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Cryopreservation of sperm in annual fish *Austrolebias minuano* (Cyprinodontiformes; Rivulidae)

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Abstract

The effects of the cryoprotectants dimethyl sulfoxide, glycerol and methyl glycol at different concentrations on *Austrolebias minuano* sperm quality parameters were evaluated in this study. The cellular kinetic parameters, determined using flow cytometry, indicated the best results with the samples cryopreserved with 7.5% methyl glycol. Dimethyl sulfoxide concentrations of 7.5% and glycerol concentrations of 10%, 12.5% and 15% demonstrated the least benefit across all evaluated sperm kinetic parameters. When assessed using flow cytometry, a concentration of 7.5% methyl glycol similarly showed better sperm kinetic parameters than 12.5% dimethyl sulfoxide. We conclude that cryoprotectants, especially methyl glycol, are effective for the preservation of sperm quality in *A. minuano*. However, high sensitivity of spermatozoa against glycerol was observed in these studies; thus, it is not recommended for cryopreservation purposes.

KEYWORDS
cryoprotectants, extinction, killifish, preservation

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1 | INTRODUCTION

Approximately 30% of the ichthyofauna threatened in Brazil are represented by annual fish of the Rivulidae family (ICMBio, 2012). Drastic loss and fragmentation of ephemeral aquatic habitats particularly threaten this group (Bonisławska, Szulc, & Formicki, 2015). Their small isolated populations make this group prone to higher rates of genetic drift and accelerated evolution and increase the frequency of allopatric speciation events (Beheregaray, Attard, Brauer, & Hammer, 2016). These findings highlight the need for immediate conservation steps to preserve the threatened lineages of this group.

According to the IUCN Red Data Book (2018), the annual fish *Austrolebias minuano* (Costa & Cheffe, 2001) is threatened for extinction in Brazil. This species belongs to the group *A. adloffi*, which can be distinguished by its body size and colour patterns characteristic of the species. *A. minuano* presents a relatively wide distribution taking into account the distribution perspectives of annual fish, occurring in ponds of the Patos Mirim lagoon system (IUCN Red Data Book, 2018). Being an opportunistic feeder, it plays a particularly important role in balancing the food chains of wetland ecosystems (Volcan, Fonseca, & Robaldo, 2011).

Ex-situ conservation strategies like germplasm banks allow for at least partial preservation of the genetic heritage of endangered species (Figueroa et al., 2015; Silva et al., 2016). Genetic resources like cryopreserved spermatozoa and oocytes allow for the reintroduction of endangered species into their natural habitats, thus, avoiding loss of biodiversity (Cabrita et al., 2010; Machado et al., 2016).

However, given the variation in the biochemical composition of sperm, individualized species-specific cryopreservation protocols are necessary for each species (Viveiros et al., 2014). An efficient and practical cryopreservation protocol for sperm needs to take into account and standardize factors like type and concentration of cryoprotectants, diluents, osmolarity, freezing and thawing temperatures of spermatozoa and duration of each processing step. Dimethyl sulfoxide (DMSO), glycerol and methyl glycol (MG) are common intracellular cryoprotectants used for cryopreservation of fish spermatozoa (Viveiros et al., 2012). Indeed, alcohol-based cryoprotectants are widely used for cryopreservation of sperm from freshwater fish: Piracanjuba (*Brycon orbignyanus*; Perry et al., 2019), Tambaqui (*Colossoma macropomum*; Garcia et al., 2015; Varela Junior et al., 2012), Curimba (*Prochilodus lineatus*; Miliorini et al., 2011; Viveiros et al., 2014), Pacu (*Piaractus mesopotamicus*; Pires et al., 2018).

DMSO shows cryopreservative capabilities in freezing of fish semen due to its high solubility in an aqueous medium, easily penetrating the plasma membrane, and engaging the free hydroxyl groups to inhibit the formation of reactive oxygen species (Viveiros et al., 2014). Glycerol is another common agent used for cryopreservation of male gametes (Ahn, Park, & Lim, 2018), although certain factors, such as like its high molecular weight, viscosity and impermeability, make it less favourable for use as a cryoprotectant (Miliorini et al., 2011). A number of studies indicate that MG is an ideal cryoprotectant for fish spermatozoa, based on investigations in *Brycon insignis* (Viveiros et al., 2011), *Piaractus brachypomus* (Ramirez-Merlano, Velasco-Santamaría, Medina-Robles, & Cruz-Casallas, 2011), *Piaractus mesopotamicus* and *Piaractus lineatus* (Viveiros et al., 2014), *Colossoma macropomum* (Carneiro, Azevedo, Santos, & Maria, 2012) and *Steindachneridion scriptum* (Ribeiro et al., 2019).

