5-1-2024

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Recommended Citation

Arregui, L., Koch, J., & Tiersch, T. (2024). Transitioning from a research protocol to a scalable applied pathway for Xenopus laevis sperm cryopreservation at a national stock center: The effect of cryoprotectants. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution, 342* (3), 291-300. https://doi.org/10.1002/jez.b.23228

HHS Public Access

Author manuscript

J Exp Zool B Mol Dev Evol. Author manuscript; available in PMC 2024 May 15.

Published in final edited form as:

J Exp Zool B Mol Dev Evol. 2024 May ; 342(3): 291–300. doi:10.1002/jez.b.23228.

Transitioning from a research protocol to a scalable applied pathway for *Xenopus laevis* sperm cryopreservation at a national stock center: The effect of cryoprotectants

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Abstract

Sperm cryopreservation is a critical tool for safeguarding and managing valuable genetic resources. Protocols for cryopreservation of *Xenopus laevis* sperm were available but lacking sperm quality evaluation and scalability and the outcomes were inconsistent. The goal of this study was to begin developing a center-level cryopreservation pathway for this species by integrating French straws as containers that would facilitate germplasm repository development. The objectives were to analyze the effect of: (1) three sperm concentrations (33, 50, and 100 × 10^6 sperm/mL) on post-thaw fertilization, (2) three final concentrations (2.5%, 5%, and 10%) of dimethyl sulfoxide, methanol, and dimethylformamide (DMFA) on sperm membrane integrity of fresh and frozen samples, (3) two concentrations (5% and 10%) of DMFA with and without 5% sucrose at four cooling rates (5, 10, 20, and 40°C/min) on sperm membrane integrity and motility, and (4) egg exposure to different concentrations of DMFA on fertilization. Few differences in sperm viability were found among fresh samples incubated in cryoprotectants, but thawed samples frozen in methanol or DMFA presented higher membrane integrity. Samples frozen in 10% DMFA at 20°C/min showed higher membrane integrity (60 ± 7%) than other DMFA concentrations and cooling rates, and the same total motility (30 ± 7%) as at 10°C/min. Higher DMFA concentrations (10%–13%) were detrimental for embryo development compared to lower concentrations (<6%). This study provided a reliable protocol for sperm cryopreservation in *Xenopus laevis* to yield an application pathway with potential for high throughput that can be used as a roadmap for work with other species.

Keywords

amphibian; biobank; biomedical model; genetic resources; germplasm repository

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

1 | INTRODUCTION

National stock centers for biomedical models play a major role in supporting access of the scientific community to research animals by maintaining and distributing wild type, transgenic, inbred, and mutant lines. In the United States of America, five national stock centers hold the main aquatic biomedical models: the *Ambystoma* Genetic Stock Center, the National Resource for *Aplysia*, the National *Xenopus* Resource (NXR), the *Xiphophorus* Genetic Stock Center, and the Zebrafish International Resource Center. The requirements to produce and maintain an increasing number of transgenic and mutant lines of these species have led to a rapidly expanding need for cost-effective and efficient ways to keep and protect these genetic resources.

Storage of cryopreserved sperm in germplasm repositories, or biobanks, can provide a way to substantially reduce the number of live animals maintained, offers long-term preservation of valuable genetic resources, and allows reconstitution of lines on demand. A germplasm repository has multiple integrated components that involve processes and information, equipment, and personnel (Tiersch & Green, 2011). For historical reasons, these five existing aquatic animal stock centers have had to add and integrate cryopreservation components as practical pathways into their processes years after their foundation, although it would have been easier and cheaper to integrate them at the establishment of the centers. In addition, long-term needs and applicability must be considered when making the first decisions toward the development of cryopreservation pathways and repositories to avoid inefficient and non-scalable processes and waste of invested resources.

When working with valuable genetic resources such as emerging or minor biomedical models, imperiled populations, or commercially relevant species, it is necessary to consider these decisions in the early stages of repository development. These considerations are not only applicable to repositories where a specialized centralized facility processes and holds samples. Germplasm repositories can also be developed as aggregate collections where community members collect and preserve samples themselves, but the basic processes and requirements should at least be harmonized with the centralized repository (Liu et al., 2022). This mechanism of aggregate input would benefit minor and emerging models by offering protection of valuable germplasm, and allowing the sharing of concerns, experiences, and training resources. This would enable effective use of limited resources, and most importantly, would have a germplasm repository capability in place before the rapid expansion of lines and populations.

The NXR is an established national stock center for *Xenopus laevis* (and other *Xenopus* species), a powerful model system for studying vertebrate embryology, early development, and basic cell and molecular biology that has played a key role in numerous discoveries (Harland & Grainger, 2011). A complete genome sequence (Session et al., 2016) and high-efficiency gene editing have expanded the utility of *X. laevis* to a wide range of research areas such as genomics, neurobiology, toxicology, and to model human diseases. Each year more than 7000 papers are published using *X. laevis*, and millions of dollars are invested in maintenance, production, and distribution of animal lines for basic and translational research. In the past 7 years, the number of available transgenic lines has doubled to 300

(from *X. laevis* and *X. tropicalis*) and most are held in duplication as live populations at the NXR and the European *Xenopus* Resource Center (EXRC) (M. Horb, personal communication). Sperm cryopreservation is needed at the stock centers and is an important tool for the research community. At the stock centers, cryopreservation will reduce the maintenance required for the growing number of lines. In conjunction with this, almost all research laboratories using *Xenopus* species perform in vitro fertilization (IVF) routinely to produce embryos and will benefit from a reliable and standardized sperm cryopreservation process. Overall, cryopreserved sperm will reduce costs, simplify sample transport, and facilitate embryo production by minimizing the limits of operating time during IVF.

Methods for sperm cryopreservation of *X. laevis* sperm have been published (Mansour et al., 2009; Sargent & Mohun, 2005). These methods were compared by NXR and EXRC in a subsequent study (Pearl et al., 2017) and were unified to adopt and implement a protocol to backup *Xenopus* lines (Noble et al., 2022). However, this adopted protocol showed high variability in post-thaw fertilizing capacity, with ~30% of males failing to produce sufficient embryos to recover a line (Pearl et al., 2017). This inconsistency was proposed to be driven by differences among individual animals, but sperm quality was not assessed other than by fertilization, resulting in two concerns: (1) the effect of individual steps within the cryopreservation process is unknown and (2) the outcome is a combined consequence of sperm and egg quality. In addition, 2-mL cryovials were used for cryopreservation, changing the containers used in the original studies: hematocrit tubes (Mansour et al., 2009) and 0.25-mL straws and 500-μL Eppendorf tubes (Sargent & Mohun, 2005). We should also consider that, although useful, these methods were developed at a research scale without consideration of large-scale application at the stock centers, inhibiting throughput and limiting efficiency. Problems such as these are typically not initially recognized but become increasingly evident as larger numbers of animals and samples are processed and stored.

