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Seminal characteristics and sensitivity of *Astyanax lacustris* (Characiformes: Characidae) sperm to cryoprotective solutions based on dimethylsufoxide and methylglicol

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This study aimed to determine the semen characteristics of *Astyanax lacustris* after hormonal induction and to evaluate the sensitivity of the species sperm to cryoprotective solutions based on the cryoprotectants dimethyl sulfoxide and methyl glycol. Volume, color, sperm concentration, total motility and aspects of sperm movement were analyzed using "Integrated Semen Analysis System". Three different extenders were tested: A) glucose 5%+egg yolk 10%, B) BTS® 5% and C) glucose 5% and two permeable cryoprotectants: dimethyl sulfoxide (Me_2SO) and methyl glycol (MTG). Fresh *A. lacustris* semen presented total motility of 76.6±11.2%, motility duration of 33.0±2.2s, sperm concentration of 7.22±3.2×10^9sptz/mL and seminal osmolality of 219±0.03mOsm/kg^{-1}. The toxicity test showed the highest total motility values at the MTG15%+A, Me_2SO15%+B and Me_2SO10%+C dilutions, and the Me_2SO10%+C and Me_2SO15%+C dilutions presented the highest values for curvilinear velocity, linear velocity and average velocity. The tested protocol was not effective at maintaining the viability of *A. lacustris* semen after freezing because no motility was observed in any of the dilutions. However, the Comet Assay demonstrated that cryoprotectant solutions were effective in protecting the genetic material of cells, as DNA damage levels were low, with no difference between control and Me_2SO10% + A, dilutions MTG10%+C, Me_2SO10%+B and Me_2SO15%+B.

Keywords: Characiformes, DNA integrity, Freezing, Semen quality, Yellow-tailed Lambari.

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O objetivo deste estudo foi determinar as características do sêmen de *Astyanax lacustris* após indução hormonal e avaliar a sensibilidade dos espermatozoides da espécie a soluções crioprotetoras baseadas nos crioprotetores dimetilsulfóxido e metilglicol. Volume, cor, concentração espermática, motilidade total e aspectos do movimento espermático foram analisados usando o "Sistema Integrado de Análise de Sêmen (ISAS®CASA)". Três extensores diferentes foram testados: A) glicose 5%+gema de ovo 10%, B) BTS® 5% e C) glicose 5% e dois crioprotetores permeáveis: dimetilsulfóxido (Me_2SO) e metilglicol (MTG). O sêmen fresco de *A. lacustris* apresentou motilidade total 76,6±11,2%, duração da motilidade 33,0±2,2s, concentração de espermatozoides 7,22±3,2×10^9sptz/mL e osmolalidade seminal 219±0,03mOsm/kg^{-1}. O teste de toxicidade apresentou maiores valores de motilidade total nas diluições MTG15%+A, Me_2SO15%+B e Me_2SO10%+C, e as diluições Me_2SO10%+C e Me_2SO15%+C apresentaram maiores valores de velocidade curvilínea, velocidade linear e velocidade média. O protocolo testado não foi eficaz em manter a viabilidade do sêmen de *A. lacustris* pós-congelamento, pois não foi observada motilidade em nenhuma das diluições. No entanto, o Ensaio Cometa demonstrou que as soluções crioprotetoras eram eficazes na proteção do material genético das células, pois os níveis de dano ao DNA eram baixos, sem diferença entre controle e Me_2SO10%+A, MTG10%+C, Me_2SO10%+B e Me_2SO15%+B.

Palavras-chave: Characiformes, Congelamento, Integridade do DNA, Lambari-do-rabo-amarelo, Qualidade do sêmen.

INTRODUCTION

The yellow-tailed lambari *Astyanax lacustris* (Lütken, 1875) (= *Astyanax altiparanae*), belonging to the Characidae family, is a small species native to the upper Paraná basin, with omnivorous eating habit, easy environmental adaptation, rapid growth and spawn more than once a year, so it is used as a biological model (Sabbag *et al*., 2011; Gonçalves *et al*., 2012a,b; Lucena, Soares, 2016). With great economic potential, this species can be used as a sport bait, as well as being well accepted as a snack, which is why production is growing in aquaculture (Gonçalves *et al*., 2012a).

Considering their biological and economic importance, the development of technologies that catalyze the reproductive process of captive species is necessary, in order to support the projects of environmental preservation and commercial production. Technologies such as methods for the cryopreservation of fish sperm have the objective of facilitating and maximizing the reproduction of species of commercial interest, either threatened or endangered, and favoring the processes of genetic improvement in these situations (Cárton-Garcia *et al*., 2013). However, the freezing and thawing processes can cause decreased sperm motility, loss of functional and structural integrity of the plasma membrane, morphological changes in gametes and changes in sperm fertilization capacity (Streit Jr. *et al*., 2006; Viveiros *et al*., 2009; Ciereszko *et al*., 2014; Viveiros *et al*., 2015; Yang *et al*., 2016; Galo *et al*., 2019).

Due to the problems mentioned above, cryoprotective substances are added to the semen, whose main functions are to decrease the freezing point of the cell and protect the cell membrane during the cryopreservation process (Maria *et al*., 2006).

The cryoprotectants used can be divided into two groups: impermeable (glucose, sucrose, etc), composed of large molecules and permeable ones, formed by chemical substances composed of small molecules, highlighting some types already used in semen conservation protocols of freshwater fish, such as dimethyl sulfoxide (Me_2SO) (*Brycon amazonicus* (Agassiz, 1829) (Ninhaus-Silveira *et al*., 2006a); *Colossoma macropomum* (Cuvier, 1816) (Carneiro *et al*., 2012); *Brycon orbignyanus* (Valenciennes, 1850) (Chiacchio *et al*., 2017)); and methyl glycol (*Brycon opalinus* (Cuvier, 1819) (Viveiros *et al*., 2012); *Prochilodus lineatus* (Valenciennes, 1837) (Viveiros *et al*., 2010)), this cryoprotectant being considered as the most effective in the cryopreservation of fish semen (Elliott *et al*., 2017). Viveiros *et al*. (2012) also highlighted that the use of methyl glycol as a cryoprotectant for neotropical fish semen is growing in Brazil.

