

Electric ultrafreezer ($-150\text{ }^{\circ}\text{C}$) as an alternative for zebrafish sperm cryopreservation and storage

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Abstract Zebrafish sperm cryopreservation is a fundamental methodology to manage and back-up valuable genetic resources like transgenic and mutant strains. Cryopreservation usually requires liquid nitrogen for storage, which is expensive and hazardous. Our objective was to evaluate if electric ultrafreezers ($-150\text{ }^{\circ}\text{C}$) are a viable alternative for zebrafish sperm storage. Zebrafish sperm was cryopreserved in the same conditions ($-20\text{ }^{\circ}\text{C}/\text{min}$), stored either in liquid nitrogen or in an ultrafreezer, and thawed after 1 week, 1 month, and 3 months. Sperm motility, membrane integrity, and fertilization ability were assessed. There were no significant differences in motility and hatching rate throughout storage time. Additionally, we aimed at understanding if cryopreservation directly in an ultrafreezer ($-66\text{ }^{\circ}\text{C}/\text{min}$) could improve post-thaw sperm quality. Freezing at $-20\text{ }^{\circ}\text{C}/\text{min}$ was performed

as before, and compared to samples cryopreserved with a fast cooling rate by placing directly in an ultrafreezer ($-66\text{ }^{\circ}\text{C}/\text{min}$). Sperm quality was assessed according to motility, viability, DNA fragmentation, and apoptosis (annexin V). The $-66\text{ }^{\circ}\text{C}/\text{min}$ cooling rate showed significantly higher membrane and DNA integrity, and lower number of cells in late apoptosis in comparison to the other treatments. This study showed that zebrafish sperm cryopreservation and storage in an ultrafreezer system is possible and a fast cooling rate directly in ultrafreezer improves post-thaw sperm quality.

Keywords Storage · Ultrafreezer · Zebrafish sperm · Cryopreservation · Cooling rate

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Introduction

Sperm cryopreservation is a useful tool applied in assisted reproduction in over 200 species (Tiersch et al. 2007). It constitutes a long-term storage technique that preserves structurally intact living cells (Tsai and Lin 2012). Cryopreservation usually requires liquid nitrogen for storage since it has high thermal stability in ultra-low temperatures ($-196\text{ }^{\circ}\text{C}$); however, it is expensive, with limited availability, can be hazardous for users, and carry the risk of cross contamination of the stored samples (Larman et al. 2014; Grout and Morris 2009). Consequently, the use of liquid nitrogen has been considered one of the most relevant bottlenecks for cryopreservation practical application (Larman et al. 2014; Yuan et al. 2016). Although mammalian tissue

cultures and microbial suspensions can be stored in $-80\text{ }^{\circ}\text{C}$ freezers (Polak and Pitombo 2011; Esteves-Ferreira et al. 2013), these conditions are not adequate for storage of gametes, since the traces of liquid water observed at this temperature are considered to be responsible for the low stability of samples stored under these conditions (Mazur 1984).

Nowadays, with the technological advances in the past years, electric ultrafreezer systems ($-150\text{ }^{\circ}\text{C}$) are easily available (Medrano et al. 2002; Álamo et al. 2005; Yavaş and Daşkin 2012) in the facilities dedicated to zebrafish research. Liquid water does not exist below $-135\text{ }^{\circ}\text{C}$ where diffusion rates are negligible (Mazur 1984); therefore, theoretically gametes could be stored indefinitely using these systems. Batista et al. (2009) summarize the advantages of electric ultrafreezer systems over liquid nitrogen storage. It has higher storage capacity, easier sample manipulation, unlike liquid nitrogen storage it does not require periodic reposition and the global costs of cryopreservation are more cost-efficient. The disadvantages of ultrafreezer are the limited mobility, which in zebrafish facilities is not required, and for cryopreservation the cooling rate is not programmable.

Altogether, electric ultrafreezers have the potential to be an alternative to liquid nitrogen for sperm cryopreservation and storage. There are few studies on sperm cryopreservation and storage in an ultrafreezer and the only reports found using this technique were in canine (Álamo et al. 2005; Batista et al. 2006), caprine (Batista et al. 2009, Medrano et al. 2002), and bull sperm (Yavaş and Daşkin 2012), reporting encouraging post-thaw quality results. However, there are no studies on this subject using sperm from teleost species.

Zebrafish is an important model species with increasing interest to the scientific community in the past years. Since the development of feasible genome editing technologies in the past two decades, such as Tol2 transposon and CRISPR/Cas9 (Suster et al. 2009; Liu et al. 2017), thousands of new mutant and transgenic strains were developed, posing problems in terms of facilities space and management, which cryopreservation can solve (Harvey et al. 1982; Cabrita et al. 2010; Robles et al. 2009). However, despite the fact that the first zebrafish sperm cryopreservation protocol was developed more than 30 years ago (Harvey et al. 1982), there is a lack of standardization of the methodologies (e.g., sperm collection and analysis, cryopreservation

procedure), which results in high variability on post-thaw sperm quality and in vitro fertilization success (Robles et al. 2009). The objective of this work was to evaluate if ultrafreezer is a viable alternative to liquid nitrogen for zebrafish sperm storage. Furthermore, we aimed to understand if a fast and simple cooling rate ($-66\text{ }^{\circ}\text{C}/\text{min}$) directly in an ultrafreezer is beneficial for zebrafish post-thaw sperm quality.

Material and methods

Fish rearing and sperm collection

Adult AB zebrafish males ($n = 110$) and females ($n = 363$) (6–8 months old) were selected as main broodstock, according to similar size and maintained separated by sex into 3.5 L tanks. Males were distributed in 10 aquariums, in a density of 11 males per tank. The 363 females were distributed in 33 aquariums at the same density. The fish were maintained in a ZebTEC® (Tecniplast, Italy) recirculation system. The fish room had a controlled photoperiod with a 14:10 h light/dark cycle, an independent air conditioning system ($26 \pm 1\text{ }^{\circ}\text{C}$) and an air extraction system to guarantee the air renewal in the room, maintaining the humidity close to 60%. The water rearing system was partially replaced (10%) daily and the water system maintained at $28.5 \pm 0.5\text{ }^{\circ}\text{C}$, $700 \pm 50\text{ }\mu\text{S}$ and pH 7.5 ± 0.1 . The fish were fed twice a day with *Artemia nauplii* (AF480, INVE, Belgium) and ZEBRAFEED® diet (Sparos Lda, Portugal) ad libitum. Food consumption was visually controlled, and the debris removed daily.

