

Spermatic abnormalities of piracanjuba *Brycon orbignyanus* (Valenciennes, 1849) after cryopreservation

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(With 3 figures)

Abstract

The objective of this research was to verify the presence of spermatic abnormalities on semen of *Brycon orbignyanus* after cryopreservation. Semen was collected from ten four-year-old males who presented secondary reproductive characteristics for migrating fish. Sperm was evaluated for motility, vigor and spermatic morphology before and after cryopreservation. A cryoprotectant solution was made of 20 mL of yolk egg, 5.0 g of glucose and dimethyl sulfoxide diluted in distilled water (10 mL: 90 mL). The diluted semen (1:3, semen:solution) was submitted to nitrogen steam for 24 hours and then to liquid nitrogen (-196 °C) for 60 days. Cryopreservation decreased the percentage of normal spermatozoa from 62.20% to 54.60%. Consequently, the percentage of spermatozoa with secondary abnormalities increased from 8.50% to 15.00%. However, there was no difference in primary abnormalities. Both spermatic motility and vigor were decreased in cryopreserved semen compared with fresh semen. In conclusion, cryopreservation of semen of *B. orbignyanus* increased the percentage of secondary abnormalities and decreased the spermatic motility and vigor.

Keywords: freezing, spermatozoa, morphology, Bryconinae.

Anormalidades espermáticas de piracanjuba *Brycon orbignyanus* (Valenciennes, 1849) após a criopreservação

Resumo

O objetivo deste trabalho foi verificar a presença de anormalidades espermáticas no sêmen de *Brycon orbignyanus* após a criopreservação. Dez machos com quatro anos de idade e que apresentavam características reprodutivas secundárias para peixes migradores tiveram o sêmen coletado. O sêmen foi avaliado através da motilidade, vigor e morfologia espermática antes e após a criopreservação. A solução crioprotetora continha 20 mL de gema de ovo, 5,0 g de glicose e dimetil sulfóxido diluído em água destilada (10 mL: 90 mL). O sêmen diluído (1:3, sêmen:solução) foi submetido ao vapor de nitrogênio por 24 horas e então ao nitrogênio líquido (-196 °C) por 60 dias. A criopreservação reduziu o percentual de espermatozoides normais de 62,20% para 54,60%. Consequentemente, o percentual de espermatozoides com anormalidades secundárias aumentou de 8,50% para 15,00%. Porém, não foi observada diferença nas anormalidades primárias no sêmen “in natura” e pós-criopreservação. Ambos motilidade e vigor espermático foram inferiores aos observados no sêmen “in natura”. Conclui-se que a criopreservação do sêmen de *B. orbignyanus* aumentou o percentual de anormalidades secundárias e reduziu a motilidade e o vigor espermático.

Palavras-chave: congelamento, espermatozoide, morfologia, Bryconinae.

1. Introduction

Brycon orbignyanus, popularly known as piracanjuba, is a reophylic species (Godoy, 1975) and is considered a highly migratory fish because it migrates over long distances in order to reproduce. Abundant in past decades, the population of *B. orbignyanus* is now reduced due to various factors such as the obstruction of rivers using hydroelectric dams, riparian forest destruction, reduction of lateral lakes and predatory fishing. Nevertheless, some individuals can still be found in the drainage basin of the Uruguai and Paraná rivers (Zaniboni-Filho, 1999). It is a fish with a high commercial value and is well-known by the riverside communities. It is also appreciated by those who fish for being a “fighting” fish, but it no longer counts in commercial fishing statistics (Baldiasserotto and Gomes, 2005).

This species has aroused great interest among research institutions over the last few years, because of the excellent meat quality, as well as the diet in the natural environment, preferentially consisting of seeds and fruit (Murgas et al., 2003). Moreover, fast growth and weight gain demonstrated in experimental farming show that this species is a good alternative to aquaculture developing in all regions of Brazil (Vasques, 1997).

Different strategies are necessary in order to attenuate the decline of the fish populations in natural environments (Sirol and Britto, 2005). Genoma banks, such as the ones with cryopreserved semen, can be a suitable component for conservation efforts in Brazil, especially for endangered species like *B. orbignyanus*. These banks are especially important by ensuring the genetic diversity in breeding programs in captivity in order to preserve the species until other conservation acts take place (Carolsfeld et al., 2003).