To develop a cryoprotectant solution that maintains the seminal characteristics and fertilization capacity of the sperm species, it is necessary to evaluate the tolerance of sperm cells to exposure to different cryoprotectant substances, so that there is effective sperm protection during the cryogenic process, since this tolerance varies according to the species of fish used (Judycka *et al*., 2018; Pereira *et al*., 2019).

Based on the assumptions that there is no concrete scientific information about seminal characteristics of *Astyanax lacustris* and no effective cryopreservation protocol has been established for semen of the species, this work aimed to determine the characteristics of *A. lacustris* semen after hormonal induction, and to evaluate the sensitivity of the species' sperm to cryoprotectant solutions based on dimethyl sulfoxide and methyl glycol cryoprotectants, in order to contribute to the development of a cryopreservation protocol for semen of this species.

MATERIAL AND METHODS

Animals and obtaining gametes. Thirty-eight sexually mature specimens were used of *Astyanax lacustris*, with a total mean length of 10.4 ± 0.5 cm, a standard length of 8.6 ± 0.5 cm and an average mass of 16 ± 3.1 grams, belonging to the Laboratório de Ictiologia Neotropical (L.I.NEO), Universidade Estadual Paulista "Júlio de Mesquita Filho", Ilha Solteira Campus. The fish were kept in 4,500L circular PVC tanks, at a ratio of 2:1 (male/female), with an average temperature of 27.7°C, pH 7.6 and ammonia of 0.00 ppm.

Astyanax lacustris males were hormonally induced using single-dose Ovopel® (GnRh - analogue + Dopamine) (3 mg/kg live fish) (Yasui *et al*., 2015). After 226 hours/degrees, the fish were anesthetized with benzocaine solution (Sigma-Aldrich E1501) (100mg/L), and semen was collected by craniocaudal abdominal massage of the body, with the aid of 10–100 µl micropipettes (Kasvi-K1-100B). Contamination with blood or feces was carefully avoided.

Determination of fresh sperm characteristics. Thirty-eight males were used to determine sperm characteristics. Staining was visually estimated considering the translucent and white patterns and the ejaculated volume measured by micropipettes.

To determine seminal plasma osmolarity, semen was centrifuged at 3,000 rpm for 15 minutes, and the supernatant was collected and analyzed using an osmometer (OSMOMAT® model 030, Berlin, Germany). Sperm concentration (sperm/mL) was measured using the Neubauer hematimetric chamber. For this, semen was diluted in formalin-saline solution at a ratio of 1:1000 (Bashiyo-Silva, 2014). Sperm motility characteristics were evaluated using the CASA (Computer Assisted Sperm Analysis) system (ISAS® Integrated Semen Analysis System, Proiser, Valencia, Spain) coupled to a phase contrast microscope UB200i (UOP/Proiser) with a 10x negative phase contrast objective. The images were captured with ISAS 782C camera (Proiser, Spain) and processed with CASA software, recorded at 25 image per seconds (fps). Sperm activation was performed by adding 30 µl of distilled water to 0.5 µl of semen using a Makler ™ camera (Sefi Medical Instruments Ltd, Israel), and after the analyzes were performed every 10 seconds. Total motility (MOT, %), progressive motility (PRG, %), porcentagem fast sperm (VAP=50–100 µm/s), medium (VAP=25–50 µm/s) and slow (VAP=10–25 µm/s), curvilinear velocity (VCL, µm/s), linear velocity (VSL, µm/s), and mean velocity (VAP, µm), the linearity coefficient (LIN, %), the rectilinearity coefficient (STR, %), the average oscillation of the sperm spatial trajectory (WOB, %), the lateral displacement amplitude of the head (ALH, µm) and the beat cross frequency (BCF, Hz) were also evaluated. Straightness percentage (STR) >80% was used for sperm to be considered progressive. Sperm with VCL <10 µm/s were considered immovable.

Cryoprotectant toxicity test. Two internal cryoprotectants were tested, dimethyl sulfoxide (Me_2SO) and methyl glycol (MTG) (Sigma-Aldrich), at concentrations of 10 and 15%, in combination with three diluent solutions: A) composed of 5% glucose + 10% egg yolk; B) consisting of 5% Bestlvile Thawing Solution - BTS® (composition: 79.9% glucose, 12.71% sodium citrate, 2.65% EDTA, 2.65% sodium bicarbonate and potassium chloride 1.59%, gentamicin sulfate 0.5%) (Minitub, Tiefenbach, Germany), and C) composed of only 5% glucose, thus resulting in twelve treatments, in addition to the control group (fresh semen). Before the toxicity test, the osmolality of solutions A, B and C and the twelve treatments were measured (Tab. 1).

TABLE 1 | Composition of the treatments used for the toxicity test.

