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Effect of osmotic immobilization on refrigerated storage and cryopreservation of sperm from a viviparous fish, the green swordtail *Xiphophorus helleri* ☆

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Abstract

In this study, refrigerated storage and cryopreservation of sperm from the green swordtail *Xiphophorus helleri* were investigated. Previous cryopreservation research in this species utilized motile sperm because unlike in most fish species, *Xiphophorus* sperm can remain continuously motile after collection for a week with refrigerated storage. However, this species reproduces by internal fertilization, and given the significant requirements for motility within the female reproductive tract and potential limitations on sperm energetic capacities, immobilization of sperm prior to insemination could be used to improve fertilization success. Thus, the goal in this study was to use osmotic pressure to inhibit the motility of sperm after collection from *X. helleri*, and to test the effect of immobilization on refrigerated storage and cryopreservation. The objectives were to: (1) estimate the motility of sperm at different osmotic pressures, and determine an osmotic pressure suitable for immobilization; (2) cryopreserve the immobilized sperm, and estimate the motility after thawing with or without dilution, and (3) compare motility of non-immobilized and immobilized sperm after thawing, centrifugation, and washing to remove cryoprotectant. Motility was determined when sperm were suspended in 11 different osmotic pressures (24–500 mOsmol/kg) of Hanks' balanced salt solution (HBSS). Motility was observed between 116 and 425 mOsmol/kg. Sperm were not motile when the osmolality was lower than 116 or higher than 425 mOsmol/kg. Motility of the immobilized (non-motile) sperm could be activated by changing the osmotic pressure to 291–316 mOsmol/kg, and motility of immobilized sperm from hypertonic HBSS ($\geqslant$425 mOsmol/kg) was significantly higher than that from hypotonic HBSS ($\leqslant$145 mOsmol/kg) after 48 h of storage. At an osmolality of 500 mOsmol/kg, HBSS was used as extender to maintain immobilized sperm during cryopreservation with glycerol as the cryoprotectant. High motility (~55%) was obtained in sperm after thawing when cryopreserved with 10–15% glycerol, and dilution of thawed sperm in fresh HBSS (1:4; V:V) was found to decrease the motility significantly. No difference was found in the motility of thawed sperm cryopreserved with 14% glycerol and extended in 310 and 500 mOsmol/kg HBSS. Washing by centrifugation prolonged the motility of thawed

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sperm from 24 to 72 h in HBSS at 310 and 500 mOsmol/kg. This study showed that sperm from *X. helleri* could be immobilized by use of specific osmotic pressures, and that the immobilization did not affect sperm motility after thawing. The immobilization of sperm by osmotic pressure could minimize reduction of the energetic capacities necessary for insemination, traversal, and residence within the female reproductive tract, and fertilization.

Keywords: Sperm cryopreservation; Osmotic pressure; Green swordtail *Xiphophorus helleri*

Fish of the genus *Xiphophorus* (family Poeciliidae) currently include 26 species, categorized into three broad phylogenetic groups: platyfishes, northern swordtails, and southern swordtails [20]. Hybrids between selected species within this genus can be produced by natural mating or artificial insemination. Hybrid offspring often show distinct phenotypic characteristics that are valuable in the aquarium trade, and can spontaneously develop malignant tumors, or show susceptibility to induction of neoplasia [30,36,42]. Additionally, *Xiphophorus* are used in other research fields such as evolution [24,25], sex determination [18], endocrinology [4,21], ecology [34], and toxicology [44].

Members of the genus *Xiphophorus* possess many unique characteristics as models for genetic and biomedical research because of the spontaneous or inducible tumor formation among interspecies hybrids. A melanoma model of *Xiphophorus* was first established in 1928 by Gordon and Kosswig [1], and proved that certain cancers were inherited diseases. Since then, numerous studies have utilized *Xiphophorus*, and this interest has continued to increase in recent years. A microsatellite linkage map was created [43], specific strains have been developed [20], and inbred lines have been used to study gene structure and expression [6,13,19,23,31]. Currently, the *Xiphophorus* Genetic Stock Center (Texas State University, San Marcos, Texas), holds 25 *Xiphophorus* species and 64 pedigreed lines of *Xiphophorus*. Some strains have been maintained for more than 90 generations and represent clonal lines (see the website: www.xiphophorus.org). In addition, ornamental *Xiphophorus* strains with specific body coloration have been developed by commercial farmers (e.g., www.ekkwill.com). To maintain these valuable strains and models, gamete (sperm) cryopreservation is needed.

