

Optimization of Curimba (*Prochilodus lineatus*) Sperm Cryopreservation: Efficacy of Trehalose as a Cryoprotectant

Otimização da criopreservação do sêmen de *Prochilodus lineatus*: eficácia da trealose como crioprotetor

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ABSTRACT - Semen cryopreservation is essential for the conservation and maintenance of germplasm stocks from several species, each of which has specific requirements for successful cryopreservation. The objective of this study was to develop a sperm preservation protocol for *Prochilodus lineatus* (curimba) using the sugars trehalose, sucrose, lactose, and raffinose. Because synergy among cryoprotectants is not always beneficial, each cryoprotectant was tested separately at three concentrations (50, 100, and 150 mM), and dimethyl sulfoxide (DMSO) was used as the control. After thawing, sperm kinetic parameters were analyzed using computer-assisted sperm analysis (CASA), and cellular functional parameters were assessed by flow cytometry. In the sperm kinetic analyses, only the amplitude of lateral head displacement did not differ statistically from the control ($p > 0.05$), whereas the other parameters were lower than those of the control. In the motility-period analysis, 100 mM trehalose was superior to the control ($p < 0.05$), supporting its use in the protocol. However, the results for the different parameters analyzed showed that the other sugars tested were not superior to the control (10% DMSO), indicating that they were not effective for cryopreservation. Trehalose at 100 mM showed the most favorable response among the tested sugars for *P. lineatus* sperm cryopreservation.

RESUMO - A criopreservação do sêmen é essencial para a conservação e manutenção de estoques de germoplasma de diversas espécies, cada uma com requisitos específicos para o sucesso do procedimento. O objetivo deste estudo foi desenvolver um protocolo de preservação espermática para *Prochilodus lineatus* (curimba) utilizando os açúcares trealose, sacarose, lactose e rafinose. Como a sinergia entre crioprotetores nem sempre é benéfica, cada crioprotetor foi testado separadamente em três concentrações (50, 100 e 150 mM), e o dimetilsulfóxido (DMSO) foi utilizado como controle. Após o descongelamento, os parâmetros cinéticos espermáticos foram analisados por análise espermática assistida por computador (CASA), e a funcionalidade celular foi avaliada ao longo do tempo por citometria de fluxo. Nas análises cinéticas espermáticas, apenas a amplitude do deslocamento lateral da cabeça não diferiu estatisticamente do controle ($p > 0,05$), enquanto os demais parâmetros foram inferiores aos do controle. Na análise do período de motilidade, a trealose a 100 mM foi superior ao controle ($p < 0,05$), sustentando seu uso no protocolo. Entretanto, os resultados dos diferentes parâmetros analisados mostraram que os demais açúcares testados não foram superiores ao controle (10% DMSO), indicando que não foram eficazes para a criopreservação. A trealose a 100 mM deve ser utilizada para a criopreservação do sêmen de *P. lineatus*.

Keywords: Curimba. Freshwater fish. Sugars. Flow cytometry. Sperm kinetics. Cryopreservation.

Palavras-chave: Curimba. Peixe de água doce. Açúcares. Citometria de fluxo. Cinética espermática. Criopreservação.

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INTRODUCTION

Considerable interest has been directed toward fish sperm cryopreservation in recent decades because of the need to preserve valuable genetic resources, support aquaculture production, and assist conservation and restocking programs. The development of cryopreservation protocols has been particularly relevant for species with commercial, ecological, and reproductive importance because these protocols enable the long-term storage of viable gametes while maintaining post-thaw sperm functionality (ASTURIANO; CABRITA; HORVÁTH, 2017; MARTÍNEZ-PÁRAMO et al., 2017; MAGNOTTI et al., 2018; WYLIE et al., 2025). Semen cryopreservation provides several advantages in this context, including flexibility in reproductive management, synchronization of artificial fertilization procedures, conservation of genetic resources, and reduced dependence on seasonal reproduction (MAGNOTTI et al., 2018).

Artificial fertilization associated with semen cryopreservation has become an important approach for increasing reproductive efficiency in aquaculture because it allows greater control of breeding programs and facilitates the dissemination of desirable genetic traits (WANG et al., 2021). This approach is particularly relevant for freshwater fish species of economic importance in South America, such as *Colossoma macropomum* (tambaqui), *Prochilodus lineatus*

(curimba), and *Oreochromis niloticus* (tilapia), which are widely used in commercial production and restocking programs (MURGAS et al., 2007; SOTNIKOV et al., 2024).

P. lineatus is a Neotropical migratory fish widely distributed in South American river basins and has considerable ecological and commercial importance. This species contributes substantially to nutrient cycling and is frequently used in aquaculture and fish stocking programs because of its reproductive potential and adaptability to captive conditions (SCARABOTTI et al., 2021; SOUZA et al., 2018). *P. lineatus* also has good seminal quality during the reproductive season and tolerance to handling, which makes it an attractive biological model for reproductive biotechnology studies (SOUZA et al., 2018).

Semen cryopreservation in *P. lineatus* has been investigated in several studies, with emphasis on the effects of extenders, cryoprotectants, cooling protocols, and post-thaw sperm quality. The feasibility of cryopreserving *P. lineatus* semen using different extenders, activators, and cryoprotectants was demonstrated by Murgas et al. (2007). Cryoprotectant combinations were subsequently reported to significantly affect sperm motility and morphology after thawing, which reinforced the importance of optimizing extender composition (FELIZARDO et al., 2010; VASCONCELOS et al., 2015). A morphological classification of sperm damage induced by cryopreservation in *P. lineatus* was proposed by Miliorini et al. (2011), with structural alterations associated with freezing injury described, whereas supplementation strategies were shown by Navarro et al. (2014) to improve post-thaw sperm quality. Cooling rates were more recently reported to substantially influence sperm functionality in *P. lineatus* semen, emphasizing that sperm cryosurvival is highly dependent on protocol conditions (JESUS PAULA et al., 2019).

