



## Protein profile as a quality indicator of cryopreserved semen from Tambaqui *Colossoma macropomum* (Cuvier, 1818)

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(With 2 figures)

### Abstract

The aim of this study was to evaluate the association between proteins in the seminal plasma of tambaqui *Colossoma macropomum* (Cuvier, 1818) with seminal quality indicators after thawing. The semen was cryopreserved with a dilution based on BTS with 8% DMSO. A 200  $\mu$ L sample of semen from each animal was diluted in 800  $\mu$ L BTS, centrifuged at 800 rpm, and the supernatant was cryopreserved to further analyze of the protein profile of seminal plasma through one-dimensional electrophoresis (SDS-PAGE). After 15 days of cryopreservation, a cryopreserved semen straw was thawed to analyze both qualitative and quantitative parameters. When considering all collections, the SDS-PAGE identified 15 protein bands in the seminal plasma of tambaqui. When the interaction (presence or absence) between proteins observed in the seminal plasma and the post thawed spermatic parameters was evaluated, we observed a great influence of the presence of proteins on spermatic quality. A greater ( $P<0.05$ ) fertilization rate was observed with the presence of proteins 12, 34, 44, 85, and 90 kDa. Proteins in seminal plasma of tambaqui influenced the spermatic quality after thawing, and thus, they can be utilized as an indicator of sperm quality, especially the proteins with a molecular weight  $\leq 50$  kDa.

**Keywords:** *Colossoma macropomum*, cryopreservation, spermatic motility, proteins, fertilization rate.

### Perfil protéico como indicador qualitativo do semen criopreservado de Tambaqui *Colossoma macropomum* (Cuvier, 1818)

#### Resumo

O objetivo desse estudo foi de avaliar a associação entre a presença de proteínas no plasma seminal do tambaqui *Colossoma macropomum* (Cuvier, 1818) com indicadores de qualidade seminal pós-descongelamento. O semen foi criopreservado com diluidor a base de BTS com 8% DMSO. Uma amostra de 200  $\mu$ L de semen de cada animal foi diluída em 800  $\mu$ L de BTS, e centrifugada em 800 rpm, e somente o sobrenadante foi criopreservado para posterior análise do perfil proteico do plasma seminal, através da eletroforese unidimensional (SDS-PAGE). Decorridos 15 dias da criopreservação, uma palheta com semen criopreservado foi descongelado para análise dos parâmetros quali-quantitativos. Considerando todas as coletas, o SDS-PAGE identificou 15 bandas proteicas no plasma seminal do tambaqui. Quando se avaliou a interação (presença ou ausência) das proteínas encontradas no plasma seminal, com os parâmetros espermáticos pós-descongelamento, observou-se grande influência da presença das proteínas na qualidade espermática. Observou-se maior taxa de fertilização ( $P<0,05$ ) com a presença das proteínas 12, 34, 44, 85 e 90 kDa. As proteínas do plasma seminal de tambaqui influenciaram na qualidade espermática após descongelamento, podendo ser utilizadas como indicadores para a qualidade espermática após descongelamento, principalmente as proteínas com peso molecular  $\leq 50$  kDa.

**Palavras-chave:** *Colossoma macropomum*, criopreservação, motilidade espermática, proteínas, taxa de fertilização.

## 1. Introduction

Gamete conservation is an important tool for fish reproduction and of great interest for fish-farming (Magnotti et al., 2018). Also, it facilitates the transport of genetic material between laboratories in a country and in another countries, as well as a greater caution to prevent disease transmission, thereby allowing for new strain introductions with a low risk of pathogens in cultivated fishes (Tiersch, 1995). Growing interest in improving the technique has led to an increase in the number of studies on this subject (Tiersch, 2011; Maria and Carneiro, 2012).

When considering the effect of the freezing process on semen quality, the study of techniques to identify variation in response to freezing among several males and to develop tests to find the freezing potential becomes important. Thus, the protein composition of the seminal plasma should be deemed as an important factor that influences fertility in males (Autiero et al., 1991; Strzeżek et al., 2005) a glycoprotein in the seminal plasma with a high molecular weight (120 kDa) was identified as a factor in the immobilization of spermatozooids (Mochida et al., 1999, 2002) in Nile tilapia (*Oreochromis niloticus*). Some of these proteins can be used as biochemical markers to identify individuals with higher or lower potential for fertility and freezing of semen (Jobim et al., 2004; Zilli et al., 2005; Asadpour et al., 2007).

The seminal plasma, in the majority of the teleost, is a product that is synthesized in the scrotum and spermatic ducts (Lahnsteiner et al., 1994) which should be considered since several species did not present with accessory glands. Some components of the seminal plasma are not secreted, but they can be produced by spermatic cells during decomposition (Ciereszko et al., 2000). The seminal plasma of teleost fishes is of great importance in the sperm physiology, because its mineral composition inhibits the spermatic motility inside of the sperm ducts, provides an isotonic and equilibrated environment for the sperm (Morisawa and Suzuki, 1980; Cosson et al., 1999), as well as monosaccharides and lipids for energetic resources of the spermatozooids (Lahnsteiner et al., 1994). According to Loir et al. (1990), proteins are the greatest organic component in the seminal fluid of teleost fish. Recent studies highlighted the importance of plasma proteins for spermatozooids. For example, in rainbow trout (*Oncorhynchus mykiss*) where the sperm were stored in a saline solution similar to the seminal fluid composition, the motility rate and swimming speed, which can be activated, increased when the stored solution contained seminal plasma proteins, thereby indicating that these proteins establish and enable the sperm (Lahnsteiner et al., 2004).