Sperm cryopreservation has been studied with different protocols in more than 200 fish species, such as salmon, catfish, and carp [22,40]. However, few aquarium fish, such as the genus *Xiphophorus*, which are characterized by small body sizes (2–5 cm) and microliter volumes of sperm, have been studied [41]. Currently, sperm cryopreservation studies in aquarium fishes have been mainly confined to zebrafish *Danio rerio* [8,12,29], medaka *Oryzias latipes* [2], and *Xiphophorus* [14–16], all of which are valuable research models [37]. In contrast to zebrafish, medaka, and most other fish species, *Xiphophorus* reproduce by internal fertilization and their sperm have specialized morphology, physiology, and characteristics. After release into the female genital tract, sperm can be stored alive for months before fertilization of mature oocytes [33], and these females may produce 4–5 broods after a single insemination [39]. These features make sperm cryopreservation and fertilization trials with cryopreserved sperm far more difficult in *Xiphophorus* than in those species that utilize external fertilization.

In previous studies on sperm cryopreservation in this genus, two species, *Xiphophorus helleri* and *Xiphophorus couchianus*, have been investigated [14,16]. High motility after thawing (40–78%) was obtained using glycerol as cryoprotectant and Hanks' balanced salt solution (HBSS) at 310–320 mOsmol/kg as the extender. However, initial fertilization trials with thawed sperm were not successful [7], and presented questions about the relationship of motility prior to insemination and subsequent fertility. Generally, the sperm of most fish with external fertilization are motile after release into the aquatic environment, and the motility persists less than 1–2 min [3]. Therefore, it is considered critical to prevent activation of sperm prior to use for fertilization for successful cryopreservation [40]. Osmotic pressure and specific ions in the extender (diluting solution) have been found to immobilize the sperm motility of these fishes [26], and have been widely used to extend sperm for cryopreservation. However, sperm from *Xiphophorus* fishes with internal fertilization are in many ways more like the sperm of mammals, in which motility and fertility are necessary within the female reproductive tract. Additionally, sperm of *Xiphophorus*

fishes are maintained in the female and fertilization can occur for several months after mating [32,33,38].

We hypothesized that immobilization of sperm by osmotic pressure could minimize pre-insemination reductions in the energetic capacity necessary for insemination and fertilization. Thus, the goal of this study was focused on immobilization of the sperm by osmotic pressure, and testing the effect of immobilization on refrigerated storage and cryopreservation. The objectives were to: (1) estimate the motility of sperm at different osmotic pressures, and determine osmotic pressures suitable for immobilizing sperm; (2) cryopreserve the immobilized sperm and estimate the motility after thawing with or without dilution; and (3) compare motility of non-immobilized and immobilized sperm after thawing, centrifugation, and washing to remove cryoprotectant.

Materials and methods

Sperm collection

Male *X. helleri* used in this experiment were shipped overnight from the *Xiphophorus* Genetic Stock Center in Texas State University (San Marcos, TX). A total of 10 *X. helleri* males were used in this study. The average total body length (mean ± SD) was 40.0 ± 6.0 mm, the average body weight was 1.33 ± 0.92 g, and the average weight for dissected testis was 12.5 ± 6.6 mg. Sperm were collected by crushing of the testis. Briefly, males were anesthetized in 0.01% tricaine methane sulfonate (MS-222, Western Chemical, Ferndale, WA), rinsed in fresh water, and blotted with a paper towel to dry the body. Before dissection, body length (from snout tip to the end of the tail) and body weight were measured. The testis was dissected at 10× magnification, separated from surrounding lipid tissues, and transferred to a 1.5-ml centrifuge tube for weighing. Hanks' balanced salt solution at 310 mOsmol/kg (HBSS310: 0.137 M NaCl; 5.4 mM KCl; 1.3 mM $CaCl_2$; 1.0 mM $MgSO_4$; 0.25 mM Na_2HPO_4; 0.44 mM KH_2PO_4; 4.2 mM $NaHCO_3$; 0.1% glucose, and pH of 7.2) was used as an extender unless other specific osmolalities were tested for experiments. The sperm were suspended by gentle crushing of the testis in HBSS, and the sperm suspensions were held on ice before use in experiments. Hereafter, HBSS of specific osmolalities such as 310 mOsmol/kg will be abbreviated as HBSS310.