Despite these advances, optimized cryopreservation protocols for *P. lineatus* remain difficult to establish because sperm cryotolerance is highly species-specific and strongly influenced by cryoprotectant composition, osmotic balance, freezing rate, and thawing conditions (ASTURIANO; CABRITA; HORVÁTH, 2017; MARTÍNEZ-PÁRAMO et al., 2017). Cryopreservation may induce structural and functional damage, including disruption of plasma membrane integrity, mitochondrial dysfunction, oxidative imbalance, DNA fragmentation, and reduced sperm kinetics, which may ultimately compromise fertilization success (MILIORINI et al., 2011; PIRES et al., 2018; VARELA JUNIOR et al., 2022). Therefore, cryoprotective agents capable of minimizing cryoinjury and preserving sperm functionality after thawing remain a priority for freshwater fish species.

Dimethyl sulfoxide (DMSO) is among the most widely used intracellular cryoprotectants because of its rapid permeability across cell membranes and its ability to reduce intracellular ice crystal formation during freezing (LEE et al., 2021). However, extracellular cryoprotectants, such as sugars, may also have protective effects during cryopreservation. Sugars such as trehalose, sucrose, lactose, and raffinose contribute to osmotic regulation by promoting controlled cellular dehydration and reducing intracellular ice formation. Furthermore, these compounds may stabilize membrane phospholipids and proteins, thereby preserving plasma membrane integrity and organelle functionality during freezing and thawing (SARVI et al., 2006; PIRES et al., 2018; VARELA JUNIOR et al., 2022).

Although the beneficial effects of sugars have been described in several freshwater fish species, including *Colossoma macropomum* (tambaqui) and *Piaractus mesopotamicus* (pacu) (PIRES et al., 2018; VARELA JUNIOR et al., 2022), limited information is available on their effects on post-thaw sperm quality in *P. lineatus*, particularly sperm kinetics, membrane functionality, oxidative profile, mitochondrial function, and DNA integrity. Therefore, the objective of this study was to develop a sperm preservation protocol for *P. lineatus* by evaluating the effects of three disaccharides (trehalose, sucrose, and lactose) and one trisaccharide (raffinose) on post-thaw sperm quality, with emphasis on sperm motility, kinetic parameters, membrane functionality, oxidative profile, mitochondrial membrane potential, membrane lipid disorder, and DNA fragmentation.

MATERIALS AND METHODS

Sample Processing

Location, Collection, and Selection

Semen samples were collected from fish reared at the Panamá fish farm (27°57'39.1"S, 48°45'29.1"W), in Paulo Lopes, Santa Catarina, Brazil. Fifteen sexually mature male *Prochilodus lineatus* (curimba), with mean body weight of 1.28 ± 0.05 kg and mean total length of 48.2 ± 1.3 cm, were used in the experiment. Fish were hormonally induced by intramuscular injection of carp pituitary extract (1 mg kg^{-1} body weight). Approximately 12 h after hormonal induction, semen was collected individually from each animal by gentle abdominal massage in the craniocaudal direction, with care taken to avoid contamination with urine, feces, blood, or water. Semen samples were collected in sterile Falcon tubes and maintained in a cooler at 8°C until processing.

An initial semen screening was performed under a phase-contrast microscope with glass slides and coverslips to verify the absence of contamination and assess baseline sperm motility. This evaluation was used exclusively as a quality-control step before cryopreservation. Only semen samples with $\geq 80\%$ subjective motility at 10 s after activation, consistently evaluated by the same trained technician, were selected.

No semen pools were prepared. Semen from each male was processed individually and distributed among all experimental treatments. Cryopreservation procedures were performed separately for each animal, and each male constituted one biological replicate ($n = 15$).

Addition of Extenders and Cryoprotectants

All chemicals used in the sperm freezing and thawing procedures were obtained from Sigma Chemical Company (St. Louis, MO, USA). Collected semen was diluted in Beltsville Thawing Solution (BTS; pH 7.2; osmolality, 330 mOsm kg^{-1}) at a 1:9 (v/v) ratio in the laboratory. The control treatment consisted of BTS supplemented with 10% dimethyl sulfoxide (DMSO). Experimental treatments consisted exclusively of BTS supplemented with the sugars trehalose, sucrose, lactose, or raffinose at 50, 100, and 150 mM (VARELA JUNIOR et al., 2022), without DMSO addition, totaling 12 experimental treatments plus one control

treatment. Semen previously diluted in BTS was distributed individually among the treatments before cryopreservation.

Packaging and Freezing

Samples were packaged in 0.25-mL straws, properly labeled, and sealed. The straws were stored in metal racks and kept at room temperature for 2 min to allow sperm equilibration with the cryoprotectants. Subsequently, the samples were placed in a dry shipper with nitrogen vapor ($-70\text{ }^{\circ}\text{C}$) for 12 h and then transferred to liquid nitrogen at $-196\text{ }^{\circ}\text{C}$ (VARELA JUNIOR et al., 2022).

Sperm Kinetics

The straws were removed from liquid nitrogen and thawed in a water bath at $45\text{ }^{\circ}\text{C}$ for 7 s. After thawing, the semen content was transferred to 1.5-mL tubes. Sperm activation was performed by mixing 1 μL of semen with 3 μL of distilled water immediately before analysis (VARELA JUNIOR et al., 2022).

Sperm kinetics were evaluated using one thawed straw per male and treatment, according to Varela Junior et al. (2022). When accidental straw loss occurred during thawing (e.g., rupture), an additional cryopreserved straw from the same male and treatment was used exclusively as a replacement. Analyses were performed using a single activated sample drop placed on a microscope slide, from which 10 nonoverlapping microscopic fields were evaluated sequentially. A minimum of 1,000 sperm cells was analyzed per sample. The evaluated variables included total motility (%), progressive motility (%), straight-line velocity (VSL, $\mu\text{m s}^{-1}$), average path velocity (VAP, $\mu\text{m s}^{-1}$), curvilinear velocity (VCL, $\mu\text{m s}^{-1}$), linearity [$\text{LIN} = (\text{VSL}/\text{VCL}) \times 100$], and beat-cross frequency (Hz) (DZIEWULSKA et al., 2011). Motility duration (s) was defined as the time from sperm activation to the end of progressive movement (VARELA JUNIOR et al., 2015; JESUS PAULA et al., 2019).