When seminal plasma is incorporated into cryopreservation process, it reduces the harmful effects of other cryoprotectants and exerts a cryoprotectant and antioxidant activity which protects sperm function in fish (Alvarez et al., 2008; Figueroa et al., 2017). A series antioxidant substances are found in the seminal plasma of freshwater fish (ascorbic acid, carnitine, glutathione, methionine, tacopheryl acid

and uric acid) as well as oxidative defence enzymes (catalase, glutathione reductase, peroxidase and superoxide dismutase) which could help preserving the motility and fertilising capacity of this cells (Lahnsteiner, 2007b, 2009; Lahnsteiner and Mansour, 2010; Lahnsteiner et al., 2010)

Currently, the tambaqui *Colossoma macropomum* (Cuvier, 1818) is the endemic species that is the most highly produced in Brazil (Brasil, 2013). The freezing of semen from this species as a function of its commercial importance has been studied, and the protocol of cryopreservation was defined in the study by Varela Junior et al. (2012).

This study was conducted to identify the association between the presence of proteins in the seminal plasma of *C. macropomum* with indicators of seminal quality after thawing.

## 2. Material and Methods

### 2.1. Animals and experiment

Twenty-seven males ( $6.4 \pm 0.4$  kg and  $4 \pm 2$  years) and six females ( $8.3 \pm 0.5$  kg and  $6 \pm 2$  years) of tambaqui (*C. macropomum*) were kept in nurseries under ambient conditions and were fed with a commercial ration containing 36% crude protein and 2,900 kcal of digestible energy  $\text{kg}^{-1}$  of ration. During the reproductive period, the semen was obtained by inducing the males with extract of pituitary hormone from carp (PHC; 5.5 and 2.5 mg PHC  $\text{kg}^{-1}$  fishes, females and males, respectively). The chemicals were procured from Sigma Aldrich, St. Louis, MO, USA.

### 2.2. Semen collection and analysis

Seven hours after the hormone induction, the semen was collected using a conical tube of 15 mL (one for each animal) while recording the volume of samples and avoiding contamination by feces or urine (Billard et al., 1995). Immediately after the collection, the seminal evaluations were performed. The parameters were analyzed according to (Galo et al., 2011) as follows:

**Spermatic motility:** the spermatic motility was observed through optical microscopy (40X) by diluting 20:400  $\mu\text{L}$  (semen:distilled water) in a slide under a coverslip and was classified with a score from 0 to 100%.

**Motility time:** a chronometer was used after the dilution (20:400  $\mu\text{L}$  of semen:distilled water) to analyze the spermatic motility. The time until the last spermatozoid stopped moving in the optical field (40X) was recorded.

Using the dilution of 1:1000 (semen:formaldehyde-saline buffered solution), extensions were produced and colored by the Rose Bengal Method (Streit-Junior et al., 2004), and 100 spermatozooids extension<sup>-1</sup>animal<sup>-1</sup> were counted through optical microscopy with phase contrast (40X). Primary abnormalities were considered to be a broken tail, rolled tail, and degenerated tail, while the secondary abnormalities were a free head, free tail, and rolled tail.

### 2.3. Semen cryopreservation

After spermatic evaluations, the fresh semen was cryopreserved in a diluted solution based on Beltsville Thawing Solution (BTS) (Pursel and Johnson, 1975).

The cryopreserved solution was composed by using BTS with 8% Dimethyl-Sulfoxide DMSO (Varela Junior et al., 2012). The semen and the cryopreserved solution were homogenized at a proportion of 1:4 (semen:solution), bottled in identified 0.25 mL straws, and stored in a dry shipper cylinder (Taylor-Wharton, model CP 300 dry shipper) for subsequent cooling. After 24 h in the dry shipper, the semen was transferred to the liquid nitrogen cylinder (MVE, model CP-34) (Taitson et al., 2008) and stored for 15 days.

#### 2.4. Thawing and analyses of cryopreserved semen

One straw with cryopreserved semen was thawed by immersion in a water-bath ( $45\text{ }^{\circ}\text{C}$  5 seconds<sup>-1</sup>) (Streit-Junior et al., 2006) for spermatic motility, motility time, and morphology of spermatozooids analyses (Galo et al., 2011).