Motility estimation

For estimation of sperm motility, 2 μl of sperm suspension were placed on a glass slide, 20 μl of HBSS310 (unless otherwise noted) was added to activate the sperm, and the motility was observed at 200× magnification using dark-phase microscopy (Optiphot 2, Nikon, Garden City, NY). The motility was expressed as the percentage of sperm that moved actively in a forward direction. Sperm vibrating in place were not considered to be motile.

Experiment 1: Sperm storage in HBSS with different osmotic pressures

A series of HBSS dilutions with different osmolalities was prepared by mixing HBSS900 with different ratios of double-distilled water to yield solutions of 24, 61, 116, 145, 211, 265, 310, 365, 425, 466, and 500 mOsmol/kg. Testes from three fish were dissected and used for this experiment. Sperm were released by crushing the testes without HBSS, and sperm suspensions were prepared by adding 0.2 μl of sperm to 20 μl of HBSS at the different osmolalities. Motility was evaluated within 20–30 s after sperm were suspended at the different osmolalities of HBSS, and after 3, 24, and 48 h of storage at 4 °C by placing 2 μl of sperm on a glass slide and viewing at 200× magnification.

Also, at 3, 24 and 48 h of storage at 4 °C, sperm at the different osmolalities of HBSS were evaluated by addition of 20 μl of HBSS310 in 2 μl of sperm suspension (as described above) on a glass slide. Sperm motility was estimated, and the osmolality of these mixtures was measured directly by placing 10 μl in a vapor pressure osmometer (Model 5520; Wescor, Logan, UT).

Experiment 2: Cryopreservation of sperm suspensions in HBSS500 with glycerol

Based on the results of Experiment 1, sperm suspended in 500 mOsmol/kg HBSS were cryopreserved with 5, 10, and 15% glycerol as cryoprotectant. Sperm from three males were used in this experiment. Sperm suspensions were prepared by crushing the testis in HBSS500 with a volume of 40 times the testis weight. This volume was separated into three parts, and each was mixed with an equal volume of diluted glycerol (prepared at double strength in the appropriate volume of

HBSS500) to yield final concentrations of 5, 10, and 15% (v/v) glycerol–HBSS. After 10 min of equilibration with glycerol–HBSS, the sperm suspensions were loaded into 250-μl French straws, and cooled from 5 to −80 °C at 20 °C per minute in a programmable freezer (Kryo 10 series II; Planer Products, Sunbury-on-Thames, UK). The straws were transferred to liquid nitrogen for 3-day storage.

To estimate motility, the sperm were thawed at 42 °C for 7 s in a water bath, and poured into 1.5-ml centrifuge tubes. To evaluate the effect of glycerol toxicity in the sperm suspensions after thawing, 20 μl of thawed sperm from each group was removed and mixed with 80 μl of fresh HBSS500, with the original glycerol concentrations of 5, 10, and 15% thus decreased to 1, 2, and 3%. Motility was observed (by activating 2 μl of sperm suspension in 20 μl of HBSS310) within 30 s after thawing, and after 1 and 24 h of storage at 4 °C.

Experiment 3: Comparison of HBSS310 and HBSS500 as an extender for sperm cryopreservation

In this experiment, testes from four fish were used. After dissection, the testis from each fish was separated into two lobes, with one-half suspended in HBSS310 and the other half in HBSS500 in a volume of 40 times the testis weight. Sperm suspensions in HBSS310 and HBSS500 were mixed with an equal volume of double-strength glycerol–HBSS (28%) to yield a final concentration of 14%, equilibrated for 10 min, loaded into 250-μl French straws, and cryopreserved using the programmable freezer at a cooling rate of 20 °C/min from 5 to −80 °C, and were stored in liquid nitrogen for 3 days.

The cryopreserved samples were thawed at 42 °C for 7 s in a water bath. From each sample, 100 μl of thawed sperm was placed in 1.5-ml centrifuge tubes and was centrifuged at 1000*g* for 5 min at 4 °C. The supernatant was discarded and the sperm pellet was resuspended in 100 μl of fresh replacement HBSS (either HBSS310 or HBSS500).

