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Effects on fertility of motile sperm to egg ratio with use of cryopreserved *Rhamdia quelen* semen at different post-activation times

Giovano Neumann*, Paulo Vanderlei Sanches, Robie Allan Bombardelli

Laboratório de Tecnologia da Reprodução de Animais Aquáticos Cultiváveis (LATRAAC), Insituto de Pesquisas em Aquicultura Ambiental, Universidade Estadual do Oeste do Paraná – UNIOESTE, Rua da Faculdade 645, Jardim La Salle, Toledo, Paraná, Brazil

A R T I C L E I N F O

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A B S T R A C T

The aim in the present study was to evaluate the effects of motile sperm:oocyte ratio and the use of thawed spermatozoa at different post-activation times in artificial reproduction of gray catfish (*Rhamdia quelen*). Cryopreserved sperm samples were evaluated for sperm motility and velocity using Computer Assisted Sperm Analysis (CASA). The sperm activation curves were generated using a non-linear statistical model and were used to assess the spermatozoa velocity after thawing. Thus, the oocytes were mixed with thawed sperm at a motile sperm:oocyte ratio of 70,000, 90,000, 110,000, 130,000, 150,000 and 170,000. The thawed sperm were used at 11, 16 and 30 s after spermatozoa activation. At these times, the sperm velocities corresponded to 52, 37 and 21 μm/s. The effects of experimental factors (spermatozoa:oocyte ratio and time after sperm activation) on oocyte fertilization, egg hatching, and percentage of normal larvae were evaluated. The response surface analysis indicated there was no interaction ($P > 0.05$) between the motile spermatozoa:oocyte ratio and time after sperm activation on fertilization, hatching or percentage of normal larvae. The time after sperm activation, however, affected ($P < 0.05$) in a directly proportional waythe oocyte fertilization and egg hatching rates. The time after sperm activation affected the sperm velocity and oocyte fertilization and egg hatching rates. Thus, the use of thawed sperm immediately after sperm activation or with the greatest sperm velocities (11s; 52 μm/s; 62.59% motility) at a relatively lesser motile sperm:oocyte ratio (70,000:1) allows for acceptable fertilization (48.68% for fertilization; 29.61% for hatching) in *Rhamdia quelen*.

1. Introduction

Sperm cryopreservation is an important technique for developing cryopreserved sperm banks that may contribute to biodiversity conservation and genetic improvement programs in aquaculture (Martínez-Páramo et al., 2017). Sperm cryopreservation of fish is used experimentally in temperate climates (Cabrita et al., 2010) with saltwater fish (Magnotti et al., 2018) and in at least 17 neotropical species (Maria and Carneiro, 2012) such as the tambaqui (*Colossoma macropomum*) (Gallego et al., 2017). In all species, cryopreservation results in cell damage and leads to losses of sperm viability (Cabrita et al., 2014). These losses distract from the implementation of the use of cryopreserved semen at a large-scale and can limit the potential development of some sperm banks (Adames et al., 2015). Strategies to optimize the use of sperm for artificial fertilization, therefore, will allow for a reduction of the sperm:oocyte requirement and facilitate the use of cryopreservated sperm for large scale aquaculture.

* Corresponding author.
E-mail address: giovanoneumann@hotmail.com (G. Neumann).

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The damages caused by cryopreservation result from the formation of intracellular ice crystals (Barbas and Mascarenhas, 2009). Sperm cryopreservation also results in changes in the fluidity and the functional properties of cell membranes (Castelo, 2010) due to osmotic changes (Martínez-Páramo et al., 2012), addition of alcohol cryoprotectants (Arruda, 2000) or processes of freezing and thawing (Wang et al., 2010). This cellular damage usually reduces the quality of sperm (Rurangwa et al., 2004), affecting the membrane integrity, motility rate, velocity, and linearity index (Martínez-Páramo et al., 2012).

Motility rate (Gallego and Asturiano, 2018) and the velocity of sperm (Viveiros et al., 2010) are widely used to assess the effects of semen processing on the viability and fertility. Among these, sperm velocity is directly related to oocyte fertilization, because sperm with rapid motility are more likely to reach the oocyte and participate in the fertilization process (Gage et al., 2004; Liljedal et al., 2008; Gasparini et al., 2010). Furthermore, sperm density (Stoltz and Neff, 2006; Boschetto et al., 2011) and velocity may have an interactive effect on the probability that gametes will contribute to the process of fertilization (Browne et al., 2015) because the use of a greater number of sperm for artificial insemination with lesser motility will not overcome the suppression on fertility that results from use of sperm with relatively lesser motility as compared with those with relatively greater motility.

Research in the area of sperm cryopreservation is relevant to *Rhamdia quelen* because there is a typical requirement for the use of about 90,000 spermatozoa per oocyte to ensure satisfactory rates of fertilization with use of artificial fertilization (Bombardelli et al., 2006). In addition, results from previous studies indicate there is a possibility to reduce this ratio when sperm motility is relatively greater with the use of activation solutions enriched with sugars (Adames et al., 2015). Studies on *Rhamdia quelen* are relevant to aquaculture because the fish have been widely used in fish farming in Central and South America (Bombardelli et al., 2015). *Rhamdia quelen* have desirable growth rates during development, and its meat is valued by the consumer (Barcellos et al., 2004; Bochi et al., 2008). Because of these qualities, this species has been utilized in several farming systems (Barcellos et al., 2004; Freitas et al., 2011) and the importance of use of cryopreserved semen has been highlighted for enhancement of economic profitability. The reduction of cost of reproduction and establishment of the genetic improvement programs would be facilitated with the use of artificial fertilization (Caffey and Tiersch, 2011; Martínez-Páramo et al., 2017) with similar implementation as to what has occurred with salmon (Buchanan and Barbosa-Solomieu, 2011; Yang et al., 2018).

The present study, therefore, was conducted to assess the relationship between sperm velocity of cryopreserved sperm and the requeriment of motile sperm in the artificial fertilization of *Rhamdia quelen* oocytes by applying non-linear models for prediction of sperm motility and velocity.

2. Material and methods

2.1. Fish handling and gamete collection

The fish used were from a 2-year-old broodstock, which was reared in a masonry tank with soil at the bottom (200 m^2), with a water replacement system that allowed for recovery of the water through use of infiltration and evaporation procedures. Fish were fed daily, twice a day, at a rate of 2% of the total biomass with commercial feed containing 32% crude protein and 13.38 kJ crude energy.