Cryopreservation, when applied within a pathway, represents fully integrated applied protocols that are efficient, easily learned, robust, and reproducible and, for biomedical models, cost effective and scalable (Tiersch, 2011). The first requirement to develop a cryopreservation pathway is to establish methods to assess sperm quality (or sample quality) throughout the steps of the process that can be integrated into a quality management program. Quality management is the combination of quality evaluation, quality control (data used to make decisions about the samples), quality assurance (approaches to maintain the quality of the process such as calibrating equipment and verifying reagents), and training (Torres et al., 2016). Sperm motility and membrane integrity are common quality evaluation parameters used to assess sperm quality in fresh and thawed samples in all classes of animals including amphibians (Germano et al., 2013; Shishova et al., 2011).

Following the development of quality evaluation methods, the first decision that should be made is to choose the container. There are a wide diversity of cryopreservation containers which vary in multiple characteristics such as geometry, surface area-to-volume ratio, materials, and thicknesses. As such, container choice can have substantial effects on the outcomes of processing and of cryopreservation. For example, the container and cryoprotectant affect the cooling rate due to physical–chemical properties, and, therefore,

the interaction of container, cryoprotectant, and cooling rate should be tested together. Plastic French straws (0.25 and 0.5 mL) are commonly used containers for sperm storage because they present several advantages over other containers, including their compatibility with existing automated equipment for high-throughput developed to support the livestock industry. In addition, open hardware offers inexpensive and standardized alternatives such as a freezing device compatible with French straws that yields reproducible cooling rates using a Styrofoam box with liquid nitrogen (Hu et al., 2017). Although straws were tested for sperm cryopreservation with *Xenopus* sperm in previous studies (Sargent & Mohun, 2005), they were not implemented by the centers (Noble et al., 2022).

Other factors such as sample concentration must be considered early in the development of a cryopreservation pathway but may be optimized later. Sample concentration affects several cryopreservation factors such as sample-cryoprotectant interactions, ice nucleation events, and sperm-to-egg ratios, and has shown effects on post-thaw motility and fertilization success in different animal classes (Alvarez et al., 2012; Dong et al., 2007; Dziewulska & Domagała, 2013; Iaffaldano et al., 2009).

After decisions for sample quality evaluation, container type, and sample concentration have been made, the role of cryoprotectants and cooling rates can be analyzed. Several cryoprotectants have been tested for cryopreservation of *X. laevis* sperm, such as glycerol, methanol, and dimethyl sulfoxide (DMSO). It was found that DMSO was less toxic, and when cooling at 20°C/min, DMSO showed better recovery of motility and membrane integrity than faster cooling rates (Mansour et al., 2009). Sugars and egg yolk have also been tested, with sucrose being chosen for the protection of motility when cooling at 10°C/min (Sargent & Mohun, 2005). However, dimethylformamide (DMFA) has never been tested in *Xenopus* species. In fact, DMFA was first used to cryopreserve hormonally induced sperm from an anuran in 2011 and has shown better recovery of motility and fertilization than DMSO (Shishova et al., 2011). Since then, DMFA has been used widely for cryopreservation of anuran sperm (Arregui et al., 2020; Burger et al., 2021; Hinkson et al., 2019).

Overall, the expanding number of novel mutant and transgenic lines has challenged the NXR to increase scalability and reliability of their current cryopreservation protocol and reduce costs and resource needs at the center. French straws provide an opportunity for NXR to address these needs. But, as indicated above, changing the container would involve stepping backward into the development of new integrated approaches and questions arise that should be addressed at the beginning of the development of a cryopreservation pathway: Which sperm concentration will offer the most advantages? What cryoprotectant type and concentration will work best for sperm? What cooling rate will be most suited for using that cryoprotectant? What is the effect of the cryoprotectant on fertilization? In this study, we address these questions and therefore the objectives were to analyze the effect of: (1) sperm concentration on post-thaw fertilization, (2) cryoprotectants on sperm viability, (3) cryoprotectants and cooling rates combinations on sperm quality, and (4) egg exposure to cryoprotectants on fertilization. In answering these questions, we aim to provide a useful roadmap and example to other resource centers and to emerging and minor model systems for the establishment of future germplasm repositories and cryopreservation pathways.

2 | RESULTS

2.1 | Experiment 1: Effect of sperm concentration on post-thaw fertilization

Sperm frozen at higher concentration (50×10^6 total sperm) used in IVF resulted in a higher percentage (20%–30%) viable embryos than sperm frozen at lower concentrations (25×10^6 or 17×10^6 total sperm) at any time period (NF8 $p \leq 0.01$, NF19 $p \leq 0.01$, and NF40 $p \leq 0.01$). There were no differences in percent viable embryos when using 17×10^6 or 25×10^6 total number of sperm ($p > 0.05$). For each sperm concentration, the average number of normally developing embryos decreased over time (from fertilization day to 3 days after fertilization with an average decrease of 15%–20%). The average number of eggs per Petri dish was 40.0 ± 12.7 ($n = 21$) (Figure 1).

2.2 | Experiment 2A: Effect of cryoprotectants on fresh sperm viability

Membrane integrity of fresh samples (72 ± 1.5%, $n = 3$) was higher ($p < 0.001$) than samples held in cryoprotectants for any analyzed time, but there were no differences between samples incubated for 15 min (66 ± 4%) and 30 min (65 ± 5%). Samples from one male were held in the cryoprotectants for 5 h on ice and membrane integrity remained unchanged (65 ± 4%). With fresh samples, there were no differences in membrane integrity among samples kept at different concentrations of methanol-based or DMFA-based cryoprotectants ($p > 0.05$, Figure 2a). Higher concentrations of DMSO (10%) presented lower sperm viability than lower concentrations of DMSO or any concentration of methanol or DMFA ($p \leq 0.031$, Figure 2a).

2.3 | Experiment 2B: Effect of cryoprotectants on thawed sperm viability

After thawing, differences in sperm viability among cryoprotectants and concentrations were more visible (Figure 2b). The low methanol concentration was the least effective for maintenance of membrane integrity ($p \leq 0.015$). No differences were found among samples frozen with different DMSO or methanol concentrations ($p > 0.05$). The addition of sucrose to DMSO or methanol showed no improvement in maintaining membrane integrity ($p > 0.05$). Contrary to DMSO and methanol, the addition of sucrose to DMFA reduced membrane damage during cryopreservation compared with the same concentration of DMFA without sucrose ($p < 0.001$). The highest concentration of DMFA (10%) was better for preserving sperm membranes than any concentration of DMSO ($p < 0.001$) but was not different to methanol (5 or 10%). Methanol (5 or 10%) showed no differences with DMSO (5% or with sucrose) or DMFA with sucrose ($p > 0.05$).