Flow Cytometry

An Attune Acoustic Focusing® flow cytometer (Life Technologies, Carlsbad, CA, USA) equipped with blue (argon, 488 nm) and violet (UV, 405 nm) lasers was used for flow cytometric analysis. The cellular structures and parameters analyzed included DNA fragmentation, membrane lipid disorder, membrane functionality, mitochondrial functionality, lipid peroxidation, cellular disruption, and reactive oxygen species concentration.

Results were obtained using version 2.1 software (Life Technologies). Hoechst 33342 dye at 16.2 mM was used for all analyses except DNA fragmentation. Negative FSC $\times$ SSC and Hoechst scatter plots were generated to eliminate non-sperm events (ALVES et al., 2016). Cells were stained with fluorophores added to calcium-free phosphate-buffered saline (PBS; 0.2 g KCl, 0.2 g KH_2PO_4 , 1.15 g Na_2HPO_4 , and 8 g NaCl in 1 L of deionized water; pH 7.2), and 10,000 sperm cells were counted per analysis after debris exclusion.

Membrane Functionality

Membrane functionality was assessed using the fluorophores propidium iodide (PI) and SYBR-14 (Minitube,

Tiefenbach, Germany). An aliquot of thawed sperm was incubated for 10 min in a solution containing 0.25 μM SYBR-14 and 7.5 μM PI, according to the manufacturer's instructions (Minitube). Spermatozoa were classified (PIRES et al., 2018) as undamaged with a functional membrane (SYBR-14⁺/PI⁻) or damaged with or without a nonfunctional membrane (SYBR-14⁺/PI⁺, SYBR-14⁻/PI⁺, or SYBR-14⁻/PI⁻). The percentage of cellular disruption was determined by classifying PI⁻ cells as intact and PI⁺ cells as disrupted.

Reactive Oxygen Species Concentration

Intracellular reactive oxygen species (ROS) concentration was assessed using 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA ; 1.0 μM), a fluorogenic probe that emits green fluorescence upon oxidation, together with propidium iodide (PI; 7.5 μM) to exclude nonviable spermatozoa. Briefly, a 10- μL aliquot of thawed semen was incubated with the probes for 10 min in the dark. ROS production was quantified by flow cytometry as the median green fluorescence intensity of viable spermatozoa (PI-negative cells), thereby excluding cells with compromised plasma membranes, according to Pires et al. (2018). ROS values were normalized against the corresponding control treatment (10% DMSO) and expressed as relative fluorescence intensity to minimize variability among ejaculates and allow comparison of the relative effect of each cryoprotectant medium. The normalized ROS index was calculated using the following equation: $\text{normalized ROS} = \text{median fluorescence intensity of the treatment} / \text{median fluorescence intensity of the corresponding control}$. Thus, values > 1 indicate higher intracellular ROS production relative to the control treatment, whereas values < 1 indicate lower ROS production.

Lipid Peroxidation

Sperm lipid peroxidation was assessed immediately after thawing. BODIPY C11 dye was added to a 10- μL sample aliquot to achieve a final concentration of 1 μM , followed by incubation for 2 h at $20\text{ }^{\circ}\text{C}$. Only viable spermatozoa were analyzed. The lipid peroxidation rate was calculated as follows: $[(\text{median green fluorescence intensity of peroxidized lipids}) / (\text{median green fluorescence intensity} + \text{median red fluorescence intensity of nonperoxidized lipids})] \times 100$, according to Pires et al. (2018).

Mitochondrial Membrane Potential

Mitochondrial membrane potential was assessed by incubating a 10- μL aliquot of thawed sample with 3.1 μM rhodamine 123 (green fluorescence) and 7.5 μM PI for 10 min. Intact spermatozoa (PI-negative cells) were classified as having high functionality (high fluorescence due to rhodamine accumulation) or low functionality (low fluorescence and low rhodamine accumulation), according to Liu et al. (2015). The percentage of spermatozoa with high mitochondrial membrane potential was calculated using the following formula: $[(\text{number of spermatozoa with high mitochondrial membrane potential}) / (\text{number of spermatozoa with high mitochondrial membrane potential} + \text{number of spermatozoa with low mitochondrial membrane potential})] \times 100$, according to Alves et al. (2016).

DNA Fragmentation Index

DNA integrity was assessed using the sperm chromatin structure assay (SCSA) with acridine orange staining. For this analysis, a 10- μ L aliquot of thawed sperm suspension was mixed with 5 μ L of TNE buffer (0.01 M Tris-HCl, 0.15 M NaCl, 0.001 M EDTA; pH 7.2) and 10 μ L of 1% Triton X-100 at 30-s intervals. Subsequently, acridine orange was added, followed by incubation for 30 s to 2 min before flow cytometric analysis. Spermatozoa with fragmented DNA exhibited red fluorescence, whereas spermatozoa with intact DNA exhibited green fluorescence. The DNA fragmentation index (%) was calculated as red fluorescence intensity / total fluorescence intensity (red + green) $\times$ 100, according to Alves et al. (2016).

Membrane Lipid Disorder in Viable Spermatozoa

Membrane lipid disorder was assessed using merocyanine 540 (M540), according to Pires et al. (2018), with minor adaptations. Briefly, a 10- μ L aliquot of thawed semen was incubated with 2.7 μ M M540 and 0.1 μ M YO-PRO-1 (Invitrogen, Eugene, OR, USA) for 10 min in the dark. Merocyanine 540 was used to identify alterations in membrane phospholipid organization, whereas YO-PRO-1 was used to exclude spermatozoa with compromised plasma membranes.

Flow cytometric analysis was performed only on viable spermatozoa (YO-PRO-1-negative cells). Membrane lipid disorder was quantified as the median M540 fluorescence intensity in viable spermatozoa, according to Pires et al. (2018).

