JOURNAL OF THE
WORLD AQUACULTURE SOCIETY

Vol. 47, No. 4
August, 2016
doi: 10.1111/jwas.12293

High-throughput Cryopreservation of Sperm from Sex-reversed Southern Flounder, *Paralichthys lethostigma*

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Abstract

The Southern flounder, *Paralichthys lethostigma*, is a valuable aquaculture fish with established markets in the USA. All-female production in this species is an important technology for aquaculture because the females usually have body sizes twice those of males at the same age, and sex-reversed males (genotypic XX neomales) are used for all-female production by crossing with genetically normal females. However, sperm volume from the neomales is usually small (<0.5 mL) and limits their application for all-female fish production. Cryopreservation of sperm from these sex-reversed neomales will provide access on demand with increased efficiency to extend the application of neomales. The goal of this study was to develop a protocol for cryopreservation of sperm from the Southern flounder by using an automated high-throughput processing system. The objectives were to: (1) determine the effect of osmolality on activation of sperm motility; (2) evaluate the effect of extender solutions on sperm motility capacity; (3) evaluate the acute toxicity of cryoprotectants (dimethyl sulfoxide [DMSO], propylene glycol, and polyethylene glycol) on sperm motility, and (4) estimate the effect of cooling rate on sperm cryopreservation and post-thaw fertilization. Sperm motility was activated when osmolality was 400 mOsmol/kg or higher. Of the three extender buffers tested, HEPES4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) at 300 mOsmol/kg resulted in better protection for sperm motility than did Hanks' balanced salt solution and Mounib solution at 300 mOsmol/kg during 7 d of refrigerated storage. After 30 min equilibration with the cryoprotectant of 15% DMSO, sperm motility was 24 ± 21% (fresh sperm motility without any cryoprotectants was 42%). After cooling at a rate of 20 C/min, post-thaw sperm motility was 8 ± 5% and fertilization was 63 ± 40% evaluated at the 32–64 cell stage (5×10^5 sperm per egg). Overall, a protocol was developed for sperm cryopreservation in the Southern flounder with high-throughput processing, which provides a tool to preserve the valuable genetic resources from neomale flounders, and enables germplasm repository development for the Southern flounder.

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The Southern flounder, *Paralichthys lethostigma*, is an emerging aquaculture species along the east coast in the USA (Daniels 2000) and has been promoted for broader aquaculture application (Hundley 2006). Females can grow twice as fast as males (Fischer and Thompson 2004) and are a favored product for the commercial market. The Southern flounder has an X-Y sex determination mechanism in which females possess the XX genotype and males possess the XY genotype (Luckenbach et al. 2003). With development of sex manipulation methods, neomales with an XX genotype were produced by inducing sex-reversal at juvenile stages of normal genotypic XX females produced by gynogenesis (Luckenbach et al. 2003; Morgan et al. 2006). These XX-type neomales can be used for production of all-female fingerlings by crossing with normal females (XX genotype). Given the growth rate of all-female populations, fish can reach market size earlier with more uniform growth and improved production efficiency (Turner 2008). This breeding strategy makes the Southern flounder profitable for aquaculture. Unfortunately, the collectable sperm volume from this flounder is limited (<0.5 mL), posing challenges for breeding programs (Daniels 2000; Daniels et al. 2010; Brown et al. 2013). To enhance Southern flounder aquaculture, sperm cryopreservation can be applied to preserve sperm samples from valuable XX neomales, enable full use of this limited resource, and increase efficiency of artificial fertilization for hatchery management.

Sperm cryopreservation has been studied in other flounder species such as the yellowtail flounder, *Limanda ferruginea* (Richardson et al. 1999); winter flounder, *Pseudopleuronectes americanus* (Rideout et al. 2003); Japanese flounder, *Paralichthys olivaceus* (Zhang et al. 2003); and summer flounder, *Paralichthys dentatus* (Brown et al. 2013). However, there are no reports on sperm cryopreservation of the Southern flounder. To develop a protocol for sperm cryopreservation with a new species, the basic pathway is to start from sperm activation for selection of extender solutions to maintain sperm motility for transportation and processing (temporary storage) (Tiersch 2011a). For sperm cryopreservation, cryoprotectants and cooling rates are essential factors. In previous studies on other flounder species, dimethyl sulfoxide (DMSO) and propylene glycol (PG) at 10% were the most used cryoprotectants (Richardson et al. 1999; Rideout et al. 2003; Zhang et al. 2003). Also, the cooling process was mostly performed by use of liquid nitrogen vapor, although the actual cooling rates were not reported (Richardson et al. 1999; Rideout et al. 2003; Zhang et al. 2003). In a recent study (Brown et al. 2013), three cooling rates were tested using a medium-sized controlled rate freezer for cryopreserving sperm from the summer flounder in 2-mL cryo-vials, and a relatively slow cooling rate (5 C/min) produced the same post-thaw sperm viability (measured as membrane integrity) as that of fresh sperm. With respect to commercial-scale application, an automated system for sample packaging with 0.5-mL CBS straws, which performs filling, sealing, and labeling, was used for sperm cryopreservation of the blue catfish, *Ictalurus furcatus* (Hu et al. 2011).