The present study was aimed to generate the first standardized protocol for the cryopreservation of spermatozoa from *A. minuano* using three different cryoprotectants and evaluate the feasibility of this technique for the preservation of this endangered species.

FIGURE 1 Species of *Austrolebias minuano*, withdrawal from the type locality Ilha do Leonídio, state of Rio Grande do Sul—Brazil. The male of the species (a) is characterized by pairs of dark lines in its body and the female (b) for its smooth lines in the body

2 | MATERIAL AND METHODS

2.1 | Animals and semen collection

Immersion nets were used to capture adult males of *A. minuano* (Figure 1) from a previously reported type locality of the species at the Ilha do Leonídio (52° 22′79.49″S and 32° 05′04.78″W; Costa, 2006), Rio Grande, RS, Brazil. To avoid anaesthetics affecting the results, animals were euthanized by spinal cord transection (Resolution nº 1000, of 05/2012).

Gonads from three males were pooled together to make up 15 samples, totalling 45 animals. Semen was diluted in Beltsville Thawing Solution (BTS) at pH 7.2 and osmolarity 380 mOsm/L in a 1:9 volume/volume (v/v) ratio. Motility was estimated with the help of Computer-Assisted Sperm Analysis (CASA; Dziewulska, Rzemieniecki, Czerniawski, & Domagała, 2011) by diluting 1 μl of semen in 4 μl of water from the fish's natural habitats. Motility of sperm was only seen 10 s post-activation, and all samples exhibited ≥80% motility without contamination by urine or faeces.

2.1.1 | Experimental design

Freezing was carried out on the 15 pools in 15 treatments each per pool using DMSO, MG and glycerol in concentrations of 5%, 7.5%, 10%, 12.5% and 15%. BTS extender solution was used for all preparations (Varela Junior et al., 2012) at a pH of 7.2 and osmolarity of 380 mOsm/L. Aliquots of 250-µl were made, sealed with polyvinyl alcohol and cooled in metal racks at 5°C for 20 min. After this, a 12-hr stay in a liquid nitrogen shipper (Taylor Wharton, Model CP 300) was maintained and subsequently transferred to a larger liquid nitrogen storage unit (MVE, Model CP-34) for 45 days prior to thawing and analysis of the samples.

2.2 | Analysis of sperm kinetics

The activation of spermatozoa was carried out by dilution with puddle water (filtered with Millipore syringe filters, Millex® JBR610021/0.22 µm) in a 1:4 ratio and the parameters recorded by CASA. Ten fields with a minimum of 1,000 cells were captured. Certain parameters, such as total motility (MT, %), progressive motility (MP, %), average distance travelled (DAP, µm), curvilinear distance (DCL, µm), average path velocity (VAP, %), rectilinearity (STR, %), linearity (LIN, %), oscillation (WOB), head lateral displacement (ALH, µm) and cross flagellar beating frequency (BCF, Hz), were determined. The duration of motility was considered from the moment of activation until the progressive movement stopped in the spermatozoa (Varela Junior et al., 2015).

2.3 | Flow cytometry analyses

An Attune Acoustic Focusing® cytometer version 2.1 (Life Technologies) equipped with a blue (Argon 488 nm) and violet laser (UV 405 nm), with a VL-1 450/40 filter was used for flow analysis. The cells were stained with a 16.2 mM solution of Hoechst 33342, except for DNA fragmentation (Martinez-Alborcia et al., 2012). The selection of sperm cell population was done by filtering out non-sperm cells based on FSC × SSC and Hoechst-negative scatter plots. All flow readings were taken by resuspending cells in calcium-free phosphate-buffered saline (PBS, 80 g NaCl, 11.5 g KCl, 24 g Na_2HPO_4, 2 g KH_2PO_4 in 1 L deionized water) using a minimum of 10,000 spermatozoa per analysis.