Treatments	Composition
T1	Egg yolk 10% + Glucose 5% + Me_2SO 10%
T2	Egg yolk 10% + Glucose 5% + Me_2SO 15%
T3	Egg yolk 10% + Glucose 5% + MTG 10%
T4	Egg yolk 10% + Glucose 5% + MTG 15%
T5	BTS® 5% + Me_2SO 10%
T6	BTS® 5% + Me_2SO 15%
T7	Glucose 5% + Me_2SO 10%
T8	Glucose 5% + Me_2SO 15%
T9	BTS® 5% + MTG 10%
T10	BTS® 5% + MTG 15%
T11	Glucose 5% + MTG 10%
T12	Glucose 5% + MTG 15%

Semen was diluted 1:25 (semen/cryoprotectant solution) in all treatments and, after 15 minutes (equilibration time), 1µl of diluted semen was placed in a Makler chamber, activated with distilled water and analyzed using the sperm analysis program (ISAS®, CASA). All motility parameters mentioned in item 2.4 were measured using four repetitions (fish) for each treatment.

Freezing. For freezing, semen was diluted 1:25 (semen: solution) in eight cryoprotectant solutions selected in the toxicity test (Zanandrea *et al*., 2016). This dilution was used due to the low amount of semen of the species, approximately 30 µl. It was then placed into 0.25mL plastic straws (Minitub) at room temperature (28°C); after a 15 minute equilibration period, the straws (8) were placed on a steel mesh tray about 1 cm away from the surface of liquid nitrogen (Ninhaus-Silveira *et al*., 2006a) contained in a 33 liter Styrofoam insulating box for seven minutes until reaching a temperature of -70°C (Thermocouple - OMEGA® RDXL4SD) (Viveiros *et al*., 2000). Subsequently, the samples were immersed in liquid nitrogen and transferred to a cryogenic liquid container (ABS - Pecplan, model ABS20 - MVE). After one month, the samples were thawed in a water bath at 36°C for seven seconds (Ninhaus-Silveira *et al*., 2006b), and motility was checked using the integrated system (ISAS® CASA). Four fish (repetitions) were also used per treatment.

DNA damage assessment. The Comet Assay methodology for determining the DNA integrity of cryopreserved sperm was based on the protocol proposed by Singh *et al*. (1988) and Klaude *et al*. (1996), and analyzed three repetitions (fish) by treatments. To perform the analyses, histological glass slides were prepared with a thin layer of normal melting agarose (NMP). Next, 10µl of diluted semen and 120µl of low melting temperature agarose (LMP) were added to a microtube. These samples were placed on slides previously prepared with normal agarose, covered with a coverslip and refrigerated for about 30 minutes to solidify. After this time, the slides were immersed in pH 10 lysis solution (2.5M NaCl, 100mM EDTA, 10mM Tris, 35mM Lauril) for one hour at 4°C.

Subsequently, the slides were placed in an electrophoresis vat that was filled with pH 13 solution (200mM EDTA, 10N NaOH), and kept for 30 minutes at 4°C. Then, the electrophoresis process was performed for a period of 20 minutes at 25V/300mA. After this process, the slides were covered with pH 7.5 neutralization solution (0.4M Tris) for five minutes (3x), fixed by immersion in ice-cold absolute ethanol for five minutes, and dried at room temperature. To visualize the comet, the slides were stained by adding 100µl of ethidium bromide solution onto each slide, covered with coverslips and analyzed in a 40x objective with the aid of a fluorescence microscope (Olympus, BX-FLA, Japan). In total, 100 cells per sample were analyzed and grouped into damage class according to tail size and intensity as follows: class 0 - no apparent damage, and class 1 to 3 - increasing DNA damage, related to tail length and intensity (Kobayashi *et al*., 1995) (Fig. 1).

The damage index (total DI) was obtained by multiplying the number of comets in each class by the denominator digit of each class, according to the formula below, where "n" represents the number of cells in each damage class:

DI total = 0 x (n Class 0) + 1 x (n Class 1) + 2 x (n Class 2) + 3 x (n Class 3)

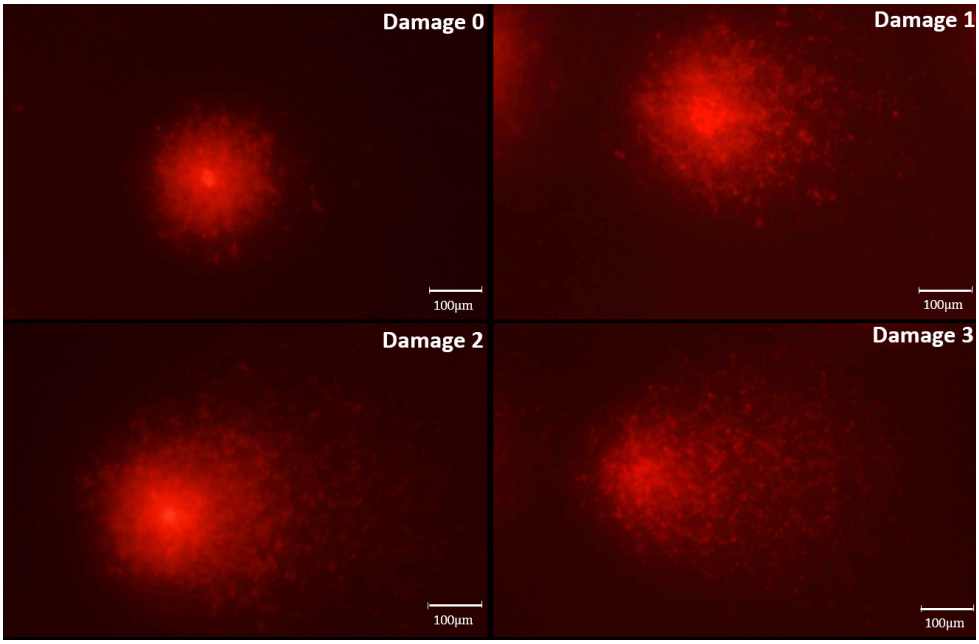

FIGURE 1 | Fluorescence photomicrograph of *Astyanax lacustris* (100X magnification) of nuclei with different types of damage assessed by the Comet Assay: damage 0 - no apparent damage; damage 1 - little damage; damage 2 - average damage; damage 3 - major damage.