The goal of this study was to develop a protocol for cryopreservation of sperm from the Southern flounder by using an automated high-throughput processing system. The objectives were to: (1) determine the effect of osmolality on activation of sperm motility; (2) evaluate the effect of extender solutions on sperm motility capacity; (3) evaluate the acute toxicity of cryoprotectants (DMSO, PG, and polyethylene glycol [PEG]) on sperm motility, and (4) estimate the effect of cooling rates on sperm cryopreservation and post-thaw fertilization. Here, we report the first protocol suitable for high-throughput for cryopreserving the sperm of Southern flounder.

Materials and Methods

Fish

Fish used in this study were 3-yr-old F_3 neomales (XX genotype) descended from an original stock produced by gynogenesis (Morgan et al. 2006). Each generation of fish was cultured according to the established protocols (Daniels et al. 2010) in a commercial-scale

recirculating system at the Lake Wheeler Field Laboratory at North Carolina State University (Raleigh, NC, USA). Mature males were removed from the growout tanks when they reached 12 mo of age. Male broodstock were placed into 2.5-m diameter fiberglass tanks at least 3 mo prior to the start of these trials. The broodstock system water was prepared with artificial sea salts (Crystal Sea Marinemix, Marine Enterprises International Inc., Baltimore, MD, USA) at a salinity of 33 ppt. Fish were fed every other day with BioBrood pellets (Bio-Oregon®, Longview, WA, USA; 52% protein, 15% fat). Males were conditioned to spawning stage by manipulation of photoperiod and temperature to extend winter and summer conditions (Daniels and Watanabe 2002; Watanabe et al. 2006). Feeding was stopped 2 d before spawning.

Sperm Collection

Mature males ($n = 23$) with body weights of 0.41 ± 0.23 kg (mean $\pm$ SD) were anesthetized with (40 mg/L) tricaine methanesulfonate (MS-222; Argent Chemical Laboratories, Redmond, WA, USA) and blotted dry. Sperm were collected by gently pressing along the abdomen from caudal fin to the anal fin and were aspirated into 1-mL pipette tips and transferred into 5-mL microcentrifuge tubes. Extra care was provided to avoid potential contamination from urine. Dry sperm were packed in a Styrofoam box with frozen gel packs at 0 C (Polar Pack, Daigger™, Vernon Hills, IL, USA) and shipped overnight to the Aquaculture Research Station of the Louisiana State University Agricultural Center in Baton Rouge, Louisiana (Tiersch 2011b).

Motility Estimation and Concentration Adjustment

Sperm motility was observed and estimated using a dark-field microscope (Olympus CX41RF, Olympus Corporation, Japan) at ×200 magnification. Sperm suspension (1 µL) was activated by adding 20 µL hatchery sea water and mixing on the glass slide. Estimation of motility was based on viewing at least three different fields within 20 sec. Sperm concentration was measured using absorbance at 600 nm with a microspectrophotometer (Nanodrop® 1000, Thermo Scientific, Wilmington, DE, USA). An equation was deduced from a standard curve between the absorbance and sperm concentration with $R^2 = 0.987$:

Sperm concentration (cells/mL) = Absorbance $\times 9.77 \times 10^8 - 7.68 \times 10^7$ (Cuevas-Uribe and Tiersch 2011)

Sample Packaging and Freezing

Before freezing, the sperm suspensions were adjusted to a concentration of 2×10^8 cells/mL, and cryoprotectants were prepared at double-strength of the target concentrations. The sperm suspensions and cryoprotectant solution were mixed at a ratio of 1:1 (v/v) to yield a final sperm concentration of 1×10^8 cells/mL for packaging into 0.5-mL CBS straws by use of an automated system (MAPI, CryoBioSystem, Paris, France) for sperm sample filling, sealing, and labeling. After packaging, the straws were arrayed on horizontal racks (40 straws each) and were frozen in a commercial-scale programmable freezer (Micro Digitcool, IMV, Paris France). The time from mixing of samples with cryoprotectant to the initiation of the freezing cycle at 5 C was recorded as equilibration time and was maintained as 30 min. When the temperature of the freezer chamber reached −80 C at the programmed cooling rate, the samples were held for an additional 5 min and then transferred into liquid nitrogen. The straws were sorted by individual males and were stored in 12-compartment containers (Daisy goblets, reference number: 015144, CryoBio System) for long-term storage in liquid nitrogen.