2.3.1 | Mitochondrial functionality

Mitochondrial functionality was assayed after staining 10 µl of the thawed sample with 3.1 µM Rhodamine and 7.5 µM Propidium Iodide (PI) for 10 min. Intact spermatozoa were gated using PI negativity, after which they could be classified into high or low mitochondrial membrane potential based on either high or low Rhodamine accumulation respectively (Liu et al., 2015). Mitochondrial functionality rate was calculated by the formula: [Number of High Rhodamine-positive events/Total number of Rhodamine-positive events] × 100 (Alves et al., 2016).

2.3.2 | Membrane functionality and Cellular disruption

Aliquots of thawed semen were incubated for 5 min with fluorescent probes containing 0.25 µM Sybr14 and 7.5 µM PI (Minitube, Tiefenbach, Germany) following the manufacturer's instructions. Spermatozoa with functional membranes were gated as Sybr14+/PI−; the other populations (Sybr14+/PI+, Sybr14−/PI+, Sybr14−/PI−) were classified as lesions (Figueroa et al., 2015). Cellular rupture status was verified as the percentage of cellular rupture, with PI− cells classified as intact and PI+ cells classified as ruptured.

2.3.3 | Membrane fluidity

Ten (10) µL of the thawed sample was stained with 2.7 µM of a hydrophobic merocyanine dye 540 (M540) and 0.1 µM of YO-PRO-1 (Invitrogen-Eugene) for 5 min. High M540-positivity (high membrane fluidity) and low M540-positivity (low membrane fluidity) were evaluated only for intact spermatozoa (YO-PRO-1 negative; Fernández-Gago, Domínguez, & Martínez-Pastor, 2013). Membrane fluidity rate was defined as: [Number of high M540-positive events/Total number of M540-positive events] × 100.

2.3.4 | DNA fragmentation index

The sperm chromatin structure assay (SCSA) was used to evaluate DNA integrity. A 30-s incubation of 10 µl thawed spermatozoa diluted in 5 µl of TNE (0.01 M Tris-HCl, 0.15 M NaCl, 0.001 M disodium EDTA, pH 7.2) with addition of 10 µl of 1% (v/v) Triton X-100 was performed, immediately following which, acridine orange dye was added and flow cytometer reading taken within 2 min. Spermatozoa were classified as intact (green) or fragmented (orange/red; Jenkins, Draugelis-Dale, Pinkney, Iwanowicz, & Blazer, 2015) with the rate of DNA fragmentation calculated as: [Number of spermatozoa with fragmented DNA/(Number of spermatozoa with intact DNA + Number of spermatozoa with fragmented DNA)] × 100.

2.3.5 | Concentration of reactive oxygen species (ROS)

ROS levels in sperm cells were analysed by measuring the median intensity of green fluorescence after staining with 1.0 µM of 2′7′-dichlorofluorescein diacetate (H2DCFDA) and gating for live spermatozoa (PI+, 7.5 µM) (Domínguez-Rebolledo et al., 2011).

2.3.6 | Lipid peroxidation (LPO)

Spermatozoa shortly after thawing were incubated for 2 hr at room temperature (20°C) with 1 µM BODIPY® C11 stain for assessing lipid peroxidation (Hagedorn, McCarthy, Carter, & Meyers, 2012) in 10 µl of the sample, and live spermatozoa were analysed.

TABLE 1 Analyses of total motility, progressive motility, mean distance travelled, curvilinear distance, rectilinear distance, lateral head displacement and frequency of cross flagellar beating of *Austrolebias minuano* (Costa & Cheffe, 2001), with dimethylsulphoxide, glycerol and methyl glycol alcohols at different concentrations (mean ± standard error)