Some of the advantages of semen cryopreservation are: reduction in the number of male breeders that have to be stocked for fry production (Herman et al., 1994), easy transportation of the semen for a possible interchange among aquaculture facilities and reduction of reproductive asynchrony problems. Moreover, cryopreservation is an effective method for storage viable spermatozoa for a long time and can provide a gamete supply all year long (Zhang et al., 2003).

Fish semen production is influenced by environmental factors (mainly temperature and dissolved oxygen), species and age resulting in great variations in characteristics (Rodríguez et al., 2001). Semen quality can be determined by evaluating parameters such as: spermatic motility, spermatic vigor, pH, spermatozoa concentration and spermatic morphology (Cosson et al., 1999). Studies of spermatic cell morphology and its relationship with male infertility reached higher importance by using artificial insemination in mammals, especially in bovine species (Kavamoto et al., 1999). In addition, these studies are important for qualitative evaluation of semen, since the increase in abnormalities leads to a decrease in spermatic vigor and motility (Lahnsteiner et al., 1998; Cosson et al., 1999) reducing the fecundation capability. Nevertheless, in

fish, studies including these parameters are still restricted to structural changes in spermatozoa after cryopreservation (Yao et al., 2000; Taddei et al., 2001).

Over the last few years, production and preservation of semen of some Brazilian South American native species, such as the *Salminus maxillosus* (Streit Junior. et al., 2008), *Brycon orbignyanus* (Maria et al., 2006) and the *Piaractus mesopotamicus* (Streit Junior et al., 2007) have been increased due to the economical and environmental importance of these species. Taking this into account, new techniques have been applied in order to successfully reproduce, grow in captivity and preserve native species.

The objective of this study was to verify the presence of abnormalities in *B. orbignyanus* semen after cryopreservation.

2. Materials and Methods

The research was developed in the Hydrology and Aquaculture Station of Duke Energy Brazil, in Salto Grande, São Paulo, Brazil in association with the Animal Reproduction Laboratory of Maringá State University (UEM), Paraná, Brazil.

2.1. Selection of males and semen collection

Ten four-year-old males of *B. orbignyanus*, weighing 2.1 ± 0.12 kg who presented secondary reproductive characteristics for migrating fish, and who were males releasing semen after gentle pressure on the abdomen and females with a soft and rounded abdominal region and a turgid and reddish urogenital orifice, were selected from breeder stocks of Duke Energy Brazil.

In order to collect the semen, the fish were wrapped in a damp towel to reduce stress, their urogenital region was dried and the semen was released after gentle pressure. The semen was collected into 5.0 mL syringes (Billard et al., 1995). Immediately after having been collected, the semen was evaluated and cryopreserved.

2.2. Spermatic evaluation

Spermatic motility and vigor: 20 μ L of semen was diluted in 160 μ L of distilled water at 27 ± 0.5 °C in a slide glass and observed under an optical microscope (40x) for subjective evaluation of both parameters. For spermatic motility, a score from 0 to 100 was used and for spermatic vigor the score ranged from 0 to 5 points. It should be mentioned that the higher it was, the better.

Spermatic morphology: a fraction of the semen (10 μ L) was diluted in 10 mL of a saline buffered formaldehyde solution (1:1000, semen:solution), then a smear from the fixed semen from each fish was taken and stained with Rosa Bengala (Streit Junior. et al., 2004), dried and analysed under an optical microscope with 40x objective. A range of 100 to 115 spermatozoa were counted for each fish. Spermatozoa were classified as normal or presenting primary (broken, coiled, corrugated or degenerated tails) or secondary abnormalities (bent tail, free head and free tail).

2.3. Criopreservation

A cryoprotectant solution was formulated according to a modified protocol of Carolsfeld et al. (2003): 20 mL of fresh chicken egg yolk, 5.0 g of glucose and 10 mL of dimethyl sulfoxide (DMSO) added to 100 mL of distilled water.

Semen and solution were homogenized at a proportion of 1:3 mL (semen:solution) and placed into 0.25 mL straws, identified and sterilized. Straws were then submitted to nitrogen steam in a dry shipper container. After 24 hours of cooling, straws were transferred to a liquid nitrogen storage container at $-196\text{ }^{\circ}\text{C}$.

2.4. Thawing

Sixty days after freezing, the semen was thawed and straws were immersed in water at $45\text{ }^{\circ}\text{C}$ for five seconds. Immediately afterwards, the spermatic motility, spermatic vigor and morphology were evaluated (protocol described by Lahnsteiner, 2000).

