equilibrated [2]
0	99 ± 3 [a]	92 ± 6 [a]	4.9 ± 0.4 [a]	4.3 ± 0.5 [b]
20	96 ± 7 [a]	89 ± 9 [a]	4.6 ± 0.5 [a]	3.7 ± 0.6 [b]
40	91 ± 9 [a]	81 ± 11 [a]	4.0 ± 0.6 [a]	3.3 ± 0.6 [b]
60	84 ± 10 [a *]	73 ± 15 [a *]	3.6 ± 0.6 [a *]	2.9 ± 0.8 [b *]

[1]Motility velocity score was assigned using an arbitrary grading system from 0 (no movement) to 5 (rapidly swimming sperm).
[2]Sperm was diluted in a freezing medium containing methyl glycol and an extender (BTS™ for *B. orbignyanus* and glucose for *P. lineatus*) and evaluated after a 10-15 min equilibration time.
a, b Means within the same row followed by different lowercase, differ (F test, $P < 0.05$).
*Means within the same column were lower when evaluated at 60s than those evaluated at 0s post-activation (Scott-Knott, $P < 0.05$).

Post-thaw sperm motility. Post-thaw sperm motility and velocities were not different ($P > 0.05$) between ovaprim- and cPE-treated males of both fish species. Thus, to facilitate the description of these results the data were pooled per fish species.

Motility and velocities decreased fast as a function of time after activation, and were always higher ($P<0.05$) when evaluated at 10 s post-activation compared to all the other observation times. Motility rate decreased from 42 to 25% in *B. orbignyanus* and from 73 to 54% in *P. lineatus* after only 20 s of analysis (at 10 and 30 s post-activation). At 70 s post-activation, motility rate was only 11% in *B. orbignyanus* (Figure 1A) and 12% in *P. lineatus* (Figure 1B). Similarly, during the first 20 s of analysis, VCL decreased from 160 to 101 µm/s, VSL from 103 to 54 µm/s and VAP from 133 to 77 µm/s in *B. orbignyanus* (Figure 2A), while in *P. lineatus* VCL decreased from 277 to 126 µm/s, VSL from 171 to 80 µm/s and VAP from 254 to 100 µm/s (Figure 2B).

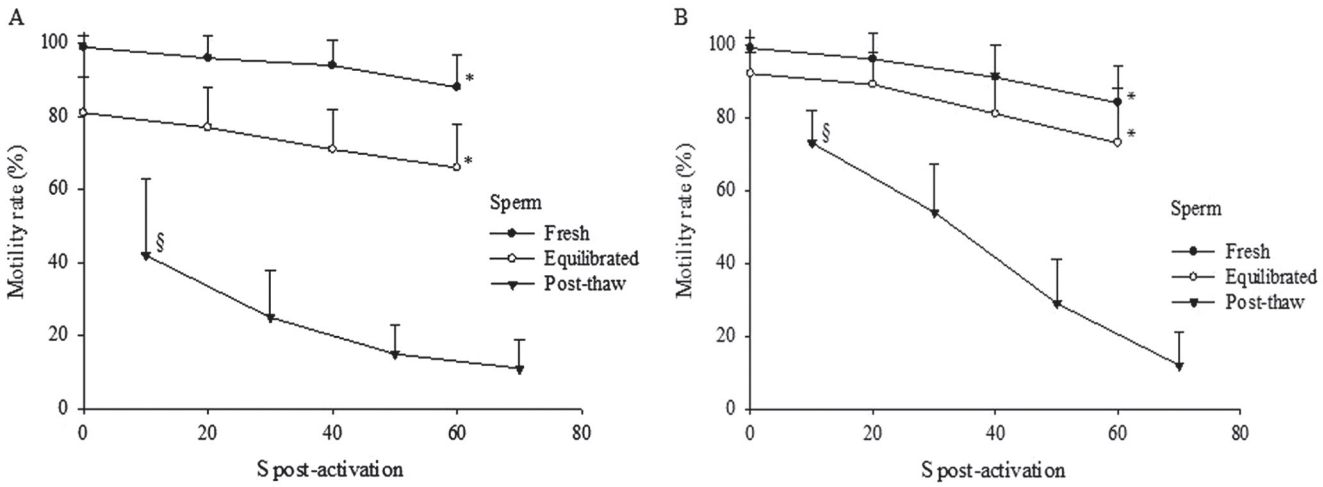

Fig. 1. Motility rate of fresh, equilibrated and post-thaw sperm of *Brycon orbignyanus* (A; *n* = 18 males) and *Prochilodus lineatus* (B; *n* = 21 males). Motility rate was evaluated after 0, 20, 40 and 60s (fresh and equilibrated sperm) or after 10, 30, 50 and 70s of activation (post-thaw sperm). *Motility evaluated at 60s was lower than that at 0s post-activation (Scott-Knott, $P < 0.05$). § Motility evaluated at 10s post-activation was the highest (Scott-Knott, $P < 0.05$).

