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Sperm cryopreservation of green swordtail *Xiphophorus helleri*, a fish with internal fertilization☆

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Abstract

Sperm cryopreservation for fishes with internal fertilization is essentially unexplored although many species of these fishes are valuable biomedical research models. To explore methods for sperm cryopreservation within the live-bearing genus *Xiphophorus*, this study used *X. helleri* to evaluate the effects of cryoprotectant, osmotic pressure, cooling rate, equilibration time, and sperm-to-extender ratio. Sperm motility and survival duration after thawing showed significant differences among different cryoprotectants with the highest motility at 10 min after thawing obtained with 14% glycerol. With subsequent use of 14% glycerol as the cryoprotectant, the highest motility after thawing was observed with Hanks' balanced salt solution (HBSS) at 300 mOsmol/kg. Samples cooled from 5 to −80 °C at 20 °C/min yielded the highest post-thaw motility although no significant difference was found in the first 4 h after thawing for cooling rates across the range of 20–35 °C/min. Evaluation of equilibration time revealed no significant difference between 20 min and 2 h, but the highest motility at 10 min after thawing was found with a 20-min equilibration. Dilution ratios of sperm-to-extender at 1:20, 1:60, and 1:120 showed no significant differences in motility and survival duration after thawing, but the dilution of sperm solutions with HBSS (320 mOsmol/kg) immediately after thawing reduced the decline of sperm motility, and significantly prolonged the survival duration. Based on these findings, the highest average sperm motility (77%) at 10 min after thawing was obtained when sperm were suspended in HBSS at 300 mOsmol/kg with 14% glycerol as cryoprotectant, diluted at a ratio of sperm to HBSS–glycerol of 1:20, equilibrated for 10 min, cooled at 20 °C/min from 5 to −80 °C before being plunged in liquid nitrogen, and thawed in a 40 °C water bath for 7 s. If diluted immediately after thawing, sperm frozen by the protocol above retained continuous motility after thawing for more than 8 days when stored at 4 °C.

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The first studies of fish sperm cryopreservation were published 50 years ago [5], and since then more than 200 fish species have been studied [26,39]. This research effort has yielded techniques that are being applied with varying levels of success for fishes around the world. However, most work has focused on large-bodied culture and sport fishes, such as salmon and trout of the family salmonidae [29], carps of the family cyprinidae [11], and catfishes of the families claridae, ictaluridae, pangasiidae, and siluridae [35]. Small teleost fishes are studied much less except for the research models zebrafish (*Danio rerio*) and Japanese medaka (*Oryzias latipes*) [1]. Published studies of sperm cryopreservation of small live-bearing fish are completely lacking except for our initial research on swordtail sperm [12]. Live-bearing fishes as a group are distinct from other fishes for many reasons, most notably for internal fertilization in which males transfer sperm in packages (spermatophores) to females which store the sperm in the *receptaculum seminis* or ''Delle'' of the genital tract for as long as several months after insemination [14,22,24,32].

Sperm of internally fertilizing fish species such as those of the genus *Xiphophorus* possess atypical features such as well-developed mitochondrial sheaths in the midpiece of spermatozoa [30] and glycolytic activity comparable to that of mammalian sperm [14]. The sperm are also different in structure (e.g., head shape) and physiology (e.g., energy metabolism) from the sperm of oviparous fishes [36] (Fig. 1). These features may be adaptations for movement or long-term survival in the female reproductive tract, suggesting physiological differences from the sperm of externally fertilizing fishes. These differences present unknown challenges and opportunities for cryopreservation.

Worldwide, there are more than 1000 species of live-bearing fish [32] and many of them have important research and commercial value. For example, swordtails and platyfish of the genus *Xiphophorus* are not only valuable models in biomedical and behavioral research, but they are also valued as ornamental fish for aquarium trade [8,20,32,40]. Their research value and the continuous decline of diversity in the wild of these fish expands the need to preserve their genetic resources. However, cryopreservation techniques that can be applied to small-bodied fishes are limited. In addition, the internal fertilization mode of live-bearing fishes presents significant challenges in sperm viability assessment after cryopreservation.

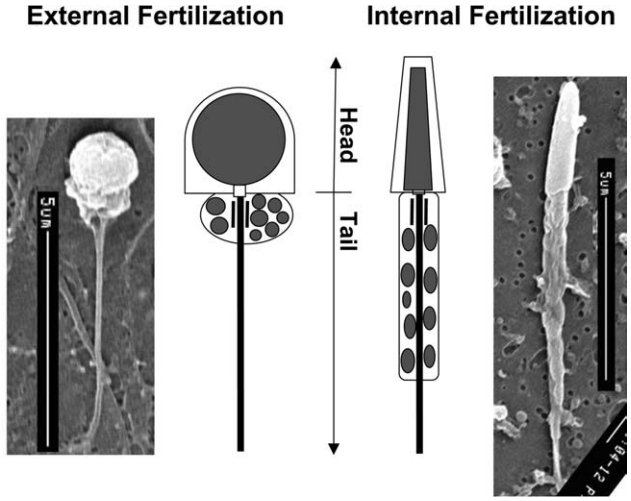

Fig. 1. Diagrammatic views and representative scanning electron micrographs of sperm cells typical of fishes that employ external fertilization (left, Nile tilapia, *Oreochromis niloticus*) or internal fertilization (right, green swordtail *X. helleri*).

Our initial studies of sperm cryopreservation of *Xiphophorus helleri* developed some basic techniques necessary for cryopreservation of sperm from small live-bearing fishes [12]. In the present study, we refined those techniques to optimize the protocols for cryopreservation of *X. helleri* sperm, with the intention that these protocols could be adapted to other species of live-bearing fish. The specific objectives of the present study were to evaluate the effects on sperm motility and storage after thawing of: (1) cryoprotectants (CPAs) and concentrations; (2) extender solution osmolality; (3) cooling rate; (4) equilibration time; and (5) dilution ratio of sperm to extender before freezing and after thawing.

