SPERM CRYOPRESERVATION FOR LIVE-BEARING FISHES OF THE GENUS *Xiphophorus*

Q. Dong, C. Huang, L. Hazlewood, R.B. Walter and T.R. Tiersch

1. INTRODUCTION

Swordtails and platyfish of the genus *Xiphophorus* are viviparous teleost of the family Poecilliidae. They are valuable models for biomedical research, especially for cancer genetics. This animal model was one of the first to prove that certain cancers are inherited diseases. In addition, they are also valued as ornamental fish for the aquarium trade because of vibrant body coloration and a long sword-like tail (in males). Species of this genus usually attain sexual maturity at 10-12 weeks of age [1]. The sperm of *Xiphophorus* fishes are different in structure (e.g., head shape) and physiology (e.g., energy metabolism) from the sperm of oviparous fishes. Sperm of internally fertilizing species possess atypical features such as well-developed mitochondrial sheaths in the midpiece of spermatozoa and glycolytic activity comparable to that of mammalian sperm, which may be adaptations for movement or long-term survival in the female reproductive tract.

The increased research and commercial value, and the continuous decline of diversity in the wild of these fish, expand the need to preserve their genetic resources. Despite study of sperm cryopreservation in some 200 species of freshwater and marine fishes, sperm cryopreservation has just begun in live-bearers including *Xiphophorus* [2]. Cryopreservation protocols have been developed to address their small body size and limited sperm volume (e.g., 5-10 µl per fish), and post-thaw motility as high as > 70% has been observed with *X. helleri* [3] and *X. couchianus* [4]. Recently, live young were produced with cryopreserved sperm from *X. helleri* (our unpublished data).

2. PROTOCOL FOR FREEZING AND THAWING

2.1 Equipment

- Sperm collection: 1 liter beakers, Petri dish, Kimwipes, stereomicroscope, tweezers, straight microscissors, styrofoam box with crushed ice, permanent marker, resealable, plastic bags, plastic goblet, 1.5 ml centrifuge tubes and top-loading balance.
- Motility estimation: micropipette and tips (10 µl), glass microscope slide, microscope with darkfield and 20 x objective.
- Straw filling: 0.25 ml straws, goblets, cane holders, PVC powder, paper towels, and filling tool (Fig. 1).

- Freezing: a controlled-rate freezer with accompanying low-pressure liquid nitrogen cylinder, liquid nitrogen storage dewar, and cryogloves.
- Sample shipping: shipping Dewar (CP35, Taylor-Wharton, Theodore, Alabama), plastic cable tie wraps, and tape.
- Thawing: Safety glasses, water bath with temperature controls (Model 1141, VWR Scientific, Niles, Illinois), long tweezers, scissors, paper towels, and 1.5 ml microcentrifuge tubes.
- Artificial insemination: 1 liter beakers, Petri dish, cotton batting, micropipette tips, scalpel, artificial insemination tool (Fig. 2), glassware, and 10 liter aquaria.

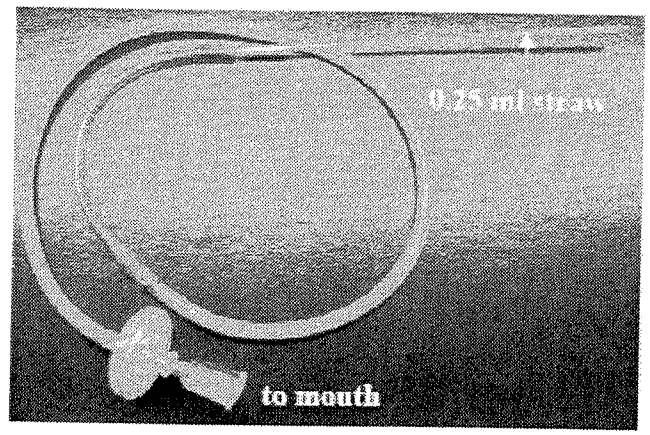

Figure 1 - Device used to fill straws.