Spermatic kinetics							
Treatments (%)	**MT**	**MP**	**DAP**	**DCL**	**DSL**	**ALH**	**BCF**
DMSO 5	8.41 ± 0.9^{bcdef}	4.64 ± 0.7^{cd}	5.69 ± 0.7^{efg}	7.46 ± 0.9^{de}	4.41 ± 0.6^{ef}	0.88 ± 0.1^{abcde}	10.71 ± 1.1^{def}
DMSO 7.5	7.22 ± 0.9^{def}	3.22 ± 0.7^{d}	3.96 ± 0.6^{ghij}	5.38 ± 0.7^{efg}	3.30 ± 0.5^{fgh}	0.69 ± 0.1^{cdefg}	8.32 ± 1.1^{fg}
DMSO 10	11.13 ± 0.9^{ab}	7.27 ± 0.8^{b}	7.81 ± 0.6^{bcd}	10.86 ± 0.9^{bc}	6.20 ± 0.5^{bc}	0.81 ± 0.1^{bcdef}	13.61 ± 1.1^{bcd}
DMSO 12.5	7.36 ± 0.6^{bcdef}	3.55 ± 0.4^{d}	5.20 ± 0.5^{fgh}	7.44 ± 0.7^{de}	3.66 ± 0.3^{fg}	0.65 ± 0.1^{defg}	10.83 ± 1.1^{def}
DMSO 15	9.94 ± 0.9^{abcd}	5.77 ± 0.8^{bc}	6.05 ± 0.6^{def}	8.81 ± 0.9^{cd}	4.48 ± 0.4^{def}	0.85 ± 0.1^{abcdef}	10.06 ± 1.0^{ef}
GLY 5	5.86 ± 0.9^{f}	3.48 ± 0.7^{d}	4.91 ± 0.6^{fghi}	6.83 ± 0.8^{def}	3.80 ± 0.4^{fg}	0.63 ± 0.1^{defg}	11.03 ± 1.3^{cdef}
GLY 7.5	8.73 ± 0.9^{bcde}	4.74 ± 0.7^{cd}	7.20 ± 0.9^{cde}	10.24 ± 1.1^{bc}	5.89 ± 0.8^{bcd}	1.00 ± 0.1^{ab}	12.12 ± 1.1^{bcde}
GLY 10	5.97 ± 0.8^{ef}	2.80 ± 0.7^{d}	3.38 ± 0.4^{ij}	4.54 ± 0.5^{fg}	2.61 ± 0.3^{gh}	0.61 ± 0.1^{efg}	8.37 ± 1.1^{fg}
GLY 12.5	6.20 ± 0.7^{def}	3.20 ± 0.5^{d}	3.51 ± 0.4^{hij}	4.87 ± 0.6^{fg}	2.63 ± 0.3^{gh}	0.66 ± 0.1^{def}	6.43 ± 0.9^{g}
GLY 15	6.46 ± 0.8^{def}	2.69 ± 0.5^{d}	2.71 ± 0.4^{j}	4.01 ± 0.6^{g}	2.01 ± 0.3^{h}	0.60 ± 0.1^{fg}	6.37 ± 0.9^{g}
MG 5	7.97 ± 0.8^{cdef}	4.23 ± 0.7^{cd}	4.41 ± 0.6^{fghij}	5.88 ± 0.8^{efg}	3.69 ± 0.5^{fg}	0.53 ± 0.1^{g}	8.56 ± 1.1^{fg}
MG 7.5	14.45 ± 1.8^{a}	9.62 ± 0.9^{a}	10.28 ± 0.7^{a}	14.63 ± 1.0^{a}	7.84 ± 0.5^{a}	1.11 ± 0.1^{a}	16.86 ± 1.1^{a}
MG 10	11.42 ± 0.8^{ab}	7.43 ± 0.7^{b}	9.55 ± 0.7^{ab}	14.96 ± 1.2^{a}	6.96 ± 0.5^{ab}	1.05 ± 0.1^{ab}	17.10 ± 1.2^{a}
MG 12.5	11.24 ± 0.9^{ab}	6.85 ± 0.7^{b}	7.14 ± 0.5^{cde}	10.58 ± 0.8^{bc}	5.31 ± 0.4^{cde}	0.90 ± 0.1^{abcd}	14.06 ± 1.1^{abc}
MG 15	10.91 ± 0.9^{abcd}	7.36 ± 0.8^{b}	8.04 ± 0.6^{bc}	11.58 ± 0.9^{b}	5.99 ± 0.4^{bc}	0.94 ± 0.1^{abc}	14.96 ± 1.1^{ab}

Abbreviations: ALH, lateral head displacement; BCF, frequency of cross flagellar beating; DAP, mean distance travelled; DCL, curvilinear distance; DMSO, dimethylsulphoxide; DSL, rectilinear distance; GLY, glycerol; MT, total motility; MP, progressive motility; MG, methyl glycol. Different letters indicate differences ($p < .05$).