Males and females of *Rhamdia quelen* reproduce between September and March (Barcellos et al., 2002). So, the fish were caught in December 2015 using a trawl (18 mm mesh). Males (n = 6; 246.80 ± 61.74 g; mean ± standard error) were selected for the study that released milt upon gentle abdominal massage (Tessaro et al., 2012) and the females (n = 18; 361.39 ± 25.25 g) were selected that had a swollen abdomen and reddish urogenital papilla, which released oocytes upon slight abdominal pressure. The males were subsequently subjected to hormonal treatments with 3.0 mg carp pituitary extract (CPE)/kg applied intramuscularly (Sanches et al., 2011). The females were treated intramuscularly with 5.5 mg CPE/kg in two doses (0.5 and 5.0 mg CPE/kg) at an interval of 12 h between doses (Sanches et al., 2011).

Sperm and oocytes were collected 10 h (water at 24 °C) after the last hormonal treatment. The gametes were collected by applying a gentle abdominal massage in the head-to-tail direction (Sanches et al., 2011). The first drop of released sperm was discarded to avoid possible contamination with urine, mucus, or feces (Khara et al., 2012). The sperm samples were collected directly into a graduated test tube with an accuracy of 0.1 mL. Immediately after the collection, the sperm samples were stored at 12.00 ± 0.32 °C (Kanuga et al., 2012) in an ice box with the temperature being monitored using a digital thermometer, during the period of sperm manipulation. Oocytes were collected before the fertilization assays were conducted and oocytes were placed directly on Petri dishes (Sanches et al., 2013).

The sperm from each male (6.23 ± 1.30 mL) was evaluated immediately after collection to verify the occurrence of sperm activation in undiluted fresh semen. These evaluations were conducted by observation using an optical microscope with a 40× objective (Nikon, Eclipse E200; with phase contrast) with the presence or absence of motile spermatozoa being assessed (Viveiros et al., 2012). These procedures were conducted to avoid the use of sperm samples with less motility that had been induced by unintentional sperm activation as a result of the way the sperm samples were processed. *Rhamdia quelen* spermatozoa, as compared to sperm of some other species, have a relative short duration of motility after application of the activation solution (40.50 ± 1.65 s). Sperm samples were only used from males with immotile spermatozoa immediately after sperm collection. The samples from six males were mixed so that a pooled semen sample was available for artificial insemination.

The Ethical Committee of Animal Use of Universidade Estadual do Oeste do Paraná (Brazil) approved the experimental procedures (CEUA Protocol n° 4212).

2.2. Assessment of fresh sperm

In the fresh sperm samples, only those containing motile sperm were evaluated because these variables were used to select the sperm samples which would be submitted for cryopreservation and subsequently used for fertilization assays. The other variables were not evaluated because these were not relevant for the fertilization assays because only cryopreserved sperm were used. To evaluate spermatozoa movement, an aliquot (1 μL; $n = 6$) from the pool of fresh sperm was diluted in 1000 μL of distilled water at 24.00 ± 0.16 °C (dilution of 1:1000; sperm:distilled water; v/v; there was no addition of bovine serum albumin). Immediately after the sperm dilution, 10 μL was deposited in a Neubauer hemocytometer chamber, overlapped by a coverslip, to evaluate the spermatozoa movement using an optical microscope (Nikon, Eclipse E200; with phase contrast) with a 10× objective, connected to a digital video camera (Basler, A602fc, Firewire, 656 × 490 pixels, 100fps). Assessments of sperm motility were made at 8 s after the induction of sperm activation and then every 1 s until there was cessation of sperm motility.

The AMCap software was used to record the spermatozoa movement videos, at a capture rate of 100 fps (Adames et al., 2015). There were assessments of sperm motility rate (MOT), curvilinear velocity (VCL), average path velocity (VAP), straight-line velocity (VSL), straightness (STR), linearity (LIN), wobble (WOB), beat/cross frequency (BCF), and duration of sperm movement using the Computer Assistant Sperm Analyzer (CASA) plugin free software (ImageJ 1.48; National Institutes of Health, USA, http://rsb.info.nih.gov/ij/) (Wilson-Leedy and Ingermann, 2007), adapted to the species (Adames et al., 2015). There was use of the following input variable values in the CASA plugin: a = 1, b = 30, c = 98, d = 12, e = 3, f = 10, g = 15, h = 5, I = 1, j = 15, k = 15, l = 25, m = 80, n = 80, o = 50, p = 60, q = 98, r = 943.3962, s = 0, t = 0, u = 0).

2.3. Sperm cryopreservation

The sperm cryopreservation was conducted using the procedures previously reported by Carolsfeld et al. (2003) with a modified dilution ratio of milt and extender composition. Thus, immediately after the collection of milt, samples from the fresh sperm pool were diluted at a ratio of 1:3 (sperm:extender; v/v) in an extender containing 5.0% D-fructose (Synth), 5.0% milk powder (Ninho fortified; Nestle), and 10% methanol (Synth) (3016 mOsm/kg; pH 6.88) (Adames et al., 2015). After dilution, the semen was immediately filled into 0.25-mL straws (IMV Technologies, L'Agile, France) frozen in vapor-phase nitrogen, and stored in dry shipper container (MVE®, mod. MVE SC4/2E) for 18 h (Carolsfeld et al., 2003). This procedure allows for achieving a temperature of −170 °C at a freezing rate of approximately −35.6 °C min (Maria et al., 2006). Straws were subsequently transferred and stored in liquid nitrogen at −196 °C (Adames et al., 2015). After 24 h, cryopreserved semen was subjected to the thawing protocol by immersing straws in water at room at 25 °C for 10 s (Adames et al., 2015).

2.4. Assessment of thawed sperm

After thawing, there was evaluation of sperm characteristics for the relevant sperm variables (motility rate, curvilinear velocity, average path velocity, straight-line velocity, straightness, linearity, wobble, beat/cross frequency, and duration of movement) in fresh semen as previously described. The analysis of sperm motility was conducted by dilution of sperm 1:250 (sperm:destilled water; v/v). The activation of thawed sperm was induced at a different dilution rate from fresh sperm to compensate for the sperm dilution in the extender (1:3) (sperm:extender;v/v) previously to the cryopreservation. These procedures allowed for the maintenance of the same number of spermatozoa on the view field during the CASA procedures, when compared to analysis of the fresh sperm. Six different straws were evaluated.

Aliquots of thawed semen were subsequently fixed in a buffer saline formalin solution (containing formaldehyde, NaCl, Na_2HPO_4 and KH_2PO_4) at a dilution of 1:1000 (v/v) (Sanches et al., 2013) to assess the sperm concentration. The sperm concentration assessments were conducted using a hemocytometer (Neubauer chamber) placed on a light microscopy stage for direct cell counting at 400X of magnification (Goes et al., 2017). After cell counting, the sperm concentration was calculated using the following equation:

$$SC\,(SPZ/ml) = \left(\frac{\sum SPZ}{10q.\ c.}\right) \times \frac{50q.\ t.\ \times dilution \times 1000}{camara\ depth\ (mm)}$$

Where:

SC = sperm concentration;
SPZ = spermatozoa;
ΣSPZ = total number of spermatozoa counted;
q.c. = squares counted;
q.t. = total squares;
camara depth = 0.10 mm;
dilution = factor of dilution of the semen by the fixative.