The mitochondrial functionality analysis was performed through fluorescent coloration by adding 10  $\mu\text{L}$  of thawed semen with 40  $\mu\text{L}$  work solution of Rhodamine 123 (13  $\mu\text{M}$ ) and incubating the mixture at  $20\text{ }^{\circ}\text{C}$  10 min<sup>-1</sup> (Varela Junior et al., 2012). The DNA integrity was evaluated by using acridine orange catheter and adding 45  $\mu\text{L}$  of thawed semen in 50  $\mu\text{L}$  TNE (0.01 M Tris-HCl, 0.15 M NaCl, 0.001 M EDTA, and pH 7.2); after 30 seconds, 200  $\mu\text{L}$  of Triton solution (1X) was added. After another 30 seconds, 50  $\mu\text{L}$  of acridine orange (2 mg per mL in deionized water) was added, and after 5 minutes, the sample was evaluated without exceeding 1 minute of exposition. Through the use of acarboxy fluoresce indiacetate fluorescent catheter (CDFC) and propidium iodide (PI), the membrane integrity of the spermatozooids was obtained by adding 10  $\mu\text{L}$  of thawed semen and 40  $\mu\text{L}$  of work solution incubated at  $20\text{ }^{\circ}\text{C}$  10 minutes<sup>-1</sup>. The mitochondrial functionality, integrity of DNA, integrity of spermatic membrane, and cell feasibility for semen of tambaqui were analyzed according to (Billard et al., 1995) by using anepifluorescence microscope (Olympus® BX 51, America INC, São Paulo, Brazil) to evaluate 200 cells sample<sup>-1</sup> from 1  $\mu\text{L}$  of solution with spermatozooids in microscopic slide under a cover slip. The rates were expressed through the percentage of integral/functional cells compared to the total number of cells.

#### 2.5. Reproductive efficiency test

The *cell feasibility* was obtained by using eosin-nigrosin histochemical dye, and the protocol was adapted from (Morrison et al., 1997). A fraction of 10  $\mu\text{L}$  of semen diluted in BTS was added to 10  $\mu\text{L}$  of coloration solution (1.6 g Eosin Y and 6 g nigrosin BTS), was homogenized, and after 2 minutes, the smear was performed. After the slide was dry, the spermatozooids were observed in a light field (BX41 Olympus®) with the oil immersion objective (100x), and the dead spermatozooids were colored in red.

When evaluating the use of cryopreserved semen on the fertilization and hatching rates of 27 animals, the semen samples were divided into two groups: 1) Semen from 10 animals that fertilized oocytes of 3 females; 2) Semen from 17 animals that fertilized oocytes of 3 new females,

there by leading to a total of 30 and 51 combinations (semen  $\times$  oocytes). A 0.25 mL pellet was utilized for each 2 g aliquot of oocytes, which were homogenized, activated, and hydrated with 8 mL of water. Another two aliquots of oocytes were fertilized with fresh semen collected from two other males at the same time (control with fresh semen), thereby adding up to 83 combinations. For these samples, 50  $\mu\text{L}$  of fresh semen were utilized so the number of spermatic cells oocytes<sup>-1</sup> would be equal to that of the frozen treatments that were already diluted.

After fertilization, the eggs were deposited in 3-liter incubators individualized for each combination (semen  $\times$  oocytes), and after 6 h of incubation ( $T = 28 \pm 1\text{ }^{\circ}\text{C}$ ), an aliquot of the eggs from each incubator was removed and the fertilization rate was measured. This operation was performed three times while counting 100 eggs/sample, and thus, the mean was the fertilization rate. After 12 h of measuring the fertilization rate at the mean water temperature of  $28 \pm 1\text{ }^{\circ}\text{C}$ , visual counting of the hatching rate was performed, where all viable larvae and eggs were counted to determine the percentage.

#### 2.6. Protein analysis

A 200  $\mu\text{L}$  sample of semen from each animal was diluted in 800  $\mu\text{L}$  BTS and centrifuged at 800 rpm (rotation/minute), after which the residue (spermatozooids) was discarded and only the supernatant (seminal plasma - proteins) was cryopreserved in liquid nitrogen. After thawing, the supernatant was re-centrifuged at  $10.000 \times g$  10 min<sup>-1</sup> to obtain only the seminal plasma. From the plasma, a 10  $\mu\text{L}$  aliquot was removed and 30  $\mu\text{L}$  of deionized water and 20  $\mu\text{L}$  of sample buffer were added; the buffer consisted of 20% glycerol, 10% Tris-HCl 0.6173 M, pH 6.8 (Gibco-Invitrogen, Grand Island, NY, USA), 2%  $\beta$ -Mercaptoethanol (Sigma Aldrich, St. Louis, MO, USA), 20% Dodecilsodium sulfate at 10% - SDS (Fisher Scientific, Suwanee, GA - USA), 2.5 mg bromophenol blue (Synth, Diadema, Sao Paulo, Brazil), and deionized water. Then, the samples were heated at  $100\text{ }^{\circ}\text{C}$  10 min<sup>-1</sup> for protein denaturation. The one-dimensional electrophoresis (SDS-PAGE) was performed through the BIORAD Mini-Protean 3 Cell® system (Bio-Rad Laboratories, California, USA) according to (Laemmli, 1970) in polyacrylamide gels (Gibco-Invitrogen, Grand Island, NY, USA). It was concentrated at 8 and 15% (Maňásková and Jonáková, 2008) and the molecular marker Bench Mark Protein Ladder® (Gibco-Invitrogen, Grand Island, NY, USA) was utilized. The gels were colored with Coomassie Brilliant Blue (Bio-Rad Laboratories, California, USA) for 20 minutes (Syntin et al., 1996). The discolored process of the gels was obtained by using a discolored solution consisting of 40% methanol (Synth, Diadema, São Paulo, Brazil), 10% glacial acetic acid (Synth, Diadema, São Paulo, Brazil), and 50% deionized water for 1 h in a water bath at  $75\text{ }^{\circ}\text{C}$ . The analysis was based on the visualization and distinction of protein bands formed during electrophoresis. Data obtained from electrophoresis was analyzed by TotalLab TL100® software, version 2006 (Nonlinear Dynamics, UK).