Statistics. Statistical analysis was performed following the subdivided parcel model by analysis of variance (ANOVA). For results showing a significant difference ($p<0.05$), the Scott-Knott test was applied to compare control and treatments, and verify the difference between treatments. For nonparametric data, the Kruskal-Wallis test was applied ($p<0.05$). Data were analyzed using the R Studio statistical program (RStudio Team ,2015).. The fish was considered as the repetition with five fish per treatment.

RESULTS

Features of Fresh Semen. Fresh semen was translucent, with little viscous consistency and a seminal volume of about 30 µL. The parameters for osmolality, sperm motility and concentration for *Astyanax lacustris* semen are presented in Tab. 2.

Cryoprotectant toxicity test. All solutions used in the toxicity test were hyperosmotic to the seminal plasma of the species (Tab. 3). Despite this, diluent solutions A, B and C without the addition of cryoprotectants activated sperm. And treatments composed of 5% BTS + 10% methyl glycol (T9), 5% BTS + 15% methyl glycol (T10), 5% glucose + 10% methyl glycol (T11) and 5% glucose + 15 % methyl glycol were toxic for *A. lacustris* semen because, after dilution, no sperm motility was observed.

The total motility (MOT) differed statistically between the control and treatment groups, but no statistically significant differences were observed in the comparison between treatments. Treatments T4, T6 and T7 were the ones that provided higher

TABLE 2 | Seminal characteristics of fresh *Astyanax lacustris* semen after hormonal induction.

Parameters	Fresh semen
Sperm concentration (× 10^9 spermatozoa ml^{-1})	7.22±3.2
Seminal plasma osmolality (mOsm kg^{-1})	219±0.03
Total motility (%)	76.6±11.2
Progressive motility (%)	55.3±10.8
Fast spermatozoa (%)	46.0±22.7
Medium spermatozoa (%)	24.7±15.3
Slow spermatozoa (%)	5.8±4.1
Duration of motility (sec.)	33.0±2.2
Curvilinear velocity (µm s^{-1})	51.1±13.6
Straight line velocity (µm s^{-1})	41.4±11.9
Average path velocity (µm s^{-1})	48.2±13.5
Linearity (%)	80.7±6.7
Straightness (%)	85.9±4.5
Wobble (%)	93.8±3.5
Amplitude of lateral head displacement (µm)	1.1±0.1
Beat cross frequency (Hz)	7.0±0.3

TABLE 3 | Osmolality of the solutions used in the toxicity test.

Solution	Composition	Osmolality (mOsm/kg)
A	Egg yolk 10% + Glucose 5%	339
B	BTS® 5%	347
C	Glucose 5%	318
T1	Egg yolk + Glucose + Me_2SO 10%	2.766
T2	Egg yolk + Glucose + Me_2SO 15%	3.125
T3	Egg yolk + Glucose + MTG 10%	2.531
T4	Egg yolk + Glucose + MTG 15%	2.920
T5	BTS® 5% + Me_2SO 10%	2.529
T6	BTS® 5% + Me_2SO 15%	3.056
T7	Glucose 5% + Me_2SO 10%	2.226
T8	Glucose 5% + Me_2SO 15%	3.028
T9	BTS® 5% + MTG 10%	2.343
T10	BTS® 5% + MTG 15%	2.843
T11	Glucose 5% + MTG 10%	2.164
T12	Glucose 5% + MTG 15%	2.754

values of total motility (Fig. 2A). Regarding progressive motility (PRG), the highest values were obtained in treatments T7, T8 and T6, respectively; however, there was no statistical difference between them (Fig. 2A).

Regarding the percentage of fast, medium and slow sperm, there was a marked decrease in fast sperm and an increase in medium and slow sperm after dilution in cryoprotective solutions (Tab. 4). However, no statistical difference was observed

between the control and treatments T7, T8, T1, T2 and T5 for fast sperm, but treatment T7 had the highest number of fast spermatozoids, highlighting that treatments T3, T4 and T6 had less than 10% of fast spermatozoa differing statistically from the control. In the average sperm there was no difference between any of the treatments, but there was an increase in the percentage of average sperm compared to the control in all treatments with the exception of T7 and T8. The same pattern was observed for slow sperm that significantly increased in relation to the control, with the highest percentage found in the T3 treatment that differed statistically from the control (Tab. 4).