2.4 | Experiment 3: Effect of cryoprotectant and cooling rate on sperm viability and motility

All cryoprotectants and cooling rates showed recovery of more than 20% sperm with intact membranes. The statistical model showed that cryoprotectant composition, cooling rate, and their interaction were significant predictors of sperm membrane integrity, total motile sperm, and sperm moving forward ($p < 0.001$). Therefore, there was a different effect of cooling rate on sperm quality among cryoprotectants (Figure 3).

The fastest (40°C/min) and slowest (5°C/min) cooling rates showed lower preservation of sperm membrane integrity and motility. There were no differences in membrane integrity between 5% or 10% DMFA when cooling at 10°C/min ($p > 0.05$), while 10% DMFA presented more than 15% higher membrane integrity than 5% DMFA when cooling at 20°C/min ($p < 0.008$). Similarly, there were more sperm moving in samples frozen in 10% DMFA than in 5% DMFA when cooling at 10°C/min ($p < 0.001$) or 20°C/min ($p = 0.037$). Cooling in a higher DMFA concentration (10%) and at 20°C/min was better for maintaining membrane integrity than cooling at 10°C/min ($p < 0.006$). For motility, there were no differences between cooling at 10 or 20°C/min when using 10% DMFA ($p > 0.05$). The addition of sugar only increased the recovery of membrane integrity compared to the same concentration of cryoprotectant (5% DMFA) without sugar when cooling at 5°C/min. Sperm membrane integrity in fresh samples (72 ± 2%) was higher than that of the samples showing the best recovery of viability, that is sperm frozen in 10% DMFA and at 20°C/min (62 ± 3%, $t = 3.989$, $p < 0.05$, $n = 8$). Similarly, fresh samples presented a higher percentage of total motility (37 ± 3% vs. 30 ± 2%, $n = 8$) and forward motility (28 ± 4% vs. 18 ± 3%, $p = 0.04$, $n = 8$) than did thawed samples.

2.5 | Experiment 4: Effect of egg exposure to cryoprotectants on fertilization

Approximately 50% of embryos were viable 1 day after IVF (NF19) and 10% of the total number of eggs showed abnormal morphology at the NF40 stage (Table 1). Both, the composition of cryoprotectant and total number of sperm, were predictors of live embryos at NF19 and normal embryos at NF40 ($p \leq 0.06$). Methanol and the higher concentration of DMFA (10%–13.3% final concentration) negatively affected embryo survival compared with lower concentrations of DMFA ($p \leq 0.029$). All other treatments showed a minimum of 40% of normal embryo development at the NF40 stage. Cryoprotectants without sucrose presented similar percentages of viable or normally developing embryos compared with the same cryoprotectants with sucrose ($p > 0.05$). Fertilizing with double the total number of sperm increased the fertilization rate by 15% ($p = 0.02$). The number of eggs per petri dish was 42.3 ± 13.9.

3 | DISCUSSION

The process of sperm cryopreservation can be characterized as a series of interrelated steps (Mazur et al., 1970). As previously mentioned, sample concentration, container, cryoprotectant, and cooling rate must be examined together due to their interactions. If optimization of the process is performed independently at each step, when the steps are combined into a pathway there can be incompatibilities, unpredictable results, and an overall failure of the pathway. To protect valuable genetic resources for their community, the NXR and EXRC evaluated the available *Xenopus* cryopreservation methods (Pearl et al., 2017), and adopted a protocol for application in their stock centers. This protocol provided a foundation, but its outcome was inconsistent and was limited by the lack of quality evaluation and scalability as it was created to back up the scarce available lines at that moment. As such, there was a great need to reevaluate the cryopreservation protocol and raise it to the level of a pathway (which included quality management) to enable scalability and reduce sources of technical inconsistency.

This study began that process by selecting a new container, the French straw. This required a new round of protocol development that evaluated steps independently and synergistically. French straws were chosen as containers for sperm cryopreservation because they allow standardization of freezing and thawing rates due to their large surface area to volume ratio. Reducing the variation of freezing and thawing rates could help to minimize the technical variation causing the inconsistency found by NXR. In addition, straws enable manual and automatic sample processing (filling and sealing) permitting scalability. Finally, the use of space for storage and shipping is more efficient with straws than with other containers. Centers working with valuable genetic resources can learn from the NXR experience that scalability must be considered early in the development of the cryopreservation pathway. A process that starts with few males and few samples becomes more demanding with advances in research and technical knowledge. Similar observations and decisions were made at the Zebrafish International Resource Center before and during pathway development (Yang et al., 2022).

The sperm concentration chosen for cryopreservation affects many downstream decisions and is an essential consideration in an applied pathway; although it should be noted that sample concentration does not need to be optimized at the beginning of pathway development. Cryopreservation is a physical–chemical process and high sperm concentration can interact with this process at multiple levels such as sperm–sperm and sperm–cryoprotectant relationships. Low sperm concentration affects other aspects of cryopreservation, including reduction in efficiency and increase in costs. Standardizing sperm concentration before the addition of cryoprotectant and keeping a constant dilution ratio with cryoprotectants allows regulation of sperm–cryoprotectant ratios, reducing the technical variation in a protocol. The sperm concentration presented herein is not a final optimization. The sperm concentration chosen in this study was used to provide information on sperm quality to inform concentrations used in these experiments. Further analyses must be conducted to derive a sperm dose: a specific number of frozen sperm that can fertilize a specific number of eggs. This provides valuable information about the number of straws that need to be frozen to account for future use.

Just as sperm concentration is a vital consideration, cryoprotectant choice is as well. This includes the type and the concentration of cryoprotectant. In the present study, DMFA was chosen as the optimal cryoprotectant based on experimental results. DMFA was not used as a cryoprotectant agent for *X. laevis* sperm previously. Using DMFA as a cryoprotectant also resulted in practical advantages compared to the cryoprotectant used previously at the NXR. This cryoprotectant is simpler, composed of a single component compared to four components in the current NXR protocol. In addition, it is easier and faster to prepare and store because it does not contain egg yolk (Noble et al., 2022). Unlike previous publications (Mansour et al., 2009) *Xenopus* sperm were tolerant to incubation in cryoprotectants on ice, and methanol did not affect sperm membrane integrity more than other cryoprotectants. This study showed a higher recovery of sperm with intact membranes when frozen using DMFA (60%) than the protocol at the NXR (10%), and was similar to previous results with DMSO with sucrose (Mansour et al., 2009; Morrow et al., 2017). However, higher motility was found when combining DMSO with sucrose (46%) compared with DMFA (22%). Further studies are needed to assess the fertilization capability of sperm frozen with DMFA as the

relationship between sperm motility and fertility capacity can vary in anurans (Edwards et al., 2004).