2.5. Experimental design and statistics

A block design was used where each fish semen sample generates two experimental units, fresh or after freezing. Therefore, out of 10 original samples, a total of 20 experimental units were analyzed. A binomial negative distribution of the residuals with a canonical link was carried out. For analysis of variance, the GENMOD procedure of SAS (SAS Institute, Cary, NC – USA) was used. For Spearman Correlation analysis, the CORR of SAS procedure was used.

3. Results

In general, cryopreserved semen presented a worse quality compared to the fresh semen (Table 1). While spermatic motility in fresh semen was 90.02%, in cryopreserved semen it decreased to 14.87% ($p < 0.05$). Moreover, spermatic vigor decreased from 3.0 in fresh semen to 1.8 in cryopreserved semen ($p < 0.05$).

In morphological evaluation, there was no difference ($p > 0.05$) in the percentages of normal spermatozoa (Figure 1a) or in the total and primary abnormalities between fresh and cryopreserved semen (Table 1). Indeed, some

of the abnormalities were recurrent, such as degenerated tails (Figure 2a) and free heads (Figure 2b) which were increased ($p < 0.05$) from 11.47% to 22.89% and from 3.70% to 40.80% in fresh and cryopreserved semen, respectively. For other secondary abnormalities (free and bent tails) and primary (like broken and coiled tails) there were no differences between fresh or cryopreserved semen. However, there was a high occurrence of spermatozoa with broken tails in cryopreserved semen of 13.20% versus 9.02% fresh semen (Figure 3).

Spermatic motility presented positive correlation with spermatic vigor ($r = 0.95$; $p < 0.05$). As well as secondary abnormalities, shown in Table 2, there was a negative correlation with spermatic motility ($r = -0.55$) and with spermatic vigor ($r = -0.63$).

4. Discussion

Values of spermatic motility in fresh semen observed in this study were similar to those found by Murgas et al. (2003) for the same species: 90.02% and 97.77%, respectively. But for cryopreserved semen, those authors found a higher value (73.33%) compared to our study (14.87%). Possibly the difference between the values from these two studies is related to the spermatozoa activator used in the present study, sodium bicarbonate. Indeed, the loss of seminal quality from the cryopreservation is reassured here by the difference in spermatic vigor.

A decrease in the percentage of normal spermatozoa was expected. Although there were no changes in total abnormalities, there was an increase in ($p < 0.05$) secondary abnormalities in cryopreserved semen (15%), which was almost twice the percentage found in fresh semen (8.5%). According to Herman et al. (1994), in mammals, secondary abnormalities are related to factors linked to temperature, nutrition, spermatic duct problems, semen extrusion and smear production. Spermatic deformations are associated to functional deficiency leading to a reduction in motility and fertilization capability. For example, fish spermatozoa exposed to mercury ion (Hg^{2+}) are characterized by having broken tails (Van Look, 2001; Van Look and Kime, 2003), decreased motility and less capability of fertilization (Rurangwa et al., 1998).

Table 1. Percentage averages and standard deviations of spermatic motility, spermatic vigor, normal spermatozoa and primary, secondary and total abnormalities in fresh or cryopreserved semen of *Brycon orbignyana*.

Parameters	Semen	
	Fresh	Cryopreserved
Spermatic motility (%)	90.02 \pm 1.58 ^a	14.87 \pm 11.66 ^b
Spermatic vigor (points)	3.00 \pm 0.00 ^a	1.80 \pm 0.44 ^b
Normal spermatozoa (%)	62.20 \pm 17.86 ^a	54.60 \pm 4.16 ^a
Primary abnormalities (%)	29.10 \pm 13.86 ^a	30.6 \pm 4.71 ^a
Secondary abnormalities (%)	8.50 \pm 6.32 ^b	15.00 \pm 3.66 ^a
Total abnormalities (%)	37.71 \pm 17.86 ^a	45.60 \pm 4.15 ^a

Averages in the same line with the same letter don't differ significantly to each other.