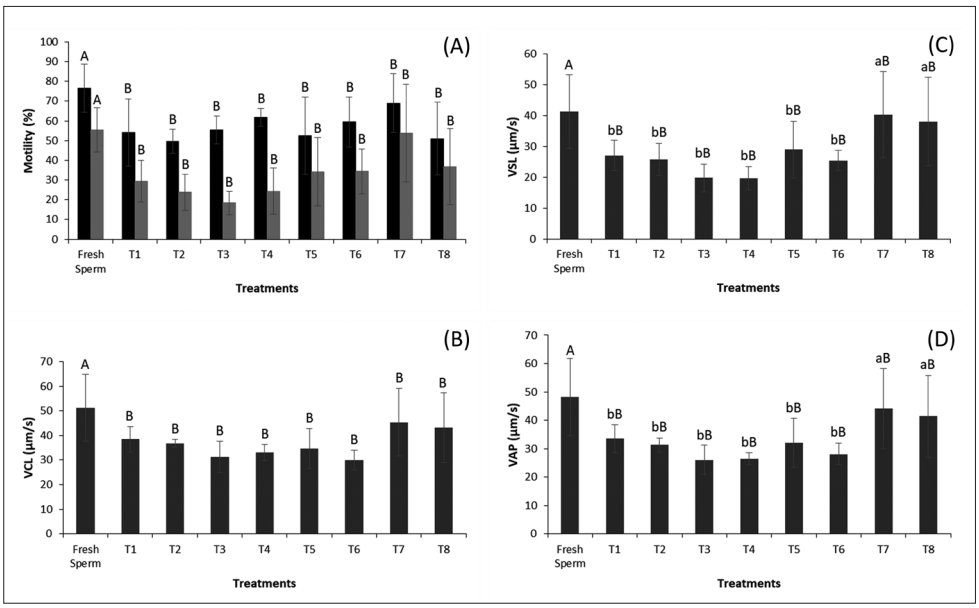

FIGURE 2 | Comparative analysis of sperm motility of *Astyanax lacustris*. Total and progressive motility (A), curvilinear velocity (B), straight line velocity (C) and average path velocity (D). Different letters indicate significant differences ($p < 0.05$) by the Scott-Knott test: Upper case relationship between control and treatments; tiny relationship between treatments.

TABLE 4 | Percentage of fast, medium and slow sperm of *Astyanax lacustris* in relation to total motility.

Treatments	Motility (%)	Fast (%)	Medium (%)	Slow (%)
Fresh sperm	76.6±11.2	46.0±22.7^{a}	24.7±14,7	5.8±4.1^{b}
T1	54.2±16.9	16.1±7.7^{ab}	27.0±8.2	10.9±3.5^{ab}
T2	49.6±6.0	10.2±1.6^{abc}	25.3±3.2	14.1±2.5^{ab}
T3	55.4±7.1	5.0±4.5^{bc}	28.0±7.4	22.3±11.3^{a}
T4	61.7±4.6	5.8±4.1^{bc}	38.15±6.8	17.8±2.2^{ab}
T5	52.3±19.5	10.0±9.1^{abc}	26.9±13.8	15.2±7.3^{ab}
T6	59.4±12.7	3.6±3.6^{c}	33.6±10.1	22.0±3.0^{ab}
T7	68.9±14.8	34.0±27.5^{a}	23.1±5.4	11.8±9.9^{ab}
T8	51.0±18.3	21.8±16.4^{ab}	17.4±6.9	11.7±6.2^{ab}

For the VCL, VSL and VAP parameters, a significant difference was found between the control and treatment groups (Figs. 2B–D). Regarding VCL, treatments T7, T8 and T1 presented the highest values, but did not differ statistically from the other treatments (Fig. 2B). For VSL and VAP, T7 and T8 represented the treatments with higher velocities, being statistically different from the others. The lowest velocities for both VSL and VAP were obtained at T3 and T4 (Figs. 2C–D).

For LIN and STR, there was only a statistical difference between treatments, with no difference between control and treatment groups, while for WOB there was a statistical difference in the control-treatment comparison and between treatments. For these three parameters, T5, T6, T7 and T8 presented the highest values, differing significantly ($p<0.05$) from the other treatments; it can also be shown that T4 was the treatment that provided the lowest values (Figs. 3A–C). An increase of LIN values was also observed for treatments T6, T7 and T8 in relation to the control. This increase was also found for STR in treatments T5, T6, T7 and T8, but only in treatment T7 for WOB.

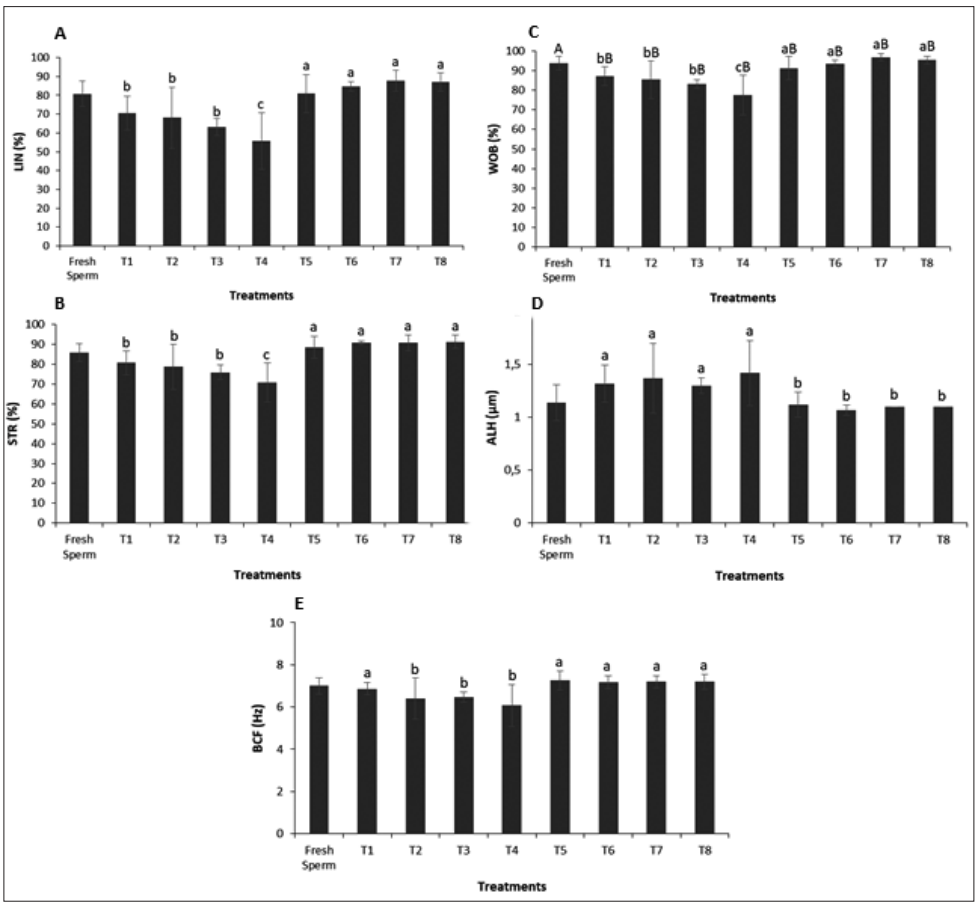

FIGURE 3 | Linearity (A), Rectilinearity (B), Oscillation (C), Head lateral displacement amplitude (D) and beat cross frequency (E). Different letters indicate significant differences ($p<0.05$) by the Scott-Knott test: Upper case relationship between control and treatments; tiny relationship between treatments.