For sperm collection, males were anesthetized in 0.168 mg/ml tricaine sulfonate solution (MS-222) (Sigma-Aldrich) according to Westerfield (2000), rinsed with phosphate buffered saline (PBS) solution and abdominal massage was performed to collect the sperm, using a glass capillary tube attached to a mouth piece. The collected sperm from each individual (1 to 3 μL) was immediately diluted in 10 μL of sterilized and filtered (0.20 μm) Hank's Balanced Salt Solution (HBSS) (Jing et al., 2009; Hagedorn et al. 2012) and pooled after quality analysis and sample selection. The sperm samples were maintained at $4\text{ }^{\circ}\text{C}$ in the dark until analysis and cryopreservation was performed (no longer than 1 h).

Two sets of experiments were performed.

Experiment 1—effect of zebrafish sperm storage throughout time in an ultrafreezer (− 150 °C) and in liquid nitrogen

To understand the viability of zebrafish sperm storage in an ultrafreezer, we conducted an experiment where sperm samples from the broodstock established previously ($n = 110$), with total motility over 50% (at 10 s post activation) and cell concentration over 3×10^7 cells/mL were selected to perform 3 pools ($n = 12$), each pool contained sperm from 4 males. A control cooling rate ($- 20$ °C/min) (Yang et al. 2007) was applied to all samples in a programmable biofreezer (Asymptote Grant EF600, UK). Sperm was cryopreserved with final concentration of 10% N-N dimethylformamide (DMF) in HBSS (Asturiano et al. 2015), with a dilution rate of pre-diluted sperm to extender of 1:1, in a final volume of 10 μ L and stored in 2 ml cryovials (VWR® Low Temperature Freezer Vials). The samples were stored either in a liquid nitrogen tank (LN) or in an ultrafreezer (UL). Thawing was performed in a 40 °C water bath during 8 s (Yang et al. 2007). Samples ($n = 3$ pools) were thawed after 1 week, 1 month, and 3 months post-storage. Sperm quality was evaluated through sperm motility, membrane integrity and in vitro fertilization success.

Experiment 2—effect of a fast cooling rate (− 66 °C/min) on zebrafish post-thaw sperm quality

To understand if zebrafish sperm can be directly cryopreserved using a ultrafreezer system, an experiment was set up where sperm samples from the broodstock established previously ($n = 110$), with total motility over 50% (at 10 s post activation) and cell concentration over 3×10^7 cells/mL, were selected to perform 7 pools ($n = 35$). Each pool contained sperm from 5 males. The first treatment was a fast cooling rate of $- 66$ °C/min performed by placing the samples directly in the ultrafreezer. This method is not programmable, the cooling rate obtained was verified through a thermocouple (Hanna Instruments, USA). Two control treatments were performed, both had a $- 20$ °C/min cooling rate in a programmable biofreezer. However, one treatment was stored in a liquid nitrogen tank and the other in an ultrafreezer system. The samples were thawed in a 40 °C water bath during 8 s. Sperm quality was evaluated in

terms of sperm motility and membrane integrity. Additionally, other cell quality tests such as cell apoptosis (annexin V assay) and DNA integrity (Comet assay) were performed to ensure the viability of this process.

Sperm concentration and motility

Sperm concentration and motility was evaluated using computer-assisted sperm analysis (CASA) system (ISAS Integrated System for Semen Analysis, Proiser, Valencia, Spain) coupled to a phase contrast microscope (Nikon E-200, Nikon, Tokyo, Japan) with a x10 negative phase contrast objective. The images were captured with a Basler camera A312f (Basler AfC, Germany) and processed with CASA software. The settings of CASA system were adapted for this species. For sperm concentration, pre-diluted sperm (individual males and pools) was diluted 1:19 in HBSS and 3 fields were sampled to determine sample concentration. For motility analysis, 0.5 μ L of sperm was placed on a Mackler chamber and immediately activated with 5 μ L of filtered (0.20 μ m) and sterilized system water at 28 °C. Each pool was measured twice. Sperm motility was characterized at 10 s post-activation for each pool and treatment according to total motility (TM; %), progressive motility (PM; %), curvilinear velocity (VCL; μ m/s), straight-line velocity (VSL; μ m/s) and linearity (LIN; %). Only sperm samples with VCL > 10 μ m/s were considerate motile.

Membrane integrity

Sperm membrane integrity was assessed through flow cytometry using SYBR 14 (Invitrogen, Spain) and propidium iodide (PI) (Sigma Aldrich, Spain) labeling. SYBR 14 is a permeant nucleic acid stain that crosses plasma membrane and PI is a membrane impermeable dye that label cells with disrupted membrane. Cells with disrupted membrane are labeled in red from PI and viable cells are labeled in green from SYBR 14 (Daly and Tiersch 2012). SYBR 14 was prepared diluting 5 μ l of stock solution in 120 μ l of sterilized and filtered HBSS and PI was used undiluted. The pre-diluted sperm samples were re-diluted (1:200) in HBSS and each stain was added in a final concentration of 6.7 nM of SYBR 14 and 3.3 M of PI. Analysis were performed after 5 min of incubation in the dark at room temperature (21 to 25 ± 1 °C), in a flow cytometer (BD FACSCalibur™, BD

Biosciences, Spain) adjusted for the detection of SYBR 14 through a 530 nm bandpass filter (FL1) and PI was detected with a 670 nm long pass filter (FL3). Flow cytometer settings were previously adjusted using a positive (100% dead cells) and a negative control (fresh sperm). For negative control spermatozoa were exposed to cycles of freezing thawing (Cabrita et al. 2005). A total of 5000–10,000 events were counted for each sample.