Materials and methods

Sperm collection and dilution

A total of 26 male *X. helleri* were shipped weekly by overnight delivery from the *Xiphophorus* Genetic Stock Center (XGSC) of Southwest Texas State University to the Aquaculture Research Station (ARS) of the Louisiana State University Agricultural Center in January and February of 2003. Fish were anesthetized in 0.01% tricaine–methane sulfonate (Western Chemical, Ferndale, WA) for 2 min, and standard lengths (tip of snout to the crease of the caudal peduncle) and wet weights were measured. Sperm were collected by surgical removal of the testis. Adherent tissue was dissected away and testes were placed in resealable plastic bags (NASCO whirl-pak, MBCOCT, New Haven, CT) and weighed. Hanks' balanced salt solution (HBSS) was added before crushing of the testis to release sperm. Dilutions with HBSS were based on the testis weight. Except for the experiment used to evaluate different ratios of testis to HBSS, the ratio of testis to HBSS (mass:volume) was always 1:100 with the sperm density of $\sim 5 \times 10^7$ cell/ml. Based on our preliminary research [12], HBSS at 320 mOsmol/kg was used for sperm suspension after collection. Within this manuscript, HBSS at specific osmolalities such as 320 mOsmol/kg are abbreviated as HBSS 320.

Motility estimation

The motility of *Xiphophorus* sperm is different from that of other teleosts. The sperm were motile upon collection and they were continuously motile after suspension in HBSS, and therefore activation solutions were not necessary for motility estimates. For estimation, a 5-μl aliquant was removed from each sample and placed on a glass microscope slide. When the ratio of sperm to HBSS–CPA was below 1:100, 2μl of sperm suspension were diluted with HBSS 320 at 1:100 before estimation. Sperm motility was estimated visually at 200× magnification using darkfield microscopy (Optiphot 2, Nikon, Garden City, New York) and was expressed as the percentage of cells actively moving in a forward direction (e.g., [23]). Sperm vibrating in place were not considered to be motile. After thawing, samples were stored at 4 °C and motility was monitored at various intervals based on experimental design until motility ceased or the entire sample volume was depleted.

Freezing procedures

Aliquants (80 μl) of sperm suspensions with cryoprotectant (detailed below) were drawn into 0.25-ml French straws (IMV International, MN, USA) and held (equilibrated) for 8 min at room temperature (23–25 °C) and 2 min at 4 °C before cooling in a controlled-rate freezer (Kryo 10 Series II; Planer Products, Sunbury-on-Thames, UK) at 45 °C/min from 5 to −80 °C. The straws were transferred to a liquid nitrogen storage dewar after the temperature reached −80 °C. After a minimum of 12 h, the straws were thawed for 7 s in a 40 °C water bath (Model 1141, VWR Scientific, Niles, IL, USA).

Effect of cryoprotectants and concentrations

There were four trials in this experiment. In the first trial, sperm samples from six males were used. Sperm-extender suspensions were prepared from each fish at a ratio of sperm to HBSS 320 of 1:50, and were divided into fifteen 40-μl sub-samples. Each sub-sample was mixed with equal volumes of

freshly prepared HBSS 320 (control) or HBSS–CPA solution. The cryoprotectants studied were: dimethyl sulfoxide (DMSO), *N*-dimethyl formamide (DMF), *N*-dimethyl acetamide (DMA), glycerol, propylene glycol (P-glycol), methanol, and sucrose. All chemicals were of reagent grade (Sigma Scientific, St. Louis, MO), and each was evaluated with final concentrations of 6 and 10% (v/v). To minimize differences in final osmolality, suspensions were prepared with HBSS 320 for DMF, P-glycol, and methanol, while HBSS 240 was used for the other cryoprotectants because higher osmolalities were found for the same concentrations of these cryoprotectants, which can affect sperm motility [12]. Motility was estimated at 10 min, 4, 16, and 40 h after thawing.

Based on the results of the first trial, the second trial compared DMSO and glycerol at 6, 10, and 14% with sperm of two males. Two samples from each fish were used for each concentration and CPA combination. Motility was estimated at 10 min, 4, 12 h, and daily for 6 days after thawing or until motility ceased. The subsequent two trials expanded the concentration range further from 5 to 20% for DMSO and glycerol with sperm from two males used for each cryoprotectant. In the third trial, the final concentrations of DMSO were 5, 8, 10, 12, 14, 17, and 20%, and motility was estimated at 10 min, 4, 16, 28, 40, and 64 h after thawing. In the fourth trial, the final concentrations of glycerol were 5, 8, 11, 14, 17, and 20%, and motility was estimated at 10 min, 4, 16, 40, 64, 88, 112, 136, 160, 184, and 208 h after thawing.

Effect of osmolality of HBSS

Based on the results of the previous experiments, glycerol at 14% was used for subsequent studies. Four males were used for osmolality evaluation in this experiment. The final osmolality of the sperm–HBSS–glycerol mixtures was prepared by adjusting the ratio of HBSS 800 to distilled water. One sample from each of four fish was used to evaluate the nine osmolalities: 150, 180, 210, 240, 270, 300, 330, 360, and 390 mOsmol/kg. Motility was estimated at 10 min, 4, 16, 28, 40, 64, 88, and 112 h after thawing.

Effect of cooling rate

Based on the results of the previous experiments, HBSS 300 was used for subsequent studies. There were two trials in this experiment. In the first trial, sperm from two males were used to evaluate four cooling rates: 5, 25, and 45 °C/min from 5 to −80 °C before plunging into liquid nitrogen, and direct plunging of the samples into liquid nitrogen. Three samples from each of two fish were used for each cooling rate. Motility was estimated at 10 min after thawing and daily for 6 days or until all motility ceased.

To more accurately identify the appropriate cooling rate, the second trial used sperm from three males and evaluated rates at a narrower interval: 15, 20, 25, 30, and 35 °C/min from 5 to −80 °C. Motility was estimated at 10 min, 4, 18, 42, 66, and 90 h after thawing.