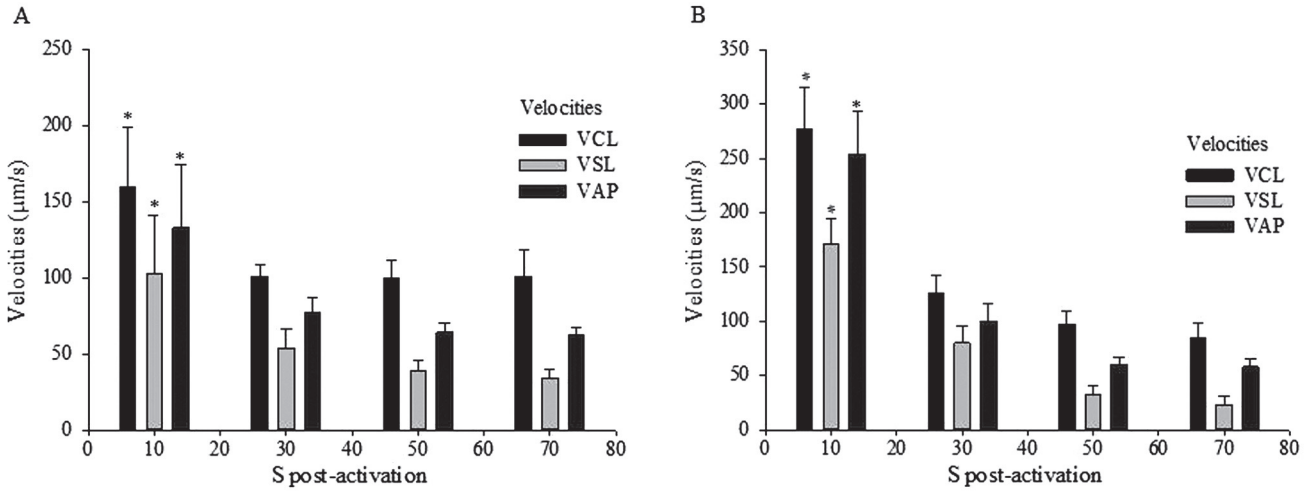

Fig. 2. Post-thaw sperm velocities (curvilinear = VCL; straight-line = VSL; average path = VAP) of *Brycon orbignyanus* (A; *n* = 18 males) and *Prochilodus lineatus* (B; *n* = 21 males) evaluated after 10, 30, 50 and 70s after activation. * Mean at 10s was the highest (Scott-Knott, $P < 0.05$).

Discussion

The present study demonstrates for the first time the efficiency of Ovaprim™ therapy to facilitate semen collection of *B. orbignyanus*. Furthermore, we demonstrated that single and reduced doses of Ovaprim™ are as effective as cPE therapy in terms of sperm quality, even after cryopreservation, in both *B. orbignyanus* and *P. lineatus*.

Ovaprim™ stimulates the release of endogenous pituitary gonadotropins, while the exogenous gonadotropins present in cPE stimulate the gonads directly. Both therapies facilitated semen release and collection in all males, of both fish species. The values observed for sperm quality were all within the range previously reported for both fish species (Maria *et al*., 2006; Gonçalves *et al*., 2013; Nascimento *et al*., 2012; among others). The recommended dose of Ovaprim™ is set at 0.5 mL/kg, yet both *B. orbignyanus* and *P. lineatus* males responded to lower doses, such as ½ and ¼ of the recommended dose, without any loss of sperm quality when compared to the routine method used in the Fish Culture, the cPE. This is a great economy as one 10-mL bottle of Ovaprim™ contains hormones to induce spermiation of 80 kg of fish (at least in these two species) when administered at 0.125 mL/kg, rather than only 20 kg of fish using the recommended dose.