Cell apoptosis

The Muse™ Annexin V & Dead Cell Assay (Thermo Fisher Scientific, Spain) analysis quantifies live cells, early and late apoptosis, and necrotic/dead cells by other mechanisms. The pre-diluted sperm samples were re-diluted (1:200) in HBSS with 1% of Bovine Serum Albumin (BSA) and the labelling was conducted according to manufacturer's specifications. Samples were acquired in a flow cytometer equipped with a 488-nm laser for excitation a 530/30 BP filter and a 690/50 nm BP filter for fluorescence emission. A total of 5000–10,000 events were counted for each sample. For the annexin V apoptosis tests, the total events were collected as the relation of forward scatter (FSC; cell size characterization) and side scatter (SSC; cell granularity) plots. The gating (R1) of sperm population was used to exclude non-sperm events and it was based on the FSC and SSC profile of zebrafish fresh sperm (Fig. 1a) The annexin V component has high affinity to the phosphatidylserine in the outer leaflet of the membrane in apoptotic cells and 7-AAD (7-amino-actinomycin D) is a dead cell marker. Early apoptotic cells are labeled with annexin V, late stage apoptotic and dead cells have both 7-AAD and annexin V labelling, necrotic/dead cells by other mechanism are labeled with 7-AAD and non-apoptotic cells (viable) are not labeled. This allows the detection of 4 subpopulations corresponding to viable cells (lower left, LL), cells in early apoptosis (lower right, LR), cells in late apoptosis (upper right, UR) and necrotic/dead cells by other mechanism (upper left, UL) (Fig. 1b). Flow cytometer settings were previously adjusted using a positive (100% dead cells) and a negative control (fresh sperm) where controls were incubated with each dye (annexin V or 7-AAD), separately and in combination.

DNA fragmentation

DNA integrity was evaluated through Comet assay adapted from Reinardy et al. (2013) with slight modifications. After sample thawing, 3 μ L of sperm was diluted in 60 μ L of low melting point agarose (0.5%), distributed into pre-coated slides with 0.5% of agarose (dried overnight) and covered with a coverslip 15 min at 4 °C. A positive control (2 μ L sperm + 2 μ L 100 μ M H₂O₂, incubated for 20 min at 4 °C) was set up to induce DNA fragmentation. The coverslip was removed, and the slides were incubated in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris and 1% Triton X-100) for 1 h at 4 °C. Subsequently, the samples were placed in an alkaline electrophoresis solution (300 mM NaOH and 1 mM EDTA, pH 13), 20 min followed by 20 min of electrophoresis (25 V, 280–300 mA). The slides were washed twice with neutralization solution (0.4 M Tris-HCl, pH 7.5) for 5 min and fixed in ethanol during 15 min. For slide observation, DNA was labeled with 10 μ L of PI (1 mg/mL) and slides observed at \times 600 in a fluorescence microscope (Olympus IX 81, Olympus, Japan) with blue excitation 450–480 nm. Images were captured and recorded with a digital camera (F-view, Olympus, Japan) and processed with the Cell^F image software (Olympus, Japan). At least 100 cells per slide were scored and then analyzed using Kinetic Imaging Komet 5.5 software (Andor Technology Ltd., United Kingdom). DNA fragmentation was expressed in terms of DNA in tail (%) since it relates the amount and size of the DNA fragments.

In vitro fertilization

Females used for in vitro fertilization were maintained in a breeding tank separated from males for 16 h previously to the experiments. Females were anesthetized with MS-222 rinsed with sterile PBS (pH 7.4) and placed in a 35 mm Petri dish. To collect the oocytes, an abdominal massage was carefully performed and if the clutch had good quality characteristics (Carmichael et al. 2009; Bobe and Labbé 2010), 100 μ L of AquaBoost® OvaCoat (Cryogenetics, USA) was immediately added to avoid oocyte dehydration and prolong oocyte fertilization ability up to 30 min. Only good quality clutches ($n=99$) with 100–200 oocytes were selected to test all treatments. For each sperm sample (fresh or thawed), in vitro fertilizations were performed immediately and simultaneously for all oocyte clutches

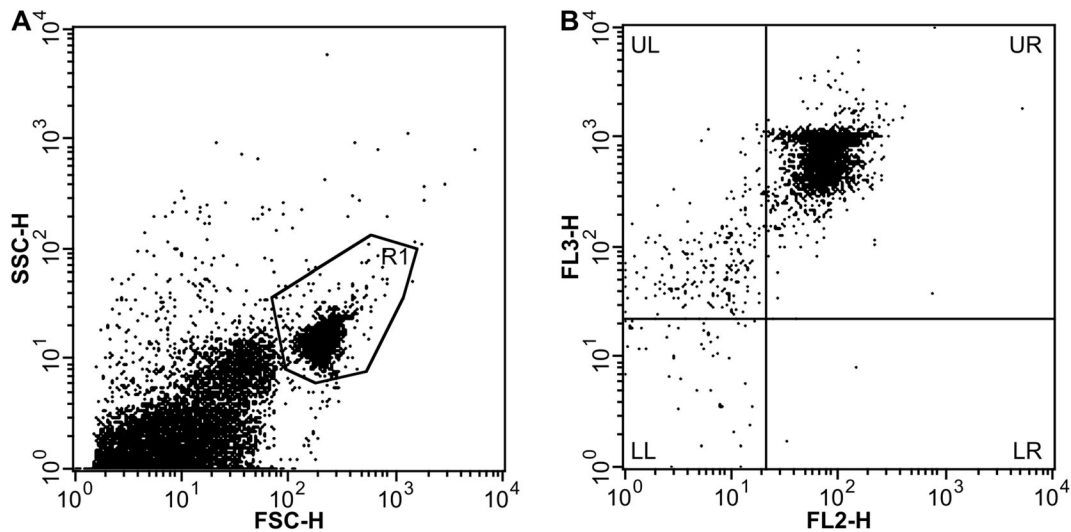


Fig. 1 Flow cytometry analysis. **a** Sperm cell population gating (R1) used for annexin V and plasma membrane integrity. **b** Annexin V sperm cell subpopulations corresponding to viable

cells (lower left, LL), cell in early apoptosis (lower right, LR), cells in late apoptosis (upper right, UR), and necrotic/dead cells by other mechanism (upper left, UL)

(3–6). The AquaBoost® OvaCoat was removed with a pipette before fertilization and 1×10^6 spermatozoa (Hagedorn and Carter 2011) of either fresh or post-thaw samples was added to the oocytes ($0.5\text{--}1 \times 10^4$ spermatozoa/oocyte) and immediately activated with 360 μL of sterilized and filtered (20 μm) system water at 28 $^{\circ}\text{C}$. After 5 min, 5 mL of system water was added to the Petri dish. The embryos were maintained in an incubator at 28 $^{\circ}\text{C}$ with the same photoperiod as in the zebrafish facilities (14 L: 10D). The fertilization rate was measured 3 h post-fertilization (3 hpf), at morula stage. All the dead embryos were removed, and the viable embryos transferred to 100 mm Petri dishes. Survival and hatching rates were calculated at 24 hpf and 72 hpf, respectively, according to the initial number of oocytes of each clutch. For each treatment and sampling point, each sperm pool was used to fertilize 3 to 6 clutches of oocytes. A total of 99 fertilizations were performed, where at least 13 fertilizations were done per treatment.