Effect of equilibration time

Based on the results of the previous studies, five equilibration periods were tested with two samples from each of three fish for a total of six samples for each period. Samples were equilibrated for 10, 20, 30, 60, and 120 min at 4 °C before cooling at a rate of 20 °C/min from 5 to −80 °C. Before the addition of HBSS–glycerol, sperm suspensions were stored at 4 °C. Motility was estimated at 10 min, 12, 24, 48, and 72 h after thawing.

Effect of dilution ratio: before freezing and after thawing

Sperm from two males were used in this experiment. To obtain the final ratios of sperm to HBSS–glycerol of 1:20, 1:60, and 1:120, initial ratios of sperm to HBSS of 1:10, 1:30, and 1:60 were prepared. Two samples from each of two fish were used for each dilution ratio. Samples with a sperm-to-HBSS–glycerol ratio of 1:20 were divided into three sub-samples immediately after thawing: one of which was diluted three times with the final ratio of 1:60, another was diluted six times yielding a final ratio of 1:120, and the third remained undiluted with a final ratio of 1:20 (Table 1). Samples with a sperm-to-HBSS–glycerol ratio

Table 1
Final sperm-to-extender ratios with single dilution (before freezing) and a second dilution (after thawing)

Single dilution factor	Second dilution factor	Final ratio	Dilution number
1:20	0	1:20	Single
	1:3	1:60	Double
	1:6	1:120	Double
1:60	0	1:60	Single
	1:2	1:120	Double
1:120	0	1:120	Single

of 1:60 were divided into two sub-samples: one was diluted twice yielding a final ratio of 1:120, and the other remained undiluted (1:60). Samples with a sperm-to-HBSS–glycerol ratio of 1:120 remained undiluted after thawing. Motility was estimated at 10 min, 12, 24, 72, 96, 144, 168, and 192 h after thawing. Samples with dilutions before freezing only also refer to single dilution, and samples with dilutions after thawing refer to double dilution (Table 1).

Data analysis

General linear model (GLM) repeated measure analysis was used to test for differences ($P = 0.05$) among results for different cryoprotectants, concentrations of cryoprotectant, osmolality of HBSS, cooling rates, equilibration times, and among the different volume ratios of milt to extender-cryoprotectant before freezing, and for dilution with HBSS 320 immediately after thawing. Results were provided as means ± SD. Data for sperm motility were arcsine transformed prior to analysis when heterogeneity of variance occurred. The software used was SPSS 10.0 for Windows, 1999.

Results

Effect of cryoprotectants and concentrations

Although it was low, the motility of fresh sperm before addition of cryoprotectants (30–40%) was satisfactory for this initial range-finding experiment. Except for the control group (without addition of cryoprotectant), samples with cryoprotectants were motile at 10 min after thawing (Fig. 2). The highest motilities were found in the samples with 10% glycerol (15 ± 6%) and 10% DMSO (15 ± 4%), followed by 6% glycerol (10 ± 4%), 6% DMSO (9 ± 2%), and 6% sucrose (3 ± 1%). Throughout the experiment, sperm motility in the samples with DMSO or glycerol was significantly higher ($P < 0.000$) than those with the other cryoprotectants. No significant difference was found between 6% DMSO and 6% glycerol ($P = 0.163$) or between 10% DMSO and 10% glycerol ($P = 0.052$), but the motility was signifi-

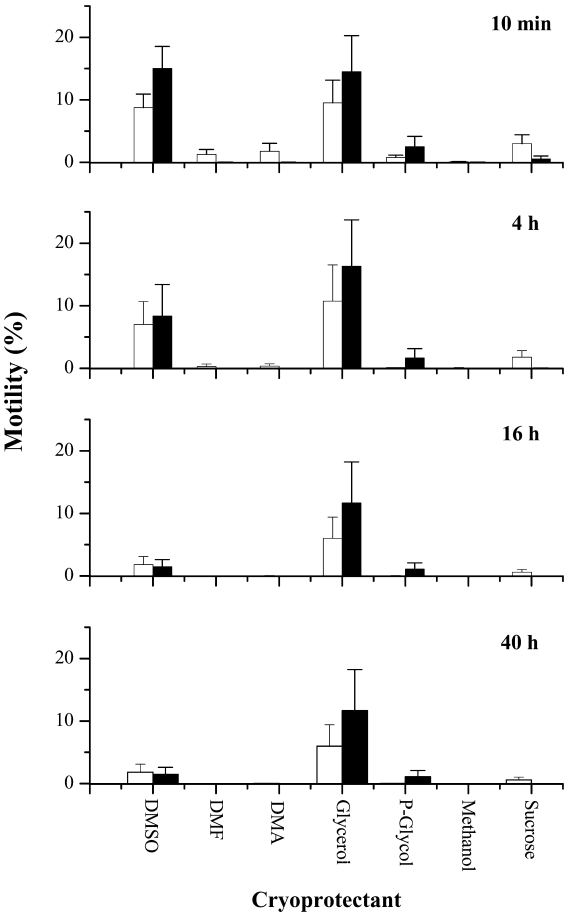

Fig. 2. Motility (mean ± SD) after thawing of *X. helleri* sperm cryopreserved using dimethyl sulfoxide (DMSO), *N*-dimethyl formamide (DMF), *N*-dimethyl acetamide (DMA), glycerol, propylene glycol (P-glycol), methanol, and sucrose at concentrations of 6% (white bars) and 10% (black bars) during storage at 4 °C for 40 h. All control samples (without any cryoprotectant) had no motility upon thawing.

cantly higher ($P = 0.020$) in 10% glycerol than that in 6% DMSO. The average motility in 6 and 10% methanol, 10% DMF, and 10% DMA were all below 1%, and no significant difference was found among them and the control treatment ($P \geqslant 0.141$).