To compare the effect of HBSS310 and HBSS500 as extenders, the motility of fresh sperm, fresh sperm mixed with 14% glycerol, thawed sperm, and thawed sperm centrifuged and resuspended in HBSS310 and HBSS500 was observed (by activating 2 μl of sperm suspension in 20 μl of HBSS310) during storage at 4 °C at the following times: immediately, 0.5, 3 h, 1, 2, and 3 days.

Data collection

Data were analyzed using SYSTAT 11 (Systat Software, California, USA). The effects of treatments were tested by use of either *t* test or ANOVA. Percentage data were arcsine transformed before analysis. The significance level was set at $P < 0.050$ unless otherwise noted.

Results

Sperm motility and refrigerated storage in HBSS with different osmotic pressures

The sperm were stored as packets within the testis. After the testes were crushed without HBSS, the sperm packets were broken and the sperm were not motile. When these sperm were suspended in HBSS of different osmotic pressures, motility was observed at between 116 and 425 mOsmol/kg, and was inhibited at osmalities of lower than 116 and higher than 425 mOsmol/kg (Fig. 1). In solutions within the range of 211–365 mOsmol/kg, average motility was 87–90%. After storage at 4 °C for 3 h, the motility of sperm at most osmolalities did not change except for those at 211, 265, and 310 mOsmol/kg which dropped from 87–90 to 80–82% ($P \leqslant 0.023$). After 24 h of storage at 4 °C, the motility dropped to 30–40% ($P \leqslant 0.033$), and to 20–30% after 48 h of storage ($P \leqslant 0.010$). Interestingly, some sperm (<5%) immobilized at 466 and 500 mOsmol/kg HBSS became motile after 48 h of storage at 4 °C, and the movement of the sperm was slower than that stored at 211–365 mOsmol/kg.

After 3, 24, and 48 h of storage at 4 °C, sperm samples suspended in the different osmolalities were

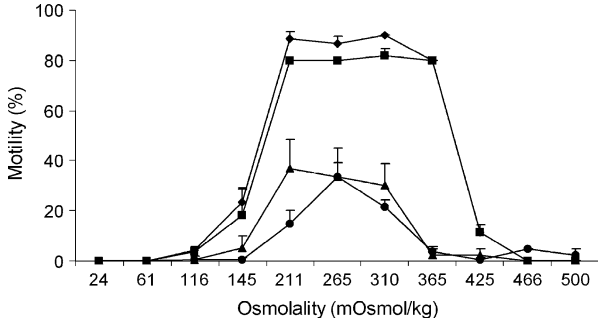

Fig. 1. Motility (mean ± SD) of sperm from *Xiphophorus helleri* ($n = 3$) in different osmolalities of Hanks' balanced salt solution for 0 h (diamonds), 3 h (squares), 24 h (triangles), and 48 h (circles) of storage at 4 °C.

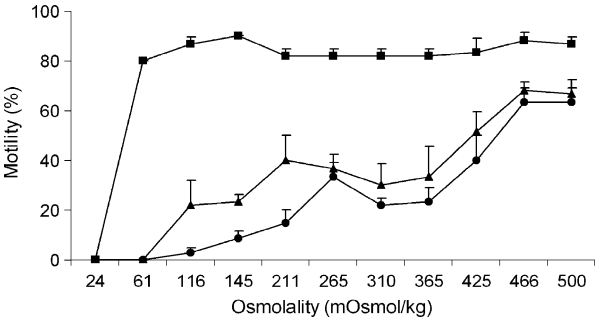

Fig. 2. Motility (mean ± SD) of sperm from *Xiphophorus helleri* ($n = 3$) when diluted in 310 mOsmol/kg Hanks' balanced salt solution (HBSS) after storage at different osmolalities of HBSS (24–500 mOsmol/kg) at 4 °C for 3 h (squares), 24 h (triangles), and 48 h (circles).