Data analysis

IBM SPSS Statistics 25.0 software was used for statistical analysis. Data were expressed as means \pm SD (Standard Deviation) and normalized by logarithmic, or arcsine transformation when results were expressed as percentages.

To check the robustness of obtained data within our sample dimension ($n = 3$), a hierarchical cluster analysis was applied to the data obtained in experiment 1. The Ward's method (Ward 1963) was applied since this methodology is appropriate for small samples. To apply Ward's method, the squared Euclidean distance was fixed computationally. This methodology is a mechanism of agglomerative hierarchical clustering procedure to classify pools, according to a multivariate perspective. All the variables were considered in this statistical treatment adjusted according to sperm motility, viability and in vitro fertilization measures. This analysis is represented through a dendrogram (Online Resource 1), labeled by pools of sperm, which resulted in a mixture per clustering group. When we consider a rescale distance cluster combined inferior to 5, we can observe 5 clusters of sperm pools with both storage methods. The cluster representation allows the observation of sperm pools groups formation without differentiation between LN and UF storage methods. This fact re-ensured the lack of differentiation per storage treatments, according to the considered variables (motility, viability and in vitro fertilization) and therefore one-way and two-way ANOVA was performed to compare storage time and treatments and *t* test to compare fresh samples and post-thaw samples.

The data of cooling rate experiment (Experiment 2, $n = 7$) was subjected to one-way ANOVA. Statistical

differences between treatments were detected by post hoc Student-Newman-Keuls (SNK) multiple comparison tests ($P < 0.05$).

Results

Experiment 1—effect of zebrafish sperm storage throughout time in a ultrafreezer ($-150\text{ }^{\circ}\text{C}$) and in liquid nitrogen

The pools of fresh sperm yielded an average of 55% of total motility (at 10 s post activation). As expected, the percentage of total motility, VCL, and VSL (Fig. 2a–c) of cryopreserved sperm was significantly lower when compared to fresh sperm. However, linearity was not significantly different between fresh and cryopreserved samples (Fig. 2d). Most importantly, there were no significant differences between treatments throughout

storage time in all motility descriptors (Fig. 2). In agreement with this information, we observed that membrane viability was not significantly different between samples stored in liquid nitrogen or ultrafreezer at each sampling point (Fig. 3). Nevertheless, we observed a decrease of membrane viability throughout time in both storage conditions, where membrane viability was significantly lower at 3 months post-storage when compared to 1 week of storage.

An average of 88% fertilization rate was obtained when using fresh sperm for in vitro fertilization (Fig. 4a) and it was not significantly different when using cryopreserved sperm. There was a decrease in the embryo survival at 24 hpf and in the hatching rate, when compared to the fertilization rate at 3 hpf. However, the hatching rate had similar values to the observed for survival at 24 hpf (Fig. 4b, c). There were no significant differences in the hatching rate and survival at 24 hpf between liquid nitrogen and ultrafreezer storage throughout time.

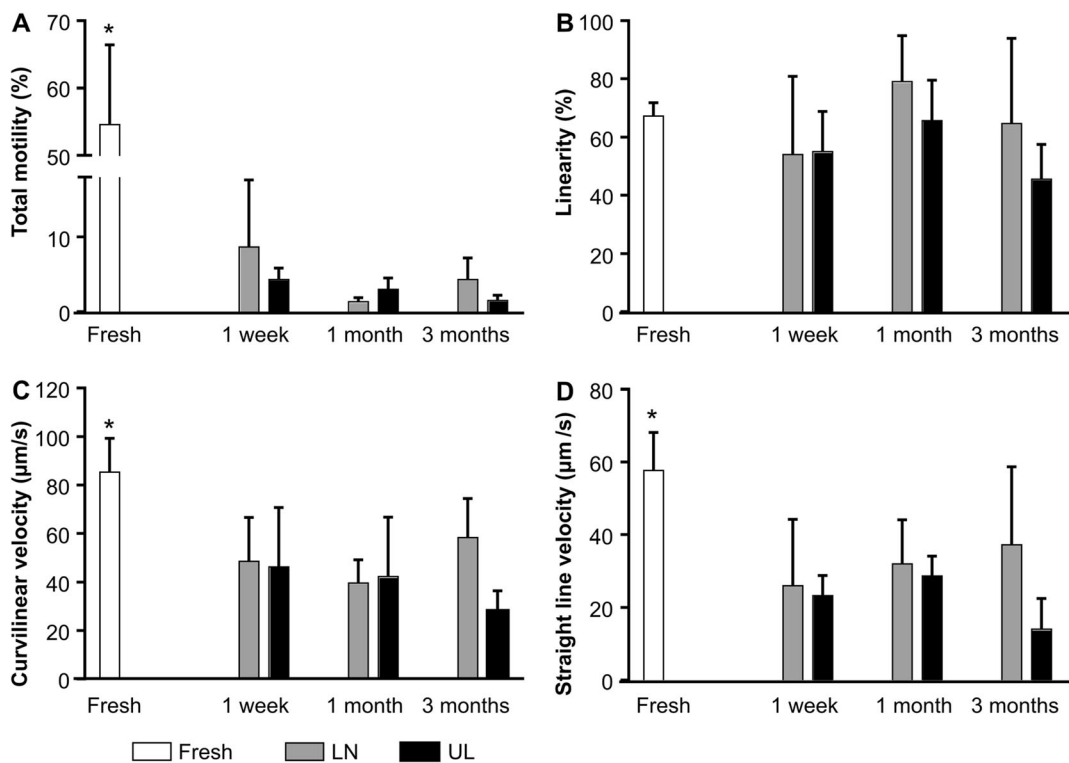


Fig. 2 Motility of zebrafish sperm ($n = 3$ pools) cryopreserved at $-20\text{ }^{\circ}\text{C}/\text{min}$ and stored in liquid nitrogen or in ultrafreezer. The samples were thawed 1 week, 1 month, and 3 months after storage and characterized in terms of **a** total motility (%), **b**

linearity (%), **c** curvilinear velocity ($\mu\text{m}/\text{s}$), and **d** straight line velocity ($\mu\text{m}/\text{s}$). Data is expressed as mean values \pm SD. Statistical differences (t test, $P < 0.05$) between fresh and cryopreserved sperm are represented with asterisk