In the second trial, sperm motilities before the addition of cryoprotectants were 60 and 80%. After thawing, no significant difference was found between sperm motilities in 6% glycerol and 6, 10, and 14% DMSO ($P \geqslant 0.129$) (Fig. 3). However, the sperm motilities in high concentrations (10 and 14%) of glycerol were significantly higher ($P \leqslant 0.050$) than those with DMSO, but no significant difference was found between 10 and 14% glycerol ($P = 0.551$). At 10 min after thawing, motility was highest with 14% glycerol ($35 \pm 15\%$), followed by 10% glycerol ($30 \pm 9\%$), 10% DMSO ($21 \pm 7\%$), and 14% DMSO ($13 \pm 2\%$). Although motility in all samples declined continuously over time after thawing, samples with 14% DMSO declined most rapidly, followed by 10% DMSO. Motility after thawing ceased at 72 h for samples with 14% DMSO, at 96 h for 10% DMSO, and at 120 h for 6% DMSO. Motility was retained in the samples with glycerol after 120 h, but ceased at 148 h after thawing.

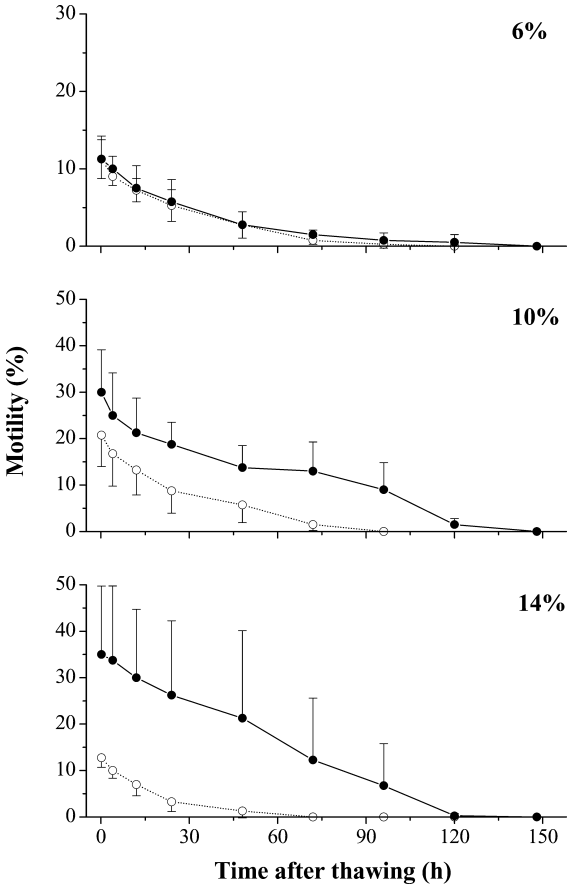

Fig. 3. Motility (mean ± SD) after thawing of *X. helleri* sperm cryopreserved using dimethyl sulfoxide (○) and glycerol (●) during storage at 4 °C for 148 h. Each cryoprotectant was tested at concentrations of 6, 10, and 14%.

In the third trial with DMSO as cryoprotectant, the sperm motilities before addition of DMSO were 70 and 80%. At 10 min after thawing, the highest sperm motility ($35 \pm 11\%$) was found for the 10 and 12% concentrations, followed by 14 and 8% ($26 \pm 8\%$), and the lowest motility was found with 5% DMSO (Fig. 4). Over time, however, motilities of sperm samples in higher concentrations declined faster than did those in lower concentrations. After 16 h, no motile sperm were found in 20% DMSO. At 28 h, the highest motility ($13 \pm 8\%$) was found with 10% DMSO, while no motile sperm were found with 17% DMSO. At 40 and 64 h, the highest motility was found with 8% DMSO. Sperm motilities were significantly higher ($P \leqslant 0.022$) in 10 and 12% than those in 5% and above 12%, although no significant difference was found between 8 and 10–17% ($P \geqslant 0.253$), 5 and 14–20% ($P \geqslant 0.077$). Moreover, no significant difference was found between 14 and 17% ($P = 0.169$), and 17 and 20% ($P = 0.127$).

In the fourth trial with glycerol as cryoprotectant, sperm motility before addition of glycerol was 70%. At 10 min after thawing, sperm in the samples with 14% glycerol showed the highest motility ($58 \pm 7\%$), while the lowest motility ($15 \pm 4\%$) was found with 5% glycerol (Fig. 5). Throughout the experiment, the sperm motilities were significantly higher ($P \leqslant 0.047$) in 11 and 14% than those of the other four concentrations. Although higher motilities were found for concentrations above 14% than for those below 14% at 10 min after thawing, with prolonged storage the motility of sperm in higher concentration declined faster than did that of sperm in lower concentrations as found with DMSO (Fig. 4). For example,