2.2 Reagents

- Anaesthetic: 0.01% tricaine-methane sulfonate (Western Chemical Inc., Ferndale, WA)
- Hanks´ balanced salt solution (HBSS) at 300 mOsm/kg (8.0 g NaCl, 0.4 g KCl, 0.16 g $CaCl_2 \cdot x\,2H_2O$, 0.2 g $MgSO_4 \cdot x\,7\,H_2O$, 0.06 g Na_2HPO_4, 0.06 g KH_2PO_4, 0.35 g $NaHCO_3$, and 1.0 g $C_6H_{12}O_6$ in sufficient distilled water to yield a final volume of 1,000 ml)
- Cryoprotectant: glycerol

2.3 Semen collection

- Transfer the fish from breeding tank to 1 liter beaker and anesthetize the fish in 0.01% tricaine-methane sulfonate for 2 min.
- Dry the fish with Kimwipes and place them in a Petri dish.
- Carefully open the abdomen with the straight microscissors under a stereomicroscope and use tweezers to remove adherent tissue around the testis.

Mature testes usually have a milky appearance and are located above the gonopodial area.

- Use the tweezers to grasp the posterior tubular portion of the testes and remove them from the body cavity while taking care to avoid sperm release.
- Place the testes in resealable plastic bags, weigh, add sufficient HBSS based on a 1:10 ratio of testis weight to HBSS volume, seal the bag, and crush the testis with the plastic goblet to release sperm.
- Transfer the sperm solution from the plastic bag to 1.5 ml centrifuge tubes, and place it on crushed ice prior to use.

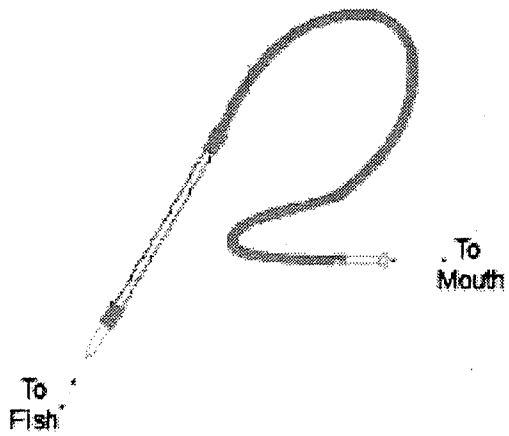

Figure 2 - Artificial insemination tool (*pthoto:* Steven Kazianis, Texas State University- San Marcos, with permission).

2.4 Sperm motility estimation

- Take 10 ml HBSS from a stock solution stored at –20°C and allow it warm to room temperature.* Evaluate the HBSS with microscopy for presence of bacterial contamination.
- Dilute samples to attain a concentration of 10^7 cells/ml before motility estimation. Generally a dilution ratio of sperm to HBSS (fresh samples) or HBSS-cryoprotectant (thawed samples) of 1:100 will yield the desired concentration.
- Equilibrate sperm suspensions in the centrifuge tubes at room temperature for 5 min before motility estimation for samples after thawing or refrigeration.
- Gently mix the sperm suspension with HBSS with the pipette tip and evaluate motility by estimating at 200 x magnification, using darkfield microscopy, the percent of sperm that are actively moving in a forward direction.
- Estimate the percent motility in increments of 5%. Samples with motility below 5% that have motile sperm are recorded as 1%.

*It is recommended to use the same activation solution throughout a single working season. HBSS can be aliquotted into small volumes (such as 10 ml) and stored at –20°C.