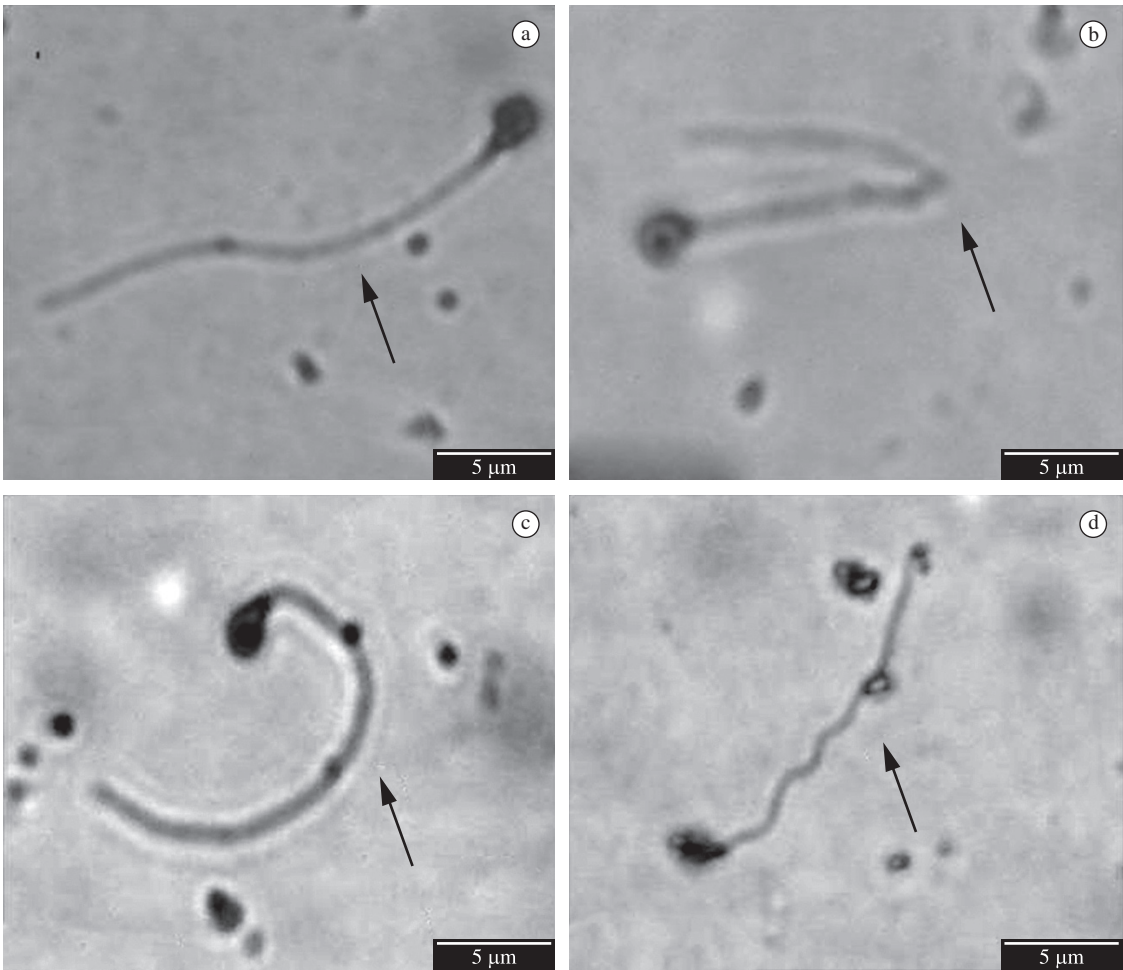


Figure 1. Illustrations of morphologies observed in the semen of *Brycon orbignyanus* after cryopreservation. a) normal spermatozoa; b) spermatozoa with bent tail; c) spermatozoa with coiled tail; d) spermatozoa with corrugated tail.

Table 2. Spearman correlations between characteristics of spermatic quality of fresh and cryopreserved semen of *Brycon orbignyanus*.

Parameters	Motility	Vigor	Normal	Abnormalities		
				Primary	Secondary	Total
Motility	1	0.95**	0.18 ^{ns}	0.05 ^{ns}	-0.55**	-0.18 ^{ns}
Vigor		1	0.27 ^{ns}	-0.03 ^{ns}	-0.63**	-0.27 ^{ns}
Normal spermatozoa			1	-0.80**	-0.50*	-1.00**
Primary abnormalities				1	0.06 ^{ns}	0.80**
Secondary abnormalities					1	0.50*
Total abnormalities						1

**p < 0.05; *p < 0.01; ^{ns}p > 0.05

The main problem caused in fish semen by cryopreservation is related to a decrease in spermatic motility and vigor and, as a consequence, to the possible loss of fertilization capability (Lahnsteiner et al., 1996, 1998; Cosson et al., 1999). In this study, it is worth mentioning that there were no statistical differences for total pathologies, both before and after cryopreservation of the semen and

that the decrease in the spermatic motility from 90.05% to 14.87% and the spermatic vigor from 3.0 to 1.8 points, can be related to the increase in morphological abnormalities, especially the secondary ones.