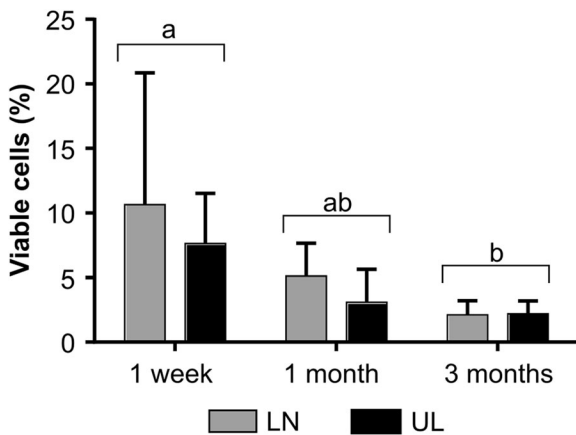


Fig. 3 Plasma membrane integrity of zebrafish sperm ($n = 3$ pools). Samples were cryopreserved with a $-20\text{ }^{\circ}\text{C}/\text{min}$ and stored in liquid nitrogen or in an ultrafreezer and thawed 1 week, 1 month, and 3 months after storage. Data is expressed as mean values \pm SD. Statistical differences within periods of storage (two-way ANOVA with post hoc SNK, $P < 0.05$) are represented with different letters. No differences were found between the two storage methods

Experiment 2—effect of a fast cooling rate ($-66\text{ }^{\circ}\text{C}/\text{min}$) on zebrafish post-thaw sperm quality

Sperm cryopreserved with a cooling rate of $-66\text{ }^{\circ}\text{C}/\text{min}$, by placing the samples directly in an ultrafreezer, had significantly higher total motility than sperm cryopreserved with a cooling rate of $-20\text{ }^{\circ}\text{C}/\text{min}$ and stored in ultrafreezer (Fig. 5a). Sperm cryopreserved with a $-66\text{ }^{\circ}\text{C}/\text{min}$ cooling rate did not show any significant differences in total motility when compared with sperm cryopreserved with a $-20\text{ }^{\circ}\text{C}/\text{min}$ rate and stored in liquid nitrogen (Fig. 5a). There were no significant differences between treatments in terms of progressive motility (PM) and velocity (VCL and VSL) (Fig. 5b–d), however there were no progressive cells in the sperm cryopreserved at $-20\text{ }^{\circ}\text{C}/\text{min}$ and stored in ultrafreezer. Sperm cryopreserved with a $-66\text{ }^{\circ}\text{C}/\text{min}$ cooling rate had no significant differences in linearity when compared to $-20\text{ }^{\circ}\text{C}/\text{min}$ followed by liquid nitrogen or ultrafreezer storage (Fig. 5e). The membrane viability was significantly

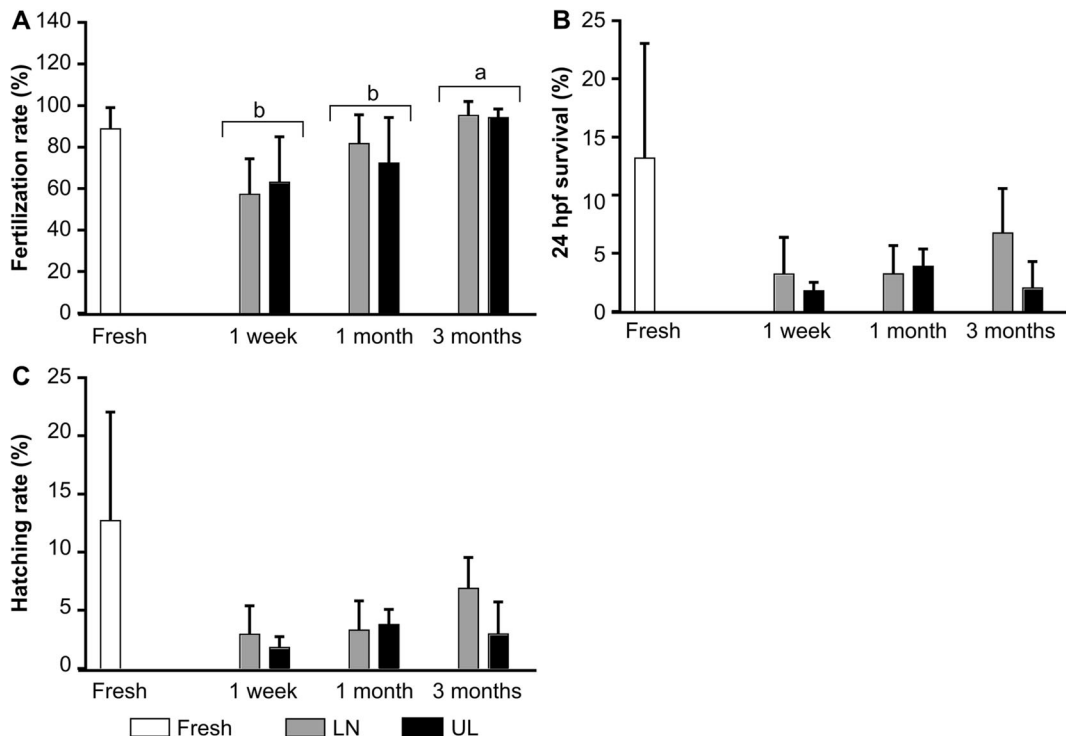


Fig. 4 In vitro fertilizations performed with zebrafish sperm ($n = 3$ pools) cryopreserved at $-20\text{ }^{\circ}\text{C}/\text{min}$ and stored in liquid nitrogen or in an ultrafreezer. The samples were thawed 1 week, 1 month, and 3 months after storage and the fertilization success was evaluated according to **a** fertilization rate at 3 hpf (%) (blastula stage),

b embryo survival (%) at 24 hpf, and **c** hatching rate (%) at 72 hpf. Data is expressed as mean values \pm SD. Statistical differences within periods of storage (one-way ANOVA with post hoc SNK, $P < 0.05$) are represented with different letters. No differences were found between the two storage methods