Regarding the amplitude of lateral displacement of the head (ALH), treatments T1, T2, T3 and T4 presented the highest values, not differing significantly from each other ($p<0.05$); in the other treatments (Fig. 3D), these values were also larger than those obtained in the control (1.14 μm). For the beat cross frequency (BCF), T1, T5, T6, T7 and T8 presented the highest values, without differing significantly from each other ($p<0.05$). It is important to highlight that these values were also higher than the value found in the control (7.0 Hz), where T4 was the group that provided the lowest value for BCF (Fig. 3E).

Semen post-thawing motility. After the thawing of *A. lacustris* semen, no mobile sperm were observed in any of the treatments tested.

DNA integrity. DNA integrity analysis showed that, in terms of possible DNA degradation, there was no difference between the control and T1, T3, T5 and T6 treatments, but the T5 treatment was responsible for presenting a lower DNA damage index (ID) (51 ± 34.5) (Fig. 4). The highest IDs were found in treatments T7 and T8, showing lower DNA protection compared to other treatments.

For the T4 treatment, compaction of the sperm nucleus was observed (Fig. 5). This compaction is possibly due to the chemical components of this treatment, which did not allow the exposure of nuclear DNA and, consequently, an evaluation of its damage.

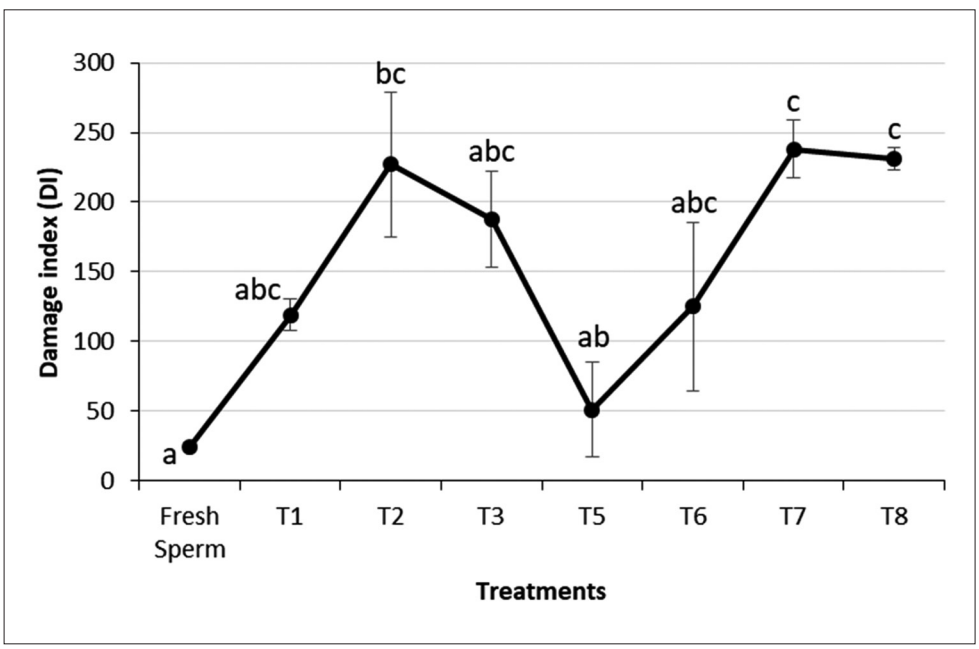

FIGURE 4 | Index of DNA damage generated by the cryopreservation process of *Astyanax lacustris* semen. Control - fresh semen, T1 - 10% egg yolk + 5% glucose + Me_2SO10%, T2 - 10% egg yolk + 5% glucose + Me_2SO15%, T3 - 10% egg yolk + 5% glucose + MTG010%, T5 - BTS5% + Me_2SO10%, T6 - BTS5% + Me_2SO15%, T7 - 5% Glucose + Me_2SO10% and T8 - 5% Glucose + Me_2SO15%. Different letters indicate significant differences ($p<0.05$) by the Kruskal-Wallis test.

FIGURE 5 | Compressed cell nucleus found in T4 of cryopreservation process of *Astyanax lacustris* semen.

DISCUSSION

The seminal characteristics of the species are of great importance for assessing the quality of the breeders. In this study, the total motility of the fresh semen was low when compared to other species of Characiformes (Chiacchio *et al*., 2017; Paula *et al*., 2019) and the spermatozoa proved to be slower since the *A. lacustris* spermatozoa showed VCL values (51.1 µm/s) VSL (41.4 µm/s) and VAP (48.2 µm/s) lower than those of other Characiforms, such as *Colossoma macropomum* (VCL - 111.5 µm/s; VSL - 77.2 µm/s; VAP - 100, 6 µm/s), *Prochilodus brevis* Steindachner, 1875 (VCL - 108.2 µm s; VSL - 45.8 µm/s; VAP - 84.4 µm/s), *Brycon henni* Eigenmann, 1913 (VCL - 115.4 µm/s; VSL - 64.8 µm/s; VAP - 84.0 µm/s) and *Prochilodus lineatus* (VCL - 245 µm/s; VSL - 127 µm/s; VAP - 204 µm/s), which demonstrates the specificity of each species (Lopes *et al*., 2014; Pineda-Santis *et al*., 2015; Viveiros *et al*., 2017; Lopes *et al*., 2018).