### 2.7. Spermatozoid ultrastructural analysis: Transmission Electronic Microscopy (TEM)

Samples of semen (fresh and frozen) from each animal were fixed in 2.5% glutaraldehyde in a phosphate buffer (0.1 M, pH 7.2) and post fixed in 1% osmium tetroxide in a phosphate buffer (0.1 M, pH 7.2) for 3 h. After dehydration, the material was included in araldite (Durcupan ACM, Fluka, Sigma-Aldrich, St. Louis, USA), and after, 0.5µm sections were colored with toluidine blue for the choice of the area to be evaluated. Then, sections between 50 to 70 nm were contrasted in uranyl acetate and lead citrate for further analysis and photo documentation through electronic microscopy transmission, mark CM100 (Eindhoven, The Netherlands).

### 2.8. Statistical analysis

A normality analysis was performed through the Shapiro-Wilk test for all variables, for which all variables had a parametric behavior. Thus, a variance analysis (ANOVA) was preceded with a subsequent comparison

among means using the Tukey test. For these analyzes were considered at a significance level of 0.05.

## 3. Results

The spermatic parameters recorded for fresh and cryopreserved semen with DMSO of *C. macropomum* are summarized in Table 1.

When considering all collections, the SDS-PAGE identified 11 protein bands in the 8% polyacrylamide gel (12, 25, 29, 34, 37, 44, 50, 65, 75, 90, and 100 kDa) and 4 bands in the 15% polyacrylamide gel (40, 70, 78, and 85 kDa), totaling 15 bands in the seminal plasma of tambaqui *C. macropomum* (Figure 1).

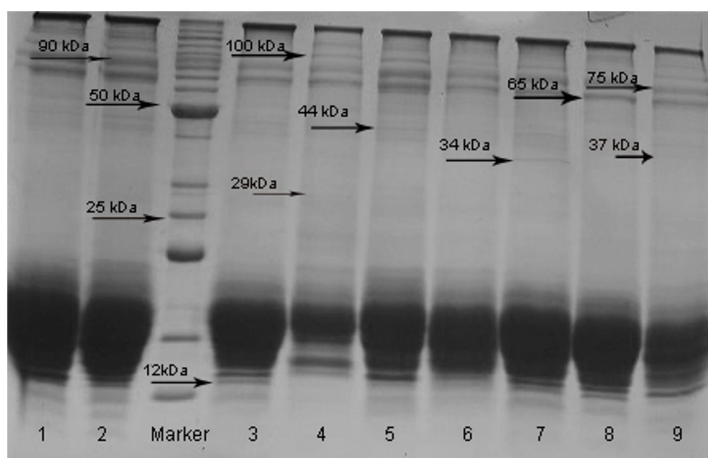
When the interaction (presence or absence) of proteins found in the seminal plasma and the spermatic parameters of *C. macropomum* frozen with DMSO was evaluated, an effect of proteins on spermatic quality was observed (Tables 2 and 3).

The majority of the proteins in the seminal plasma of *C. macropomum* positively influenced ( $P < 0.05$ )

**Table 1.** Seminal parameters of fresh and cryopreserved semen from *Colossoma macropomum*.

PARAMETERS	SEMEN	
	Fresh	Cryopreserved
Spermatic motility (%)	87.50 ± 12.25	14.40 ± 9.15
Motility time (seconds)	50.75 ± 81.90	30.60 ± 10.71
Normal spermatozoids (%)	31.90 ± 1.72	22.23 ± 1.72
Total abnormalities (%)	68.12 ± 1.72	77.77 ± 9.07
Primary abnormalities (%)	50.00 ± 2.72	55.76 ± 10.25
Secondary abnormalities (%)	18.12 ± 2.58	22.01 ± 7.90
Fertilization rate (%)	87.67 ± 15.81	26.73 ± 13.57
Hatching rate (%)	83.25 ± 23.60	27.65 ± 16.76
DNA integrity (%)	-	95.43 ± 3.96
Membrane integrity (%)	-	55.18 ± 18.39
Mitochondrial functionality (%)	-	53.83 ± 30.01
Cell feasibility (%)	-	19.22 ± 16.91

Mean ± SEM (standard error).



**Figure 1.** Electrophoresis of polyacrylamide gel (8% SDS-PAGE colored with Coomassie blue) of seminal plasma from *Colossoma macropomum* showing 9 animals (with molecular weight from 12 to 100 kDa).