Artificial Fertilization of Post-thaw Sperm

Fertilization trials were performed at the North Carolina State University Lake Wheeler Facilities. Hormones used to induce a spawning of females were 0.5 mL/kg of Ovaprim® including 10 µg/kg of salmon gonadotropin-releasing hormone analogue plus 10 µg/kg of Domperidone® (Syndel International Inc., Vancouver, Canada). At 3–4 d before egg collection, mature females ($n = 10$, weight 1.09 ± 0.28 kg) were injected with a priming dose of Ovaprim (10% of the

total dose based on weight), and followed with a resolving dose on the next day (90% of the total dose based on weight). Eggs were obtained by stripping of the females and egg morphology was observed by use of microscope before fertilization. For fertilization, eggs were aliquoted into 20-mL plastic containers with approximately 100 eggs (0.1 mL) in each. Frozen straws were thawed individually in a 40 C water bath for 20 sec, and sperm were released and mixed with a single aliquot of eggs. The estimated sperm-to-egg ratio was 5×10^5 sperm per egg (estimated 2×10^5 motile sperm per egg for fresh collected sperm and 4×10^4 motile sperm per egg for thawed sperm). After mixing of the gametes for about 10 sec, 2–3 mL of sea water from the system (at 16 C and approximately 33 ppt salinity) were added, and the eggs were allowed to incubate for 4–6 h. The percent fertilization was recorded as the number of embryos at the 32–64-cell stage of the total eggs used. On the same day, fresh sperm from 2 to 3 males were collected, and 2–3 aliquots of 100 eggs were fertilized by addition of 30 μL of sperm per each aliquot for evaluation of egg quality.

Experiment 1. Activation of Sperm Motility at Different Osmolalities

Three buffer types, HBSS, Ca^{2+}-free HBSS, and NaCl solution, were used for activation of sperm motility (Table 1). The composition for HBSS was 8 g/L NaCl, 0.4 g/L KCl, 0.14 g/L $CaCl_2$, 0.17 g/L $MgSO_4 \bullet 7H_2O$, 0.075 g/L $Na_2HPO_4 \bullet 7H_2O$, 0.066 g/L KH_2PO_4, 0.35 g/L $NaHCO_3$, and 1 g/L glucose (pH = 7.2). For each solution, 10 osmolalities ranging from 100 to 1000 mOsmol/kg with an interval of 100 mOsmol/kg were prepared by diluting with various volume of water. Fresh sperm from three males were used individually at each osmolality for the three solutions, and sperm motility was evaluated by the use of microscope as described above.

Experiment 2. Effects of Extenders on Sperm Refrigerated Storage

At the time of sperm collection, the sperm were packaged in two ways: as undiluted sperm or diluted with HBSS at 300 mOsmol/kg (HBSS300) with 1:3 ratio (volume of sperm: volume of HBSS). After overnight shipping, the undiluted sperm were distributed and suspended in four different extender solutions: HEPES buffer (Extender B, 6 g/L NaCl, 3.15 g/L KCl, 0.15 g/L $CaCl_2 \bullet 2H_2O$, 0.20 g/L $MgSO_4 \bullet 7H_2O$, 4.69 g/L HEPES, pH 7.8, 13.3 g/L bovine serum albumin, and 5.00 g/L sucrose) (Lahnsteiner 2000), Mounib solution (42.78 g/L sucrose, 2.00 g/L reduced glutathione, and 10 g/L $KHCO_3$) (Mounib 1978), HBSS at 200 mOsmol/kg (HBSS200), and HBSS300 with two different dilution ratios: 1:3 and 1:99 (volume of sperm: volume of HBSS) (Table 1). The remaining undiluted sperm and 1:3 diluted sperm were tested in parallel as control groups with the other treatments. In this experiment, sperm samples from three males were used for each treatment. Sperm motility of each sample was estimated daily by use of a microscope for 7 d except for d 5.