Membrane lipid disorder values were normalized against the corresponding control treatment (10% DMSO) and expressed as relative fluorescence intensity to minimize variability among ejaculates and allow comparison of the relative effect of each cryoprotectant medium. The normalized membrane lipid disorder index was calculated as follows: normalized membrane lipid disorder = median fluorescence intensity of the treatment / median fluorescence intensity of the corresponding control. Values > 1 indicate greater membrane lipid disorder relative to the control treatment, whereas values < 1 indicate lower membrane lipid disorder.

Statistical Analysis

All variables were tested for normality using the Shapiro–Wilk test, followed by analysis of variance (ANOVA) and Tukey's post hoc test. Sugar type and concentration were considered independent variables, whereas total motility, progressive motility, motility duration, average path velocity, straight-line velocity, linearity, beat-cross frequency, membrane integrity, mitochondrial functionality, membrane lipid disorder, DNA fragmentation index, lipid peroxidation, and cell disruption were considered dependent variables. Before statistical analysis, intracellular ROS and membrane lipid disorder values were normalized against the corresponding control treatment to reduce variability among ejaculates and allow comparison of the relative effect of each cryoprotectant medium. Statistical analyses were performed using Statistix 2010 software. Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Sugar addition to BTS supplemented with 10% DMSO significantly influenced sperm quality after cryopreservation in the present study. Sugar type influenced progressive motility, membrane integrity, intracellular reactive oxygen species (ROS) levels, mitochondrial membrane potential, and sperm kinetic parameters, whereas concentration and the sugar $\times$ concentration interaction influenced motility-related traits, oxidative parameters, and sperm movement dynamics. These findings indicate that sperm cryosurvival in *Prochilodus lineatus* (curimba) depends not only on the presence of sugars but also on the interaction between sugar type and concentration.

Motility and Sperm Movement

Motility-related parameters were significantly influenced by the treatments (Figure 1). Progressive motility was enhanced in semen cryopreserved with trehalose, particularly at 100 mM, whereas raffinose, sucrose, and lactose generally had lower values at the same concentration (Figure 1A). Similarly, total motility was higher in sperm treated with trehalose at 100 and 150 mM, whereas the remaining sugars had more modest responses (Figure 1B). Motility duration, an essential parameter in freshwater fish fertilization, was also maintained at higher levels in groups treated with trehalose, particularly at 100 mM, whereas lactose at 100 and 150 mM tended to result in shorter durations (Figure 1C).

These findings are biologically relevant because sperm activation in freshwater fish is rapid and fertilization occurs within a narrow temporal window after gamete release. Thus, maintenance of sperm movement for longer periods may directly influence fertilization efficiency (MURGAS et al., 2007). Post-thaw sperm quality in *P. lineatus* has been reported to decline substantially depending on cryoprotectant composition and freezing conditions (MURGAS et al., 2007; FELIZARDO et al., 2010; JESUS PAULA et al., 2019), emphasizing the importance of maintaining both sperm motility and motility duration.

The higher values observed with trehalose may be explained by its physicochemical characteristics as a nonpermeating cryoprotectant. Trehalose functions mainly through extracellular osmotic regulation and membrane stabilization, thereby reducing the deleterious effects of cellular dehydration and extracellular ice crystal formation during freezing (MAGNOTTI et al., 2018).

Additionally, trehalose may interact with membrane phospholipids, reduce membrane phase transitions, and preserve bilayer organization during thawing, which may contribute to the maintenance of sperm movement (VARELA JUNIOR et al., 2022).

Beneficial effects of trehalose supplementation on sperm quality were demonstrated by Pires et al. (2018) in a study evaluating the combined use of DMSO and sugars during *Piaractus mesopotamicus* (pacu) sperm cryopreservation. Likewise, trehalose added to BTS extenders was reported to improve post-thaw sperm motility, membrane integrity, and fertilization rates in *Colossoma macropomum* (tambaqui) (VARELA JUNIOR et al., 2022). Studies evaluating sugar-based extenders in other fish species also

provide evidence that extracellular sugars reduce cryodamage and preserve sperm function after thawing (YANG et al., 2022; FADHILLAH et al., 2022).

Sperm motility and motility duration in fresh semen were identified by Carvalho et al. (2017), using canonical correlation analysis, as the variables most strongly associated with post-thaw sperm quality, indicating that these parameters

may predict cryosurvival in *P. lineatus* spermatozoa. This finding is consistent with the biological relevance of the longer motility duration observed in the present study, particularly in groups treated with trehalose, because maintenance of sperm movement after thawing is considered one of the main determinants of fertilization success in freshwater fish.