Because the evaluation of fresh and equilibrated sperm was carried out in the field, only subjective motility analysis was performed. Post-thaw motility, on the other hand, was evaluated in the lab, thus using the CASA system. Yet, we can pull out some information concerning motility rate among fresh, equilibrated and post-thaw sperm. In *B. orbignyanus*, motility rate significantly decreased from fresh (99%) to equilibrated sperm (81%), and then dropped after thawing (42%). Similarly, motility velocity score (the subjective analysis of sperm velocity) decreased after sperm was equilibrated in the freezing medium compared to fresh samples. The freezing media utilized in this study has been tested after 30 min of equilibration (Nascimento *et al*., 2012) and after freezing and thawing (Maria *et al*., 2006) with better results compared to other media. Yet the medium composed of BTS™ and methyl glycol seems inadequate for *B. orbignyanus* sperm as motility significant decreased after only 10-15 min of contact, when compared to fresh sperm. Thus, we suggest that other freezing media should be tested during the cryopreservation of *B. orbignyanus* sperm.

On the other hand, in *P. lineatus*, motility rate was similar between fresh (99%) and equilibrated sperm (92%) and decreased only after thawing (73%), but not to values as low as those observed for *B. orbignyanus* sperm. Motility velocity score, however, was lower in equilibrated samples compared to fresh ones. The freezing media utilized in this study has been tested after 30 min of equilibration (Nascimento *et al*., 2012) and after freezing and thawing (Viveiros *et al*., 2009, 2010) with better results compared to other media. In our laboratory, we consider high post-thaw sperm quality when motility rate is above 60% and VCL is above 140 μm/s. Thus, the freezing medium composed of glucose and methyl glycol was suitable for the cryopreservation of *P. lineatus* sperm.

In the present study, motility decreased as a function of time after activation, and this decrease was significant after 60 s in fresh samples. It is interesting to observe that, although significant, this decrease was not that intense as more than 80% of the sperm were still moving after 60 s, and with a velocity score as high as 3.6-3.7. In most of the fish species, fresh sperm motility lasts from 30 s to few min and initial velocity is high (up to 300 μm/s) (Cosson 2008). The duration of fresh sperm motility of *P. lineatus* can last up to 2 min, as previously reported (Orfão *et al*., 2010). However, the freezing and thawing processes exert a strong and negative effect on the duration of sperm

motility of both fish species. In frozen samples, motility (both rate and velocities) significantly decreased as soon as after 30 s post-activation. Despite the fast decrease of motility post-activation, this fact may not interfere with the fertilization ability of frozen sperm. It has been reported that Bryconinae sperm needs only few seconds (perhaps < 20 s) of motility to fertilize an oocyte as several sperm were observed on the opening of the micropyle and more than one inside the micropylar canal 20 s after water was added (Isaú *et al*., 2013). However, in order to be on the safe side, we recommend the use of a higher spermatozoa:oocyte ratio when using frozen sperm for fertilization, compared to fresh sperm. In our previous study, *P. lineatus* sperm was frozen using the same freezing medium and methods as described here; after thawing, a ratio of 5 x 10^5 thawed spermatozoa per oocyte was used and a successful mean fertilization rate of 74% was achieved (Viveiros *et al*., 2009).

These results indicate that Ovaprim™ at a lower dose of 0.125 mL/kg is a suitable therapy to facilitate the release and collection of high quality sperm in both *P. lineatus* and *B. orbignyanus*. Sperm obtained from ovaprim-treated males can be cryopreserved without any loss of quality, compared to sperm collected after cPE therapy. The methodology described above to cryopreserve *P. lineatus* sperm is efficient and maintain good sperm quality; however, other freezing media should be tested in *B. orbignyanus* sperm as motility decreased after dilution, even before freezing. Fertilization of oocytes using frozen sperm should be carried out with greater attention as, after activation, both sperm motility and velocities significantly decreased rapidly.

Acknowledgments

This study received funding from the Brazilian fostering agencies CNPq (PQ 302434/2011-9; 554950/2009-0; 142816/2009-4; 471393/2011-8), ANEEL P&D Furnas (017965) and FAPEMIG (PPM 00038-13; BPD 00167-12). This research is part of A.C.S. Gonçalves' Ph.D. project. The authors thank the undergraduate students L.F.R. Pereira and M.A.G. Lemes (UFLA), and the biologists D.M. Ribeiro and M.B. Goulart (Furnas) for assistance during the experiments, and A.C. Costa (UFLA) for statistical support.

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Submitted August 01, 2014
Accepted September 20, 2014 by Bernardo Baldisserotto
Published March 31, 2015