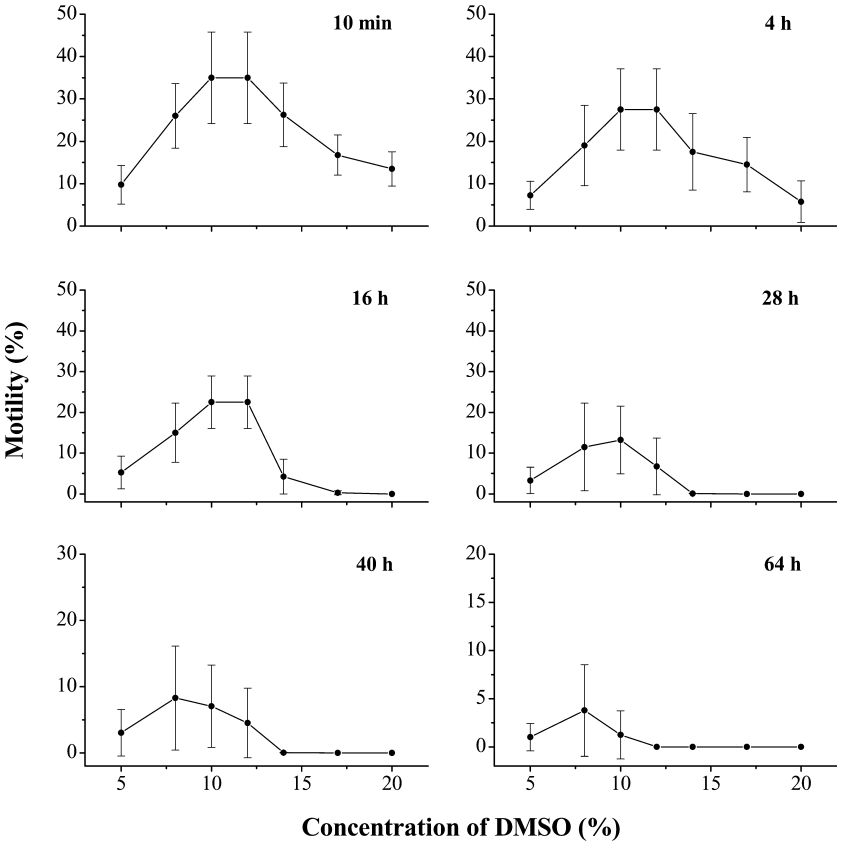

Fig. 4. Motility (mean ± SD) after thawing of *X. helleri* sperm cryopreserved using dimethyl sulfoxide (DMSO) at seven concentrations ranging from 5 to 20% during storage at 4 °C for 88 h.

sperm with 14% glycerol consistently showed the highest motility through 112 h after thawing, but was lower than that of 11 and 8% after 112 h. In this experiment when stored at 4 °C, sperm retained motility for 208 h with 5 and 8% glycerol, and for 160 h with 14% glycerol. There was no significant difference between 11 and 14% ($P = 0.172$), 8 and 17% ($P = 0.075$), and 8 and 20% ($P = 0.586$).

Effect of osmolality of HBSS

The motility of fresh semen in this experiment varied from 50 to 80%. At 10 min after thawing, the lowest motility (3 ± 1%) was found with HBSS at the lowest osmolality, 150 mOsmol/kg (Fig. 6). Sperm motility increased with osmolality of HBSS to the highest level (50 ± 12%) with 330 mOsmol/kg and declined with the increase to HBSS 360 and HBSS 390. The motility declined over time, but the same pattern persisted for 16 h. At 40 h after thawing, the motility in all samples declined below 10%, but the highest motility was at HBSS 270. Sperm suspended in HBSS 180 to HBSS 330 retained motility for 112 h after thawing. No significant difference was found among the samples with osmolalities from HBSS 270 to HBSS 360 ($P \geqslant 0.314$), but the sperm motilities in HBSS 300 were significantly higher ($P \leqslant 0.040$) than those below HBSS 270 and HBSS 390.

Effect of cooling rate

In the first trial, motility of fresh semen was around 95%. At 10 min after thawing, the highest motility (71 ± 7%) was found in samples cooled at

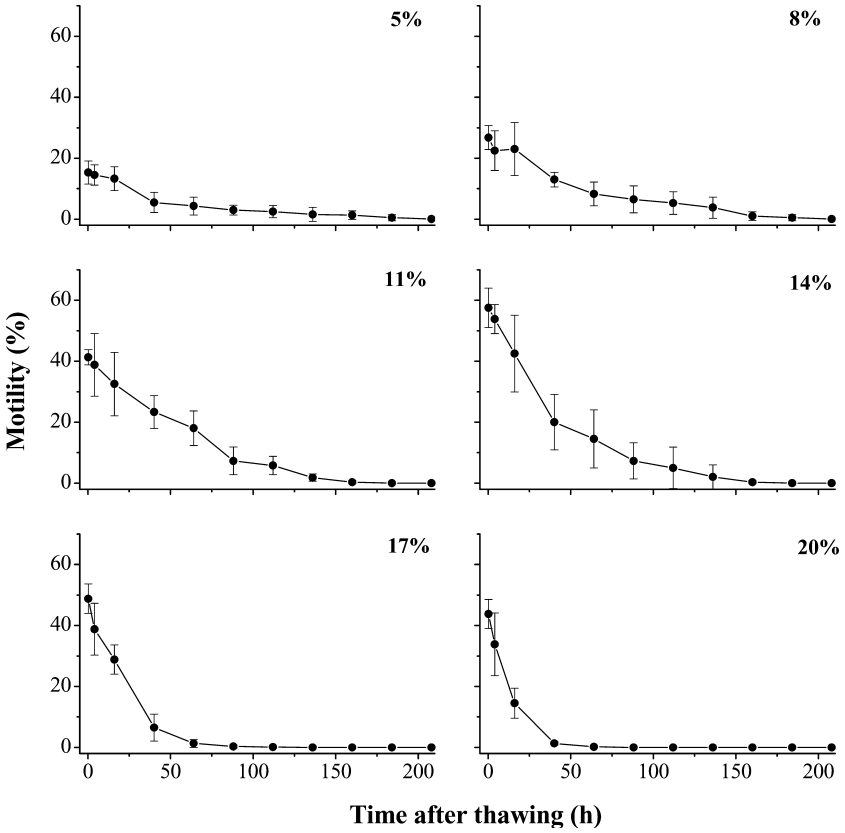

Fig. 5. Motility (mean ± SD) after thawing of *X. helleri* sperm cryopreserved using glycerol at six concentrations ranging from 5 to 20% during storage at 4 °C for 208 h.

25 °C/min, compared with 49 ± 12% at 45 °C/min and 49 ± 13% at 5 °C/min (Fig. 7). Only minimal motility (<1%) was found in control samples that were directly plunged into liquid nitrogen after equilibration and no motile sperm were found in these samples at 24 h after thawing. Sperm cooled at 25 °C/min retained significantly higher motility ($P \leqslant 0.004$) throughout the experiment than did the other cooling rates.

In the second trial, motility of fresh semen was also 95%. At 10 min after thawing, the highest motility (77 ± 3%) was found in samples cooled at 20 °C/min, followed by those at 25 °C/min (Fig. 8). Although the sperm motilities in all samples declined continuously over time after thawing, sperm cooled at 20 °C/min retained the highest motility throughout the experiment and was significantly higher ($P \leqslant 0.002$) than those at other cooling rates. Sperm cooled at 25 °C/min also retained significantly higher motilities ($P \leqslant 0.020$) than did those cooled at 15, 30, and 35 °C/min.