The same conclusion could be made when the combined effect of the concentration and composition of cryoprotectant and cooling rate were simultaneously studied. A cooling rate of 20°C/min within DMFA was less detrimental for membrane integrity and was similar to 10°C/min for motility. Similarly, sperm mixed with DMSO and cooled at 20°C/min showed better retention of viability and motility than faster cooling rates (40 and 60°C/min; Mansour et al., 2009). Although membrane integrity was higher in samples cooled at 20°C/min than at 10°C/min, sperm motility was similar. The fertilization capacity of sperm samples cooled at 20°C/min in 10% DMFA should be tested to increase our knowledge of the role that motility after thawing plays as a predictor of fertilization in *X. laevis*. Meanwhile, cooling at 20°C/min could be selected for sperm of *X. laevis* because faster processing will be more likely to result in higher throughput and productivity at the stock centers. Potentially the elongated shape of sperm in amphibians could be playing an important role in increasing the surface area-to-volume ratio, allowing a faster dehydration than a spherical cell and a less harmful effect of a faster cooling rate. However, different results have been found in sperm quality after freezing with different cryoprotectants or concentrations (Mansour et al., 2009; Sargent & Mohun, 2005) supporting the idea that cooling rate should be assessed for specific cryoprotectants and concentrations.

Cryoprotectant plays an important role in sperm cryopreservation, but it also can affect eggs or the fertilization process. After thawing, sperm can be poured directly onto the eggs or it can be washed or diluted if needed when the cryoprotectant shows detrimental effects on gametes (Dong et al., 2006) or embryo development. This study analyzed the effect of short-term exposure to different cryoprotectant concentrations on the fertilization of eggs with fresh sperm. A concentration of 10% DMFA or <3% methanol was harmful during IVF and resulted in fewer viable and normally developing embryos when compared with 5% or 2.5% DMFA. Previous studies tested the effect of DMSO. When sperm was frozen in 5% DMSO with 73 mM sucrose diluted 1:2 (final DMSO concentration 1.7%) and used for IVF, a 48% hatching rate was observed (Mansour et al., 2009). However, in another study, sperm frozen in 15% DMSO and diluted 1:2 after thawing (final concentration 5%) presented no fertilization capability although sperm motility was observed after thawing (Buchholz et al., 2004). The toxic role of cryoprotectants during the fertilization process should be considered when developing post-thaw steps. The results presented herein suggest that sperm frozen in 10% DMFA (the concentration showing the best recovery of membrane integrity and motility) should be diluted at least 1:1 before IVF to avoid a negative effect on fertilization.

This study demonstrates the first steps in transitioning from a research protocol to a scalable pathway for *Xenopus laevis* sperm cryopreservation. The effect of sperm concentration, cryoprotectant type and concentration, and cooling rate were studied in conjunction with the effect of cryoprotectant on fertilization to develop a germplasm repository capability at a busy national stock center. These results will support studies necessary to establish a quality management program (Bodenstein et al., 2022; Hu et al., 2013). Also, these results represent a reliable sperm cryopreservation protocol for the *Xenopus* research community, but optimization of the effects of sperm concentration and fertilization conditions must

be considered. Development of germplasm repositories and cryopreservation pathways are generalizable to other centers and communities, but there must be addressed early and at multiple levels to integrate scalability, quality management, and efficiency. In addition, considerable attention should be given to the need for community-level standardization of protocols and reporting to enable direct comparison of research results and the transfer of practices as identified for motility analysis of fish sperm (Blackburn et al., 2022).

Other aquatic biomedical stock centers are in various stages of repository development: the *Xiphophorus* Genetic Stock Center and the Zebrafish International Resource Center began repository development more than 15 years ago (Huang et al., 2004; Yang et al., 2007), while the *Ambystoma* Genetic Stock Center and National Resource for *Aplysia* began their journeys in the last 3 years. Also, aquaculture-relevant species such as the Eastern oyster (*Crassostrea virginica*) have moved past protocol development and started the development of sperm repositories (Bodenstein et al., 2022; Yang et al., 2012, 2021). The experiences and lessons from the *Xenopus* stock centers and this study provide an important resource and guide for communities that want to develop cryopreservation pathways and comprehensive germplasm repositories to protect valuable genetic resources. These efforts will be strengthened considerably by integration with open hardware to improve access to cryopreservation capabilities maintaining reproducibility (Huene et al., 2022).

4 | MATERIALS AND METHODS

4.1 | Ethics and animal care

All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the Louisiana State University Agricultural Center. *Xenopus laevis* were purchased from Nasco and housed in recirculating tank systems at 18–19°C with a 12 h:12 h (L:D) photoperiod. Animals were provided commercial feed (Nasco frog brittle) three times per week, and water quality was measured weekly.

4.2 | Sperm collection and assessment of viability

Animals were euthanized by immersion in sodium bicarbonate buffered 0.4% tricaine methanesulfonate (Syndel) for 20 min followed by cervical transection (severing the spine). Testes were dissected and gently rolled on a paper towel to remove blood. Each testis was transferred to a 1.5- mL Eppendorf tube with 400 μL of ice-cold 1.2× Marc's Modified Ringer's solution (MMR; Noble et al., 2022), macerated using a plastic pestle (70 mm, Bio Basic) and both solutions were combined. This concentration of MMR (1.2× at 250 vs. 1× at 200 mOsmol/kg) was selected because sperm macerates in 1× MMR showed partial activation, often with >20% of sperm vibrating with some forward progression (<1%). Sperm concentration was estimated using a Neubauer hemocytometer after dilution in 1.2× MMR and quantified under phase contrast at ×200 magnification on an Optiphot-2 microscope (Nikon).

Sperm membrane integrity (e.g., an estimator of viability) was evaluated by dye exclusion by adding 5 μL of 0.4% eosin Y (Sigma-Aldrich) diluted in Simplified Amphibian Ringer (Browne et al., 1998) to 10 μL of sperm suspension. Two hundred spermatozoa were

classified using brightfield microscopy at ×400 (Microphot-SA, Nikon). Membranes were considered to be damaged when sperm exhibited a pinkish color, while those without color were scored as having intact membranes (Figure 4).

