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Seminal Analysis, Cryogenic Preservation, and Fertility in Matrinxã Fish, *Brycon cephalus* (Günther, 1869)

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ABSTRACT

Aiming to improve fish reproduction techniques, the characterization and cryopreservation of semen of Brycon cephalus were performed. The seminal characteristics observed were: an almost transparent, milky semen with a mean volume of 4mL, and sperm concentration of 9.617±1.630 x 10^6 spermatozoa/mm^3. Spermatozoa (length = 31.288 ±4.47μm) were of the aquasperm type and displayed a small, round head (length = 1.727 ±0.18 μm; width = 1.752 ±0.17μm) without acrosomal vesicle, nucleus with highly condensed chromatin forming coarse clots and centriolar complex located in the nuclear fossa; a midpiece (length = 2.561±0.44μm), narrowed rearward, with a cytoplasmic canal; and a flagellum (length = 29.521 ±4.37μm). Fertilization tests with thawed semen demonstrated a significant effect (α= 0.05) on the increase of thawed semen fertility with diluent type B in both 0.5mL and 4.0 mL straws. No significant effect (α= 0.05) on hatching rate was observed in both straw sizes used.

Key words: Semen characteristics, Cryopreservation, *Brycon cephalus*, Ultrastructure, Fish

INTRODUCTION

Cryopreservation of fish semen has contributed to the development and application of methods of reproductive control by favoring genetic manipulation, broodstock selection and reduction of male stock since it provides gametes for undetermined periods. In addition, these freezing methods may be used in hybridization programs, preservation of genetic material from endangered species and gene banking. The development of methods of semen preservation and the implementation of effective artificial reproduction protocols require a good knowledge of the reproductive biology and seminal characteristics (sperm concentration, semen color and volume, sperm morphology, etc.) of the targeted species.

In the neotropical species of interest for fish breeding, "ex situ" genetic preservation has been tested. Within this context, the efforts made by some investigators are noteworthy: the evaluation of fresh semen of the catfish *Rhamdia hilarii* (Valenciennes, 1840) and the cryopreservation semen in dry ice by Fogli da Silveira et al. (1981; 1985); the studies by Coser et al. about the conditions for the preservation of curimbatá (*Prochilodus scrofa*) semen (1984) and also the freezing and cryopreservation of piau (*Leporinus silvestrii*) semen (1987); the freezing of pacu (*Piaractus mesopotamicus*) semen by Carosfeld et

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al. (1990); and the sperm evaluation, cryogenic preservation and semen fertility of *P. mesopotamicus* also carried out by Fogli da Silveira et al. (1990).

The semen characterization, as well as the cryopreservation techniques used in neotropical fish species are still incipient. For their development and qualitative control, specific information including data on the structure of spermatozoa is required. Among fish, different reproduction strategies and spermatozoa of varied morphology are described. In fishes of internal fertilization, spermatozoa usually display an elongated head and a more elaborate and structured midpiece. In species with external fertilization, spermatozoa commonly have a round, or oval head, and a small midpiece. Among Teleostei, spermatozoa exhibit no acrosome. Such condition is associated to the presence of a developed micropyle in the eggs of the species (Jamieson, 1981).

Brycon cephalus is a Brazilian migratory fish from the Amazon region, of annual maturation and total spawning. It is omnivorous and presents great adaptability to confined systems. Its flesh is of excellent quality and it reaches slaughter age at one year-old (Pereira Filho, 1994; Mendonça, 1994). It is remarkably valuable at the fish market as well as in the sport fishing trade. The purpose of this study was to determine the seminal characteristics of *B. cephalus*, including volume, sperm concentration, spermatozoa morphology and morphometry, and to establish an efficient and easy method for semen cryopreservation of this species.

MATERIAL AND METHODS

Adult specimens (three years-old) of *B. cephalus* from the broodstock of CEPTA - Centro de Pesquisa e Gestão de Recursos Pesqueiros Continentais - IBAMA, Pirassununga, São Paulo, Brazil were used as semen donors. They were kept in 1000-m^2 tanks and fed with commercial pellets (30% protein content) until the beginning of reproduction. Spermiation was induced by one injection of 1mg of carp pituitary extract (CPE) per kg of body weight. After 6 h, semen was collected by gentle abdominal pressing and stored in sterilized test tubes.