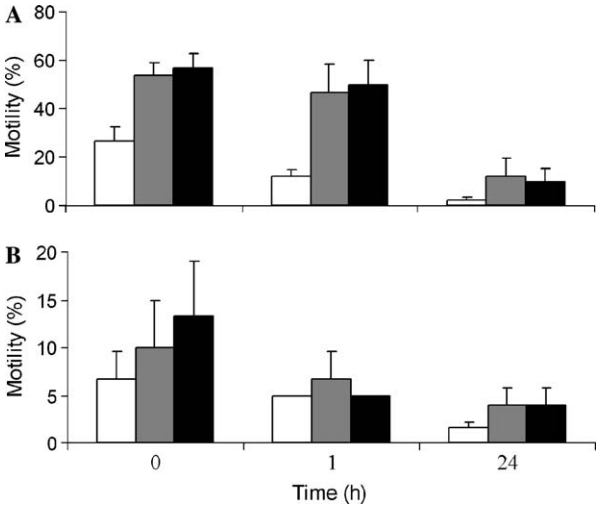

Fig. 3. Motility (mean ± SD) of thawed sperm from *Xiphophorus helleri* ($n = 3$) after suspension in 500 mOsmol/kg Hanks' balanced salt solution (HBSS500) as extender and cryopreserved using 5% (white), 10% (grey), and 15% (black) glycerol as cryoprotectant at a cooling rate of 20 °C/min from 5 to −80 °C. (A) Thawed at 42 °C for 7 s; (B) thawed and diluted with fresh HBSS500 at 1:4 volume ratio.

also diluted with HBSS310 (Fig. 2). The osmolalities of these mixtures after dilution were 292–318 mOsmol/kg. Except for the sperm held at 24 mOsmol/kg, motility of sperm immobilized in HBSS at osmolalities lower than 145 mOsmol/kg and higher than 425 mOsmol/kg was activated by the addition of HBSS310. After 3 h of storage at 4 °C, motility of sperm held at all osmolalites (except 24 mOsmol/kg) was above 80%. With additional storage time, the motility of the sperm initially stored in hypotonic HBSS ($\leqslant$145 mOsmol/kg) dropped to around 10–25% at 24 h and to less than 10% at 48 h. The sperm in 61 mOsmol/kg HBSS completely lost motility after 24 h of storage. However, the sperm initially held in hypertonic HBSS ($\geqslant$425 mOsmol/kg) showed 40–65% motility after 24 and 48 h of storage, which was significantly higher than the motility of sperm held in hypotonic HBSS ($\leqslant$145 mOsmol/kg) ($P \leqslant 0.004$). Moreover, the motility of sperm initially held at 466 and 500 mOsmol/kg was higher than that initially held at 211–365 mOsmol/kg after 48 h of storage at 4 °C ($P \leqslant 0.017$).

HBSS500 used as extender to cryopreserve sperm with different concentrations of glycerol

After thawing and being activated in HBSS310, the sperm cryopreserved with 10%, and 15% glycerol showed significantly higher motility (~55%) than that with 5% glycerol (27%) ($P \leqslant 0.006$), and no difference in sperm motility was found between 10 and 15% glycerol ($P = 0.519$) (Fig. 3A). After 1 h of storage at 4 °C, the motility of thawed sperm did not change ($P \geqslant 0.100$), but after 24 h of storage, the motility dropped to less than 3% ($P \leqslant 0.027$) in all the three glycerol concentrations.

When sperm were thawed and diluted in fresh HBSS500 at a ratio of 1:4, the motilities of sperm in 5, 10, and 15% glycerol were 7, 10, and 13% after being activated in HBSS310 (Fig. 3B); all significantly lower than the motility of thawed sperm without dilution ($P \leqslant 0.016$).

After suspension in 500 mOsmol/kg HBSS, the sperm were immotile in all replicates. However, sperm began to vibrate in place after mixing with glycerol at 5, 10, and 15%, which yielded final osmolalities of 1354, 1724, and 2608 mOsmol/kg, and after thawing, the sperm were also observed to vibrate in place in all three concentrations of glycerol.

Comparison of HBSS310 and HBSS500 as extenders for sperm cryopreservation

After sperm were suspended in HBSS310 and HBSS500, and cryopreserved with 14% glycerol, the motility was estimated for fresh sperm, fresh sperm with 14% glycerol, sperm after thawing, and thawed sperm washed once by centrifugation (Fig. 4). The motilities of fresh sperm in HBSS310 and HBSS500 were not significantly different within 24 h of storage at 4 °C. At 48 and 72 h of storage, the motility of