4.3 | Egg collection, IVF, and embryo evaluation

For induction of ovulation, females of *X. laevis* were subcutaneously injected with 5 IU of human chorionic gonadotropin (hCG, Sigma-Aldrich) per gram of body weight the night (12–18 h) before collection. Females were kept in individual containers overnight. Eggs were collected by gently massaging the abdomen and were expressed into the center of a 100 × 25 mm Petri dish. Sperm and eggs were mixed using a standard microbiology plastic inoculation loop modified into a needle by cutting the loop end off and tapering the end down to a blunt point with a piece of sandpaper. After mixing, sperm and eggs were left for 5 min, and the dish was flooded with 0.1× MMR. Embryos were held at room temperature (21–23°C) and 0.1× MMR was changed daily. Embryo development was estimated as the percentage of live embryos at approximately stage NF 19 (21–31 h after IVF, end of neurulation and initiation of embryo elongation), and the percentage of normal embryos at stage NF 40 (3 days after IVF, blood circulation visible in gills, some embryos floating face up in the surface; Zahn et al., 2022).

4.4 | Experiment 1: Effect of sperm concentration on post-thaw fertilization

Three sperm concentrations were tested to choose a working concentration for subsequent experiments. Both testes of three animals were collected, suspensions from both testes were pooled and evaluated as described previously. Fresh sperm concentration was adjusted to 67×10^6, 100×10^6, and 200×10^6 sperm/mL with 1.2× MMR. Sperm samples were diluted 1:1 with DMFA to a final concentration of 5% (all cryoprotectants in this study were prepared in 1.2× MMR) and 125 µL was loaded into 0.25-mL French straws kept on a rack on ice (2–5 min at 7°C), equilibrated for an additional 10 min at 4°C inside a programmable freezer (IceCube 14M, SY-LAB), and cooled at 10°C/min to −80°C. Frozen samples were held at −80°C for 5 min before transfer and storage in liquid nitrogen. After a minimum of 1 week in liquid nitrogen storage, samples were thawed by immersion in a 40°C water bath for 5 s, and the contents of each straw were expelled into 1.5-mL Eppendorf tubes kept on ice. Then, 100 µL of sperm were mixed with 100 µL of ice cold 0.1× MMR and IVF was performed and evaluated as described previously. The total numbers of sperm cells used per Petri dish were 17, 25, and $50 \times 10^{6.}$ Sperm from three males were used to fertilize eggs from one female, and sperm from two of the males were used with eggs from two additional females. Approximately 7 h after IVF, embryos not showing division at late morula and early blastula stages were removed by cutting the connections among embryos using surgical self-opening scissors (8 cm). Embryo development was evaluated as described previously.

4.5 | Experiment 2: Effect of cryoprotectants on sperm viability

Two experiments were performed to reduce the number of cryoprotectants and concentrations to be analyzed.

4.6 | Experiment 2A: Effect of cryoprotectants on fresh sperm viability

The effect of three cryoprotectants (DMSO, methanol, and DMFA) at three final concentrations (2.5%, 5%, and 10%) on sperm viability was tested. The addition of 5% sucrose to 5% DMSO, methanol, or DMFA was also evaluated. The right testes of three males were macerated in 1.2× MMR. Based on Experiment 1, sperm concentration was adjusted to 100×10^6 sperm/mL by adding the corresponding amount of 1.2× MMR. Aliquots of 30 μL of sperm were mixed 1:1 with all cryoprotectants (final volume of 60 μL and a final sperm concentration of 50×10^6 sperm/mL) and held on ice. Sperm viability was evaluated as described previously before the addition of cryoprotectants, and after 15 and 30 min of incubation with cryoprotectants on ice. In addition, samples from one male were evaluated after 5 h of incubation with cryoprotectants.

4.7 | Experiment 2B: Effect of cryoprotectants on thawed sperm viability

The left testes from animals used in experiment 2 ($n = 3$) were held in 1.5-mL Eppendorf tubes with 1 mL of 1.2× MMR in the refrigerator (4°C) until processing (~3 h after collection). Testes were macerated, and sperm concentration was evaluated and adjusted to 100×10^6 sperm/mL. Aliquots of 25 μL sperm were mixed 1:1 with all cryoprotectants (a final volume of 50 μL and a final sperm concentration of 50×10^6 sperm/mL) and loaded into 0.25-mL French straws. Straws were held on a rack on ice (10 min at 7°C), equilibrated for an additional 10 min at 4°C inside a programmable freezer (IceCube 14M, Sy-Lab) and cooled as described in Experiment 1. Straws were thawed after at least 10 days of storage in liquid nitrogen, and sperm membrane integrity was assessed within 3 min of thawing as described above.

4.8 | Experiment 3: Effect of cryoprotectant and cooling rate on sperm viability and motility

The effect of four cryoprotectants (5% DMFA, 5% DMFA + 5% sucrose, 10% DMFA, and 10% DMFA + 5% sucrose) and four cooling rates (5, 10, 20, and 40°C/min) was evaluated. Testes from six animals were used to test the four cryoprotectants and three cooling rates (5, 10, and 20°C/min) and testes from three additional males were used to evaluate the effect of the two faster cooling rates (20 and 40°C/min) with three of the cryoprotectants (5% DMFA, 5% DMFA + 5% sucrose and 10% DMFA). Both testes were collected from each male, suspensions from both testes were pooled, and sperm concentration was adjusted to 160×10^6 sperm/mL with 1.2× MMR. Samples were diluted 1:1 with all cryoprotectants and 100 μL was loaded into 0.25-mL French straws, held on a rack on ice (2 min at 7°C), equilibrated for an additional 10 min at 5°C and cooled and thawed as described in Experiment 1. Straws were thawed after at least 7 days of frozen storage. For evaluation of motility, 100 sperm cells in thawed samples without dilution were classified within 3 min of thawing from videos as displaying: forward movement, twitching movements but stationary, and nonmotile. The total motile sperm was calculated as the addition of sperm moving forward and twitching. Videos were recorded using α7S III (Sony). Sperm viability was assessed as previously described within 5 min after thawing.

4.9 | Experiment 4: Effect of egg exposure to cryoprotectants on fertilization

The effect of five cryoprotectants (initial concentration 5% DMFA, 5% DMFA + 5% sucrose, 10% DMFA, 10% DMFA + 10% sucrose, 20% DMFA, and 5% methanol) was tested. Cryoprotectants were loaded into French straws and frozen over liquid nitrogen. Then, straws were thawed as previously described and cryoprotectants were held in Eppendorf tubes on ice. Testes from three males were obtained and sperm concentration was adjusted to 60×10^6 sperm/mL and held on ice. Eggs from three females were collected as described previously. An aliquot of 100 μL of each cryoprotectant was added to the eggs and mixed with an inoculation needle. Then, 50 μL of fresh sperm (3×10^6 total number of sperm and 3.3%, 6.7%, and 13.3% final concentration of DMFA) were added to the eggs and mixed, and after 5 min the Petri dish was flooded with 0.1× MMR. In addition, sperm samples of two of the males were used to fertilize eggs from two females using an aliquot of 100 μL (6×10^6 total number of sperm and 2.5%, 5%, and 10% final concentration of DMFA).