Aliquots from the same samples of fixed sperm were used to determine the percentage of sperm without morphological abnormalities, by evaluating 300 cells using a light microscope (400×), as described by Tessaro et al. (2012).

2.5. Assessment of sperm kinetic variables

Thawed sperm were evaluated for the dynamics of spermatozoa movement during the period in which the sperm remained motile after motility induction. The sperm motility and sperm velocity were previously assessed at different times post-activation induction therefore, data were subjected to a non-linear model (Sanches et al., 2013), which generated a sinusoidal representation as follows:

$$\text{Variable} = \frac{1}{(1 - h0)e^{-r(t)}}$$

Where:

Variable = motility (%) and/or average path velocity (μm/s) calculated for a given time (t (s));

h0 = initial variable value (time 0 s);

r = parameter related to the rate of decline of the variable.

This model was selected because it was adapted and validated in previous studies by Sanches et al. (2013). In addition, average path velocity was considered as the main sperm velocity variable because of its close correlation with oocyte fertilization and egg hatching (Lehnert et al., 2012; Smith, 2012).

2.6. Fertilization and hatching assays

Fertilization assays were performed using a completely randomized block design with two-factors (6 × 3) and three replicates. Thus, the oocytes from 12 females (342.58 ± 27.57 g) were mixed to produce a pool of oocytes with fertilization occurring with use of cryopreserved sperm of 70,000, 90,000, 110,000, 130,000, 150,000, and 170,000 motile sperm per oocyte. To achieve each sperm:oocyte ratio, the number of motile spermatozoa was corrected using the procedure of Adames et al. (2015) which included calculating values with the following equation:

$$IV = \frac{ID}{[SPZ_{cryop}]xMOTxDFC} \text{ or } IV = \frac{(SPZ\colon OOC)x(OOC)}{[SPZ_{fcryop}]xMOTxDFC}$$

Where:

IV = sperm volume to insemination (μL);

ID = total insemination dose (number of sperm cells);

SPZ:OOC = number of motile sperm required to fertilize each oocyte (units);

OOC = number of oocytes in each experimental incubator (units);

$[SPZ_{cryop}]$ = sperm concentration in cryopreserved semen (sperm/mL of semen);

MOT = percentage of motile sperm after thawing (%);

DFC = semen dilution factor in cryopreservation solution.

For each sperm:oocyte ratio used in the fertilization assays, thawed sperm were assessed at 11 s, 16 s or 30 s after activation which provided estimates for sperm velocities of 21, 37, and 52 μm/s, respectively. These sperm velocities were determined using the non-linear model to evaluate the sperm kinetics in the way that was previously described in this manuscript. Each replicate consisted of a pool of oocytes formed by gametes from four females. The experimental units were the incubators (2.5 L volume) containing 2507 ± 96 eggs.

To perform the fertilization assays, the sperm in the samples of thawed semen were activated with 20 mL distilled water (24.00 ± 0.16 °C), and the oocytes were subsequently added at 11 s, 16 s or 30 s after activation. There was a subsequent mixing action imposed on the gametes for 60 s which was followed with oocytes and spermatozoa being transfer to the incubator.

In addition, there was a control treatment that consisted of the oocytes fertilized with use of cryopreserved sperm at a ratio of 90,000 motile sperm per oocyte. The oocyte fertilizations occurred immediately after the sperm motility activation was induced by the addition of 20 mL distilled water.

The effects of the treatment were evaluated based on oocyte fertilizations, egg hatching rates, and percentage of normal larvae. Measurements of artificial fertilization rates were performed after the closure of the blastopore, approximately 12 h after fertilization (Pereira et al., 2006). These procedures were conducted by counting three sub-samples (93.90 ± 1.33 eggs) from each experimental unit.

After hatching (38.0 h; water at 24.57 ± 0.10 °C), all larvae were fixed in 4% formalin buffered by calcium carbonate. The hatching rates were determined by the counting of all fixed larvae (Goes et al., 2017). Furthermore, the fixed larvae were used to determine the rate of morphologically normal larvae by counting and sorting 300 individuals from each experimental unit using a stereo microscope (10×). Larvae without any abnormality in the spine, yolk sac, or head were considered to be morphologically normal (Jezierska et al., 2009).

2.7. Statistical analysis

Data of fertilization rates, egg hatching, and morphologically normal larvae were subjected to arcsine square root transformation so that there was homogeneity of variance. The effects of sperm:oocyte ratio and average path velocity on the response variables were evaluated by multiple regression analysis using the response surface model. Non-significant variables ($P > 0.05$) were progressively removed using the backward stepwise method. Assumptions were checked for normality of the residuals by the Shapiro-Wilk test

Table 1
Values for spermvariables of fresh and thawed sperm of *Rhamdia quelen* at different times subsequent to induced spermatozoa activation with distilled water.

Variables	Fresh	Cryopreserved				
	11 s	11 s	16 s	30 s	min	max
Motility (%)	85.6 ± 3.0	62.6 ± 5.7	54.2 ± 4.5	12.0 ± 2.1	4.3	67.1
VCL (μm/s)	72.9 ± 2.2	70.3 ± 2.2	56.5 ± 1.4	47.2 ± 5.3	46.9	86.7
VAP (μm/s)	54.0 ± 2.0	52.1 ± 1.9	35.2 ± 0.9	21.3 ± 0.4	18.9	67.0
VSL (μm/s)	50.3 ± 1.8	50.0 ± 1.6	33.8 ± 0.8	19.7 ± 0.9	16.3	63.8
Straightness (%)	93.2 ± 0.4	96.0 ± 0.7	96.0 ± 0.4	92.4 ± 3.3	85.3	96.1
Linearity (%)	68.9 ± 1.0	71.2 ± 1.2	59.9 ± 0.9	44.3 ± 4.6	33.8	75.5
Wobble (%)	73.9 ± 0.9	74.1 ± 1.3	62.4 ± 0.9	47.2 ± 3.8	37.9	78.6
BCF (Hz)	49.4 ± 0.8	54.1 ± 1.5	60.3 ± 1.1	65.4 ± 1.6	48.8	65.4

Data expressed as mean ± standard error; VCL = curvilinear velocity; VAP = average path velocity; VSL = straight-line velocity; BCF = beat/cross frequency; min = minimum value; max = maximum value.

(Kéry and Hatfield, 2003). Furthermore, the Dunnett's mean test was used to compare the outcomes between treatment and control groups. Statistical analyses were conducted using the Statistica 7.1® software (StatSoft Inc., Tulsa, USA).