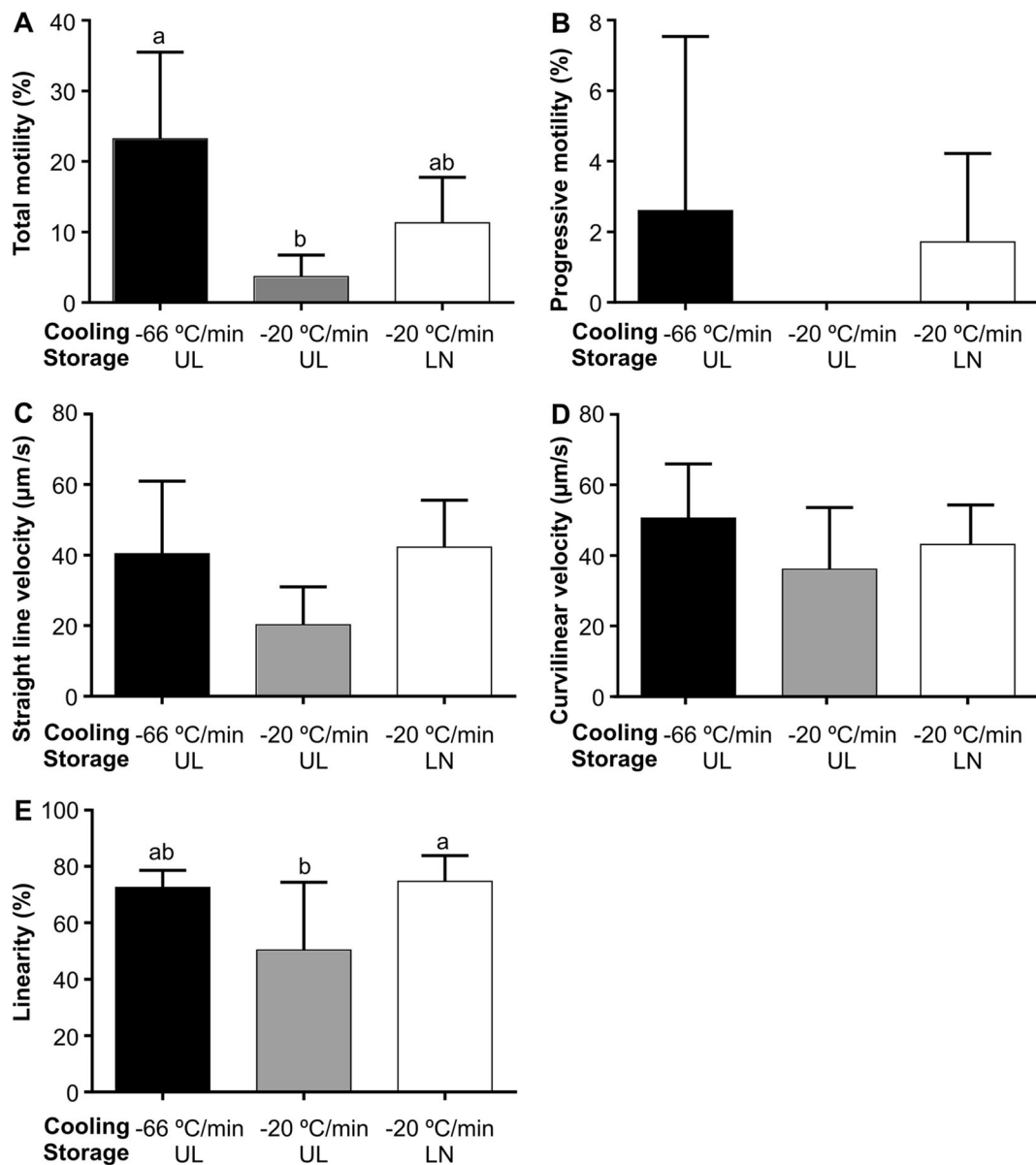


Fig. 5 Post-thaw zebrafish sperm motility ($n = 7$ pools) obtained from samples cryopreserved and stored directly in a ultrafreezer ($-66\text{ }^{\circ}\text{C}/\text{min}$, UL) and with a $-20\text{ }^{\circ}\text{C}/\text{min}$ cooling rate with storage in ultrafreezer ($-20\text{ }^{\circ}\text{C}/\text{min}$, UL) and liquid nitrogen ($-20\text{ }^{\circ}\text{C}/\text{min}$, LN). The samples were characterized in terms of **a** total motility (%), **b** progressive motility (%), **c**

straight line velocity ($\mu\text{m}/\text{s}$), **d** curvilinear velocity ($\mu\text{m}/\text{s}$), and **e** linearity (%). Data is expressed as mean values \pm SD. Statistical differences between treatments (two-way ANOVA with post hoc SNK, $P < 0.05$) are represented with different letters

improved in sperm with a $-66\text{ }^{\circ}\text{C}/\text{min}$ cooling rate when compared to the other treatments (Fig. 6a). Sperm cryopreserved with a $-66\text{ }^{\circ}\text{C}/\text{min}$ cooling rate showed significantly lower DNA fragmentation when compared to the other treatment conditions (Fig. 6b). The highest DNA fragmentation was observed in the $-20\text{ }^{\circ}\text{C}/\text{min}$ with ultrafreezer storage conditions. Sperm cryopreserved with

a $-66\text{ }^{\circ}\text{C}/\text{min}$ cooling rate had significantly higher viable cells (Fig. 7a) when compared to the other treatments as observed before, and significantly lower late apoptosis (Fig. 7b) when compared to the other treatments. However, $-66\text{ }^{\circ}\text{C}/\text{min}$ cooling rate with ultrafreezer sperm storage also had significantly higher number of necrotic/dead cells by other mechanisms (Fig. 7b) when compared to