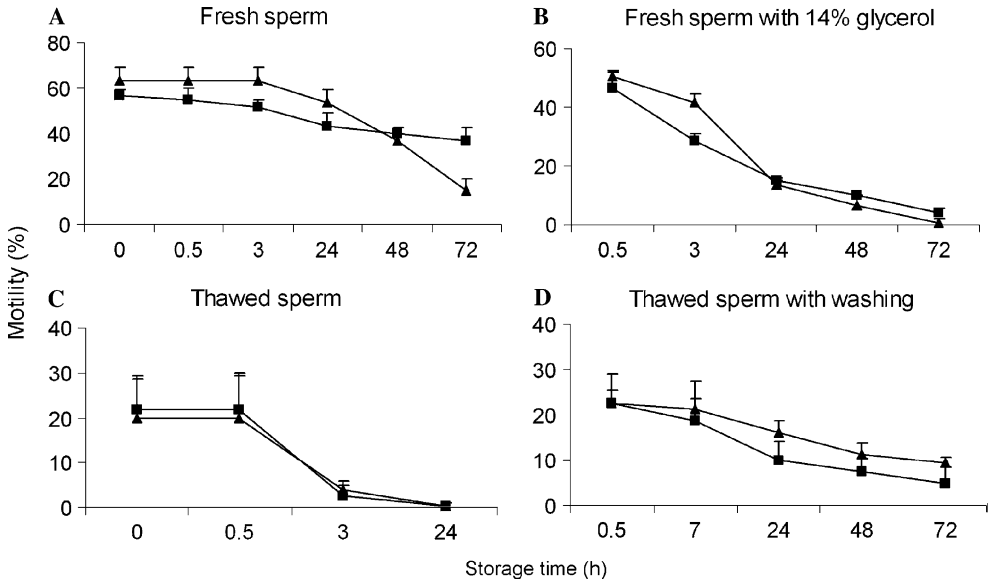

Fig. 4. Motility (mean ± SD) of sperm from *Xiphophorus helleri* ($n = 4$) when suspended in 310 (triangles), and 500 mOsmol/kg (squares) Hanks' balanced salt solution (HBSS) as extender. (A) Fresh sperm; (B) fresh sperm mixed with 14% glycerol; (C) thawed sperm at 42 °C for 7 s after cryopreserved with 14% glycerol at a cooling rate of 20 °C/min from 5 to −80 °C; (D) thawed sperm after washing once by centrifugation at 1000*g* for 5 min, and re-suspending in fresh HBSS.

sperm in HBSS310 and HBSS500 decreased significantly ($P \leqslant 0.005$). After 72 h of storage, the motility of sperm in HBSS500 was significantly higher than that of sperm in HBSS310 ($P = 0.006$) (Fig. 4A).

After mixing with 14% glycerol, the sperm in HBSS310 and HBSS500 showed similar motility at different times of storage at 4 °C (after activation in HBSS310) (Fig. 4B). No significant differences were found in motility of sperm in HBSS310 and HBSS500 ($P \geqslant 0.058$). At 3 h after mixing with glycerol, the motility of sperm decreased significantly ($P \leqslant 0.012$), and dropped to less than 5% after 72 h of storage at 4 °C.

The thawed sperm in HBSS310 and HBSS500 did not show significant differences in motility after activation in HBSS310 ($P \geqslant 0.417$) (Fig. 4C). There was no significant change in motility ($P \geqslant 0.974$) within 30 min after thawing for sperm cryopreserved in either HBSS310 or HBSS500. However, the motility of thawed sperm dropped significantly from 20–22 to 3% at 3 h after thawing, and to less than 1% at 24 h after thawing ($P \leqslant 0.017$).

The motility of thawed sperm washed by centrifugation was similar in HBSS310 and HBSS500 during 72 h of storage at 4 °C ($P \geqslant 0.061$) (Fig. 4D). In HBSS310 and HBSS500, the motility of thawed sperm after washing did not change during 7 h of storage ($P \geqslant 0.393$), but dropped significantly after 24 h ($P = 0.016$), 48 h ($P \leqslant 0.004$), and 72 h ($P \leqslant 0.003$) of storage. Compared with non-centrifuged thawed sperm (Fig. 4C), the washed sperm showed significantly higher motility after 24 h of storage ($P \leqslant 0.002$).