Experiment 3. Evaluation of the Acute Toxicity of Cryoprotectants on Sperm Motility

Fresh sperm were mixed with DMSO, PG, and PEG at final concentrations of 5, 10, and 15% for acute toxicity testing (at 1×10^8 cells/mL) (Table 1). After mixing with cryoprotectants, sperm motility was estimated every 15 min for 60 min. Fresh sperm at the same concentration were tested with same volume with cryoprotectant-free extender solution as a control treatment. Three sperm samples (different origins) were used as replicates.

Experiment 4. Estimation of the Effects of Cooling Rates on Sperm Motility and Fertility

For the first trial, DMSO and PG at 10% were used as cryoprotectants, and sperm samples were equilibrated for 30 min and cooled at a programmed cooling rate of 40 C/min (freezer chamber temperature) (Table 1). Sperm from 10 males were used in this trial. Post-thaw motility and fertility were evaluated. Artificial fertilization was performed using eggs from seven females individually.

TABLE 1. *Overview of experiment designs to determine the best cryopreservation conditions for sex-reversed Southern Flounder, Paralichthys lethostigma.*

	Factor 1	Factor 2	Factor 3	Replication
Exp. 1	Three extender solutions (HBSS, Ca^{2+}-free HBSS, NaCl)	Ten osmolality levels (0–1000 mOsm/kg)		3
Exp. 2	Four extender solutions (HEPES buffer, Mounib solution, HBSS at 200 and 300 mOsmol/kg)	Two dilution ratios (1:3 and 1:99)	7 d (Day 0–Day 6)	3
Exp. 3	Three cryoprotectants (DMSO, PG, and polyethylene glycol)	Four concentrations (control, 5, 10, 15%)	Five time points (0–60 min)	3
	Two cryoprotectants (DMSO and PG at 10%)			10×7[a]
Exp. 4	Three cooling rates (5, 20, and 40 C/min)			4×3[a]

DMSO = dimethyl sulfoxide; HBSS = Hanks' balanced salt solution; PG = propylene glycol.
[a]Male number by female number.

In the second trial, DMSO (10%) was chosen as cryoprotectant for freezing of sperm samples at cooling rates of 5, 20, and 40 C/min with an equilibration time of 30 min (Table 1). Sperm from four males were used in this trial. Motility after equilibration (before freezing) and after thawing was evaluated, and fertility after thawing was evaluated recorded by fertilization of eggs from three females.

Data Analysis

Data were organized using Microsoft Office Excel 2007 and analyzed with Statistical Analysis System 9.2 with ANOVA program. Least squares means were analyzed for specific comparisons within factors. Percentage data were arcsin square root transformed before analysis. During the statistical analysis for each experiment, no interactions of factors were tested. All datasets were utilized in analysis and no outlier screening was conducted. Differences were considered significant at $P < 0.050$.

Results

Experiment 1. Activation of Sperm Motility at Osmolalities from 0 to 1000 mOsmol/kg

Sperm were activated by all three extender solutions when osmolalities were higher than isotonic osmolality (300 mOsmol/kg) (Fig. 1). Overall, the higher the osmolality, the higher the motility (Fig. 1). With HBSS and NaCl, sperm were immotile at 300 mOsmol/kg, but

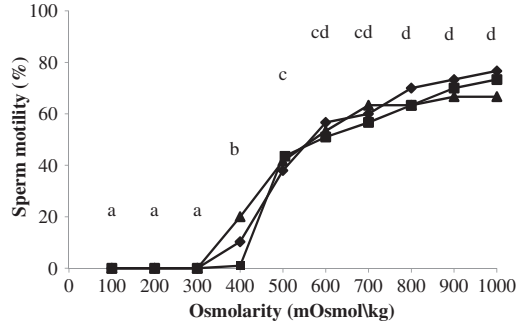

FIGURE 1. *Sperm from three Southern flounder males were tested across a range of osmolality (0–1000 mOsmol/kg) for optimal extender solution. Three extender solutions were studied: Hanks' balanced salt solution (HBSS; diamonds), calcium-free Hanks' balanced salt solution (squares), and sodium chloride (triangles). The motility at each osmolality level was measured and the average of three males is shown. There were no statistical differences in motilities among three solutions (*$P > 0.14$*). Threshold motility was 10%. The letters represent differences identified by Tukey's* post hoc *test (points sharing letters were not significantly different).*

were activated when osmolality increased to 400 mOsmol/kg ($P = 0.080$ for HBSS and $P = 0.020$ for NaCl). For Ca-free HBSS, sperm were immotile at 300 mOsmol/kg, but motility was initiated at 400 mOsmol/kg ($5 \pm 7\%$) and increased significantly to $43 \pm 21\%$ at 500 mOsmol/kg ($P = 0.030$). For each of the three extenders tested, motility did not show significant differences at osmolalities ranging from 500 to 1000 mOsmol/kg ($P < 0.050$) (Fig. 1). Also, there were no significant differences

in sperm motility among the three solutions ($P > 0.140$).