Assessment of semen characteristic

Semen was collected from twenty-five specimens of B. cephalus. The semen samples were evaluated for volume (mL), determined by emptying the gonad until bleeding occurred; color; subjective motility rate (0-5 scale, with **0** = no motility, **1** = 10 -20%, **2** = 20 -40%, **3** = 40 -60%, **4** = 60 -80% and **5**= > 80% of motile cells) (Fribourg, 1966) and, sperm concentration (spermatozoa/mm^3 = sperm/mm^3), obtained by using a Neubauer cell counting chamber. For the ultrastructural analysis of spermatozoa, an aliquot of each semen sample (fresh and thawed) was fixed in 4% paraformaldehyde and 2% glutaraldehyde in a 0.1M phosphate buffer (pH 7.3). For transmission electron microscopy (TEM), samples were post-fixed in 1% osmium tetroxide for two hours, counterstained in a water solution of 0.5% uranyl acetate, dehydrated in acetone and embedded in Araldite resin. The ultra-thin sections were counterstained in uranyl acetate (Watson, 1958), washed in 50% alcohol, once again counterstained in lead citrate (Reynolds, 1963), investigated and electromicrographed with a Phillips-CM 100 transmission electron microscope.

For scanning electron microscopy (SEM), the pre-fixed material was centrifuged (Fanem, Centimicro, 212) (3x/3min/1000 rpm) and transferred to a 13-mm-diameter slide covered by 1% poly-L-lysine, post-fixed in 0.5% osmium tetroxide, dehydrated in ethanol, critical point dried in a Balzers CPD-20 set. The samples were covered by 10nm-thick gold film in a Balzers MED-010 metalizer, observed and electromicrographed under a Phillips-SEM 515 microscope.

Spermatozoa dimensions were measured with the image analysis program Kontron Elektronik KS 300 v. 2.0. A mean value was determined for head length (HL) and head width (HW), midpiece length (ML) and flagellum length (FL) as well as total length (TL) in micrometers. Fifty-five spermatozoa from each semen sample were used for this purpose.

Semen cryopreservation

Sixteen males were used in this experiment and sperm motility (Fribourgh, 1966) was assessed at each collection. The ejaculates, in which motility was rated “5”, were later mixed for the preparation of a semen pool ($9.23 \pm 1.5 \times 10^6$ sperm/mm^3) with three replicates. The semen was diluted at a ratio of 1:3 (sperm:extender) at room temperature. The

first aliquot was diluted in the type A extender, developed by Stein and Bayrle (1978), denominated "V2e", which was also utilized by Fogli da Silveira et al. (1981; 1984). It consisted of NaCl 750mg, $NaHCO_3$ 200mg, KCl 38mg, glucose 100mg, distilled water 100mL, hen egg yolk 20mL (pH= 8.0; 313mOsm) and 10% dimethyl sulfoxide (DMSO). The second aliquot was diluted in type B extender which consisted of glucose (5.0g), hen egg yolk (10mL) and distilled water (80mL) (pH= 6.38; 356 mOsm) and 10% DMSO, according to Hugo Pereira Godinho (oral communication).

The semen was filled into 0.5mL straws or 4.0mL straws (adapted bovine insemination sheaths (I.M.V., L´Aigle, France) sealed with cotton and polyvinylic alcohol at one end). Subsequently, the straws were laid on a steel screen tray and frozen in liquid nitrogen vapor, at 1cm above the level of liquid nitrogen (-185ºC) inside an insulated Styrofoam® box. The freezing temperature was determined by a PT 100 thermocouple probe (+100ºC - -200ºC). Ten minutes later, the straws were plunged into liquid nitrogen and then transferred to a cryogenic container (Cryometal-DS- 34). Twenty-four hours after freezing, a fertilization assay was performed with eggs from six mature, three years-old females. Ovulation was induced with two injections of carp pituitary extract (0.5 mg and 5.0mg/kg body weight, respectively) at 10-hour interval. Six hours after the second injection, the eggs were obtained by gentle abdominal massage.

Thawed semen from a 0.5mL straw was used to fertilize 4.0g of eggs (approximately 4950 eggs) and a 4.0mL straw was used to fertilize 20.0g of eggs (approximately 24760 eggs). As a control, 0.2mL of fresh semen provided by fresh semen pool, collected from six males, were used to fertilize 4.0g of eggs and 0.5mL for 20.0g of eggs. Total sperm/egg ratio for all treatments is about 2,9±0.96 x 10^5 sperm/egg.