Effect of equilibration time

In this experiment, motility of fresh semen was 95%. At 10 min after thawing, the highest motility (77 ± 4%) was found with samples equilibrated for 20 min, and the lowest (62 ± 8%) was with a 2-h equilibration (Fig. 9). With storage after thawing, the motilities of samples with an equilibration time of 10 or 20 min were generally higher than those with longer equilibration times and significantly higher ($P \leqslant 0.050$) than those with an equilibration time of 1 or 2 h. However, no significant difference

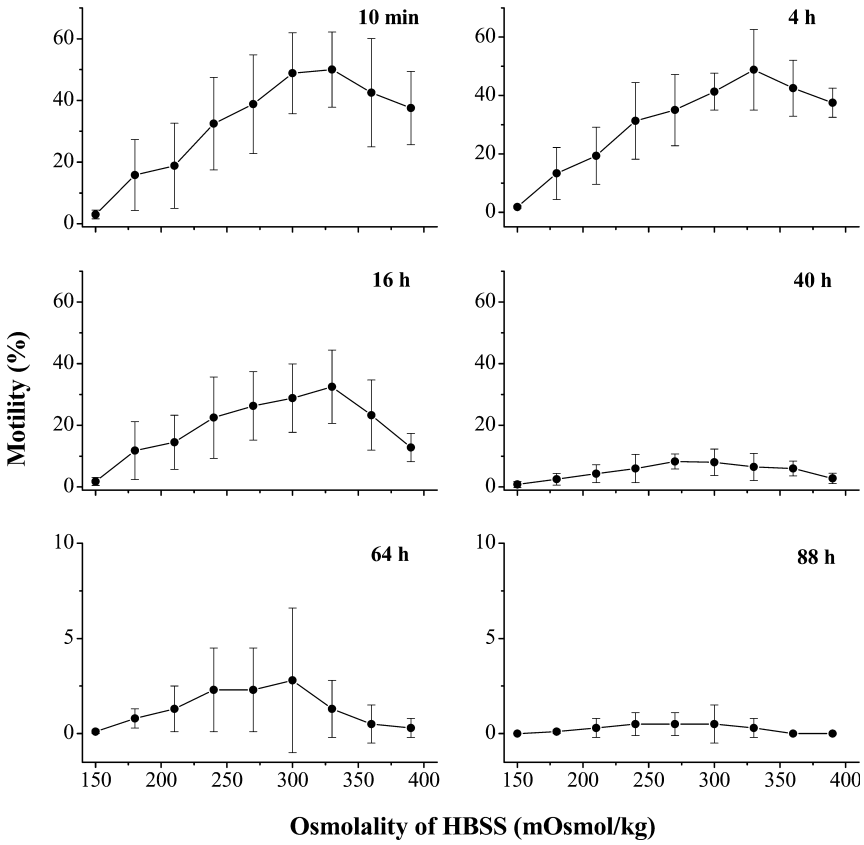

Fig. 6. Motility (mean ± SD) after thawing of *X. helleri* sperm suspended in Hanks' balanced salt solution (HBSS) at nine osmolalities ranging from 150 to 390 mOsmol/kg during storage at 4 °C for 112 h. The final concentration of glycerol for all trials was 14%.

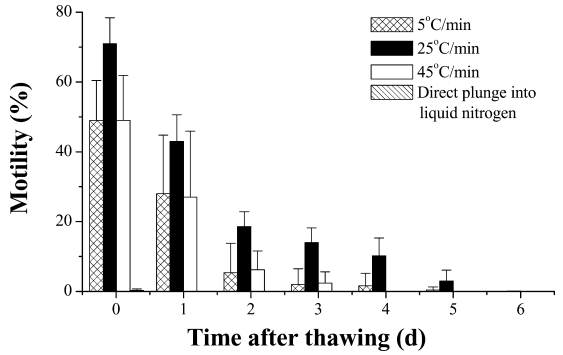

Fig. 7. Motility (mean ± SD) after thawing of *X. helleri* sperm cryopreserved with four cooling rates during storage at 4 °C for 6 days. The final concentration of glycerol for all trials was 14%.

was found among those with an equilibration time of 10, 20, and 30 min ($P \geqslant 0.065$), or between 1 and 2 h equilibration ($P = 0.853$).

Effect of dilution: before freezing and after thawing

Dilution ratio was evaluated before freezing (single dilution) and after thawing (double dilution) (Table 1). Initial motility of fresh semen was 95% for this experiment. For samples with single dilution, the highest motility (76 ± 2%) at 10 min after thawing was found with a volume ratio of sperm to HBSS–glycerol of 1:60, and the lowest (70 ± 4%) was at 1:120 (Fig. 10), although no significant difference ($P \geqslant 0.150$) was found among them (Table 1).

Motility of samples with dilution again after thawing was significantly higher than those diluted only once ($P \leqslant 0.002$) (Table 1, Fig. 10A), although there was no significant difference between the dilution factors of 1:3 and 1:6 ($P = 0.763$). The motility of samples without dilution after thawing

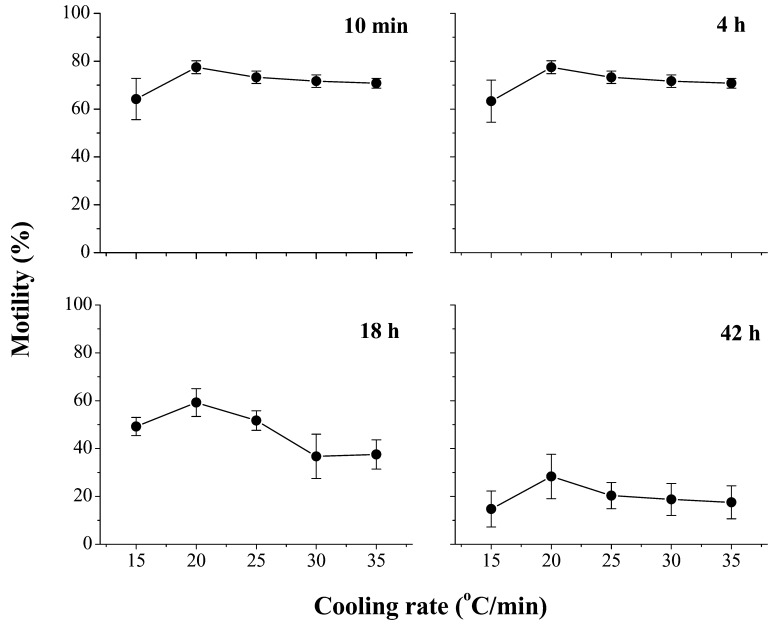

Fig. 8. Motility (mean ± SD) after thawing of *X. helleri* sperm cryopreserved at five cooling rates ranging from 15 to 35 °C/min from 5 to −80 °C during storage at 4 °C for 90 h. The final concentration of glycerol for all trials was 14%.