3. Results

3.1. Sperm motility variables of fresh and cryopreserved sperm

The fresh semen was of a high quality and had adequate sperm motility following use of the cryopreservation procedures (Table 1). In addition to the adequate sperm motility and velocity, the sperm in the fresh semen had 40.5 ± 1.7 s duration of sperm movement and there were 77.4 ± 0.9% normal spermatozoa.

The thawed sperm also had an adequate sperm motility rate, and velocity (Table 1) as well as a 42.5 ± 1.2 s duration of sperm movement, $3.4 \times 10^{10} \pm 0.3 \times 10^{10}$ spermatozoa/mL, and 70.1 ± 2.4% normal sperm.

3.2. Linking sperm:egg ratio and sperm kinetic variables with fertilization/hatching rates

Based on the equations generated using the non-linear model, there were average path velocities of 21, 37, and 52 μm/s that occurred at 30, 16, and 11 s, respectively after the induction of sperm motility (Fig. 1). At these times, the corresponding sperm motility were 16.0%, 50.9%, and 65.5%, respectively (Fig. 1).

There was no interaction effect of motile sperm:oocyte ratio and the average path velocity ($P > 0.05$) on oocyte fertilization and egg hatching. Considering the independent effects of experimental factors, however, the average path velocity was the experimental factor that was affected ($P < 0.05$) by the fertilization rates (Fig. 2A) and egg hatching (Fig. 2B). The greater rates of reproductive success, therefore, were verified when the spermatozoa with greater motility were used. Similarly, for the other sperm variables ($P < 0.05$) that affected the fertilization and egg-hatching rates there was a quadratic scheme. The exception was straightness, which affected ($P < 0.05$) the fertilization rates in a directly proportional way (Table 2).

Results from use of the Dunnett's test indicate that number of oocytes fertilized when using any sperm:oocyte ratio and relatively

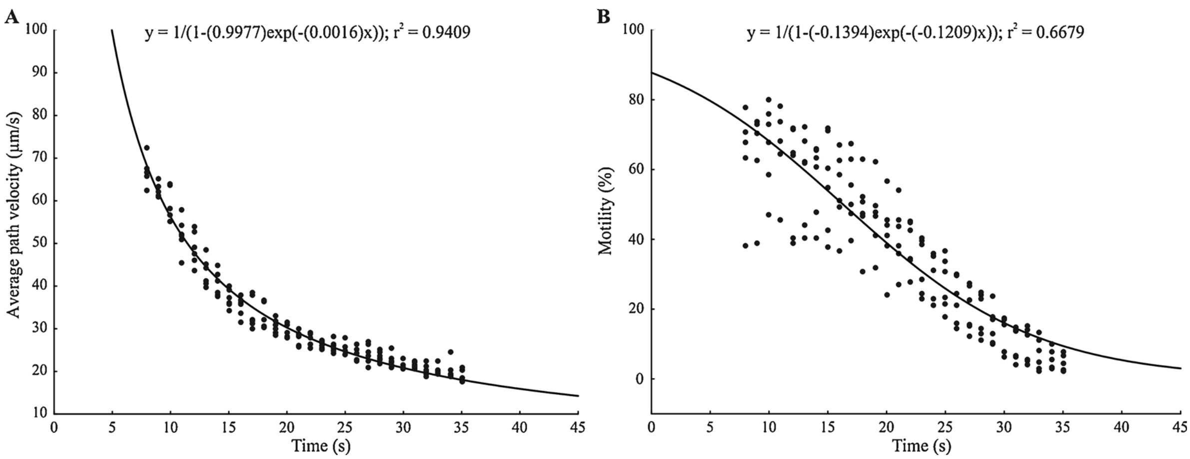

Fig. 1. Theoretical curves of spermatozoa activation kinetics of cryopreserved sperm; A) Average path velocity (μm/s), B) Sperm motility (%).

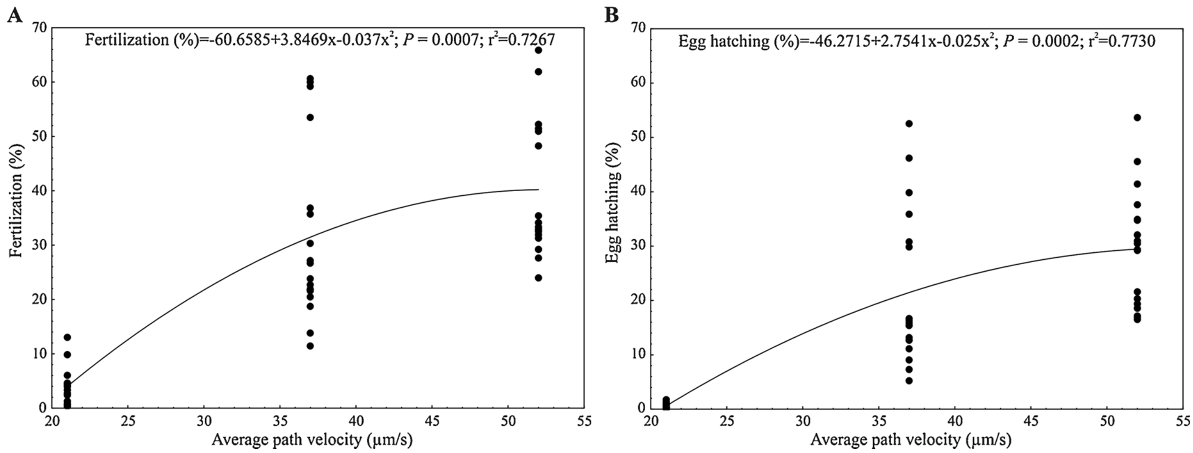

Fig. 2. A) Oocyte fertilization (%) and B) egg hatching (%) of *Rhamdia quelen* when there was fertilization with cryopreserved sperm, using three different average path velocities and six different motile sperm:oocyte ratios.

Table 2
Effects of values for sperm variables when measured using CASA on oocyte fertilization and the egg hatching rates of *Rhamdia quelen*.