Thawing was carried out by immersing the 0.5mL and 4.0mL straws in a 36ºC water bath for 10s and 30s, respectively. Subsequently, semen was spread over the eggs and gently mixed. Then, water was added in order to activate sperm. After twenty minutes, the eggs were washed with water and placed in an incubator and larvae started to hatch after 16 hours of incubation at 22ºC. The incubators were emptied, the larvae were collected and fixed in 4% formol solution and hatching rate was determined. These experiments were performed with three replicates.

In order to determine the degree of interaction between variables (straw size and diluent type) and hatching rate, the statistical software SAS (8.02) was used. Data were analyzed by ANOVA at a significance level of 5% ($\alpha = 0.05$), and data regarding significant effects were analyzed by the Turkey test ($\alpha = 0.05$).

RESULTS

Semen of *Brycon cephalus* was milky, almost transparent and showed low viscosity, high spermatic concentration (X= 9.617±1.6 x 10^6 sperm/mm^3), and a relatively large volume after hormone induction (ranging from 4mL of semen to over 10 mL). Spermatozoa were of an average length (TL) of 31.288±4.47 μm and was differentiated into a head, a midpiece and a tail or flagellum. The head was small, round shaped (HL= 1.727±0,18 μm; HV= 1.752±0,17 μm), and almost totally occupied by a nucleus. The nucleus consisted of highly dense chromatin forming dense clots, covered by a cytoplasmatic membrane without acrosome (Figures 1B and 1C). The nuclear fossa was deep and contained a proximal and a distal centriole (basal body), perpendicular to each other, and the initial portion of the flagellum (Figs. 1A, 1C, 1D). The midpiece (ML= 2.561±0.44 μm) started at the posterior region of the head, where the tail began, and narrowed toward the posterior end of the spermatozoa. In the cytoplasm of this region, mitochondria and several vesicles could be seen. Mitochondria were located in the anterior part of the midpiece and form a necklace around the initial portion of the flagellum (Figs. 1A, 1C, 1E). They were completely separated from the flagellum by the cytoplasmatic canal (Figure 1C, 1D).

The flagellum (FL = 29.521±4.37 μm) of the spermatozoon arose from the basal body, located at the nuclear fossa (Figs. 1A, 1B, 1E). It was perpendicular to the base of the nucleus, though slightly eccentrical. It was formed by a basic axoneme with typical microtubules arrangement and consisted of nine peripheral doublets and a central pair (Fig. 2E). The ultrastructural examination (SEM; TEM) of thawed spermatozoa from all treatments, compared with that of fresh spermatozoa (Figs. 2C, 2F) revealed a range of

that varied from swelling of the plasma membrane and midpiece retraction (Figs. 2A, 2B, 2D, 2G) to total destruction of the spermatozoon (Figs. 2E, 2H). Some structural alterations were present in all thawed spermatozoa observed.

The results of fertilization tests demonstrated a significant effect ($\alpha = 0.05$) on the increasing of fertility in the diluted and frozen semen using the extender of **B** type, at both 0.5mL and 4.0mL straws. No significant effect ($\alpha = 0,05$) related to the utilization of 0.5mL or 4.0mL straws in relation to the hatching percentage, according to the adopted freezing method, was observed, despite of hatching differences favorable to the utilization of 0.5mL straws (Fig. 3).

DISCUSSION

Sperm characteristics

Spermatozoa of several *Brycon* species (Romagoza, 1999; Aires, 1998; Zaiden, 2000) were very similar to each other and, therefore, similar to *Brycon cephalus* spermatozoa. Excepts by the chromatin density pattern, *Brycon sp* spermatozoa could be classified as aquasperm type. According to Jamieson (1991), aquasperms usually have a head with a round-shaped nucleus containing homogeneous and highly dense chromatin, and a midpiece and cytoplasmatic canal of varying sizes.