**Table 2.** Interaction of the presence or absence of proteins in the seminal plasma analyzed in the 8% polyacrylamide gel with the spermiatic parameters of cryopreserved semen from *Collossoma macropomum*.

Parameters	Proteins - Gel with concentration of 8%											
	12 Kda	25 Kda	29 Kda	34 Kda	37 Kda	44 Kda	50 Kda	65 Kda	75 Kda	90 Kda	100 Kda	
Spermiatic motility (%)	presence	18.5 ± 1.7 A	17.2 ± 1.6 A	17.9 ± 1.6 A	17.9 ± 1.6 A	13.6	20.5 ± 1.9 A	15.8 ± 1.5 A	13.3	16.2 ± 1.5 A	14.8	13.7
	absence	9.1 ± 1.5 B	8.0 ± 1.7 B	9.0 ± 1.7 B	9.0 ± 1.7 B	14.7	9.7 ± 1.3 B	9.2 ± 2.1 B	15.5	6.0 ± 1.9 B	10.0	14.5
Motility time (seconds)	presence	32.8	31.6	32.6	33.4 ± 1.6 A	29.6	33.4	30.8	30.2	33.6 ± 1.2 A	29.8	36.7
	absence	27.7	28.3	27.4	26.3 ± 3.0 B	31.1	28.5	29.8	31.0	19.8 ± 4.6 B	39.5	29.3
Fertilization rate (%)	presence	30.5 ± 2.4 A	28.9	28.7	30.4 ± 2.2 A	23.6	31.8 ± 2.9 A	28.0	27.6	26.2	28.5 ± 2.0 A	23.0
	absence	21.2 ± 3.3 B	20.9	23.2	20.2 ± 3.6 B	28.2	22.5 ± 2.6 B	22.5	25.8	28.9	9.3 ± 4.3 B	27.6
Hatching rate (%)	presence	32.2 ± 3.4 A	30.7 ± 3.2 A	30.4	31.2	25.3	31.8	29.9	29.8	28.4	29.2 ± 2.6 A	28.4
	absence	21.1 ± 3.2 B	19.4 ± 2.8 B	22.9	21.4	28.7	24.2	19.9	25.5	24.0	12.2 ± 4.3 B	27.5
Normal spermatozooids (%)	presence	24.5 ± 2.0 A	22.4	24.4 ± 1.8 A	24.1	24.4	24.5	22.4	22.6	23.8 ± 1.4 A	22.2	25.3
	absence	19.0 ± 1.5 B	21.8	18.4 ± 1.6 B	19.0	21.2	20.3	21.8	21.8	15.0 ± 2.8 B	23.0	21.6
Sperm abnormality (%)	presence	75.5 ± 2.0 B	77.6	75.6 ± 1.8 B	75.9	75.6	75.5	77.6	77.4	76.2 ± 1.4 B	77.8	74.7
	absence	81.0 ± 1.5 A	78.2	81.6 ± 1.6 A	81.0	78.8	69.7	78.2	78.1	85.0 ± 2.9 A	77.0	78.4
Membrane integrity (%)	presence	50.8	48.2 ± 3.0 B	48.1 ± 3.2 B	54.2	43.9 ± 5.4 B	51.1	51.0 ± 3.8 B	55.2	51.2 ± 3.3 B	57.6 ± 3.7 A	39.7 ± 9.3 B
	absence	61.4	70.1 ± 8.3 A	67.6 ± 7.6 A	56.9	60.5 ± 4.7 A	58.6	69.4 ± 9.8 A	55.2	73.0 ± 3.3 A	31.0 ± 19 B	58.6 ± 4.0 A
Mitochondrial functionality (%)	presence	45.0	41.3 ± 6.1 B	42.6 ± 6.8 B	48.6	47.1	43.3	47.7 ± 7.0 B	55.0	52.7	53.8	34.0
	absence	65.3	82.4 ± 8.0 A	71.3 ± 9.7 A	62.0	56.7	61.9	75.8 ± 9.8 A	52.5	57.8	54.5	58.0
DNA Integrity (%)	presence	96.3	96.1	96.3	96.4	96.5	96.6	95.2	94.8	95.4	96.2 ± 0.4 A	91.0 ± 5.5 B
	absence	94.2	94.0	94.2	93.9	95.0	94.6	96.2	96.2	95.6	80.0 ± 0.1 B	96.2 ± 0.5 A
Cell feasibility (%)	presence	13.8	13.6 ± 3.5 B	14.8	16.9	22.7	12.1	13.7 ± 3.1 B	16.3	19.2	19.1	20.2
	absence	26.3	32.1 ± 6.3 A	26.1	22.8	18.6	24.7	39.2 ± 6.2 A	22.3	19.4	20.0	19.0

Mean ± SEM (standard error) with different letters in the column are different (P&lt;0.05).

**Table 3.** Interaction of the presence or absence of proteins in the seminal plasma analyzed in the 15% polyacrylamide gel with the spermatoc parameters of cryopreserved semen from *Colossoma macropomum*.