Experiment 2. Evaluation of the Effect of Extender Solutions on Refrigerated Storage

Sperm motility decreased during 1 wk of refrigerated storage in all treated groups (Fig. 2). In the 1:3 dilution group, motility in HEPES buffer was significantly higher than controls ($P = 0.000$) and other solutions ($P < 0.003$) each day. The second highest motilities were provided by HBSS300, which were significantly higher than for undiluted sperm ($P = 0.010$) and the other two treatments ($P < 0.040$). In the 1:99 dilution group, HEPES buffer was also significantly higher than the control groups ($P < 0.180$) and other treatments ($P = 0.000$). The second highest motility was again provided by HBSS300, which was not significantly different from controls ($P > 0.070$) and was higher than the other two treatments ($P < 0.009$). The motility of the remaining treatments was significantly lower than the values of the controls ($P < 0.008$). Furthermore, HEPES buffer yield higher motility with the 1:3 dilution than observed with the 1:99 dilution ($P = 0.033$).

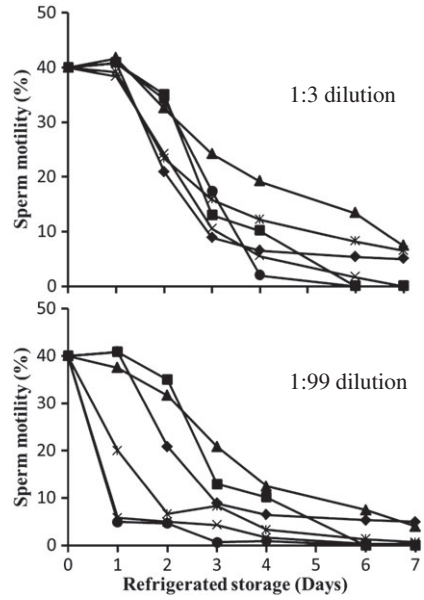

FIGURE 2. *Effects of buffer types as extender on retention sperm motility capacity during refrigerated storage at 1:3 (A) and 1:99 dilution ratio to undiluted sperm (v/v) (B). The buffer types were: Hepes buffer (triangles), Monib solution (circles), HBSS at 200 mOsmol/kg (crosses), and HBSS at 300 mOsmol/kg (stars). The control groups, undiluted sperm (diamonds), and diluted with HBSS at 300 mOsmol/kg at 1:3 ratio before shipping (squares) were measured with all other treatments.*

Experiment 3. Evaluation of the Acute Toxicity of Cryoprotectants on Sperm Motility

All three cryoprotectants (DMSO, PG, and PEG) reduced sperm motility. Motility decreased as the incubation time increased from 0 to 60 min (Fig. 3). After 30 min of refrigerated storage, the sperm motility in DMSO ($35 \pm 8\%$) and PG ($33 \pm 81\%$) was higher than PEG ($2 \pm 2\%$). Specifically, 10% DMSO yielded higher motility ($37 \pm 3\%$) than did 10% PG ($33 \pm 11\%$) ($P = 0.017$) and 10% PEG ($1 \pm 2\%$) ($P = 0.000$). However, all treatment curves were significantly different from the control by using ANOVA comparing just the factor of cryoprotectants ($P < 0.014$).

Experiment 4. Estimation of the Effects of Cooling Rates on Sperm Motility and Fertility

In the first trial, after 30 min equilibration and cryopreservation, the treatment with 10% DMSO had equilibration motility of $30 \pm 19\%$, post-thaw motility of $23 \pm 11\%$, and fertilization of $34 \pm 46\%$; the treatment with 10% PG had equilibration motility of $31 \pm 20\%$, post-thaw motility of $20 \pm 13\%$, and fertilization of $52 \pm 49\%$. There were no significant differences between the two treatments ($P = 0.239$). However, the fertilization of post-thaw sperm cryopreserved with PEG ($17 \pm 14\%$) was significantly lower than that with PG ($P = 0.003$), but not significantly different from that with DMSO ($P = 0.190$) (Fig. 4).