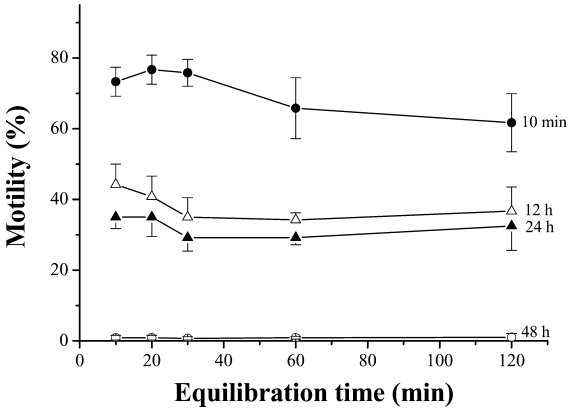

Fig. 9. Motility (mean ± SD) after thawing of *X. helleri* sperm during storage at 4 °C after thawing for 10 min (●), 12 h (△), 24 h (▲) or 48 h (○). Sperm were equilibrated for 10, 20, 30, 60, or 120 min at 4 °C before freezing, and the final concentration of glycerol for all trials was 14%.

declined to 0% within 96 h, but all samples with double dilution retained motility for longer than 192 h. Similarly, in samples with an initial volume ratio of sperm to HBSS–glycerol of 1:60, motility of samples with dilution after thawing was significantly higher ($P = 0.002$) than those of single-dilution samples (Table 1 and Fig. 10B). Sperm retained motility longer than 192 h with the second dilution and 144 h without the second dilution.

For samples with the final dilution ratio of 1:60, those with double dilution showed significantly higher ($P < 0.000$) motility than those with single dilution (Table 1 and Figs. 10A and B), and retained motilities longer when stored at 4 °C. Similar findings were observed for samples with the final dilution ratio of 1:120 although no significant difference ($P = 0.601$) was found in comparing with samples with double dilution (Table 1 and Fig. 10).

Discussion

The sperm of fishes of the genus *Xiphophorus* are different from sperm of most other teleost fishes, likely due to the physiological constraints of internal fertilization. The sperm of *Xiphophorus* are more mammalian-like in morphology and behavior [15,30]. Sperm of *X. helleri* were motile upon collection and swam continuously when suspended in HBSS [13]. The continuous swimming behavior is similar to that observed for sperm of aquatic

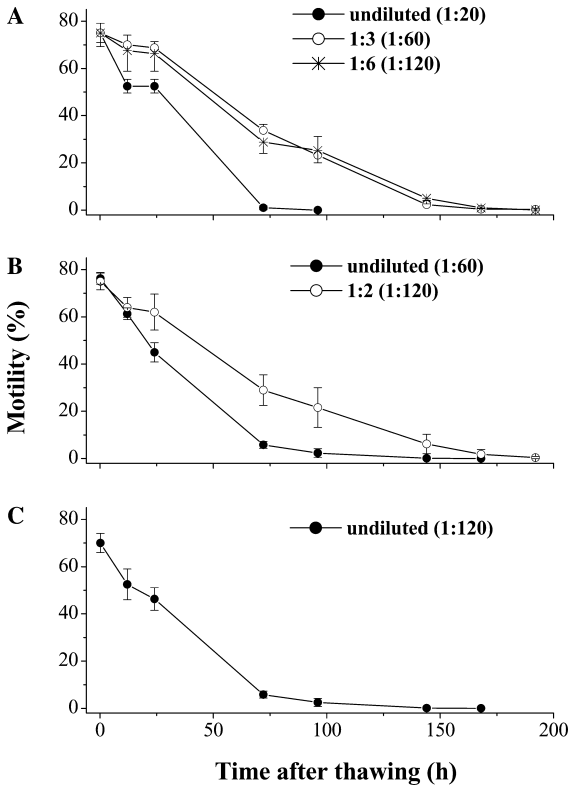

Fig. 10. Motility (mean ± SD) during storage at 4 °C for 192 h after thawing of *X. helleri* sperm suspended (dilution) at ratios of semen to extender-cryoprotectant (HBSS–glycerol) before freezing of 1:20 (A), 1:60 (B), and 1:120 (C), and diluted again immediately after thawing with HBSS at ratios of sperm suspension to HBSS of 1:2, 1:3, and 1:6. The first dilution factors are presented before the parenthesis in the figure and within the parenthesis are the final ratios after the second dilution. The final concentration of glycerol for all trials was 14%.

invertebrates such as eastern oysters *Crassostrea virginica* [7,23]. This is in stark contrast to sperm of fishes that utilize external fertilization. In those fishes, sperm motility is elicited upon contact with the external environment, and the duration of active motility is typically brief, usually lasting no longer than 1 or 2 min [2,9,10,31]. The extended motility of *Xiphophorus* sperm would seem to reflect the requirements for prolonged activity and survival in the female reproductive tract. This atypical reproductive mode also suggests that after thawing, motile sperm would be more likely to produce fertilization success than non-motile sperm, indicating the value of motility assays as a sperm quality indicator for preliminary experiments.