Parameters		Proteins - Gel with concentration of 15%			
		40 Kda	70 Kda	78 Kda	85 Kda
Spermatoc motility (%)	presence	17.5	15.0	13.4	15.0
	absence	13.7	14.2	19.0	13.3
Motility time (seconds)	presence	30.5	32.8	30.2	31.3
	absence	30.6	30.0	32.7	29.2
Fertilization rate (%)	presence	31.6	32.2	26.8	29.5 ± 2.3 A
	absence	25.6	25.1	26.4	20.7 ± 3.8 B
Hatching rate (%)	presence	38.8 ± 3.5 A	35.9	27.5	30.1
	absence	25.1 ± 2.8 B	25.2	28.5	22.5
Normal spermatozooids (%)	presence	20.5	18.8	21.3	21.5
	absence	22.6	23.2	26.5	23.7
Sperm abnormality (%)	presence	79.5	81.2	78.7	78.5
	absence	77.4	76.8	73.5	76.3
Membrane integrity (%)	presence	50.5	55.4	54.2	54.5
	absence	56.2	53.9	61.0	56.4
Mitochondrial Functionality (%)	presence	48.7	50.2	56.0	55.3
	absence	54.9	54.8	43.5	51.0
DNA Integrity (%)	presence	96.5	96.4	95.8	95.1
	absence	95.2	95.1	93.3	96.2
Cell viability (%)	presence	14.5	23.0	21.4	19.9
	absence	20.2	18.2	9.0	17.9

Mean ± SEM (standard error) with different letters in the column differed ( $P < 0.05$ ).

the progressive motility of frozen semen with DMSO (Table 2). As a consequence, a greater fertilization rate was observed with the presence of the following proteins: 12, 34, 44, 85, and 90 kDa. However, the hatching rate was improved ( $P < 0.05$ ) only with the presence of proteins 12, 25, 40, and 90 kDa.

The percentage of normal spermatozooids (%) in thawed semen of *C. macropomum* was greater ( $P < 0.05$ ) with the presence of proteins 12, 29, and 75 kDa in the seminal plasma, thus reflecting the percentage of total spermatoc abnormalities.

For DNA integrity, the presence of heavier proteins such as 90 and 100 kDa demonstrated an interaction with spermatoc parameters post thawing, with the greatest and lowest ( $P < 0.05$ ) DNA integrity, respectively. For membrane integrity (%) and mitochondrial functionality (%), greater ( $P < 0.05$ ) percentages for these spermatoc parameters were recorded with the absence of proteins 25, 29, 37, 50, 75, and 100 kDa. The absence of the proteins 25 and 50 kDa positively influenced the cell feasibility of the spermatozooids.

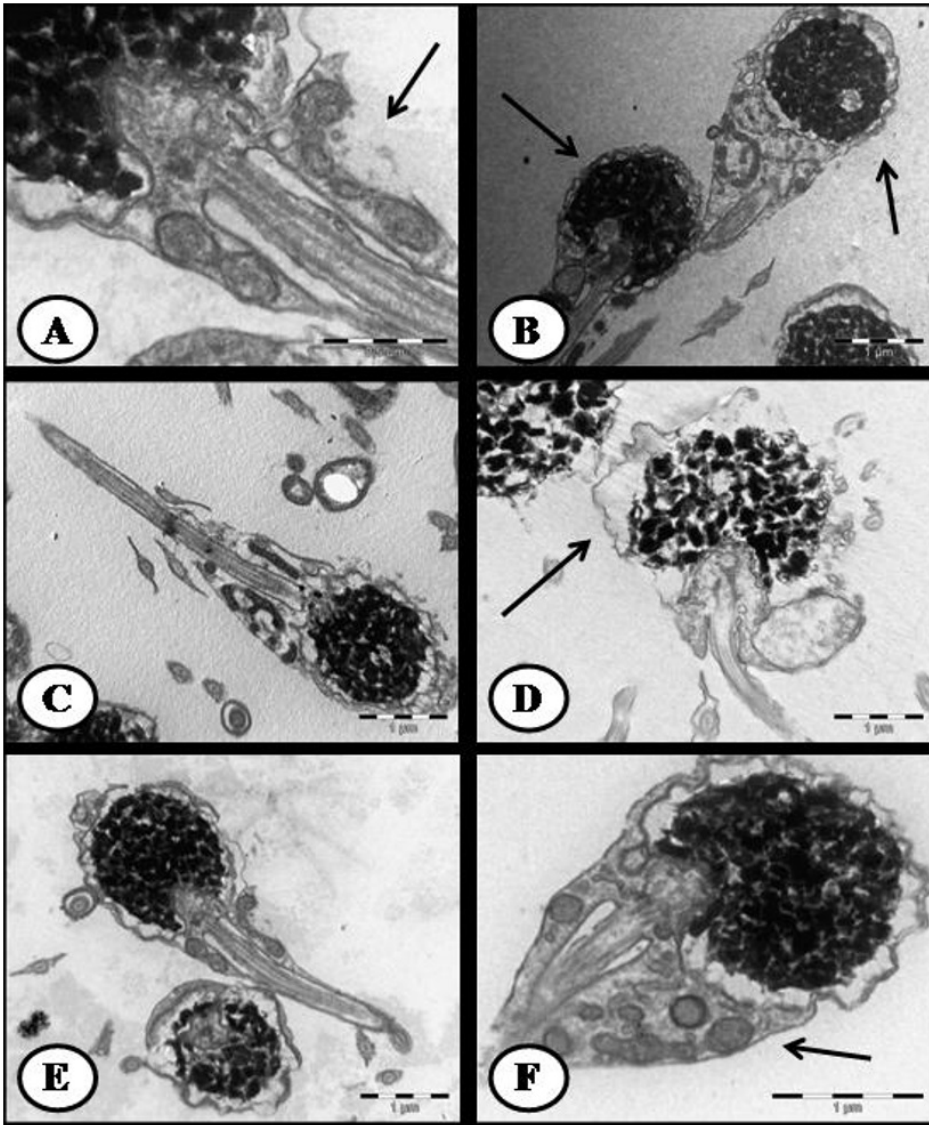
The presence of the protein 12 kDa affected ( $P < 0.05$ ) the progressive motility, percentage of normal and abnormal spermatozooids, and consequently, the fertilization and hatching rates. The interaction ( $P < 0.05$ ) with motility time (seconds) was observed only for the proteins 34 and 75 kDa, while the proteins 65, 70, and 78 kDa did not influence ( $P > 0.05$ ) the parameters of cryopreserved semen of *C. macropomum*.