A decrease in motility after cryopreservation can be correlated to structural deformation during freezing/thawing processing of spermatozoa. According to Zhang et al. (2003),

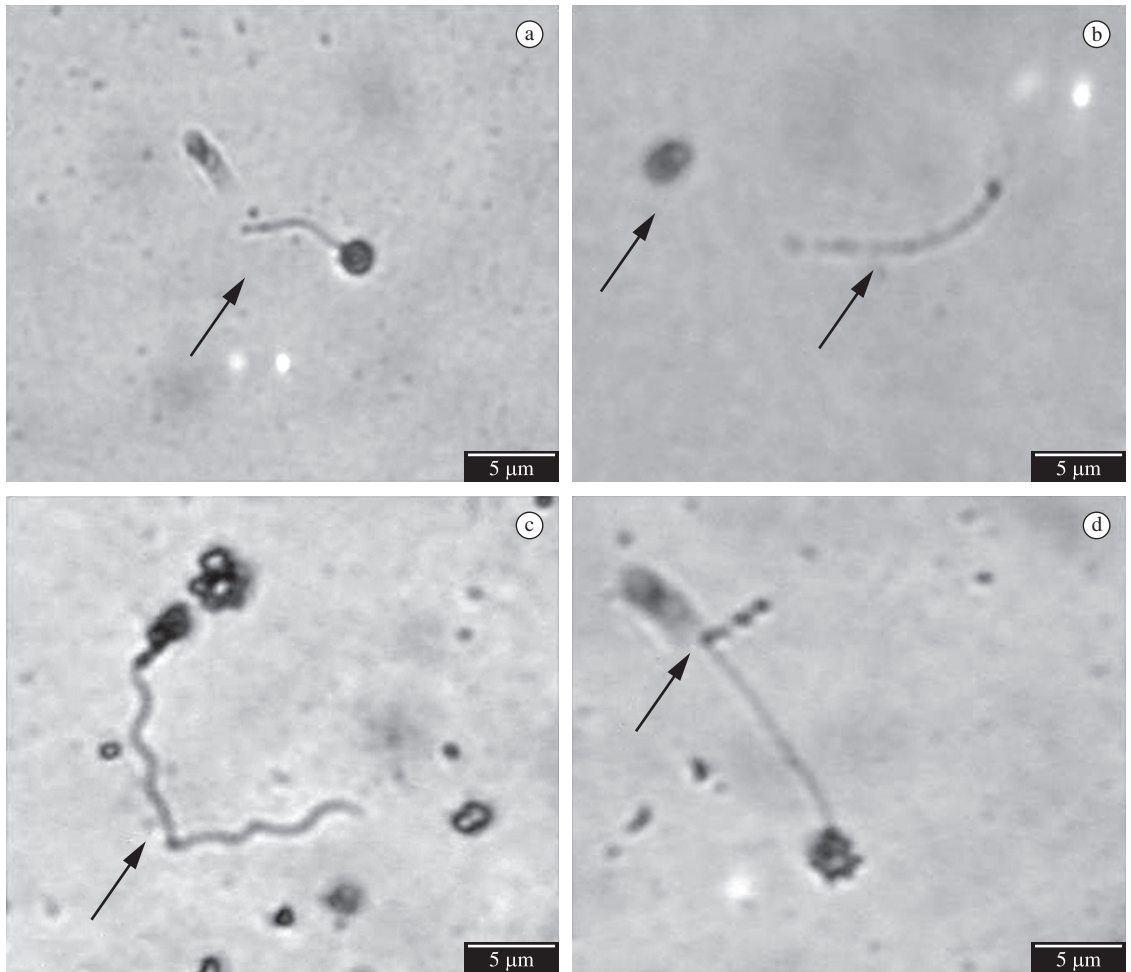


Figure 2. Illustrations of morphologies observed in the semen of *Brycon orbignyanus* after cryopreservation. a) spermatozoa with degenerate tail; b) spermatozoa with free tail and head; c) spermatozoa with the broken tail in the intermediate portion; d) spermatozoa with the broken tail in the final portion.