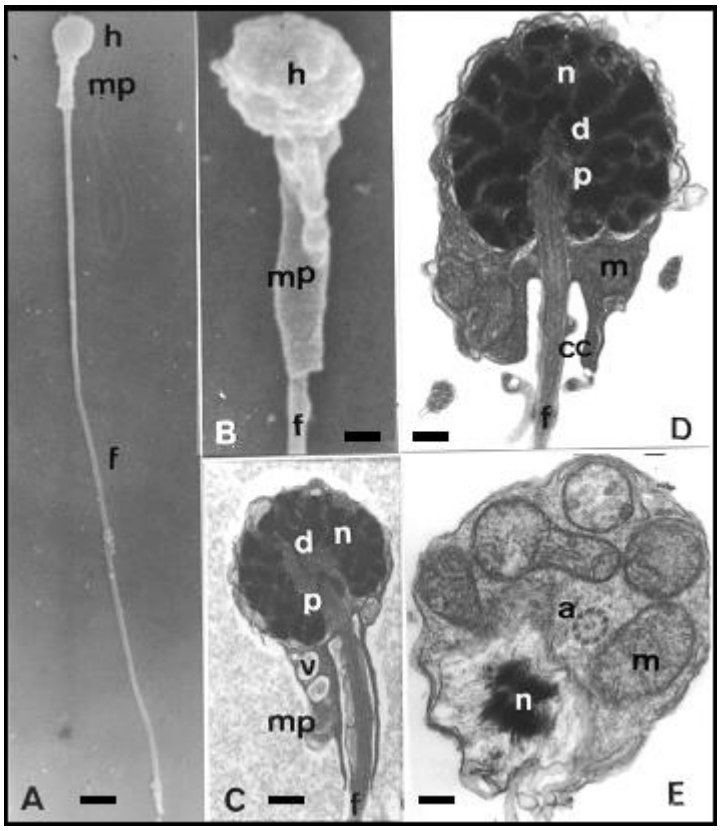

Figure 1 - Ultrastructural aspects of *B. cephalus* spermatozoa. Head (h), midpiece (mp), flagellum (f), nucleus (n), proximal centriole (p), basal body (d), mitochondria (m), vesicle (v), cytoplasmatic canal (cc), axoneme (a) Bars: A - 1.54μ; B - 0.38μ; C - 0.63μ; D - 0.29μ; E - 0.37μ

Seminal volume and fertilization spermatozoa/egg ratio

The volume of semen produced by this species after CPE induction make unnecessary the sacrifice of the animal to obtain sperm as it occured with the African catfish *Clarias gariepinus* (Viveiros et al., 2000). The large semen volume facilitated the application of

cryopreservation techniques and its high concentration allowed the use of different diluents, a better use of semen in the process of artificial fertilization and reduction of male broodstock. In studies with the African catfish, *Clarias gariepinus*, Rurangwa et al. (1998) demonstrated that both, excess of semen and low sperm concentration, reduced fertilization success. This was also observed by Levanduski and Cloud (1988) in the rainbow trout, *Oncorhynchus mykiss*. Scott and Baynes (1980) concluded that a small amount of good quality semen was enough to obtain a high fertilization rate.

The number of spermatozoa necessary to fertilize an egg among fishes is species specific. Furthermore, variations in the spermatozoa/egg ratio can occur according to egg or semen quality, among other factors (Table 1). On the other hand, regarding to thawed semen, the spermatozoa/egg ratio should be higher because, as demonstrated by Martinez and Ekwall (1998), the freezing/thawing may cause great mortality among spermatozoa besides damaging their cell structures (cell membrane, midpiece and flagellum) and thus disable them for fertilization (Fig. 2E, 2H).