**Table 4.** Frequency distribution (%) of the protein bands detected through SDS-PAGE in seminal plasma of *Colossoma macropomum*.

Bands	Molecular weight (kDa)	Frequency of bands (%) in 27 samples
1	12	40.7 (11)
2	25	33.3 (9)
3	29	40.7 (11)
4	34	40.7 (11)
5	37	66.7 (18)
6	40	81.5 (22)
7	44	59.3 (16)
8	50	18.5 (5)
9	65	44.4 (12)
10	70	77.8 (21)
11	75	18.5 (5)
12	78	22.2 (6)
13	85	40.7 (11)
14	90	14.8 (4)
15	100	77.8 (21)

Samples within parentheses: number of animals that presented with the protein.

No protein was observed in the samples of the seminal plasma. Only the protein 40 kDa was verified in 81.5% of the samples. However, the protein 90 kDa was observed in four samples (14.8%) (Table 4).



**Figure 2.** Illustrations of morphology obtained by electronic microscopy transmission of the cryopreserved spermatozoids of *Colossoma macropomum*. (A) and (E) broken membrane in the intermediate piece; (B) and (C) membrane of the integral head; (D) broken membranes in the head and intermediate pieces; (F) integral mitochondria.

The frequencies of the proteins 29 and 75 kDa in the semen samples were 40.7 and 18.5%, respectively. Thus, 55.2% of the spermatozoids were observed with full membranes, while 53.8% of the spermatozoids were observed with mitochondrial functionality in the semen of *C. macropomum* after thawing (Figure 2).

#### 4. Discussion

Currently, several proteins of seminal plasma have been identified and characterized (Moreau and Manjunath, 1999), and others have been associated with fertility in different species (Ayyagari et al., 1987; Kraus et al., 2001). The identification of the proteins in the seminal

plasma has an important merit because it can be used to predict, increase, and prevent (preservative) fertility (Killian et al., 1993).

The correlation between proteins of the seminal plasma and fertility of males was demonstrated for some species of domesticated animals such as bulls (Killian et al., 1993), rams (Jobim et al., 2005), goats (Villemure et al., 2003), stallions, and hogs (Calvete et al., 1997). The presence of protein factors in the seminal plasma was described for several species of fish, such as *Oreochromis niloticus* (Mochida et al., 2002), *Oncorhynchus mykiss* (Loir et al., 1990; Lahnsteiner et al., 2004; Lahnsteiner, 2007a), and *Cyprinus carpio* (Kowalski et al., 2003). In some studies (Mochida et al., 1999; Lahnsteiner et al., 2004; Lahnsteiner,

2007a), the identified protein factors were associated with parameters of sperm quality. However, there is little information regarding the proteins of seminal plasma for Brazilian native fishes (Campos et al., 2006).

In the present study, despite the fact that the motility time was greater only for the presence of proteins 34 and 75 kDa, the majority of the proteins with a molecular weight equal to or less than 50 kDa (12, 25, 29, 34, 44, and 50 kDa) were associated with an improvement in the progressive motility of the semen after thawing. This fact agrees with the report of (Lahnsteiner et al., 2004) who observed that the dilutor vehicle containing PSP (proteins of the seminal plasma) of *Oncorhynchus mykiss* with a molecular weight <50 kDa results in higher motility and spermatic speed rates when compared to a dilutor containing proteins with a molecular weight >50 kDa.