TABLE 2 Analyses of average path velocity, curvilinear velocity, straightness, linearity and oscillation of *Austrolebias minuano* (Costa & Cheffe, 2001), with dimethylsulphoxide, glycerol and methyl glycol alcohols at different concentrations (mean ± standard error)

Spermatic kinetics					
Treatments (%)	**VAP**	**VCL**	**STR**	**LIN**	**WOB**
DMSO 5	13.34 ± 1.6^{def}	17.72 ± 2.1^{cd}	0.29 ± 0.0^{cde}	0.23 ± 0.0^{cde}	0.29 ± 0.0^{de}
DMSO 7.5	9.35 ± 1.3^{fgh}	12.89 ± 1.7^{def}	0.23 ± 0.0^{ef}	0.17 ± 0.0^{ef}	0.21 ± 0.0^{fg}
DMSO 10	17.18 ± 1.5^{bcd}	23.77 ± 2.1^{b}	0.41 ± 0.0^{ab}	0.30 ± 0.0^{ab}	0.38 ± 0.0^{abc}
DMSO 12.5	11.57 ± 1.2^{efg}	16.66 ± 1.7^{cde}	0.28 ± 0.0^{de}	0.20 ± 0.0^{de}	0.28 ± 0.0^{def}
DMSO 15	13.86 ± 1.3^{cde}	20.35 ± 2.1^{bc}	0.32 ± 0.0^{cd}	0.23 ± 0.0^{cde}	0.31 ± 0.0^{cd}
GLY 5	10.78 ± 1.3^{efg}	15.10 ± 1.8^{cdef}	0.27 ± 0.0^{de}	0.20 ± 0.0^{de}	0.25 ± 0.0^{def}
GLY 7.5	16.89 ± 2.5^{bcd}	23.83 ± 2.99^{d}	0.37 ± 0.0^{bc}	0.26 ± 0.0^{bcd}	0.32 ± 0.0^{bcd}
GLY 10	8.31 ± 1.1^{gh}	11.26 ± 1.4^{ef}	0.23 ± 0.0^{ef}	0.18 ± 0.0^{ef}	0.22 ± 0.0^{efg}
GLY 12.5	8.45 ± 1.1^{gh}	11.73 ± 1.6^{ef}	0.22 ± 0.0^{ef}	0.17 ± 0.0^{ef}	0.22 ± 0.0^{efg}
GLY 15	6.53 ± 0.9^{h}	9.64 ± 1.5^{f}	0.17 ± 0.0^{f}	0.12 ± 0.0^{f}	0.16 ± 0.0^{g}
MG 5	9.86 ± 1.2^{efgh}	13.12 ± 1.7^{def}	0.27 ± 0.0^{de}	0.21 ± 0.0^{de}	0.25 ± 0.0^{def}
MG 7.5	22.97 ± 1.6^{a}	32.55 ± 2.2^{a}	0.47 ± 0.0^{a}	0.34 ± 0.0^{a}	0.44 ± 0.0^{a}
MG 10	20.79 ± 1.6^{ab}	32.56 ± 2.6^{a}	0.43 ± 0.0^{ab}	0.29 ± 0.0^{abc}	0.39 ± 0.0^{ab}
MG 12.5	15.81 ± 1.2^{cd}	23.50 ± 1.9^{b}	0.42 ± 0.0^{ab}	0.30 ± 0.0^{ab}	0.39 ± 0.0^{ab}
MG 15	17.86 ± 1.3^{bc}	25.74 ± 2.1^{b}	0.43 ± 0.0^{ab}	0.33 ± 0.0^{a}	0.43 ± 0.0^{a}

Abbreviations: DMSO, dimethylsulphoxide; GLY, glycerol; MG, methyl glycol; LIN, linearity; STR, straightness; VAP, Path velocity; VCL, curvilinear velocity; WOB, oscillation. Different letters indicate differences ($p < .05$).

Lipid peroxidation rate was calculated as follows: [Median red fluorescence intensity (non-peroxidized lipid)/{Median green fluorescence intensity (peroxidized lipid) + Median red fluorescence intensity}] × 100.