Discussion

Typically fish sperm are quiescent in the testis or in solutions isotonic to seminal plasma, and unless affected by the concentration of specific ions or other factors, the motility of sperm of freshwater fishes is initiated by exposure to hypotonic solutions, and that of marine fishes is initiated by exposure to hypertonic solutions [26,28]. This relates to the natural conditions in which sperm motility is inhibited by isotonic conditions in the seminal plasma in the testis and initiated by a decrease or an increase in the osmolality of the aquatic environment surrounding the sperm at spawning, and is a feature in most fish with external fertilization. However, in salmonid fishes, for example, low concentrations of potassium ion can override osmotic pressure to trigger motility [27]. Typically, once activated, fish sperm will remain motile for a short time (less than 2 min). Therefore, the inhibition of motility is needed when sperm are to be stored for artificial fertilization and cryopreservation.

In contrast to freshwater and marine fishes with external fertilization, fishes of the genus *Xiphophorus*

reproduce by internal fertilization. The activation mechanism of sperm motility is also distinct. First, the sperm are compacted into packets within the testis, and (as shown in this study) become motile when suspended in solutions isotonic to seminal plasma (300–320 mOsmol/kg), and become quiescent in either hypertonic (>425 mOsmol/kg) or hypotonic solutions (<116 mOsmol/kg). Second, the motility of sperm in solutions isotonic to seminal plasma can persist for 3–7 days of storage at 4 °C. Third, the motility of quiescent sperm in hypertonic and hypotonic solutions can be activated by changing the osmolality to that which is isotonic to seminal plasma. These characteristics observed for sperm of *X. helleri* may be common to other live-bearing fishes, and require further investigation for confirmation.

After being activated by changes in osmolality, immobilized sperm in hypertonic or hypotonic HBSS exhibited different behaviors with prolonged storage at 4 °C. At 3 h, the motility of activated sperm from hypertonic and hypotonic solutions was similar, but at 24 and 48 h, the motility of activated sperm from the hypertonic solutions was higher than that from the hypotonic solutions. Motility could not be activated in the sperm suspended in the most hypotonic solution (24 mOsmol/kg) after 3 h of storage. Motility of the sperm suspended at 61 mOsmol/kg could not be activated after 24 h of storage. These results suggest that hypotonicity is more harmful than hypertonicity for storage of *X. helleri* sperm. This agrees with studies in mammalian sperm in which hypotonicity was more harmful to sperm structure and motility than was hypertonicity, when diluted Ringer's solution caused the tails of bovine sperm to coil and cease motility [9]. In addition, motility of human sperm was substantially more sensitive to hypotonic than to hypertonic conditions [11]. In studies with marine invertebrates, sperm heads were observed to swell immediately after immersion in media hypotonic to seawater [26]. Swollen sperm heads and fragmented tails have also been noted in sperm of goldfish *Carassius auratus* [26]. Given the goal of this study was to use osmotic pressure to inhibit sperm motility, therefore, hypertonic media (500 mOsmol/kg HBSS) was chosen for use as a candidate for immobilization of sperm for further studies.

In previous studies on sperm cryopreservation in *X. helleri* [16], glycerol was selected as the cryoprotectant with a cooling rate of 20 °C/min from 5 to −80 °C. In this study, sperm were immobilized by suspension in HBSS500, and cryopreserved with three concentrations of glycerol at a cooling rate of 20 °C/min. High motility (~55%) was obtained in thawed sperm cryopreserved with 10 and 15% glycerol, comparable to the motility of thawed sperm (~75%) cryopreserved previously in HBSS310 [16]. When cryopreserved with 5% glycerol, the motility of thawed sperm was ~27%, significantly lower than that cryopreserved with 10 and 15% glycerol, but higher than the motility of thawed sperm (~15%) when HBSS310 was used as extender [16]. These results demonstrated that the immobilization of sperm did not affect the motility of thawed sperm after cryopreservation, and that HBSS500 could be used as extender to immobilize sperm for cryopreservation.

The immobilized sperm in HBSS500 were observed to vibrate in place when mixed with glycerol at final concentrations of 5–15%, in which the osmolality was increased from 500 to 1354–2608 mOsmol/kg, and these sperm were also observed to vibrate after thawing. When diluted in 310 mOsmol/kg HBSS for motility evaluation, the sperm showed normal forward swimming motion. These observations suggest that the motility activation of *X. helleri* sperm is not solely dependent on osmolality, and that other factors such as ions, or mechanical or chemical factors could also have functions. For example, in some marine teleost fishes, Ca^{2+} and K^{+} channels were found to be involved in the initiation of sperm motility [5]. Within fishes of the genus *Xiphophorus*, the reproductive style of internal fertilization has resulted in specialization of sperm structure and sperm physiology including the metabolism of carbohydrates [17,35], which are not observed in sperm of fishes with external fertilization. Such sperm characteristics in *X. helleri* may influence the mechanism of motility initiation and merit further investigation.