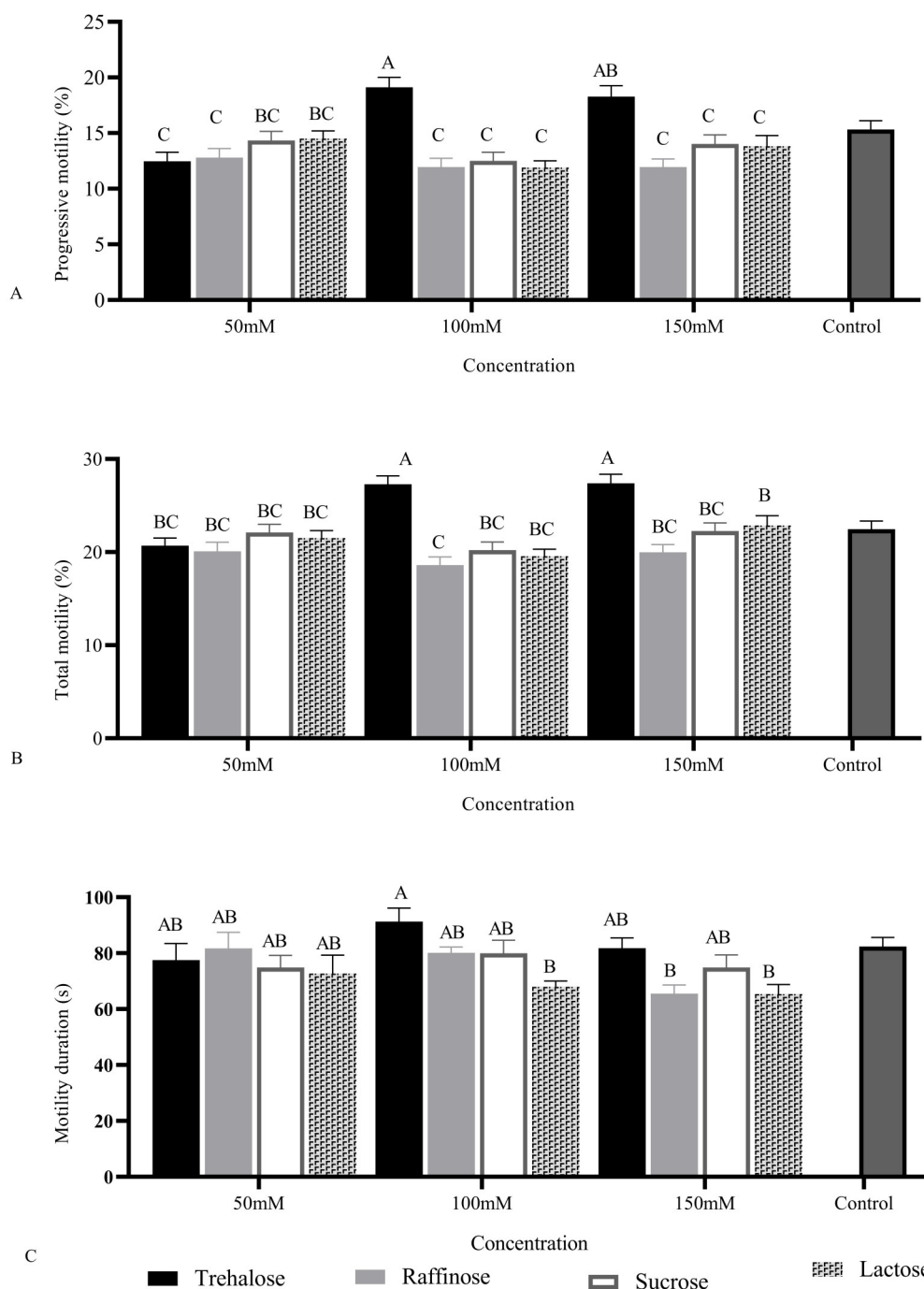


Figure 1. Effects of sugar type and concentration on sperm motility-related parameters after cryopreservation of *Prochilodus lineatus* (curimba) semen. (A) Progressive motility (%), (B) total motility (%), and (C) motility duration (s) of spermatozoa cryopreserved with trehalose, raffinose, sucrose, or lactose at 50, 100, and 150 mM. The 10% DMSO treatment was used as the control. Data are expressed as the mean $\pm$ standard error of the mean. Bars with different letters indicate significant differences among concentrations within each sugar treatment ($p < 0.05$).

Computer-assisted sperm analysis (CASA) has been widely applied in fish semen evaluation because it provides an objective assessment of sperm movement and kinetic patterns associated with fertilization capacity (GALLEGO; ASTURIANO, 2018). However, the biological significance of some CASA-derived parameters in fish species remains incompletely established, unlike in mammals, particularly for variables such as amplitude of lateral head displacement. In externally fertilizing fish, velocity-related parameters, including average path velocity and straight-line velocity, together with trajectory-related variables such as linearity and beat-cross frequency, are considered more biologically relevant because they directly influence sperm capacity to reach and penetrate the micropyle during the short fertilization period (GALLEGO; ASTURIANO, 2018; JESUS PAULA et al., 2019).

Sugar type, concentration, and the sugar $\times$ concentration interaction influenced CASA-assessed sperm kinetic parameters in the present study (Table 1). Significant effects were observed for average path velocity, straight-line

velocity, linearity, and beat-cross frequency, indicating that sperm movement efficiency was sensitive to cryoprotectant composition. Overall, groups treated with trehalose tended to maintain more favorable kinetic profiles, particularly at 100 mM, whereas lactose at 50–100 mM also resulted in higher values for some velocity parameters. Maintenance of higher average path velocity and straight-line velocity values may represent an important functional advantage after thawing because sperm velocity directly affects the probability of reaching and penetrating the micropyle in externally fertilizing fish (JESUS PAULA et al., 2019). Linearity and beat-cross frequency also provide indirect information on swimming trajectory and flagellar activity, reflecting sperm energetic status and swimming efficiency. Similar improvements in sperm kinetic parameters after cryopreservation have been associated with enhanced membrane stabilization and maintenance of cellular energy balance in freshwater fish species (VARELA JUNIOR et al., 2015; YANG et al., 2022; SOTNIKOV et al., 2024).

Table 1. Effects of sugar type and concentration on sperm kinetic parameters of cryopreserved *Prochilodus lineatus* (curimba) semen.