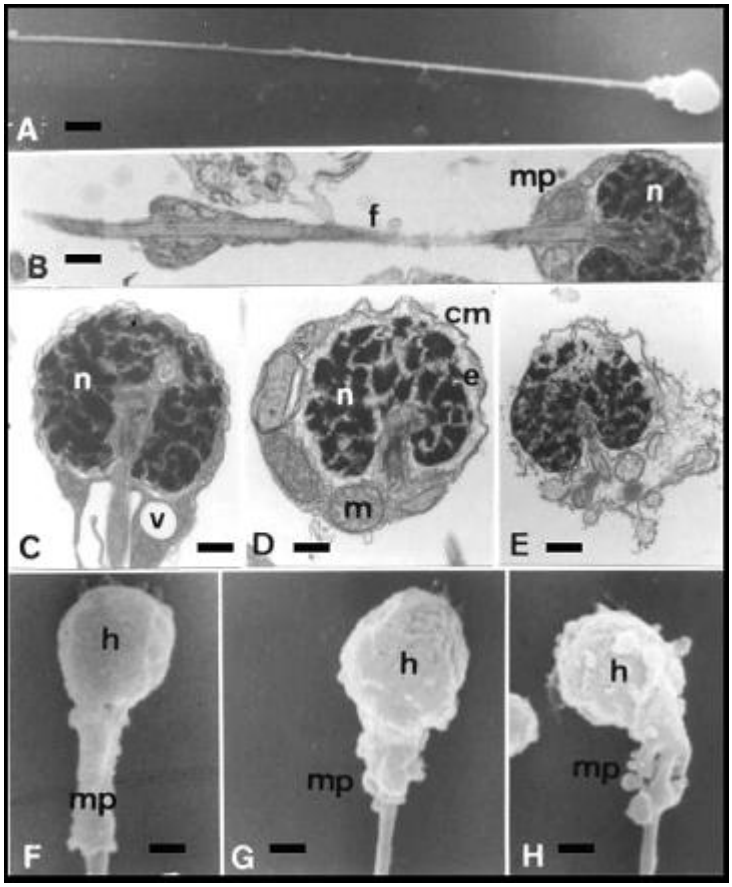

Figure 2 - Ultrastructural aspects of fresh (C and F) and thawed (A,B,D,E,G,H) *B. cephalus* spermatozoa (TEM and SEM): Head (h), midpiece (mp), flagellum (f), nucleus (n), mitochondria (m), vesicle (v), swollen cytoplasm membrane (cm), gap formed by freezing/thawing.
Bars: A - 1.41µ; B - 0.27µ; C - 0.72µ; D - 0.33µ; E - 0.44µ; F - 0.32µ; G - 0.46µ; H - 0.46µ.

By analyzing the data shown in Figure 3, it might be inferred that the preservation of sperm characteristics was effective during semen freezing/thawing. In addition, the spermatozoa/egg ratio proved to be adequate for this species as relatively high hatching rates were observed.

Straw sizes, freezing, thawing and diluents

Richardson et al. (1999), studying semen of *Pleuronectes ferrugineus* frozen in 0.25mL and 1.7mL straws, found that fertility rate in 1.7mL straws was significantly lower. They considered that this fact was caused by a long freezing point plateau (the point of residual heat release at which ice crystals was formed by a rise in the temperature inside the straw) or a slower freezing rate caused by the straw of larger volume. In present experiments with *B. cephalus*, no significant difference ($\alpha = 0.05$) in hatching rate was observed between the straws size used. This could be attributed to the low freezing temperature used (-185ºC) and the fact that thawing occurred at 36°C, thus providing homogeneous freezing and heating rates, exceeding or reducing the point of residual heat release, preventing the formation of ice crystals.

Different extenders can be used in the process of semen cryopreservation, ranging from those of a simpler composition to those compounded by several substances (Stein and Bayrle, 1978; Anderson and Mcneil, 1984; Lahnsteiner et al., 1996; Coser et al., 1987). Carosfeld et al. (2003) and Ribeiro and Godinho (2003) frozen the semen of characiforms species using extenders with simple composition, similar to the type B solution used in the present experiment, and they reached good fertilization results with the thawing semen.

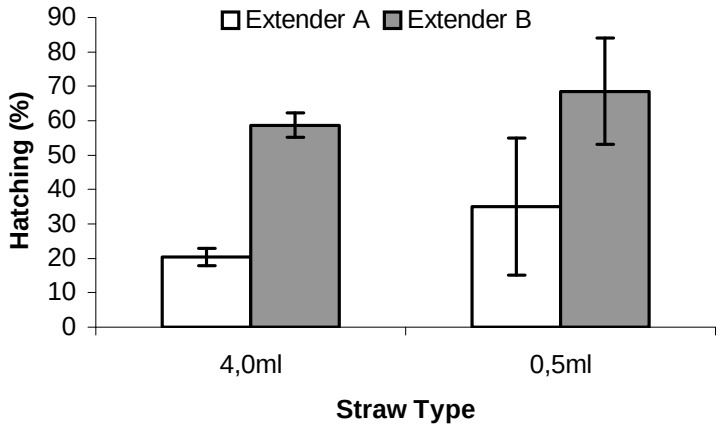

Figure 3 - Mean hatching rate, related to control test, obtained in all treatments used in the fertilization assay of frozen *B. cephalus* semen (Extenders: A and B; Straws: 0.5mL and 4.0mL) (A0.5 = 35.06±2.48 %, A4.0 = 20.42±3.61%, B0.5 = 68.56±19.99 %, B4.0 = 58.73±15.39 %)