Variables		Effect	*P* value	r^2	Equation
Fertilization (%)	VCL (µm/s)	quadratic	4.00×10^{-6}	0.7369	y = -381.9104 + 12.5329x-0.0923x^2
	VSL (µm/s)	quadratic	5.70×10^{-5}	0.7369	y = -61.7482 + 4.1415x-0.0410x^2
	Straightness (%)	linear	0.00×10^{0}	0.7149	y = -857.1793 + 9.31365x
	Linearity (%)	quadratic	3.42×10^{-3}	0.7369	y = -147.8478 + 4.6569x-0.02778x^2
	Wobble (%)	quadratic	1.56×10^{-3}	0.7369	y = -173.3501 + 5.2215x-0.0311x^2
	BCF (Hz)	quadratic	2.90×10^{-5}	0.7369	y = -886.5707 + 34.2552x-0.3156x^2
Egg hatching (%)	VCL (µm/s)	quadratic	1.00×10^{-6}	0.7730	y = -298.4961 + 9.7508x-0.0723x^2
	VSL (µm/s)	quadratic	2.00×10^{-5}	0.7730	y = -50.3172 + 3.2235x-0.0325x^2
	Straightness (%)	quadratic	1.84×10^{-2}	0.7730	y = -647.6513 + 7.0108x+0.0001x^2
	Linearity (%)	quadratic	2.29×10^{-3}	0.7730	y = -120.9316 + 3.7798x-0.0234x^2
	Wobble (%)	quadratic	9.24×10^{-4}	0.7730	y = -140.7308 + 4.2117x-0.0258x^2
	BCF (Hz)	quadratic	9.00×10^{-6}	0.7730	y = -715.2598 + 27.2775x-0.2497x^2

VCL = curvilinear velocity; VAP = average path velocity; VSL = straight-line velocity; BCF = beat/cross frequency.

more motile spermatozoa (52 µm/s) were the same ($P < 0.05$) as that with use of the control treatment (Table 3). These results ($P < 0.05$) were verified when assessing egg-hatching rates (Table 3). Hatching rates on treatments with 110,000, 130,000 and 150,000 motile sperm:oocyte with an average path velocity of 37 µm/s were also similar to those of the control treatment group (Table 3).

The sperm:oocyte ratio, average path velocity, and values for other sperm variables did not affect developmental characteristics of larvae (i.e., normality of larvae development; $P > 0.05$) which was greater than 86% for all treatment groups (Table 3). The percentage of morphologically normal larvae, however, was different ($P > 0.05$) from that of the control with use of motile sperm:oocyte ratios of 70,000, 110,000, 130,000 and 170,000, and a sperm velocity of 21 µm/s (Table 3).

4. Discussion

Results confirm the hypothesis that the thawed sperm use immediately after spermatozoa activation increases the reproductive success and that there is a relationship with sperm velocity. The loss of sperm movement as time elapses after sperm activation and the reduction of sperm velocity can be compensated for by use of a greater sperm:oocyte ratio. The increase on sperm density, however, did not compensate for the losses caused due to spermatozoa which were activated for a longer period and thus had a lesser sperm velocity before being placed with oocytes (Rudolfsen et al., 2008).

The control of the motile sperm:oocyte ratio (inseminant dose) during the artificial fertilization of fish oocytes is determinant in ensuring the reproductive success (Gallego et al., 2013). Theses doses need to be manipulated considering the sperm characteristics, reducing the cell numbers when the spermatozoa have ample motility (Adames et al., 2015) or increasing numbers when the motility is relatively less (Bombardelli et al., 2006).

Because the size of the semen depends on sperm density and number of motile spermatozoa, the sperm velocity becomes a determining factor for optimizing sperm fertility (Gage et al., 2004; Liljedal et al., 2008). The time which the spermatozoa, therefore, remain motile before being placed with the oocytes can modify the motility pattern and consequently affect artificial fertilization results (Kristan et al., 2018; Morita et al., 2014).

Table 3

Artificial fertilization rates (%), egg hatching (%) and larval normality (%) from *Rhamdia quelen* when the oocytes were fertilized with thawed sperm, using with different motile sperm:oocyte ratios and spermatozoa activated at different times after addiction of distilled water.

Motile sperm:oocyte ratio (SPZ:OOC)	Moment of fusion of gametes (s)	VAP (μm/s)	Fertilization (%)	Egg hatching (%)	Larval normality (%)
90,000	*	–	53.6 ± 17.0a	34.6 ± 7.5a	95.1 ± 1.4a
70,000	11	52	48.7 ± 8.8a	29.6 ± 5.1a	93.2 ± 2.9a
70,000	16	37	25.7 ± 5.6	19.1 ± 6.0	94.4 ± 1.8a
70,000	30	21	4.9 ± 4.1	0.4 ± 0.0	86.3 ± 3.2
90,000	11	52	36.8 ± 7.4a	28.7 ± 6.1a	94.3 ± 1.6a
90,000	16	37	24.6 ± 7.1	12.7 ± 3.7	90.9 ± 0.6a
90,000	30	21	7.2 ± 2.1	0.1 ± 0.0	100.0 ± 0.0a
110,000	11	52	47.7 ± 19.4a	34.3 ± 10.3a	94.5 ± 1.3a
110,000	16	37	34.5 ± 12.7	25.1 ± 10.6a	95.4 ± 1.9a
110,000	30	21	3.0 ± 1.3	0.4 ± 0.2	88.4 ± 6.4
130,000	11	52	43.1 ± 11.5a	28.8 ± 8.4a	92.0 ± 0.9a
130,000	16	37	35.7 ± 12.4	24.9 ± 13.8a	94.2 ± 3.0a
130,000	30	21	2.7 ± 1.1	0.7 ± 0.1	87.4 ± 6.3
150,000	11	52	40.5 ± 6.4a	29.3 ± 7.0a	92.9 ± 0.9a
150,000	16	37	32.6 ± 11.5	25.7 ± 9.6a	93.6 ± 1.1a
150,000	30	21	3.3 ± 1.6	0.6 ± 0.3	98.6 ± 1.4a
170,000	11	52	39.9 ± 5.6a	26.1 ± 4.6a	92.5 ± 2.2a
170,000	16	37	35.6 ± 12.0	21.3 ± 7.3	92.9 ± 0.5a
170,000	30	21	3.7 ± 0.6	1.1 ± 0.4	89.4 ± 4.8
Effects			*P-value* / r^2		
SPZ:OOC linear effect			9.87×10^{-1} / 0.0000	7.41×10^{-1} / 0.0021	8.30×10^{-1} / 0.0009
VAP linear effect			0.00×10^{0} / 0.6524	0.00×10^{0} / 0.7025	4.30×10^{-1} / 0.0120
SPZ:OOC quadratic effect			7.45×10^{-1} / 0.0022	8.35×10^{-1} / 0.0030	2.62×10^{-1} / 0.0255
VAP quadratic effect			7.25×10^{-4} / 0.7267	2.19×10^{-4} / 0.7730	8.53×10^{-1} / 0.0127
SPZ:OOC x VAP interactive effect			9.79×10^{-1} / 0.7285	4.96×10^{-1} / 0.7781	7.02×10^{-1} / 0.0412

*Data were collected after sperm and oocytes were placed together at the time immediately after adding the sperm activation solution; Data expressed as mean ± standard error; VAP = average path velocity; SPZ:OOC = Motile sperm:oocyte ratio; means with the same letters were not statistically different from the control values when using the Dunnett's test at 5% of probability.