Analyses	Sugar	Concentrations			
		150 mM	100 mM	50 mM	DMSO 10%
VAP ($\mu\text{m/s}$)	Trehalose	31.9 $\pm$ 0.7 ^{ABC}	33.5 $\pm$ 0.7 ^{AB}	29.5 $\pm$ 0.6 ^{BC}	35.4 $\pm$ 0.9
	Raffinose	29.1 $\pm$ 0.6 ^C	29.5 $\pm$ 0.8 ^{BC}	32.5 $\pm$ 0.8 ^{ABC}	
	Sucrose	29.2 $\pm$ 0.7 ^{BC}	30.9 $\pm$ 0.7 ^{ABC}	31.7 $\pm$ 0.8 ^{ABC}	
	Lactose	29.0 $\pm$ 0.6 ^{BC}	32.5 $\pm$ 0.6 ^{AB}	33.8 $\pm$ 1.0 ^A	
VSL ($\mu\text{m/s}$)	Trehalose	26.5 $\pm$ 0.6 ^{ABC}	27.7 $\pm$ 0.6 ^{AB}	24.8 $\pm$ 0.5 ^{BC}	30.1 $\pm$ 0.8
	Raffinose	23.9 $\pm$ 0.6 ^C	24.7 $\pm$ 0.7 ^{BC}	27.3 $\pm$ 0.6 ^{ABC}	
	Sucrose	24.5 $\pm$ 0.7 ^{BC}	25.8 $\pm$ 0.7 ^{BC}	26.2 $\pm$ 0.7 ^{ABC}	
	Lactose	24.3 $\pm$ 0.5 ^{BC}	26.9 $\pm$ 0.6 ^{AB}	28.8 $\pm$ 0.9 ^A	
LIN (%)	Trehalose	66.0 $\pm$ 0.7 ^{AB}	65.4 $\pm$ 0.6 ^{ABC}	65.1 $\pm$ 0.7 ^{ABC}	69.8 $\pm$ 0.6
	Raffinose	62.8 $\pm$ 0.5 ^{BCD}	64.8 $\pm$ 0.7 ^{ABCD}	67.8 $\pm$ 0.9 ^A	
	Sucrose	61.2 $\pm$ 0.9 ^{CD}	65.0 $\pm$ 0.9 ^{ABC}	65.4 $\pm$ 0.7 ^{ABC}	
	Lactose	62.2 $\pm$ 0.8 ^D	63.8 $\pm$ 0.7 ^{ABCD}	67.8 $\pm$ 0.7 ^A	
BCF (Hz)	Trehalose	23.8 $\pm$ 0.3 ^{ABC}	24.2 $\pm$ 0.3 ^{ABC}	23.2 $\pm$ 0.3 ^{ABC}	24.7 $\pm$ 0.4
	Raffinose	22.9 $\pm$ 0.4 ^{BC}	22.3 $\pm$ 0.4 ^C	24.2 $\pm$ 0.4 ^{ABC}	
	Sucrose	23.3 $\pm$ 0.4 ^{ABC}	24.1 $\pm$ 0.4 ^{ABC}	23.4 $\pm$ 0.4 ^{ABC}	
	Lactose	24.0 $\pm$ 0.5 ^{ABC}	24.7 $\pm$ 0.4 ^A	24.3 $\pm$ 0.4 ^{AB}	

Values are expressed as the mean $\pm$ standard error of the mean. Different letters indicate significant differences among sugar $\times$ concentration treatments according to Tukey's post hoc test ($p < 0.05$). The 10% DMSO treatment was used as the control. VAP = average path velocity ($\mu\text{m s}^{-1}$); VSL = straight-line velocity ($\mu\text{m s}^{-1}$); LIN = linearity (%); BCF = beat-cross frequency (Hz).

Membrane Integrity, Oxidative Profile, and Lipid Peroxidation

Sperm functional membrane integrity was influenced by sugar type, concentration, and the sugar $\times$ concentration interaction (Figure 2A). Although all sugars maintained viable sperm populations after thawing, trehalose at 150 mM resulted in the highest membrane integrity values, whereas

lactose at 100 mM resulted in comparatively lower values. Because plasma membrane destabilization is one of the main consequences of cryoinjury, maintenance of membrane functionality is critical because sperm membrane integrity directly affects osmotic regulation, ion exchange, and sperm viability after thawing (MILIORINI et al., 2011; NAVARRO et al., 2014).

The protective effect observed in groups treated with

trehalose may be related to the ability of this sugar to preserve membrane architecture during freezing. Trehalose has been shown to stabilize phospholipid bilayers and reduce membrane phase transitions, thereby minimizing membrane rupture and structural destabilization caused by extracellular ice crystal formation (MAGNOTTI et al., 2018). Similar

findings were reported for *Piaractus mesopotamicus* sperm cryopreservation by Pires et al. (2018) and for *Colossoma macropomum* semen by Varela Junior et al. (2022); in both studies, trehalose supplementation contributed to improved sperm functionality after thawing.