In the semen of *C. macropomum*, the 12, 29, and 75 kDa proteins demonstrated an interaction with other qualitative parameters (normal spermatozooids and spermatic abnormalities) of the semen. According to (Frazer et al., 1996), the presence of PSP in mammals, with the apparent molecular weight of 19.6 and 15.3 kDa, was related to the increase in progressive motility of the fresh and thawed sperm. Souza et al. (2007) focused on the effects of proteins with greater molecular weight such as 75 kDa, in the semen of *C. macropomum*, they studied the correlation between the characteristics of semen from dogs with the proteins of seminal plasma, and identified a positive correlation between bands 58.6 and 67 kDa with the percentage of normal spermatozooids.

The mitochondrial functionality, membrane integrity, and DNA integrity of the spermatozooids after cryopreservation of *C. macropomum* improved with the absence of proteins with molecular weights of 25, 29, 37, 50, and 75 kDa, despite the fact that motor activity (motility and motility time) were better in the presence of the same proteins. The absence of the proteins 29, 37, 50, and 75 kDa led to an integrity greater than 58%. This fact demonstrates that even though the spermatozooids had good motility, they were not ready to be functional, especially when considering the proteins 25 and 50 kDa, for which the cell feasibility was the lowest. According to (Bianchi et al., 2008), when considering in silico database, a protein group was found with a molecular weight of approximately 26 kDa called Sialoproteins that has the ability to inhibit the heads' agglutination of the spermatozooids. According to (Strzeżek et al., 2002), the deficiency of this group can compromise the potential spermatic fertilization. Using two dimensional electrophoresis (2DPAGE), Flowers (2001) demonstrated the biological importance of the proteins 26 kDa and 55 kDa in the seminal plasma of swine in relation to high birth rates (around 86%). Kraus et al. (2001) associated four proteins from the seminal plasma with bull fertility, as two proteins (26 and 55 kDa) were detected in highly fertile bulls.

The significant increase of cell destruction of the spermatozoid is a factor highly associated with the process of cryopreservation; thus, it is possible to identify a change in

membrane integrity and mitochondrial functionality. In cattle, there are proteins of seminal plasma (PSP) that participate in lipid sequester deconstructing of the membrane, which increases its fluid capacity. Then, those proteins associate with the membrane of the spermatozoid at the time of ejaculation, thereby inducing the efflux of cholesterol and phospholipids from the spermatic membrane, which can induce the non-stabilization and decrease its stability for the process of semen cryopreservation that is currently utilized (Therien et al., 1999).

The expression of the protein with a greater molecular weight (100 kDa) did not help either the membrane or DNA integrities. In the seminal plasma of pigs, Corcini et al. (2012) observed that the 100 kDa protein was also associated with a reduction in membrane integrity of fresh semen, and a reduction in motility after freezing. When the second heaviest molecular protein (90 kDa) is analyzed, despite the fact that motility and motility time were not positively altered, its presence in the semen after cryopreservation of *C. macropomum* resulted in a significant gain in the results regarding fertilization and hatching rates. Also, we have to highlight the fact that the membrane integrity and mitochondrial functionality rates were the unique positive parameters in the presence of the 90 kDa protein. An interesting fact relates to the incidence of animals that expressed the 90 kDa protein, as it was the lowest for all observations (only 4 from the total – approximately 15%).

The good results for the seminal quality associated with the 25 kDa protein in terms of plasmatic membrane integrity can be explained by the finds reported by (Bianchi et al., 2008). In this case, the authors relayed that in situ database, a protein group was observed with a molecular weight of approximately 26 kDa called Sialoproteins that have the ability to inhibit the agglutination of heads from the spermatozooids. According to Strzeżek et al. (2005), the deficiency of this group can compromise the potential spermatic fertilization. Using two dimensional electrophoresis (2DPAGE), Flowers (2001) illustrated the biological importance of the proteins 26 and 55 kDa in the seminal plasma of swine in relation to high birth rates (around 86%). Killian et al. (1993) associated four proteins of the seminal plasma with bull fertility, since two proteins (26 and 55 kDa) were detected in highly fertile bulls. The protein found in the present study (25 kDa) was the protein associated with the hatching rate, such as the proteins 40 and 90 kDa. Also, the 90 kDa protein was associated with DNA integrity. Asadpour et al. (2007) reported that in buffalo, the protein with 55 kDa was associated with feasibility of fresh semen. In this study, the proteins that demonstrated an association with cell feasibility of semen after thawing were 25 and 50 kDa.

Therefore, more detailed studies regarding the function of PSPs are necessary to elucidate the implications of the individual effects, as well as the influence of other factors such as season, nutrition, and age. The identification of potential markers related to fertility parameters should be important for breeding selection in programs using artificial



fertilization with frozen semen and for the formation of a germ plasma database of selected animals.

The proteins of the seminal plasma from *C. macropomum* influenced spermatic quality after thawing and can be utilized as markers for spermatic quality, especially the proteins with molecular weights lower than 50 kDa, such as the proteins 12, 25, 29, 34, 40, 44, and 50 kDa. However, the protein 90 kDa despite being the smallest protein found in the seminal plasma of *C. macropomum*, his presence was the one with the best parameters for sperm quality, in other words, the animal with this protein has a better predisposition to semen freezing.

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