The relationship and dynamics between these variables deserve attention, especially when the cryopreserved sperm are used for oocyte fertilization. This is because normally the thawed sperm have less motility (Viveiros et al., 2015). To compensate for this loss, it has been recommended by some to use of activators which potentiate sperm motility (Adames et al., 2015). In this case, the use of theoretical models to predict sperm kinetics are an important approach to gaining an enhanced understanding of sperm movement and to recognize the time after activation which the spermatozoa have the greatest motility or capacity for inducing the development of the oocyte micropyle in the fertilization process (Gage et al., 2004; Liljedal et al., 2008; Browne et al., 2015). These predictions are important because the pattern of sperm movement and the time in which the spermatozoa will have the greatest motility and velocity depends on the factors that regulate activation and subsequent motility (Júnior et al., 2018). This process, therefore, is dependent on the conditions that promote sperm motility and especially the duration of sperm motility after activation of motility is induced (Kristan et al., 2018; Gillies et al., 2013). It is thought that the fertilization process occurs when sperm velocity is greatest (Burness et al., 2004; Gage et al., 2004; Gallego et al., 2013).

Although fertilization rates for the *Rhamdia quelen* with use of fresh sperm are about 86% (Bombardelli et al., 2006), however, lesser rates (50% to 60%) are common with use of cryopreserved sperm (Adames et al., 2015). Another relevant point that can be drawn from the present results is the possibility of using a sperm:oocyte ratio of 70,000:1 which is less than that typically used with fresh sperm (approximately 90,000:1; Bombardelli et al., 2006) and still ensure adequate fertilization and hatching rates. There have been several reports verifying that the cryopreserved sperm of fish can be used in a sperm:oocyte ratio less than that which is typically used for fresh sperm, without reductions in fertilization rates, hatching rates or normal larvae development. These results were reported for *Ictalurus furcatus* (Hu et al., 2014), *Salmo trutta* m. *fario* L. (Nynca et al., 2014), *Oncorhynchus mykiss* (Ciereszko et al., 2014) and *Thymallus thymallus* (Horváth et al., 2015). There, however, has been a lack of assessment of the mechanisms which allow for sustaining acceptable rates for fertilization, hatching and normal larval development when the sperm:oocyte ratio is reduced. For the *Rhamdia quelen* this 22.2% reduction in sperm:oocyte ratio represents a substantial enhancement in efficiency of sperm utilization for artificial fertilization. The capacity to use the lesser sperm:oocyte ratio that resulted from assessment of sperm motility and concentration of thawed sperm in semen of *Rhamdia quelen* allowed for the feasibility of optimizing the sperm oocyte ratio. It is believed that the availability of substances from extenders, such as sugars (Adames et al., 2015), ions, or hyperosmotic inducers (Alavi and Cosson, 2005) may have modulated the regulatory events of sperm motility and consequently affected the reproductive outcomes.

Taken together, studies of sperm motility and the determination of values for variables which influence the sperm fertilization capacity are, at present, important fish reproduction and aquaculture aspects that need to be assessed (Cabrita et al., 2008). Findings in the present study are important for developing protocols to use when thawed sperm are used for artificial fertilization of *Rhamdia quelen*. Knowledge regarding the optimal number of motile spermatozoa to be used in semen doses as determined by the activation pattern of thawed sperm need to be considered as determinant factors when there is use of artificial fertilization.

5. Conclusions

The artificial reproduction of *Rhamdia quelen* can be conducted with use of cryopreserved sperm but the time after sperm activation affects sperm velocity and consequently fertilization and egg hatching rates. Thus, the use of thawed sperm immediately after sperm activation or when the sperm have the greatest velocities, at a lesser motile sperm:oocyte ratio (70,000:1) than typically used may enhance the feasibility of use of artificial reproduction of *Rhamdia quelen*.

Conflicts of interest

We declare no actual or potential conflict of interest regarding the submitted manuscript.

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References

Adames, M.S., Toledo, C.P.R., Neumann, G., Buzzi, A.H., Buratto, C.N., Piana, P.A., Bombardelli, R.A., 2015. Optimization of the sperm:oocyte ratio and sperm economy in the artificial reproduction of *Rhamdia quelen* using fructose as a sperm motility modulator. Anim. Reprod. Sci. 161, 119–128.

Alavi, S.M.H., Cosson, J., 2005. Sperm motility and fertilizing ability in the Persian sturgeon Acipenser persicus. Aquac. Res. 36 (9), 841–850.

Arruda, R.P., 2000. Avaliação dos efeitos de diluidores e crioprotetores para o espermatozoide equino pelo uso de microscopia de epifluorescência, citometria de fluxo, análises computadorizadas da motilidade (CASA) e da morfometria (ASMA). PhD Thesis. Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, SP, Brazil.

Barbas, J.P., Mascarenhas, R.D., 2009. Cryopreservation of domestic animal sperm cells. Cell Tissue Bank. 10 (1), 49–62.

Barcellos, L.J.G., Wassermann, G.F., Scott, A.P., Woehl, V.M., Quevedo, R.M., Ittzés, I., Krieger, M.H., Lulhier, F., 2002. Plasma steroid concentrations in relation to the reproductive cycle of cultured male Rhamdia quelen. J. Fish Biol. 61, 751–763.

Barcellos, L.J.G., Kreutz, L.C., Quevedo, R.M., Fioreze, I., Cericato, L., Soso, A.B., Fagundes, M., Conrad, J., Baldissera, R.K., Bruschi, A., Ritter, F., 2004. Nursery rearing of jundiá, *Rhamdia quelen* (Quoy & Gaimard) in cages: cage type, stocking density and stress response to confinement. Aquaculture 232 (1-4), 383–394.

Bochi, V.C., Weber, J., Ribeiro, C.P., Victório, A.M., Emanuelli, T., 2008. Fishburgers with silver catfish (*Rhamdia quelen*) filleting residue. Bioresour. Technol. Rep. 99 (18), 8844–8849.

Bombardelli, R.A., Morschbacher, E.F., Campagnolo, R., Sanches, E.A., Syperreck, M.A., 2006. Dose inseminante para fertilização artificial de ovócitos de jundiá cinza, *Rhamdia quelen* (Quoy & Gaimardm, 1824). R. Bras. Zooctec. 35 (4), 1251–1257.

Bombardelli, R.A., Sanches, E.A., Tessaro, L., Buzzi, A.H., Martins, C.V.B., Meurer, F., 2015. Digestible energy requirement for females of *Rhamdia quelen* on reproductive activity fed with ration based on vegetal ingredients. Lat. Am. J. Chem. Eng. 43 (3), 566–574.

Boschetto, C., Gasparini, C., Pilastro, A., 2011. Sperm number and velocity affect sperm competition successe in the guppy (*Poecilia reticulate*). Behav. Ecol. Sociobiol. (Print) 65 (4), 813–821.