The effectiveness of the sperm cryopreservation process is dependent on a number of factors such as sperm quality, diluents, cryoprotectants, dilution, and freezing and thawing velocity (Butts *et al*., 2010), all of which influence sperm motility.

Permeable cryoprotectants used in sperm freezing require low toxicity, small molecules, low molecular weight and high solubility (Chao, Liao, 2001) to reduce freezing points and cause cell dehydration, thus minimizing the cell formation of ice crystals. These substances are combined with non-permeable cryoprotectants which are large molecules that aid in cell dehydration, protect the plasma membrane and assist in osmotic homeostasis (Watson, 1995).

In the present study, the cryoprotectant toxicity test showed that the cryoprotectant solution composed of Me_2SO10% + glucose 5% best met these characteristics, presenting

the highest values for total (MOT) and progressive (PRG) motility of spermatozoa of *A. lacustris*, as well as a greater amount of fast sperm. The higher PRG obtained with this extender is an important factor, since this parameter has a high correlation with the fertilization rate (Gallego, Asturiano, 2017).

The major protection that uses this combination of cryoprotectants has been related to the species *Salminus brasiliensis* (Cuvier, 1816) (Viveiros *et al*., 2009) and *Epinephelus akaara* (Temminck, Schlegel, 1843) (Ahn *et al*., 2018). In addition, the use of Me_2SO with other diluents (Carolsfeld *et al*., 2003; Ninhaus-Silveira *et al*., 2006b; Tiba *et al*., 2009; Andrade *et al*., 2014; Fabbrocini *et al*., 2015; Shaliutina-Kolešová *et al*., 2019) is considered the most used cryoprotectant for freezing fish (Elliott *et al*., 2017).

The effectiveness of Me_2SO is attributed to its low molecular weight and ability to easily bind to sperm membrane components, such as lipids and proteins, allowing molecular membrane reorganization after thawing (Pereira *et al*., 2019). Also, regarding total and progressive motility, the use of 5% BTS with methyl glycol, regardless of concentration, was toxic to *A. lacustris* sperm, causing mortality after dilution. Viveiros *et al*. (2009) found that the use of this cryoprotective solution caused a reduction in sperm motility in *Salminus brasiliensis* from 85% to 60% after dilution, and that a total loss of sperm motility was found after thawing.

However, despite this result, other studies with Characiformes such as *Brycon nattereri* Günther, 1864 (Oliveira *et al*., 2007), *Brycon insignis* Steindachner, 1877 (Viveiros *et al*., 2011), and *Brycon orbignyanus* (Viveiros *et al*., 2015) have shown that the use of BTS® combined with methyl glycol was beneficial, with results superior or equal to the other solutions. In addition to these species, this freezing medium has also been tested for *Steindachneridion scriptum* (Miranda Ribeiro, 1918) siluriform (Pereira *et al*., 2019) and has also been positive, which further confirms the idea that cryoprotective solutions tend to be species-specific.

The toxicity caused by the cryoprotectant solution BTS® + methyl glycol also shows that there is an interaction between permeable and impermeable cryoprotectants, since the use of BTS® + Me_2SO (T5 and T6) did not cause total loss of motility, and the use of methyl glycol + yolk + glucose (T4) resulted in the second highest total motility (61%) obtained in this study. For *Brycon nattereri* and *Piaractus brachypomus* (Cuvier, 1818), semen, an interaction between Me_2SO and methyl glycol cryoprotectants with BTS® and Saad, 154mM NaCl, 200mM NaCl and 277mM glucose dilutors was also observed (Oliveira *et al*., 2007; Nascimento *et al*., 2010).

The analysis of fast, medium and slow sperm revealed that there was a negative effect of cryoprotective solutions on these aspects, characterized by an increase in medium and slow sperm, possibly this increase occurred due to the sensitivity of sperm to cryoprotectants (Chao and Liao, 2001) or as an effect of energy loss by the cell, which has a significant effect on semen quality and motility after thawing (Bondarenko *et al*., 2013; Figueroa *et al*., 2013).

The velocity values (VCL, VSL and VAP) were higher for T7 (Me_2SO10% + 5% glucose) and T8 (Me_2SO15% + 5% glucose) sperm, which clearly indicates that these extenders provide higher sperm quality when compared with the other extenders used in this work. Sperm velocity is also an indicator of semen quality (Król *et al*., 2018), and these velocities are correlated with fertilization (Gallego *et al*., 2013). VCL is one of the parameters that is most closely related to fertilization capacity, as sperm with more

circular motions are theoretically easier to find the micropile (Leite *et al*., 2018).

In addition, a higher curvilinear velocity is also considered to be characteristic of spermatozoa with faster trajectories, while higher VSL means that the sperm trajectory is more linear and regular, with less lateral movements, which would indicate the sperm's ability to move in a straight line (Chiacchio *et al*., 2017). For some treatments, LIN, STR and WOB values were not affected by extender dilution, and an increase in these parameters after dilution has already been reported for other species after freezing, such as *Acipenser baerii* Brandt, 1869 and *Acipenser ruthenus* Linnaeus, 1758 (Sieczyński *et al*., 2015).

Unlike studies already performed with other fish species (Cejko *et al*., 2012, 2013) that associate higher ALH values with higher VCL values, our study showed an opposite behavior, in which higher ALH values were found in treatments with lower VCL. According to Cejko *et al*. (2012), high ALH and BCF values would indicate better sperm quality, since high ALH values indicate sperm maturity (Cejko *et al*., 2018; Król *et al*., 2018).