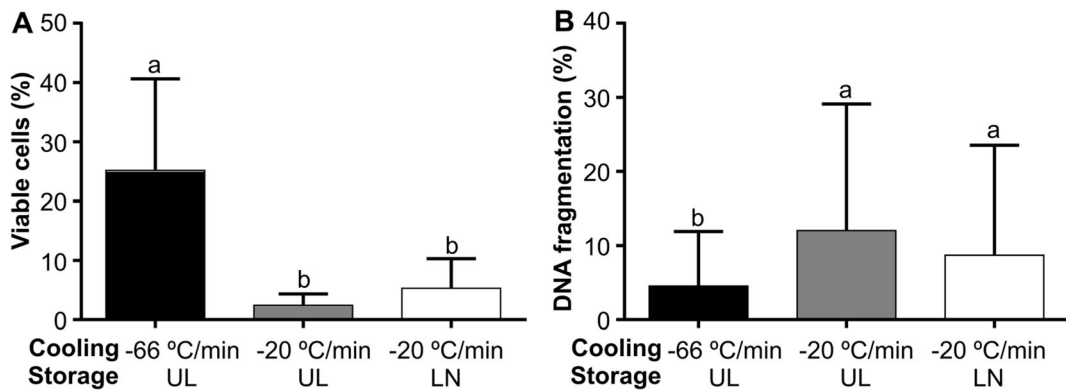


Fig. 6 Post-thaw zebrafish sperm analysis ($n = 7$ pools) of **a** plasma membrane integrity analyzed with flow cytometry and **b** DNA fragmentation detected through comet assay. The sperm was cryopreserved directly in an ultrafreezer (-66 °C/min , UL) or with a -20 °C/min cooling rate with storage in ultrafreezer

(-20 °C/min , UL) and liquid nitrogen (-20 °C/min , LN). Data is expressed as mean values \pm SD. Statistical differences between treatments (two-way ANOVA with post hoc SNK, $P < 0.05$) are represented with different letters

control method (-20 °C/min cooling rate and liquid nitrogen storage). Consequently, the main cause of cells death in cryopreserved samples with -20 °C/min was apoptosis, whereas in -66 °C/min cryopreserved samples was other cell death cause. There were no significant differences in early apoptosis values between treatments (Fig. 7a).

cryopreserved sperm from zebrafish lines in an ultrafreezer would be extremely practical and inexpensive in these types of facilities. Consequently, it was relevant to compare the effect of sample storage throughout time in liquid nitrogen (-196 °C) and in an ultrafreezer (-150 °C), to ensure the viability of the storage technique. There is no other characterization of post-thaw zebrafish sperm quality using different cryostorage periods besides the present study.

Discussion

To the best of our knowledge, this is the first report of cryopreservation and storage of teleost sperm using an ultrafreezer. The ability to store

Although all zebrafish sperm cryopreservation protocols have different methodologies, we selected the studies that used the CASA system to compare our data. While Yang et al. (2016) and Wang et al. (2015) started

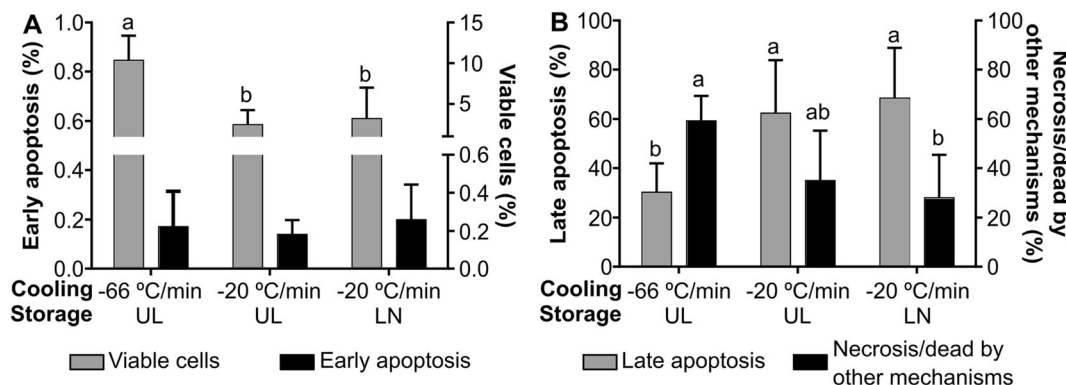


Fig. 7 Post-thaw zebrafish sperm ($n = 7$ pools) apoptosis detection through annexin V pathway of cryopreserved sperm directly in ultrafreezer (-66 °C/min , UL) or with a -20 °C/min cooling rate with storage in ultrafreezer (-20 °C/min , UL) and liquid nitrogen (-20 °C/min , LN). The sperm subpopulation were

analyzed in terms of **a** viable cells and cells in early apoptosis, and **b** cells in late apoptosis and necrotic or dead by other mechanisms. Data is expressed as mean values \pm SD. Statistical differences between treatments (two-way ANOVA with post hoc SNK, $P < 0.05$) are represented with different letters

with samples of fresh sperm ranging from 80 to 95% of motility, our fresh sperm pools had an average of 55% of motility, similar to previous data published by our group (Diogo et al. 2015). This discrepancy can be explained by the fact that our motility activation is performed with system water, set at 700 μS (13 ± 3 mOsm/Kg) for a correct approximation of the fertilization microenvironment conditions to avoid overestimations of motility, while previous studies used tap or distilled water, with lower osmolarity.

The post-thaw sperm motility registered by Wang et al. (2015) after 12 h of storage was $16 \pm 3\%$, whereas Yang et al. (2016) after 3 days of sperm storage obtained $28 \pm 15\%$ motility. In our first experiment, we obtained $8 \pm 8\%$ of motility after 1 week of storage. Although there were differences in methodologies and storage time since authors used different cryoprotectants, cooling rates and storage devices, it is obvious that the present study showed lower loss of motility after thawing (compared to the fresh sperm) when compared to these studies. This fact can be explained by the difference in the type of cryoprotectant used, since methanol and DMSO are known to affect negatively zebrafish sperm motility when compared to DMF (Hagedorn et al. 2012).

The analysis of membrane integrity by flow cytometry is considered a reliable method to evaluate sperm membrane viability at different conditions during storage (Figueroa et al. 2016). There were no significant differences between both storage techniques at each sampling point, which is in agreement with data reported for goat, canine and bull sperm (Álamo et al. 2005; Batista et al. 2006, 2009), where no significant differences were observed in motility and membrane integrity. However, there was an evident loss of membrane viability throughout storage time in both liquid nitrogen and ultrafreezer storage. Although it is generally accepted that life is on “hold” in liquid nitrogen, there are evidences of sperm quality loss over time of storage in human (Desrosiers et al. 2006) and bull sperm (Lessard et al. 2000). Lessard et al. (2000) observed a detrimental effect of storage time in the fertility marker P25b protein in bull sperm. The author hypothesized that physical vibrations at the interface between extracellular ice and plasma membrane during storage could explain P25b cryoelution. These mechanisms are still poorly understood; however, it is likely that the answer comes from the interaction of cell structures and cryosolvents. Buffers and extenders that provide different osmolarities

to the cells can interact with the isotonic cytoplasm and may influence sperm viability during cryopreservation and storage (Fuller 2004).