Browne, R.K., Kaurova, S.A., Uteshev, V.K., Shishova, N.V., McGinnity, D., Figiel, C.R., Mansour, N., Agnew, D., Wu, M., Gakhava, E.N., Dzyuba, B., Cosson, J., 2015. Sperm motility of externally fertilizing fish and amphibians (Review). Theriogenology 83 (1), 1–13.

Buchanan, J.T., Barbosa-Solomieu, V., 2011. Comercial application of fish sperm cryopreservation for management of genetic resources. In: Tiersch, T.R., Green, C.C. (Eds.), Cryopreservation in Aquatic Species, 2th edition. World Aquaculture Society, Baton Rouge, Louisiana, USA, pp. 828–833.

Burness, G., Casselman, S.J., Schulte-Hostedde, A.I., Moyer, C.D., Montgomerie, R., 2004. Sperm swimming speed and energetics vary with sperm competition risk in bluegill (*Lepomis macrochirus*). Behav. Ecol. Sociobiol. (Print) 56 (1), 65–70.

Cabrita, E., Robles, V., Herráez, P., 2008. Sperm quality assessment. In: Cabrita, E., Robles, V., Herráez, P. (Eds.), Methods in Reproductive Aquaculture: Marine and Freshwater Species. CRC, Press, Boca Ranton, pp. 93–147.

Cabrita, E., Sarasquete, C., Martínez-Páramo, S., Robles, V., Beirão, J., Pérez-Cerezales, S., Herráez, M.P., 2010. Cryopreservation of fish sperm: applications and perspectives. J. Appl. Ichthyol. 26 (5), 623–635.

Cabrita, E., Martínez-Páramo, S., Gavaia, P.J., Riesco, M.F., Valcarce, D.G., Sarasquete, C., Herráez, M.P., Robles, V., 2014. Factors enhancing fish sperm quality and emerging tools for sperm analysis. Aquaculture 432 389-104.

Caffey, R.H., Tiersch, T.R., 2011. Economics and marketing of cryopreserved Fish sperm. In: Tiersch, T.R., Green, C.C. (Eds.), Cryopreservation in Aquatic Species, 2th edition. World Aquaculture Society, Baton Rouge, Louisiana, USA, pp. 896–918.

Carolsfeld, J., Godinho, H.P., Zaniboni-Filho, E., Harvey, B.J., 2003. Cryopreservation of sperm in Brazilian migratory fish conservation. J. Fish Biol. 63 (2), 472–489.

Castelo, T.S., 2010. Efeito dos processos de centrifugação, diluição e descongelação sobre a qualidade do sêmen de catetos (*Tayassu tajacu,* Linneaeus, 1758). PhD Dissertation. Universidade Federal do Semi-Árido, Rio Grande do Norte, Brazil.

Ciereszko, A., Dietrich, G.J., Nynca, J., Dobosz, S., Zalewski, T., 2014. Cryopreservation of rainbow trout semen using a glucose-methanol extender. Aquaculture 420, 275–281.

Freitas, J.M.A., Sary, C., Luchesi, J.D., Feiden, A., Boscolo, W.R., 2011. Protein and energy in diet for catsfish raised in net cages. R. Bras. Zootec. 40 (12), 2628–2633.

Gage, M.J.G., Macfarlane, C.P., Yeates, S., Ward, R.G., Searle, J.B., Parker, G.A., 2004. Spermatozoal traits and sperm competition in Atlantic Salmon: relative sperm velocity is the primary determinant of fertilizatin success. Curr. Biol. 14 (1), 44–47.

Gallego, V., Asturiano, J.F., 2018. Fish sperm motility assessment as a tool for aquaculture research: a historical approach. Rev. Aquacult. 1–28.

Gallego, V., Pérez, L., Asturiano, J.F., Yoshida, M., 2013. Relationship between spermatozoa motility parameters, sperm/egg ratio, and fertilization and harching rates in pufferfish (*Takifugu niphobles*). Aquaculture 416, 238–243.

Gallego, V., Cavalcante, S.S., Fujimoto, R.Y., Carneiro, P.C.F., Azevedo, H.C., Maria, A.N., 2017. Fish sperm subpopulations: changes after cryopreservation process and relationship with fertilization success in tambaqui (*Colossoma macropomum*). Theriogenology 87, 16–24.

Gasparini, C., Simmons, L.W., Beveridge, M., Evans, J.P., 2010. Sperm swimming velocity predicts competitive fertilization success in the green swordtail Xiphophorus helleri. PLoS One 5 (8) e12146.

Gillies, E.A., Bondarenko, V., Cosson, J., Pacey, A.A., 2013. Fins improve the swimming performance of fish sperm: a dydrodinamic analysis of the Siberiona Sturgeon Acipenser baerii. Cytoskeleton 70 (2), 85–100.

Goes, M.D., dos Reis Goes, E.S., Ribeiro, R.P., Lopera-Barrero, N.M., de Castro, P.L., Bignotto, T.S., Bombardelli, R.A., 2017. Natural and artificial spawning strategies with fresh and cryopreserved semen in *Rhamdia quelen*: reproductive parameters and genetic variability of offspring. Theriogenology 88 (3), 254–263.

Horváth, Á., Bokor, Z., Bernáth, G., Csenki, Z., Gorjan, A., Herráez, M.P., Urbányi, B., Jesenek, D., 2015. Very low sperm-egg ratios result in successful fertilization using cryopreserved sperm in the Adriatic grayling (*Thymallus thymallus*). Aquaculture 435, 75–77.

Hu, E., Bosworth, B., Baxter, J., Tiersch, T.R., 2014. On-site evaluation of comercial-scale hybrid catfish production using cryopreserved blue catfish sperm. Aquaculture 426, 88–95.

Jezierska, B., Lugowska, K., Witeska, M., 2009. The effects of heavy metals on embryonic development of fish (a review). Fish Physiol. Biochem. 35 (4), 625–640.

Júnior, J.J.B., Jimenez, J.E., Bombardelli, R.A., Nuñer, A.P.O., 2018. Changes in external osmolality and ionic composition affect *Megaleporinus obtusidens* sperm motility. Anim. Reprod. Sci. 190, 63–74.

Kanuga, M.K., Drew, R.E., Wilson-Leedy, J.G., Ingermann, R.L., 2012. Subpopulation distribution of motile sperm relative to activation medium in steelhead (*Oncorhynchus mykiss*). Theriogenology 77 (5), 916–925.

Kéry, M., Hatfield, J.S., 2003. Normality of Raw data in general linear models: the most widespread myth in Statistics. Avian Conserv. Ecol. 4, 92–94.