4.10 | Statistical analysis

Data analyses were performed with IBM SPSS Statistics 28.0 for Windows. Generalized Estimating Equations (a generalized linear model for paired samples) was used to analyze results from all experiments and a pairwise comparison was performed using Bonferroni correction. For Experiment 4, a paired-samples *t*-test was also used to analyze the difference between fresh samples and the cryoprotectant and cooling rate showing the best retention of sperm quality. Normality was tested with the Shapiro–Wilk test. Data were expressed as mean ± SD, and $p < 0.05$ was considered significant. All figures were generated with RStudio (2022.12.0 + 353) (https://www.rstudio.com) with R (4.2.2) (https://www.R-project.org). The concentration of cryoprotectants was expressed in final concentration throughout the Results and Discussion.

ACKNOWLEDGMENTS

We thank the staff at the National *Xenopus* Resource (Dr. Marko Horb, Nikko-Ideen Shaidani, Carl Anderson, and James Parente) for their continued support of this project and for providing advice and discussion. This work was supported in part by funding from the National Institutes of Health, Office of Research Infrastructure Programs (R24-OD010441 and R24-OD028443), with additional support provided by the National Institute of Food and Agriculture, United States Department of Agriculture (Hatch project LAB94420), and LSU-ACRES (Audubon Center for Research of Endangered Species) Collaborative Program. The authors thank students at the AGGRC (Luke Turner, Lily Carter, Chandler Dunn, Victoria Byrd, and Alana Aidoo) for animal husbandry and Victoria Byrd for assistance during IVF experiments. This article was approved for publication by Louisiana State University Agricultural Center as number 2023-241-38948.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in https://github.com/LuciaArregui/Xlaevis_cryo_core.

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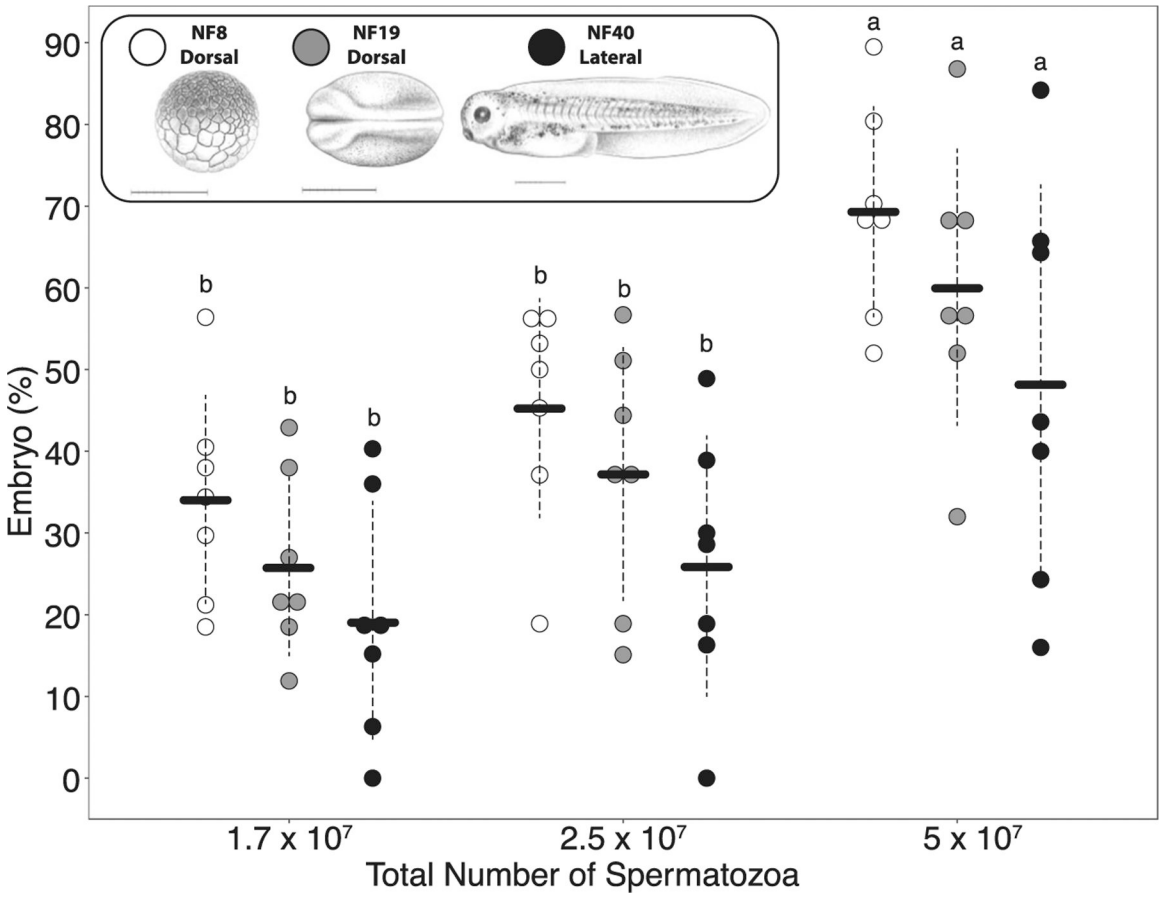

FIGURE 1.

Viable embryos at stages NF8 (white points) and NF19 (dark gray points) and normally developing embryos at stage NF40 (black points) after IVF with different total numbers of sperm. Mean ± SD are indicated with black bars and dotted lines, $n = 7$. Different letters indicate statistically significant ($\alpha = 0.05$) differences of the means among sperm concentrations at each developmental stage. *Xenopus* illustrations © Natalya Zahn (2022). Images reproduced from Xenbase (www.xenbase.org RRID: SCR_003280).