2.4 | Statistical analyses

The Shapiro–Wilk test for normality was carried out for the data, followed by an analysis of variance (ANOVA) and Tukey's post hoc

TABLE 3 Analyses of mitochondrial functionality, membrane functionality, cellular disruption, fluidity membrane, DNA fragmentation index, concentration of reactive oxygen species and lipid peroxidation of *Austrolebias minuano* (Costa & Cheffe, 2001), with dimethylsulphoxide, glycerol and methyl glycol alcohols at different concentrations (mean ± standard error)

Flow cytometry							
Treatments (%)	**MIT**	**MFU**	**CD**	**MFL**	**DNA**	**ROS**	**LPO**
DMSO 5	84.71 ± 2.1^{abc}	72.92 ± 2.1^{def}	29.95 ± 3.1^{abc}	45.64 ± 2.8^{bc}	4.62 ± 0.1^{bcd}	4,561.0 ± 747.91^{def}	15.17 ± 1.4^{abc}
DMSO 7.5	81.66 ± 2.5^{c}	81.26 ± 1.1^{abc}	31.08 ± 3.2^{ab}	48.10 ± 3.9^{bc}	4.25 ± 0.1^{de}	5,121.8 ± 569.33^{def}	15.74 ± 1.0^{ab}
DMSO 10	87.93 ± 1.1^{ab}	76.46 ± 2.1^{bcde}	21.30 ± 1.0^{f}	34.11 ± 3.2^{d}	4.32 ± 0.2^{de}	5,351.3 ± 971.40^{def}	13.70 ± 1.1^{abcd}
DMSO 12.5	88.46 ± 0.9^{a}	63.33 ± 3.9^{g}	23.58 ± 0.9^{def}	47.81 ± 2.6^{bc}	4.98 ± 0.1^{ab}	11,813 ± 3,914.6^{ab}	15.43 ± 1.4^{ab}
DMSO 15	86.13 ± 0.9^{abc}	69.26 ± 3.4^{fg}	25.16 ± 1.0^{cdef}	59.94 ± 3.0^{a}	4.66 ± 0.1^{bcd}	6,389.9 ± 924.09^{def}	12.72 ± 1.2^{bcde}
GLY 5	84.60 ± 1.3^{abc}	81.06 ± 1.1^{abc}	21.73 ± 1.0^{ef}	42.00 ± 2.7^{cd}	4.55 ± 0.0^{bcd}	5,299.6 ± 916.60^{def}	11.68 ± 1.2^{cde}
GLY 7.5	87.20 ± 1.3^{abc}	77.53 ± 2.1^{abcd}	23.45 ± 1.2^{def}	50.48 ± 3.1^{abc}	4.41 ± 0.1^{cde}	11,533 ± 1643.9^{abc}	17.03 ± 1.2^{a}
GLY 10	85.46 ± 1.5^{abc}	70.60 ± 2.0^{ef}	26.19 ± 2.1^{abcdef}	49.72 ± 3.8^{bc}	4.05 ± 0.1^{e}	4,832.3 ± 455.99^{def}	13.52 ± 0.9^{abcd}
GLY 12.5	85.28 ± 1.6^{abc}	80.50 ± 3.2^{abc}	28.16 ± 2.4^{abcd}	42.37 ± 3.7^{cd}	5.42 ± 0.1^{a}	2,539.8 ± 244.75^{ef}	9.41 ± 1.2^{e}
GLY 15	82.40 ± 1.7^{bc}	83.46 ± 2.2^{a}	27.11 ± 1.9^{abcde}	48.11 ± 3.5^{bc}	4.22 ± 0.1^{de}	5,252.7 ± 1,008.4^{def}	11.25 ± 1.2^{de}
MG 5	83.80 ± 3.1^{abc}	80.00 ± 2.9^{abc}	31.30 ± 2.9^{a}	52.33 ± 3,9^{ab}	4.62 ± 0.1^{bcd}	7,557.7 ± 1,144.3^{bcde}	13.88 ± 1.3^{abcd}
MG 7.5	84.06 ± 2.2^{abc}	79.46 ± 1.1^{abcd}	25.63 ± 1.9^{bcdef}	46.35 ± 3.5^{bc}	3.96 ± 0.2^{e}	6,694.5 ± 1,218.2^{cdef}	16.38 ± 1.2^{a}
MG 10	85.07 ± 2.4^{abc}	79.21 ± 2.3^{abcd}	23.12 ± 1.6^{def}	45.91 ± 3.5^{bc}	4.39 ± 0.2^{cde}	3,414.0 ± 308.84^{ef}	13.60 ± 1.4^{abcd}
MG 12.5	82.26 ± 3.2^{bc}	82.60 ± 1.1^{ab}	22.64 ± 1.3^{def}	45.02 ± 3.5^{bc}	4.82 ± 0.1^{bc}	9,464.4 ± 1605.1^{abcd}	16.89 ± 1.3^{a}
MG 15	83.06 ± 3.2^{abc}	75.50 ± 3.1^{cdef}	26.09 ± 1.8^{abcdef}	44.44 ± 3.5^{bc}	4.31 ± 0.1^{de}	13,812 ± 4,186.8^{a}	14.52 ± 1.4^{abcd}