Khara, H., Baradaran, S.N., Hadiseh, D., Rahbar, M., Ahmadnejad, M., Khodadoost, A., 2012. The effect of cations on sperm motility performance and fertilizing ability of silver carp Hypophtalmychtis molitrix. Acta Vet. (Beogr) 62 (5-6), 599–609.

Kristan, J., Zarski, D., Blecha, M., Policar, T., Malinovskyi, O., Samarin, A.M., Palinska-Zarska, K., Nowosad, J., Krejszeff, S., Kucharczyk, D., 2018. Fertilizing ability of gametes at different post-activation times and the sperm-oocyte in the artificial reproduction of pikeperch Sander lucioperca. Aquac. Res. 49 (4), 1383–1388.

Lehnert, S.J., Heath, D.D., Pitcher, T.E., 2012. Sperm trait diferences between wild and farmed Chinook salmon (*Oncorhynchus tshawytscha*). Aquaculture 344-349, 242–247.

Liljedal, S., Rudolfsen, G., Folstad, I., 2008. Factors predicting male fertilization success in an external fertilizer. Behav. Ecol. Sociobiol. (Print) 62 (11), 1805–1811.

Magnotti, C., Cerqueira, V., Lee-Estevez, M., Farias, J.G., Valdebenito, I., Figueroa, E., 2018. Cryopreservation and vitrification of fish semen: a review with special emphasis on marine species. Rev. Aquacult. 10 (1), 15–25.

Maria, A.N., Carneiro, P.C.F., 2012. Criopreservação de sêmen de peixes no Brasil: estado da arte e perspectivas futuras. Cienc. Anim. 22 (1), 124–131.

Maria, A.N., Viveiros, A.T.M., Freitas, R.T.F., Oliveira, A.V., 2006. Extenders and cryoprotectants for cooling and freezing of piracanjuba (*Brycon orbignyanus*) semen, and endangered Brazilian teleost fish. Aquaculture 260 398-306.

Martínez-Páramo, S., Diogo, P., Dinis, M.T., Herráez, M.P., Sarasquete, C., Cabrita, E., 2012. Sea bass sperm freezability is influenced by motility variables and membrane lipid composition but not by membrane integrity and lipid peroxidation. Anim. Reprod. Sci. 131 (3-4), 211–218.

Martínez-Páramo, S., Horváth, Á., Labbé, C., Zhang, T., Robles, V., Herráez, P., Suquet, M., Adams, S., Viveiros, A., Tiersch, T.R., Cabrita, E., 2017. Cryobanking of aquatic species. Aquaculture 472, 156–177.

Morita, M., Awata, S., Yorifuji, M., Ota, K., Kohda, M., Ochi, H., 2014. Bower-building behaviour is associated with increased sperm longevity in Tanganyikan cichlids. J. Evol. Biol. Res. 27 (12), 2629–2643.

Nynca, J., Dietrich, G.J., Dobosz, S., Grudniewska, J., Ciereszko, A., 2014. Effect of cryopreservation on sperm motility parameters and fertilizing ability of brown trout semen. Aquaculture 433, 62–65.

Pereira, C.R., Barcellos, L.J.G., Kreutz, L.C., Quevedo, R.M., Ritter, F., Silva, L.B., 2006. Embryonic and larval development of jundiá (*Rhamdia quelen*, Quoy & Gaimard, 1824, Pisces, Teleostei), a south American Catfish. Braz. J. Biol. 66 (4), 1057–1062.

Rudolfsen, G., Figenschou, L., Folstad, I., Kleven, O., 2008. Sperm velocity influence paternity in the Atlantic cod (*Gadus morhua* L.). Aquac. Res. 39 (2), 21–216.

Rurangwa, E., Kime, D.E., Ollevier, F., Nash, J.P., 2004. The measurement of sperm motility and factors affecting sperm quality in cultured fish. Aquaculture 234 (1-4), 1–28.

Sanches, E.A., Neumann, G., Baggio, D.M., Bombardelli, R.A., Piana, P.A., Romagosa, E., 2011. Time and temperature on the storage of oocytes from jundiá catfish, Rhamdia quelen. Aquaculture 319 (3-4), 453–458.

Sanches, E.A., Marcos, R.M., Okaware, R.Y., Caneppele, D., Bombardelli, R.A., Romagosa, E., 2013. Sperm motility parameters for *Steindachneridion parahybae* based on open-source software. J. Appl. Ichthyol. 29 (5), 1114–1122.

Smith, C.C., 2012. Opposing effects of sperm veability and velocity on the outcome of sperm competition. Behav. Ecol. 23 (4), 820–826.

Stoltz, J.A., Neff, B.D., 2006. Sperm competition in a fish with external fertilization: the contribution of sperm number, speed and length. Journal Compilation. J. Evol. Biol. 19 (6), 1873–1881.

Tessaro, L., Toledo, C.P.R., Neumann, G., Krause, R.A., Meurer, F., Natali, M.R.M., Bombardelli, R.A., 2012. Growth and reproductive characteristics of *Rhamdia quelen* males fed on diferente digestible energy levels in the reproductive phase. Aquaculture 326-329, 74–80.

Viveiros, A.T.M., Nascimento, A.F., Orfão, L.H., Isaú, Z.A., 2010. Motility and fertility of the subtropical freshwater fish streaked prochilod (*Prochilodus lineatus*) sperm cryopreserved in powdered coconut water. Theriogenology 74 (4), 551–556.

Viveiros, A.T.M., Orfão, L.H., Nascimento, A.F., Corrêa, F.M., Caneppele, D., 2012. Effects of extenders, cryoprotectants and freezing methods on sperm quality of the threatened Brazilian freshwater fish pirapitinga-do-sul *Brycon opalinus* (Characiformes). Theriogenology 78 (2), 361–368.

Viveiros, A.T.M., Gonçalves, A.C.S., Nascimento, A.F., Leal, M.C., 2015. Fresh, equilibrated and pos-thaw sperm quality of *Brycon orbignyanus* (Valenciennes, 1850) and *Prochilodus lineatus* (Valenciennes, 1837) treated with either salmon GnRHa and domperidone or pituitary extract. Neotrop. Ichthyol. 13 (1), 157–164.

Wang, X., Wang, F., Wu, X., Zhao, X., Liu, J., Huang, C., Dong, Q., 2010. The use of cryomicroscopy in guppy sperm freezing. Cryobiology 61 (2), 182–188.

Wilson-Leedy, J.G., Ingermann, R.L., 2007. Development of a novel CASA system based on open source software for characterization of zebrafish sperm motility parameters. Theriogenology 67 (3), 661–672.

Yang, H., Hu, E., Buchanan, J.T., Tiersch, T.R., 2018. A strategy for sperm cryopreservation of Atlantic Salmon, *Salmo solar*, for remote commercial-scale high-throughput processing. J. World Aquacult. Soc. 49 (1), 96–112.