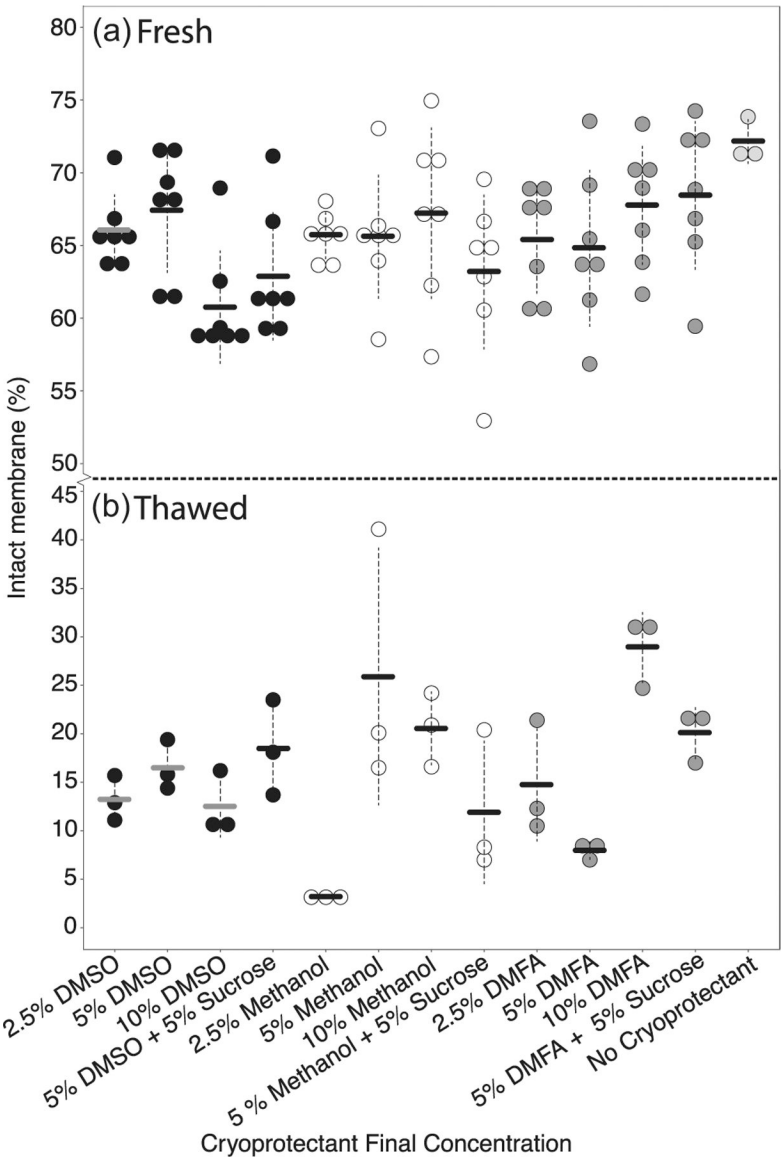

FIGURE 2.

Percentage of intact membranes of: (a) fresh sperm and (b) thawed sperm exposed to cryoprotectants. Control samples that were not exposed to cryoprotectants are represented in (a) ($n = 3$). Final cryoprotectant concentration is represented on the x-axis (after dilution 1:1 with sperm). Black points represent DMSO-based cryoprotectants, white points methanol-based, and dark gray points DMFA-based. Mean ± SD are indicated with black or gray bars and dotted lines. $n = 7$ for fresh sperm (samples exposed for 15, 30, and 300 min combined) and $n = 3$ for thawed.

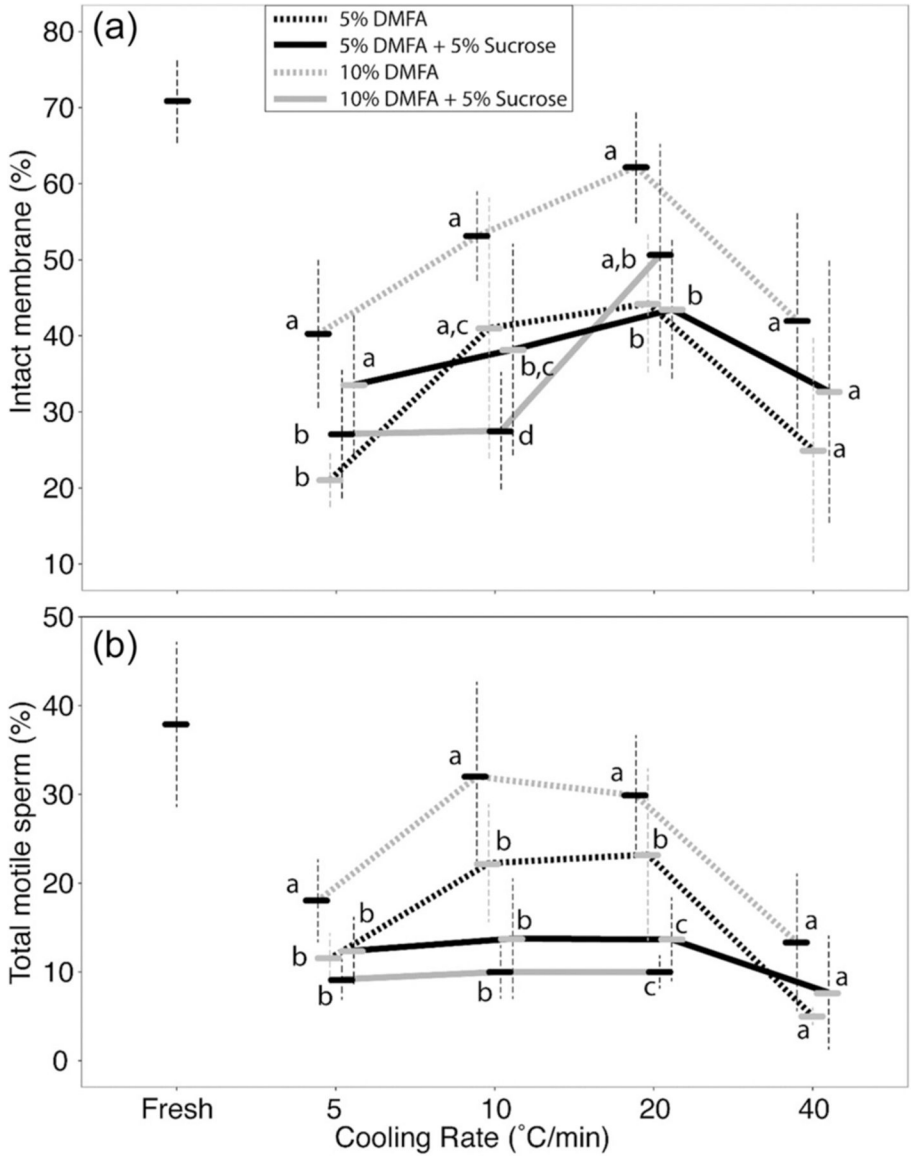

FIGURE 3.