The diluents tested here, though simply compounded, showed a good protective action on the spermatozoa of this species. However, type B diluent had a more effective interaction in the maintenance of *B. cephalus* seminal characteristics with both straw sizes used (0.5mL and 4mL). These data agreed with previous observations with the semen of the rainbow trout, which suggested that a straw/extender/semen interaction occurred during freezing/thawing and that this interaction varied accordingly to the type of diluent (extender) and straw size used.

Ultra-structural analysis of thawed spermatozoa

The comparison between the ultrastructural data and the hatching rate obtained suggested that the structural alterations resultant from the freezing/thawing process, such as swelling of the membrane and retraction of the midpiece could not affect spermatozoa viability as high hatching rates were obtained and/or because the large quantity of spermatozoa/egg used allowed the minimization of the effects caused by defective spermatozoa on fertilization.

Cell activation

In preliminary tests at 1% $NaHCO_3$ solution, Fogli da Silveira et al. (1990) successfully activated thawed semen of P. mesopotamicus. However, when this solution was used in this study during the fertilization process, the eggs clustered at the bottom of the container and hatching rate was zero. Apparently, the eggs were not hydrated. Thus, pond water was used as sperm activator as it provided a satisfactory motility and good fertilization results. The data here in presented demonstrated that the methods used for the cryopreservation of matrinxã semen were adequate for the production procedures as they were simple fast and provided good final results (production of larvae).

Table 1 - Optimal spermatozoa/eggs ratio in some teleosts updated (**) from Rurangwa (1998)

Fish species	Sperm / egg	References
Asiatic Catfish (*Clarias macrocephalu*)	4 -8,000	Tambassem-Cheong et al., 1995
European Catfish (*Silurus glanis L.)*	40,000	Redondo et al., 1989
Atlantic Croaker (*Micropogonias undulatus)*	1,000	Gwo et al., 1991
Carp (*Cyprinus carpio*)	13,000	Marcel, 1981
Pike (*Esox lucius*)	26,000	Erdhal and Graham, 1987
Brown Trout (*Salmo trutta*)	43,000	Erdhal and Graham,1987
Rainbow Trout (*Oncorhynchus mykiss*)	20,000	Billard and Carpentier, 1973
Rainbow Trout (*Oncorhynchus mykiss*)	300,000	Billard, 1975
Rainbow Trout (*Oncorhynchus mykiss*)**	200,000	Fogli da Silveira et al., 1988
Turbot (*Scophtalmus maximus)*	6,000	Suquet et al. ,1995
African Catfish (*Clarias gariepinus*)	15,000	Rurangwa et al., 1998

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RESUMO

Visando o desenvolvimento das técnicas de reprodução em peixes, foi realizada a caracterização e a criopreservação do sêmen de *Brycon cephalus.* As características seminais observadas foram: sêmen de coloração leitosa quase transparente, com um volume médio de 4mL e concentração espermática de 9.617±1.630 x 10^6 espermatozóides/mm^3. Os espermatozóides (comprimento = 31,288±4,47 μm) são do tipo "aquasperm", apresentando uma pequena cabeça arredondada (comprimento= 1,752±0,18 μm; largura = 1,752±0,17 μm) sem vesícula acrosomal, com um núcleo com cromatina altamente compactada, formando grumos grosseiros e um complexo centriolar localizado na fossa nuclear; uma peça intermediária (comprimento = 2,561±0,44 μm) que se estreita no sentido antero-posterior**,** um canal citoplasmático e um flagelo (comprimento = 29,521±4,37 μm). Os testes de fertilização com sêmen descongelado demonstraram um efeito significante ($\alpha = 0,05$) no aumento da fertilidade quando utilizado o diluente tipo B, para ambos os tipos de palhetas, 0,5mL e 4,0mL. Não foi constatado efeito significativo ($\alpha = 0,05$) entre a utilização de palhetas de 0,5mL ou de 4,0mL em relação à porcentagem de eclosão.

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