Abbreviations: CD, cellular disruption; DMSO, dimethylsulphoxide; DNA, DNA fragmentation index; GLY, glycerol; LPO, lipid peroxidation; MIT, Mitochondrial functionality; MFU, membrane functionality; MG, methyl glycol; MFL, fluidity membrane; and ROS, concentration of reactive oxygen species. Different letters indicate differences ($p < .05$).

test. The concentrations of the cryoprotectants were considered the independent variables while other variables (namely, total motility, progressive motility, DAP, DCL, DSL, VAP, STR, LIN, WOB, ALH, BCF, mitochondrial functionality, membrane functionality, cell disruption, membrane fluidity, DNA fragmentation and ROS eLPO) were considered dependent variables. Statistix® V9.0 software (Statistix, 2008) was used to perform all analyses with a *p*-value of <.05 considered for statistical significance.

3 | RESULTS

MG demonstrated the best sperm kinetic readings in cryopreserved samples at a concentration of 7.5% ($p < .05$), with a progressive motility (MP) value of 9.62 ± 0.92% (Table 1). Numerous kinetic variables analysed (MT, MP, DAP, DCL, STR, ALH, BCF, VAP, VCL, straightness, LIN and WOB) revealed that concentrations of 7.5% and 10% MG provided the most desirable motility for thawed spermatozoa ($p < .05$; Table 1). The concentrations of DMSO at 7.5% and of glycerol at 10%, 12.5% and 15% demonstrated the least favourable sperm kinetic measurements (Table 1). Likewise, the 7.5% MG concentration was sufficient in terms of flow cytometric parameters like mitochondrial functionality, membrane functionality, cellular integrity, DNA integrity and levels of ROS (Table 2). The 12.5% DMSO concentration was the least effective treatment for most of the analysed parameters (Table 2) while 7.5% MG treatment showed less ROS production than the same concentration of glycerol ($p < .05$; Table 3).

All three cryoprotectants at the concentrations of 5% and 10% showed lesser cytoplasmic membrane fluidity with well-preserved membrane permeability ($p < .05$; Table 3). When the membrane functionality of the spermatozoa was evaluated, treatment with 10% and 12.5% MG resulted in greater functionality as compared with DMSO at the same concentrations, and 10% glycerol ($p > .05$; Table 3).

4 | DISCUSSION

To the best of our knowledge, this is the first investigation testing a protocol using penetrative cryoprotectants for sperm cryopreservation in annual fish. All treatments with MG were found to be efficient in preserving almost all parameters related to sperm motility, which is a fundamental property for the reproductive success of the species (Cabrita et al., 2010; Pereira et al., 2019; Ribeiro et al., 2019). In fact, MG treatments at a 7.5% concentration showed the highest magnitude of positive results in terms of progressive motility, presenting up to three times better values than the other cryoprotectants, namely, DMSO and glycerol. Additionally, 7.5% MG treatment presented almost twofold higher values for average distance travelled (DAP) and average path velocity (VAP) than the other cryoprotectants. These findings are congruent with the reports of Ribeiro et al. (2019) for Suruvi (*Steindachneridion scriptum*), showing the efficacy of MG with BTS.