fish spermatozoa have a long tail with an intermediate piece that usually contains four to five mitochondria. After freezing and thawing, severe dilatation or a partial loss of mitochondria in the intermediate piece often occurs and the tail breaks. Loss of mitochondria may happen due to the fact that the intermediary piece swells that can push away the mitochondria from the intermediary piece or induce structural damage inside the mitochondria. These changes can adversely affect mitochondria and tail function, decreasing the lagellar movement. This phenomenon was also described by Yao et al. (2000) when observing semen of *Macrozarcus americanus*. After the cryopreservation process and posterior thawing, the authors observed severe swelling and/or a cytoplasm dehydration of the spermatozoa intermediate piece. They concluded that these alterations on mitochondrial activities cause a decrease in cellular energy supply and, as a consequence, leads to a reduction in the flagellar movement and a decrease in the spermiatic motility.

Another important factor is related to the spermatozoa activation solution that in the present study was the distilled water. Using NaCl and NaHCO₃ has brought about better results compared to distilled water. For example, Murgas et al. (2007) used the NaHCO₃ with 119 mM on semen of curimba (*Prochilodus lineatus*) and obtained the best results with semen after cryopreservation. The authors argue that the small osmotic difference between the NaHCO₃ 119 mM solution and the cytoplasm preserved the integrity of the cytoplasmic membrane of the spermatozoa. The distilled water was used in the present study because of the practicality, especially in the field where the equipment used to measure some parameters, such as osmolarity, are not usually available. It is important to mention the observation made by Murgas et al. (2007) for NaHCO₃, which is if the activator promotes toxicity on oocyte, then the fertility rate will be reduced.

The high correlation between the motility and spermiatic vigor observed in the present study can be explained by

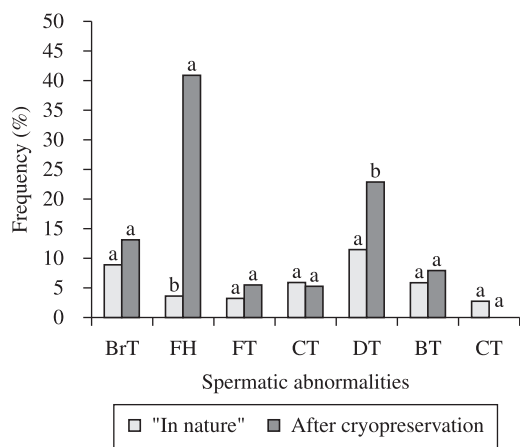


Figure 3. Average percentages of abnormalities found in fresh and cryopreserved semen of *Brycon orbignyanus*: broken tail (BrT); free head (FH); free tail (FT); coiled tail (CT); degenerate tail (DT); bent tail (BeT); corrugated tail (CT). * Averages with different letter in the same item differ ($p < 0.05$).

being both variables dependent on spermatic metabolism. Therefore, both modify when some metabolic alteration occurs. On the other hand, the negative correlation between spermatic motility and spermatic vigor with cellular alteration such as secondary abnormalities also make the loss of quality of these parameters clear, which can be observed by the tail abnormalities that are often related to the loss of normal spermatozoa movement. Ultra structural alterations to spermatozoa happen after the increase or decrease in osmolarity of the surroundings. This alterations can be physiological or pathological (Miliorini, 2006). According to Marques (2001), these modifications are limiting of motility length, which may have happened in this study.

According to the literature, motility rates around 10% to 20%, similar to the results obtained in this experiment, still provide satisfactory results for the fertilization rate. This can be confirmed by Billard et al. (2004) who obtained 73% to 93% of fertilization with sturgeon semen presenting only 10% of spermatic motility after thawing. In a similar way, Menezes et al. (2008) observed 76% of fertilization rate with semen of *Colossoma macropomum* with only 20% of spermatic motility after cryopreservation. Moreover, Ribeiro and Godinho (2003) observed a fertilization rate of 84.3% with semen of *Leporinus macrocephalus* cryopreserved with DMSO. Cryopreservation of semen of *B. orbignyanus* increased the percentage of secondary abnormalities, reducing the motility and spermatic vigor.

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