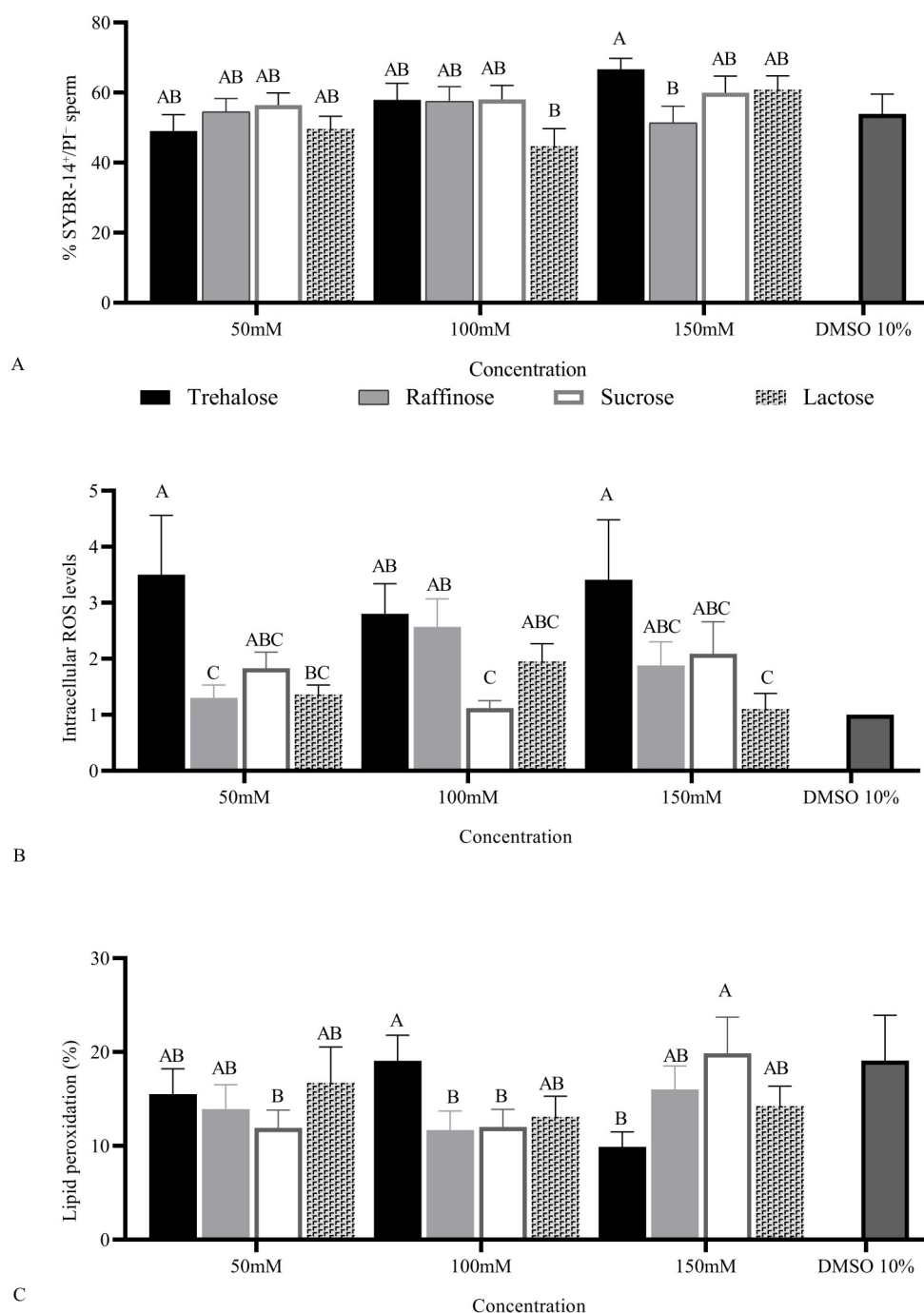


Figure 2. Effects of sugar type and concentration on sperm functional and oxidative parameters after cryopreservation of *Prochilodus lineatus* (curimba) semen. (A) Functional membrane integrity (% SYBR-14⁺/PI⁻ spermatozoa), (B) intracellular ROS levels, and (C) lipid peroxidation (%) of spermatozoa cryopreserved with trehalose, raffinose, sucrose, or lactose at 50, 100, and 150 mM. The 10% DMSO treatment was used as the control. Intracellular ROS levels were normalized against the corresponding control treatment. Data are expressed as the mean $\pm$ standard error of the mean. Bars with different letters indicate significant differences among concentrations within each sugar treatment ($p < 0.05$).

Intracellular ROS production was significantly influenced by sugar type and the sugar $\times$ concentration interaction (Figure 2B). Interestingly, treatments containing trehalose tended to have higher normalized ROS levels, particularly at 50 and 150 mM, whereas sucrose and lactose generally had lower values. Although elevated ROS production is frequently interpreted as a marker of oxidative stress, this relationship should be interpreted cautiously in sperm physiology. Moderate ROS production has essential functions in sperm signaling pathways, including energy metabolism, motility regulation, and membrane remodeling (LIU et al., 2015; SANDOVAL-VARGAS et al., 2021). Therefore, higher ROS levels do not necessarily indicate sperm damage but may reflect metabolically active sperm populations.

This interpretation is particularly relevant in the present study because the higher ROS levels observed in some trehalose treatments were accompanied by increased motility and prolonged motility duration (Figure 1), suggesting maintenance of sperm metabolic activity rather than oxidative injury. Similar findings have been described in fish semen cryopreservation, in which treatments with greater sperm movement after thawing did not necessarily correspond to the lowest oxidative activity (LEE et al., 2021; PAN et al., 2023).

Lipid peroxidation was significantly influenced by concentration and the sugar $\times$ concentration interaction (Figure 2C), although no consistent pattern indicating generalized oxidative damage was observed among treatments. At 150 mM, sucrose resulted in increased lipid peroxidation values, whereas trehalose maintained comparatively lower levels. Lipid peroxidation reflects oxidative damage to membrane phospholipids; thus, excessive increases in this parameter may compromise membrane fluidity, ion transport, and sperm fertilizing capacity (LIU et al., 2015). However, despite treatment-related variation, the absence of marked increases in lipid peroxidation in groups treated with trehalose is consistent with the hypothesis that increased ROS production in these treatments was not necessarily associated with oxidative injury.

Cryoprotectant toxicity and effectiveness were reported by Fernandez et al. (2020) to vary substantially among fish species because seminal plasma composition and sperm membrane characteristics differ considerably. Similar variability has also been reported in *Odontesthes bonariensis* (ALVES et al., 2016), *Salmo salar* (DZIEWULSKA et al., 2011), *Salmo trutta caspius* (SARVI et al., 2006), and *Austrolebias minuano* (FERNANDES et al., 2020), indicating the importance of species-specific optimization for *P. lineatus*

semen cryopreservation.

Mitochondrial Membrane Potential, DNA Fragmentation, and Membrane Lipid Disorder

Mitochondrial membrane potential differed by sugar type (Figure 3A), indicating treatment-related effects on sperm energy status. Spermatozoa cryopreserved with sucrose and lactose tended to have higher mitochondrial membrane potential at some concentrations, whereas trehalose had comparatively lower values at 50 mM and intermediate values at higher concentrations. Alterations in mitochondrial membrane potential may influence post-thaw sperm performance because mitochondrial functionality is directly associated with ATP production and sperm movement (LIU et al., 2015). However, although groups treated with trehalose did not always have the highest mitochondrial values, they maintained higher motility and motility duration, suggesting that preservation of sperm function after thawing may depend on multiple mechanisms beyond mitochondrial activity alone, including membrane stabilization and osmotic balance.

The DNA fragmentation index was influenced by the sugar $\times$ concentration interaction (Figure 3B). Although variation was observed among treatments, no consistent increase in DNA damage was detected in sperm treated with trehalose, particularly at 150 mM. Preservation of sperm DNA integrity is highly relevant because cryopreservation-induced chromatin destabilization may impair fertilization and embryo development (SANDOVAL-VARGAS et al., 2021). Sperm abnormalities caused by cryopreservation in *P. lineatus* have been associated with reduced sperm quality and post-thaw functionality (MILIORINI et al., 2011). Thus, maintenance of DNA fragmentation index values similar to those of the control suggests that trehalose did not induce additional genomic instability despite the increased ROS levels observed in some treatments.