Generally most cryoprotectants, including in glycerol, are toxic to sperm. To decrease the toxicity of glycerol used in this study, thawed sperm were immediately diluted in fresh HBSS, but the motilities after dilution were significantly lower than those without dilution. This result was somewhat unexpected. The possible reasons include a rapid change in osmolality (from 1354–2608 to 599–860 mOsmol/kg), which caused sperm volume changes. In human sperm, high osmotic sensitivity and the presence of hyperosmotic injury have been extensively studied [10]. Human spermatozoa exhibited significant lysis and damage (i.e., loss of membrane integrity) when returned to isosmotic conditions after exposure to hyperosmotic solutions. In *X. helleri*, cyroprotectant

toxicity is especially important because of the reproductive style of internal fertilization, in which sperm are injected after thawing into the female reproductive tract, and are stored for weeks to months. Dilution or washing of cryoprotectant from thawed sperm may be essential for successful fertilization. The decrease in sperm motility caused by single-step dilution of thawed sperm indicates that this is not a good choice for lowering the toxicity of cryoprotectant.

In previous studies of sperm cryopreservation in *X. helleri*, motile sperm were evaluated in isotonic extender [16]. The suggested protocol in that study was: suspension of sperm in HBSS300, use of glycerol at a final concentration of 14%, and cooling at 20 °C/min from 5 to −80 °C [16]. With this protocol sperm were cryopreserved and high motility (~77%) was obtained. However, initial artificial insemination trials with the thawed sperm did not yield offspring [7]. To compare the differences of motile and immotile sperm in cryopreservation, parallel experiments were carried out in the present study by using HBSS310 and HBSS500 as extenders. No differences were found in motility of thawed sperm, with and without washing. This means the immobilization of sperm before freezing did not affect sperm motility after thawing. Also, the thawed sperm in this study were washed by centrifugation and resuspension in fresh HBSS to remove the cryoprotectant and concentrate the sperm. This step prolonged the sperm motility to 72 h of storage at 4 °C, while thawed sperm without washing lost motility within 24 h of storage at 4 °C. This indicated that washing (centrifugation and resuspension of cells) could be a good choice for decreasing the concentration and toxicity of cryoprotectant and increasing sperm concentration in thawed samples prior to injection into the female reproductive tract.

Because fishes of the genus *Xiphophorus* reproduce by internal fertilization that can occur days to months after insemination, it is difficult to evaluate the fate of sperm within the female other than by observation of larval development (by dissection of gravid females) or the production of live young. Considering these characteristics, the following hypotheses may be useful in an understanding of fertilization by thawed sperm: (1) sperm have limited energy stores, and in the process of cryopreservation, motility in the extender could shorten the storage time of sperm in the female reproductive tract prior to fertilization; (2) in the process of cryopreservation, the suspension of sperm in extender reduces the sperm density below that necessary for fertilization; or (3) the toxicity of cryoprotectants used in cryopreservation may shorten the sperm life, or affect inseminated females, and consequently affect fertilization. In this study, we focused on addressing the question of sperm immobilization, and its effect on cryopreservation, and also evaluated the effect of sperm washing for reduction of cyroprotectant toxicity.

In summary, the sperm of *X. helleri* could be immobilized by osmotic pressures below 116 mOsmol/kg and above 425 mOsmol/kg. Motility of immobilized sperm could be activated by changing the osmotic pressure to 211–365 mOsmol/kg, and motility of sperm from hypertonic HBSS was significantly higher than that from hypotonic HBSS after 48 h of storage at 4 °C. Hypertonic HBSS at an osmolality of 500 mOsmol/kg could be used as extender to maintain immobilized sperm, and high motility (~55%) of thawed sperm was obtained when cryopreserved with 10–15% glycerol as cryoprotectant. This immobilization of sperm did not affect sperm motility upon exposure to isotonic conditions before freezing or after thawing. Washing by centrifugation could prolong the motility of sperm for storage immediately after thawing, and was an effective method to reduce cryoprotectant toxicity and concentrate the sperm. Subsequent studies should evaluate the utility of sperm immobilized by osmotic pressure in fertilization trials.

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