The overall efficacy of MG was further reinforced by the flow cytometric analysis for the parameters of sperm motility. The lower levels of DNA fragmentation and lipid peroxidation rates were also

associated with the 7.5% MG concentration. The expression levels for ROS were low across almost all treatments, which are significant given the fact that the low ROS production decreases the chances of membrane damage and consequently offers better membrane fluidity as well as mitochondrial functionality (Silva et al., 2016). Overall, most of the treatments performed similarly in terms of mitochondrial and membrane functionality, rates of cellular disruption and membrane fluidity.

Efficient cryopreservation of fish semen using MG was demonstrated in Piracanjuba (Maria, Viveiros, Freitas, & Oliveira, 2006). Derived from methanol (CH_3OH) and ethylene oxide (CH_2OCH_2; Viveiros, Oliveira, Maria, Orfão, & Souza, 2009), MG is considered a non-toxic alcohol (Pinheiro et al., 2016; Takagi, Otoi, & Suzuki, 1993). This explains its popularity as a favourite cryoprotectant in comparison to other alcohols like DMSO (Viveiros et al., 2011). Indeed, while comparing DMSO and MG at 10% concentration as cryoprotectants, better sperm motility results were reported for *Brycon insignis* with MG (77%–82%) as compared to DMSO (23%–46%). MG in concert with BTS also showed high-quality cryopreservation of semen in *Brycon nattereri* (Viveiros et al., 2012). Pinheiro et al. (2016), in a study using four different freezing media, observed that the MG-containing medium with glucose shows higher motility (70%–76%) as compared to MG and BTS-containing medium.

DMSO showed the highest sperm motility (66%) with the medium containing BTS + MG. This could possibly be attributed to the 80% glucose content in BTS that makes it a popular ingredient in cryopreservation protocols. In Suruvi (*Steindachneridion scriptum*), over 40% sperm motility was observed in samples frozen using BTS + MG (Pereira et al., 2019), and significant results were also reported in the same species using MG (Ribeiro et al., 2019).

DMSO is a common reagent in many bioassays at up to 10% v/v concentrations with no toxic effects (Galvão et al., 2014; Kais et al., 2013). DMSO as a cryoprotectant at 5%–15% concentrations in freshwater fish (Viveiros & Godinho, 2009) is considered one of the best cryoprotectants due to its efflux capacity and a less temperature-dependent process of cellular inflow than other cryoprotectants (Dziewulska & Domagała, 2013; Huang et al., 2018). In the present study, an intermediate 10% DMSO concentration performed well for almost all kinetic parameters with a lower efficacy profile at lesser concentrations. However, further investigations are required for the toxic effects of DMSO on fish in conditions of high concentrations or increased exposure time (Bozkurt, Yavaş, Bucak, & Yeni, 2019; Jang et al., 2017; Wakchaure et al., 2015).

In this study, glycerol presented the lowest values for all kinetic parameters evaluated for cryopreservation of *A. minuano* semen. The 7.5% glycerol concentration was shown to be the least toxic, with the highest kinetic parameter values, while 15% was more toxic. These findings are similar to a recent study by Bozkurt et al. (2019), where 10% glycerol presented better results as compared to methanol and DMSO. The limited efficacy of glycerol may be due to a toxic effect on the sperm cell. The presence of protein denaturation and changes in actin interactions within the spermatozoa tail may interfere with motility and, consequently, adversely affect fertilization (Shaliutina, Hulak, Gazo, Linhartova, & Linhart, 2013; Viveiros et al., 2011). Similar results were obtained for equine semen (Pace & Sullivan, 1975) and in Indian red jungle fowl (Blesbois et al., 2007; Rakha et al., 2016, 2017).

In different studies, similar results have been found on cryopreservation of semen with different cryoprotectants. We observed that for *A. minuano*, the cryopreservation presents a desirable level of motility. In other words, although low, the obtained levels of different parameters are significantly relevant when considering a species that is threatened.

In conclusion, we report the effective use of cryoprotectants for the cryopreservation of sperm from the annual fish *A. minuano*. Glycerol is recommended to be avoided for this purpose owing to its high sensitivity towards sperm. Further, our results show that the combination of BTS and 7.5% MG offers optimal results for both sperm kinetic and for cellular integrity and biochemistry parameters. We, therefore, suggest further investigations using different cryoprotectants with varying combinations of cryoprotectants of different molecular weights towards the goal of establishing a standardized cryopreservation protocol for this species.

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CONFLICT OF INTEREST

None to declare.

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