Membrane lipid disorder in viable spermatozoa, normalized against the corresponding control treatment, varied minimally among sugars and concentrations (Figure 3C). Although some differences were observed at 100 mM, no generalized pattern of membrane destabilization was detected. Membrane lipid reorganization is expected during cryopreservation because of osmotic stress and temperature shifts; thus, the relatively stable values observed in the present study suggest that sugar supplementation partially preserved membrane structural organization after thawing (PIRES et al., 2018; MAGNOTTI et al., 2018).

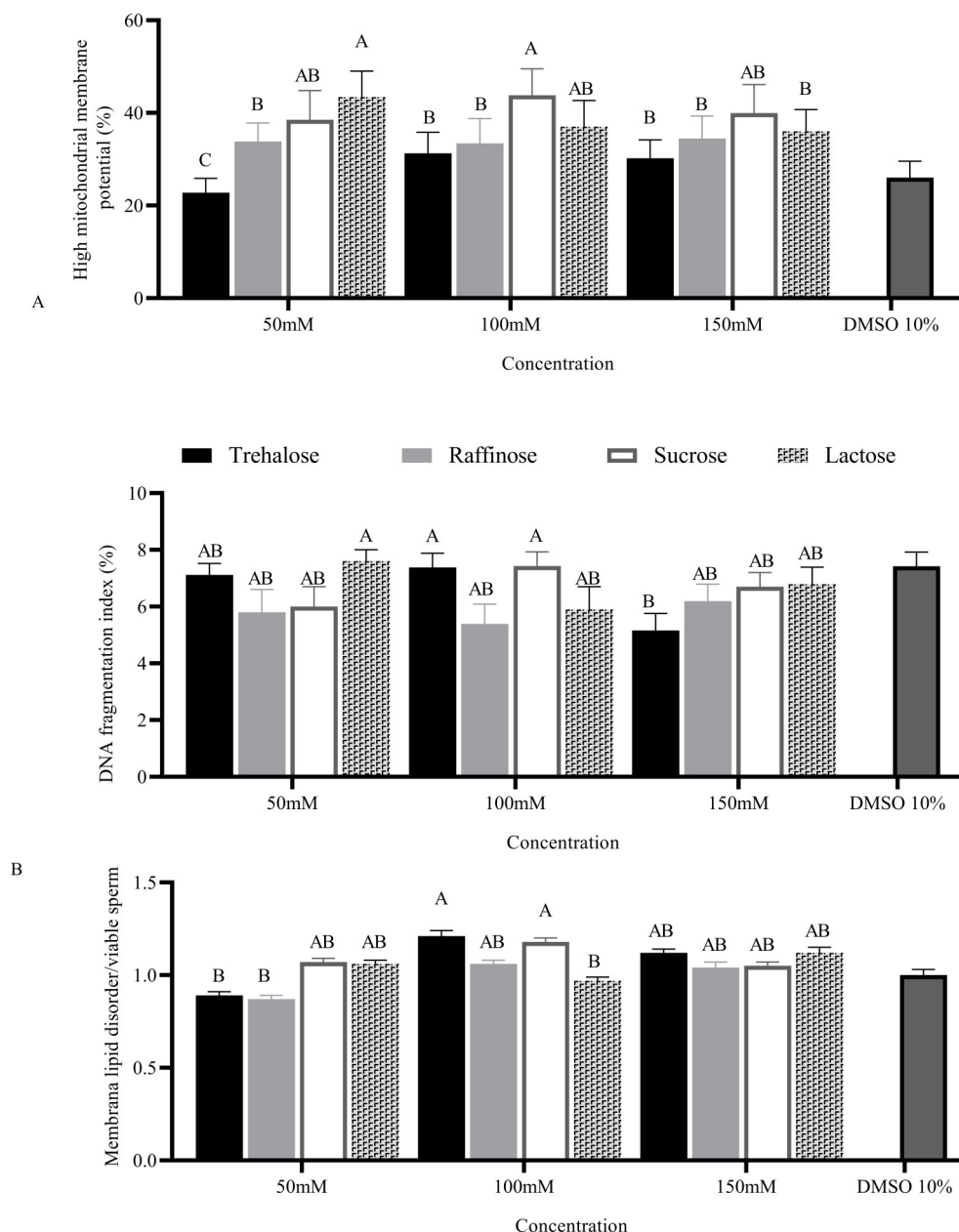


Figure 3. Effects of sugar type and concentration on sperm cellular and structural parameters after cryopreservation of *Prochilodus lineatus* (curimba) semen. (A) Spermatozoa with high mitochondrial membrane potential (%), (B) DNA fragmentation index (%), and (C) relative membrane lipid disorder in viable spermatozoa from semen cryopreserved with trehalose, raffinose, sucrose, or lactose at 50, 100, and 150 mM. The 10% DMSO treatment was used as the control. Membrane lipid disorder values were normalized against the corresponding control treatment and expressed as relative fluorescence intensity. Data are expressed as the mean $\pm$ standard error of the mean. Bars with different letters indicate significant differences among concentrations within each sugar treatment ($p < 0.05$).

CONCLUSION

Trehalose at 100 mM was associated with the most favorable motility-related profile among the tested sugars for cryopreserved *Prochilodus lineatus* (curimba) semen under the evaluated conditions, particularly for progressive motility, motility duration, and maintenance of sperm kinetic parameters after thawing. Although some cellular and

oxidative parameters remained comparable to those of the control treatment (10% DMSO), higher values with trehalose were not consistently observed across all evaluated sperm quality variables. Therefore, fertilization assays, evaluation of sperm quality over time after activation, and field validation studies are necessary before trehalose can be recommended as an alternative cryoprotectant strategy for *P. lineatus* semen cryopreservation.

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