Percentage of: (a) sperm membrane integrity and (b) total motile sperm for fresh and thawed conditions with different cryoprotectants and cooling rates. Different letters indicate statistically significant ($\alpha = 0.05$) differences of the means among cryoprotectants within a cooling rate. Black dotted lines represent 5% DMFA, black lines 5% DMFA + 5% sucrose, gray dotted lines 10% DMFA, and gray lines 10% DMFA + 5% sucrose. Mean ± SD are indicated with black or gray bars and dashed lines. Mean ± SD, $n = 6, 6, 8$, and 3.

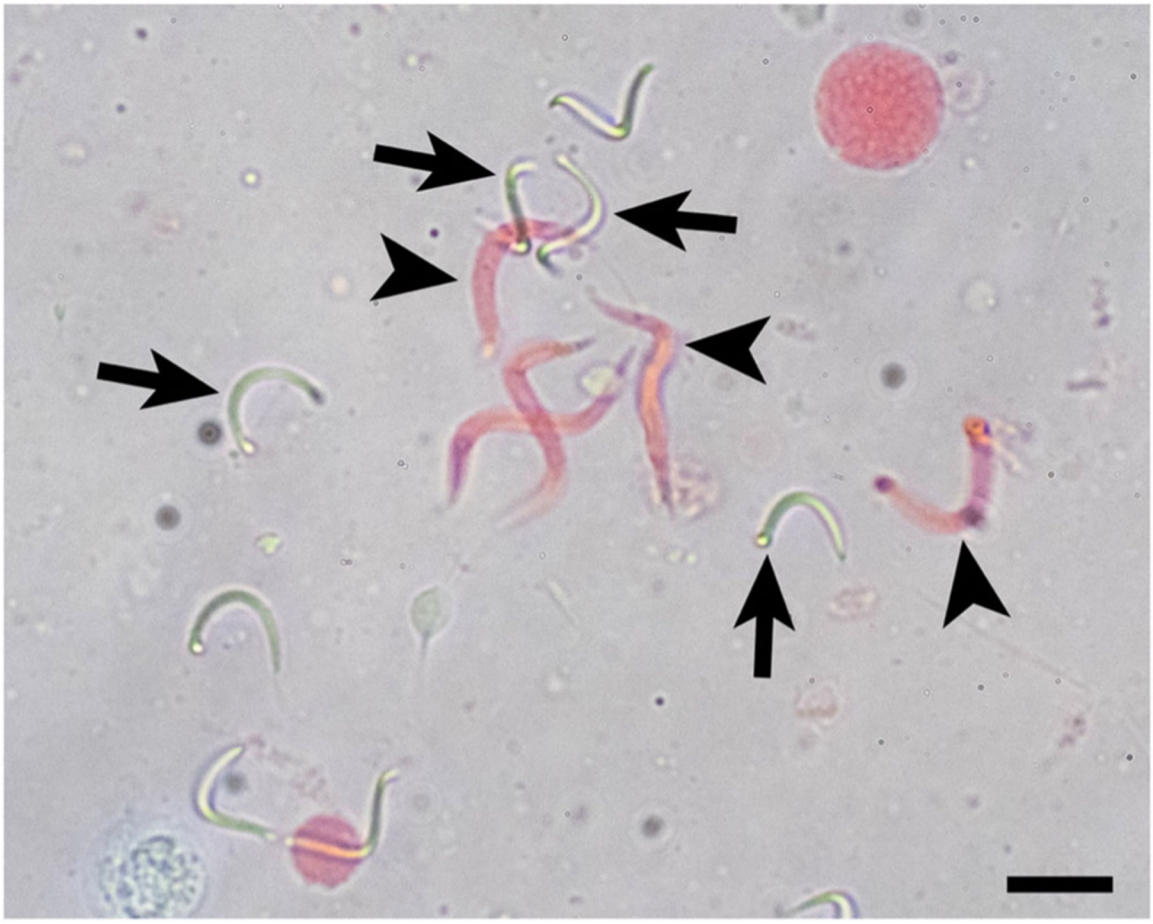

FIGURE 4.

Fresh sperm of *Xenopus laevis* obtained from macerated testes and stained with 0.4% eosin Y for assessment of membrane integrity. Sperm showing compromised membranes presented pinkish color and are indicated with arrowheads, while viable sperm were not stained and are indicated with arrows. Scale bar = 10 μm.

TABLE 1

Effect on fertilization of oocyte exposure to cryoprotectants.

	Cryoprotectant concentration			
TNS[a]	**Initial**	**Final**	**% alive NF19**	**% normal NF40**
3×10^6	10% DMFA	6.7% DMFA	66 ± 20 (92–38)[a]	44 ± 12 (69–31)[a]
3×10^6	10% DMFA + 10% Sucrose	6.7% DMFA + 6.7% Sucrose	48 ± 32 (96–5)[a],b	42 ±29 (91–4)[a],c
3×10^6	20% DMFA	13.3% DMFA	35 ± 23 (76–6)[b]	13 ± 9 (24–3)[b]
3×10^6	5% DMFA	3.3% DMFA	59 ± 26 (96–27)[a]	51 ± 26 (84–23)[a],c
3×10^6	5% DMFA + 5% sucrose	3.3% DMFA + 3.3% sucrose	60 ± 27 (95–25)[a],b	49 ±26 (90–15)[a],c
3×10^6	5% methanol	3.3% methanol	34 ± 33 (95–0)[b]	28 ± 31 (90–0)[b,c]
6×10^6	10% DMFA	5% DMFA	90 ± 8 (98–82)[a]	75 ± 10 (83–64)[a]
6×10^6	10% DMFA + 10% sucrose	5% DMFA + 5% sucrose	62 ± 14 (78–54)[b]	46 ±14 (59–31)[b]
6×10^6	20% DMFA	10% DMFA	41 ± 29 (72–15)[b,c]	29 ± 26 (55–3)[b,c]
6×10^6	5% DMFA	2.5% DMFA	73 ± 13 (81–59)[a]	47 ±23 (61–21)[a],c
6×10^6	5% DMFA + 5% sucrose	2.5% DMFA + 2.5% sucrose	82 ± 3 (84–78)[a]	70 ± 7 (75–62)[a]
6×10^6	5% methanol	2.5% methanol	21 ± 17 (40–6)[c]	14 ± 8 (22–6)[c]

Note: An aliquot of 100 μL of cryoprotectant (initial concentration) was added on top of recently expressed oocytes and mixed. An aliquot of fresh sperm (50 or 100 μL) was added, diluting the cryoprotectant to a final concentration. Mean ± SD (range), $n = 8$, for experiments using 3×10^6 sperm, and $n = 3$ for experiments with 6×10^6 sperm. Different letters indicate statistically significant ($\alpha = 0.05$) differences of the means among cryoprotectants within a sperm concentration.

[a]TNS, total number of spermatozoa (sperm concentration × sperm volume).