2.5 Freezing and thawing procedures

- Mix sperm suspensions with an equal volume of freshly prepared 28% glycerol (to yield a final concentration of 14%).
- Load the sperm suspension (80 µl) into 0.25 ml French straws with the filling tool (Figure 1). Place eight 0.25 ml straws into a 10 mm plastic goblet, and attach two goblets to a 10 mm aluminium cane. After equilibrating at 4 °C for 10 min, transfer the canes to the freezing chamber.
- Cool the samples from 5°C to –80°C at 20-25°C/min, and hold at –80°C for 5 min.
- Remove samples swiftly from the freezing chamber and immediately plunge them into liquid nitrogen in a storage dewar.
- Thaw samples after a minimum of 12 h of storage in liquid nitrogen.
- Remove individual straws from the 10 mm goblets with the long tweezers.
- Thaw in a 40°C water bath for 7 s. Wipe straws with a paper towel and cut the PVC powder sealed ends. Empty the straws into 1.5 ml microcentrifuge tubes by cutting the cotton plug end to release the contents into the tubes.
- Dilute the sperm suspension by adding an equal volume of HBSS immediately after thawing.
- Centrifuge the sperm suspension at 1000 g for 10 min at 4°C, remove the supernatant, and resuspend the sperm pellets with HBSS at the desired concentration.

2.6 Artificial insemination (AI):*

- Prepare the micropipette tips by cutting off most part of the fine end (leave ~ 2 m) with the scalpel at a 90° angle, and ensure that there are no rough edges.
- Anesthetize the virgin females in 0.01% tricaine-methane sulfonate for 2 min.
- Place the sedated female on the wet cotton pad, and position her so the urogenital opening (anterior to the anal fin) faces the operator.
- Pipette the thawed sperm suspension** into the micropipette tip and hold the solution in place (avoid air bubbles in the tip).
- Hold the female in place with one hand, and hold the artificial inseminator (Figure 2) with the other hand. Gently insert the plastic micropipette tip into the urogenital opening at a 45° angle.
- After encountering some resistance, push the micropipette tip a little bit further, and gently eject the sperm solution into the female.
- Acclimate the females in a glass beaker with equal volumes of water from the original tank and the new tank for 1 h before transfer into the new 10 liter aquarium.
- For fresh sperm controls, obtain sperm either by dissecting the testis as described above or by stripping and repeat the AI procedure.

*For detailed instructions see the website www.xiphophorus.org/ai.htm.

**Use sperm that is as concentrated as possible (10^9 cells/ml) to increase the success of AI.

3. GENERAL CONSIDERATIONS

In contrast to sperm of fishes that have external fertilization, sperm from *X. helleri* and *X. couchianus* can remain continuously motile for as long as two weeks when stored at 4°C.

Limitations of sperm volume can be overcome by pooling the milt from several fish, and by increasing the ratio of sperm-to-extender to 1:100 without significant loss of sperm motility. The use of an 80 ml loading volume in 0.25 ml straws would further help to maximize the sperm volume for evaluation of multiple parameters during protocol optimization process. Due to the need for specialized devices and technical skill to perform artificial insemination, and the fact that embryonic development in the female is not easily monitored, artificial insemination in live-bearers poses great challenges for post-thaw sperm quality assessment. One quick alternative method to evaluate the outcome of artificial insemination is to check embryonic development by dissecting females within 26-30 days after insemination. In general, thawed sperm is used for crosses with females of a different species (within the genus *Xiphophorus*), which enables verification that the offspring (hybrids) resulted from cryopreserved sperm rather than from sperm stored within the females from males (e.g., siblings) of the same species. Overall, the protocol presented here will yield high post-thaw motility for *X. helleri* and *X. couchianus*. To increase the success of artificial insemination, the use of concentrated sperm is generally recommended for fresh and thawed sperm samples. For thawed samples, sperm can be concentrated by centrifugation, which allows removal of the cryoprotectant solution and supernatant, and replacement with fresh HBSS at smaller volume.

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Complete affiliation:
Terrence R. Tiersch, Aquaculture Research Station, Louisiana Agricultural Experiment Station, Louisiana State University Agricultural Center, Baton Rouge, Louisiana 70820 USA. ttiersch@agcenter.lsu.edu.

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Edited by

Elsa Cabrita
Vanesa Robles
and Paz Herráez

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