After semen thawing, no sperm motility was observed for any of the treatments. Among the factors considered for this result, we can point out the low percentage of average total sperm motility (76.6%) and the low amount of fast sperm (46.0%); the published studies for species of the same order have a 90–100% total motility for fresh semen (Lopes *et al*., 2014, 2018; Chiacchio *et al*., 2017; Paula *et al*., 2019).

Animals were provided with a balanced feed and disposition in a controlled environment (pH, O_2, T°C, Conductivity, ammonia) during the experiment, without sudden changes that could affect sperm quality. In addition, during collections, all recommended measures to maintain sperm quality were taken, such as prior sedation, avoiding contamination with urine, feces or blood, and avoiding the early activation of sperm. As there are no data in the scientific literature regarding seminal characteristics for the species or for specimens of the genus *Astyanax*, it did not allow a comparison to determine whether these motility characteristics are inherent of this genus or species.

In addition, the amount of fast sperm decreased significantly in the diluted semen, which probably contributed to the loss of motility after freezing and thawing, since this decrease can be attributed to the loss of cell energy due to damage to mitochondria, thus limiting movement sperm. Several studies have shown that sperm viability is directly affected by damage to mitochondria and consequent loss of cellular energy (Liu *et al*., 2007; Bondarenko *et al*., 2013; Figueroa *et al*., 2013; Zilli *et al*., 2014; Yusoff *et al*., 2018).

Other factors are mentioned in the literature as possible causes of loss of motility after freezing, such as the freezing rate used which is conditioned by the volume and height of the nitrogen straws and the amount of straws by freezing because the species-specific freezing rate and affects sperm survival after thawing (Chao, Liao, 2001; Ninhaus-Silveira *et al*., 2006b; Butts *et al*., 2010).

Although the thawed semen does not show motility, the DNA integrity test demonstrated that the use of these cryoprotective solutions were effective in maintaining the DNA integrity of the sperm cell. And the treatment composed of BTS and DMSO (T5) had the lowest ID (Damage Index) among all treatments, a fact also observed for treatments T1, T3 and T6 that also had low ID. For *Steindachneridion scriptum,* the use of this extender also resulted in low DNA fragmentation (0.051%). BTS® + Me_2SO

was also used in *Prochilodus lineatus* freezing but there was DNA fragmentation for all treatments analyzed in the study of Paula *et al*. (2019), unlike in our work, which demonstrates the influence of the species as well as the freezing method used, as the protocol applied in this study was different.

The use of a simple extender composed of glucose and ME_2SO resulted in higher DNA fragmentation (T7 and T8), showing that this extender presented lower DNA protection efficiency. Nevertheless, the use of this extender for *Salmo salar* Linnaeus, 1758 was beneficial, generating low DNA fragmentation of only 4.8 ± 2.58% (Figueroa *et al*., 2016). DNA fragmentation due to the cryopreservation process using the same Me_2SO cryoprotectant has been reported for several species such as *Dicentrarchus labrax* (Linnaeus, 1758) (Zilli *et al*., 2003), rainbow trout (*Oncorhynchus mykiss* (Walbaum, 1792)) (Cabrita *et al*., 2005) and *Prochilodus magdalenae* Steindachner, 1879 (Martínez *et al*., 2012); however, the thinners used for these species differ from those used in our study.

There is some controversy as to the effect of such DNA damage on fertilization success. It is known that the beginning of embryonic development is controlled only by inherited maternal information (Zilli *et al*., 2003). Thus, the effects of DNA fragmentation would not be manifested at this stage. In addition, DNA damage can be repaired by the genetic repair mechanism during early embryogenesis (Cabrita *et al*., 2005), without affecting offspring quality. This was found for rainbow trout where, although the cryopreservation process induces increased sperm DNA fragmentation, such damage did not affect the survival or quality of the offspring (Labbe *et al*., 2001).

However, some studies show that DNA damage affects both fertilization and offspring quality. Pérez-Cerezales *et al*. (2011) found that the use of cryopreserved trout sperm with damaged DNA for fertilization caused an increase in telomere length, as well as causing alterations in gene expression in surviving embryos and larvae, thereby negatively affecting offspring development. For goldfish semen, there was a relationship between fertilization and DNA damage, where treatment with higher DNA fragmentation was responsible for the lowest fertilization rate (Nathanailides *et al*., 2011).

DNA integrity analysis also showed that only the treatment consisting of 5% glucose + 10% egg yolk + 15% methyl glycol (T4) contained highly compacted nuclei, indicating that there was no cell lysis, which consequently prevented the analysis of this treatment for DNA damage caused by the cryopreservation process. This compaction is the result of a chemical reaction caused by the increased concentration of methyl glycol.

In conclusion, in the present study, the toxicity test showed that the use of 5% glucose and 10% Me_2SO resulted in better maintenance of motility, progressivity and sperm speeds in *A. lacustris* semen. It is emphasized that there is a great sensitivity of the spermatozoa to methyl glycol combined with BTS and glucose. In addition, the cryoprotective solutions used did not lead to DNA fragmentation, thus maintaining the genetic integrity of *A. lacustris* sperm. However, *A. lacustris* sperm did not support the freezing protocol tested in this study, losing sperm viability and the ability to fertilize after thawing. Further studies are necessary to determine an effective protocol for cryopreservation the species semen.

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ETHICAL STATEMENT

All procedures were performed and approved by the Animal Use Ethics Committee - CEUA of the Engineering College, UNESP, Ilha Solteira Campus, Process CEUA-FEIS/UNESP 05/2017.

COMPETING INTERESTS

The authors declare no competing interests.

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