Although there are no universal sperm quality biomarkers, in vitro fertilization is considered one of the most reliable and integrative estimator of sperm quality, since it shows the sperm ability to fertilize the oocyte (Bobe and Labbé 2010). Yang et al. (2016) reported $62 \pm 14\%$ of fertilization rates, which is similar to the fertilization rate ($57 \pm 17\%$) after 1 week of storage reported in this study. Between 3 hpf and 24 hpf we observed a high degree of embryos abortion, predominantly in embryos with abnormal divisions observed initially. It has been previously described that non-motile post-thaw spermatozoa may obstruct the micropyle being forced to fertilize the eggs (Rurangwa et al. 2001), which can explain the high number of abortions and low hatching rates. It is also known that the oocyte has a mechanism that is able to repair, to some extent, spermatozoa DNA damage (Kopeika et al. 2004; Bobe and Labbé 2010). However, in teleosts it is reported that most embryos fertilized with damaged spermatozoa will not survive and abortion occurs between blastula and gastrula stages, where de novo gene expression starts to occur (Pérez-Cerezales et al. 2010). The fertilization rates can be calculated few hours after initial cleavage, however hatching rate is a more reliable parameter, although the results take more time to be obtained (Cabrita et al., 2009). Considering the in vitro fertilization results achieved and the fact that hatching rates were very similar to survival at 24 hpf, we propose that the survival at 24 hpf is the most simple and accurate method to evaluate sperm fertilization ability and progeny viability produced with post-thaw zebrafish sperm.

This study validated the possibility of zebrafish sperm storage in an ultrafreezer, a method that is simpler to apply in zebrafish research facilities. However, zebrafish sperm cryopreservation would be even more simplified if it could be performed by placing samples directly in an ultrafreezer system. Therefore, in our second experiment, we tested if a fast cooling rate of -66 $^{\circ}\text{C}/\text{min}$ performed directly in an ultrafreezer would benefit post-thaw sperm quality. The cooling rate is known to affect sperm survival and to interact with medium composition (Woelders et al. 1997). Fast cooling rates can reduce the time of cell exposure to the unfavorable conditions that result from ice formation and compromise cell viability (Woelders et al. 1997). In species from zebrafish family (cyprinidae) such as *Cyprinus carpio* (Bernáth et al. 2016) and *Perca*

fluviatilis (Bernáth et al. 2015), a fast cooling rate of -56 °C/min resulted in improved post-thaw sperm motility. Our results showed that -66 °C/min produced improved post-thaw sperm quality in terms of total motility, membrane viability, DNA integrity and late apoptosis events, when compared to the control cooling rate of -20 °C/min. Consequently, it seems that a -66 °C/min cooling rate in zebrafish sperm cryopreservation reduces cryodamage risks when compared to a slower cooling rate of -20 °C/min.

To perform a deeper post-thaw sperm quality characterization, we tested the effect of freezing and storage systems on zebrafish sperm DNA fragmentation and on the detection of plasma membrane phosphatidylserine externalization occurring during apoptosis mechanism. This apoptosis biomarker is a good candidate to measure damage induced by cryopreservation. Intrinsic apoptosis pathway is triggered by cell stressful factors such as radiation, toxins, hypoxia, hyperthermia, viral infections, and free radicals (Elmore 2007). Sperm cryopreservation induces intrinsic apoptosis pathway through cold exposure or free radical production. DNA integrity is a key factor in sperm quality and progeny viability (Bobe and Labbé 2010). The DNA fragmentation occurs late in apoptosis process after apoptosis inducing factors being translocated from the mitochondria to the nucleus, causing DNA fragmentation (Elmore 2007). Annexin V is a recombinant phosphatidylserine-binding protein that binds specifically to phosphatidylserine residues. The Annexin V & Dead cell marker combination used in this study enables the quantification of different populations of cells according to the type of damage: viable cells (non-stained), apoptotic cells (early or late apoptosis) and necrotic/dead cells by other mechanism. This last subpopulation has compromised plasma membrane integrity but do not show any phosphatidylserine externalization and, therefore, cell death can be attributed to mechanisms such as ice crystal injury. This subpopulation is significantly higher in sperm cryopreserved directly in the ultrafreezer ($59.27 \pm 10\%$), probably due to the rapid cooling which difficult water movement through the cell, allowing ice crystal formation that can cause plasma membrane disruption. However, this treatment also revealed the lowest values of late apoptosis and lower DNA fragmentation when compared to the other treatments. There are no references in the literature on zebrafish post-thaw sperm quality analysis in terms of apoptosis tests through annexin V and DNA damage. However, Reinardy et al. (2013) obtained 9–12% of

DNA damage in fresh zebrafish sperm, which is very similar to the values determined in our study using cryopreserved sperm (12.37% DNAt for -66 °C/min in ultrafreezer). Although slightly different values were obtained in cell viability determined by IP/SYBR 14 and the annexin-V dead kit, both methodologies are in agreement, sustaining that a faster cooling rate of -66 °C/min is more appropriate for zebrafish sperm when compared to -20 °C/min cooling rate.

These results show that sperm cells cryopreserved directly in an ultrafreezer present a decrease in cell apoptosis and DNA fragmentation, and that the main cause of cell death in this treatment have occurred through other mechanisms, such as the ones previously suggested.

In conclusion, our study demonstrates that ultrafreezers are a viable alternative for zebrafish sperm storage. Furthermore, a fast cooling rate of -66 °C/min performed directly in an ultrafreezer improved post-thaw zebrafish sperm quality. This study optimized the cooling rate of zebrafish sperm cryopreservation, which is an important contribution to support future methodological improvements. This methodology facilitates the cryopreservation process without the need of access to expensive programmable biofreezers and can be easily applied in zebrafish facilities, reducing the global costs of cryopreservation.

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