In the present study, significant differences were found among the effects of different cryoprotectants as observed in other fishes, e.g., [13,15,18, 19,21,29]. Compared with the effects of DMA, DMF, P-glycol, methanol, and sucrose, which have been found to be useful cryoprotectants in a minority of aquatic species, e.g., [1,21,38,41], the more commonly used cryoprotectants such as DMSO, and glycerol yielded significantly higher post-thaw motility in this study. For DMSO and glycerol, when the concentrations were below 10%, sperm motilities were similar immediately after thawing, but motility of sperm samples with glycerol was significantly higher with increased concentrations, and with prolonged storage at 4 °C.

Dimethyl sulfoxide is the most commonly used cryoprotectant for aquatic species and is generally useful in most species [4,25,33,34,38]. However, to date, successful sperm cryopreservation has been limited to fish species with external fertilization. *X. helleri*, used in the present study, is the first livebearing fish for which sperm cryopreservation has been developed [12]. With 10–12% DMSO, the present study yielded the highest motility of 35% and a maximum of 64 h storage duration after thawing. In contrast, by using 14% glycerol as cryoprotectant, sperm motility was increased to over 50% immediately after thawing, and motility was maintained over 160 h after thawing when stored at 4 °C. The present study also indicated that concentration was more critical for glycerol than for DMSO because for the same series of tested concentrations, significance was detected more often among the different concentrations of glycerol than those of DMSO.

The appropriate osmolality of HBSS used to suspend the sperm was also different when using different cryoprotectants. Our preliminary study found that sperm of *X. helleri* showed the highest motility in HBSS for refrigerated storage with an osmolality of around 320 mOsmol/kg, which is the same as the osmolality of blood plasma [12]. However, HBSS at the range of 200–250 mOsmol/kg would be more appropriate for sperm suspensions when using 10% DMSO as cryoprotectant [12]. In the present study, with 14% glycerol as

cryoprotectant, the appropriate range of osmolality of HBSS was from 270 to 360 mOsmol/kg, with the highest motility at HBSS 300, which is close to the osmolality of blood plasma of *X. helleri* [12]. The effect of osmolality seems less critical with glycerol than with DMSO although the osmolality of sperm suspensions would be raised to a higher level with glycerol than with the same concentration of DMSO [37].

Cooling rate is another important factor affecting motility of sperm after thawing. For fish sperm, optimal reported rates vary from 5 to 45 °C/min for cooling from 5 to −80 °C, and some species show highest post-thaw motility with a combination of different cooling rates [6,16,17,27]. Sperm of *X. helleri* showed a wide tolerance of cooling rates. With HBSS 300 as extender and 14% glycerol as cryoprotectant, sperm of *X. helleri* retained average motilities of 49% immediately after thawing across a range of cooling rates from 5 to 45 °C/min, although cooling rates of 20–25 °C/min yielded the highest motility.

An optimal equilibration time before freezing is necessary to allow permeating cryoprotectants to penetrate the sperm while minimizing toxicity. Equilibration times of 10–20 min are most commonly used for fish semen [3], although semen of sea bass (*Decentrarchus labrax*) equilibrated for 6 h with 10% ethylene glycol at 0–2 °C yielded motility on thawing comparable to that of fresh semen [28]. An equilibration time of 10 or 20 min yielded the highest post-thaw motility in the present study, although equilibration time seems to be a minor factor for glycerol in this species because no consistent significant differences were found in this experiment across the range of 10–120 min.

The small body size of *X. helleri* females limits the volume of sperm suspension that can be injected for fertilization with artificial insemination. Therefore, it is important to have high sperm motility after thawing while using the lowest dilution ratio of sperm to extender possible. In addition, a reduction in volume of the sperm suspension injected into females would also decrease the amount of cryoprotectant injected and consequent toxicity. However, male *Xiphophorus* store sperm in packets and it is difficult to obtain sperm suspensions when the dilution factor is too low (our unpublished data). Therefore, a minimal final dilution ratio of 1:10 or 1:20 of sperm to extender-cryoprotectant is desirable. Our previous study found that the sperm motility of *X. helleri* among dilutions of 1:50, 1:100, and 1:200 was not significantly different before freezing and after thawing, although the motility declined with greater dilution [12]. In the present study, the sperm motility of *X. helleri* after thawing did not show significant differences among single dilutions of 1:20, 1:60, and 1:120, but the dilution with HBSS 320 immediately after thawing (double dilutions) significantly reduced the decline of sperm motility, and prolonged the storage time at 4 °C. If artificial insemination has to be delayed for several hours, a proper dilution immediately after thawing would help sustain sperm motility. However, because any dilution would decrease the sperm density and increase the volume of sperm suspension injected into females, it would be better to finish artificial insemination using undiluted thawed sperm suspensions as soon as possible after thawing, or to dilute the sperm with a ratio of below 1:20 before freezing and a dilution factor of below 1:2 immediately after thawing. Future experiments should also address the effects of rinsing and concentrating sperm cells by centrifugation.

Based on the experiments in the present study, we improved post-thaw motility of sperm of *X. helleri* to as high as 77% (average) at 10 min after thawing when sperm were suspended in HBSS at 300 mOsmol/kg with 14% glycerol as cryoprotectant at a dilution ratio of sperm to HBSS–glycerol of 1:20, equilibrated for 10–20 min, cooled at 20 °C/min from 5 to 80 °C before plunging into liquid nitrogen, and thawed at 40 °C in a water bath for 7 s. Moreover, if diluted immediately after thawing, the sperm frozen by the above protocol retained motility for as long as 8 days when stored at 4 °C. The improvement of post-thaw motility and prolonged storage time after thawing is extremely important for live-bearing fishes due to their internal fertilization, which requires sustainable long-term storage within the female reproductive tract of viable sperm before fertilization occurs. Although an average of 77% post-thaw motility is still lower than the original 90–95% motility observed for fresh semen, it would gen-

erally be considered to be quite acceptable for cryopreservation research with typical teleost fishes. Because there may be relatively little opportunity for improvement of post-thaw motility in this species, fertilization using thawed sperm should be the goal of future studies.

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