In the second trial, no significant differences among 5, 20, and 40 C/min were found in equilibration motility ($9 \pm 9\%$, $12 \pm 13\%$, and $9 \pm 6\%$) ($P > 0.890$) or post-thaw motility ($6 \pm 5\%$, $8 \pm 5\%$, and $8 \pm 8\%$) ($P > 0.550$). The cooling rate of 20 C/min was not significantly different to other cooling rates (Fig. 5) in terms of fertilization rate at 40 C/min with

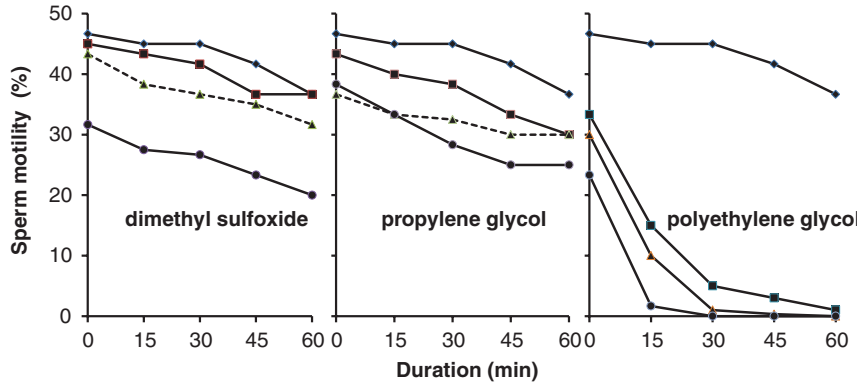

FIGURE 3. *Effects of dimethyl sulfoxide (A), propylene glycol (B), and polyethylene glycol (C) on sperm motility at final concentrations of 0% (diamonds, as control), 5% (squares), 10% (triangles), and 15% (circles) after incubating with fresh sperm for as long as 60 min. Dotted lines represent the suitable candidates with acceptable toxicity during test.*

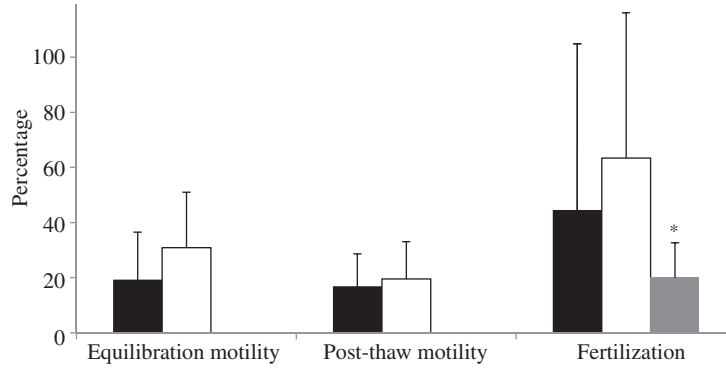

FIGURE 4. *Cryopreservation of Southern flounder sperm with 10% dimethyl sulfoxide (DMSO), propylene glycol (PG), and polyethylene glycol (PEG). The freezing was performed on 40 C/min (freezer chamber temperature). Equilibration motility and post-thaw motility of PEG were all zero. The fertilization of DMSO (black) and PG (white) was similar (*$P > 0.288$*), but PEG (gray) was significantly lower than PG (*$P = 0.003$*). *Significant difference.*

10% DMSO ($P > 0.090$). Fertilization by post-thaw sperm cryopreserved at 40 C/min was significantly higher than that at 5 C/min ($P = 0.029$).

Discussion

The Effect of Osmolality on Activation of Sperm Motility

During mating, Southern flounder release gametes into the surrounding water. Sperm cells thus move from an isotonic environment (around 300 mOsmol/kg) (Morisawa et al. 1983) to seawater with salinity about 33 ppt (1000 mOsmol/kg). This phenomenon suggested that the use of isotonic solutions as extender solutions would not activate the sperm and prevent osmotic shock (Glenn and Tiersch 2002; Tiersch et al. 2011). As this was observed, an osmolality of 300 mOsmol/kg can be recommended for maintaining sperm function during refrigerated storage. Another important aspect was the composition of extender solution. Although, balanced salt solutions have been commonly used for cell suspensions, divalent metal ions can affect sperm activation (Alavi and Cosson 2006). In this study, HBSS and Ca^{2+}-free HBSS functioned similarly with the Southern flounder sperm. Unlike medaka *Oryzias latipes* (Yang and Tiersch 2009) and other fish species (Alavi and Cosson 2006), the activation of Southern

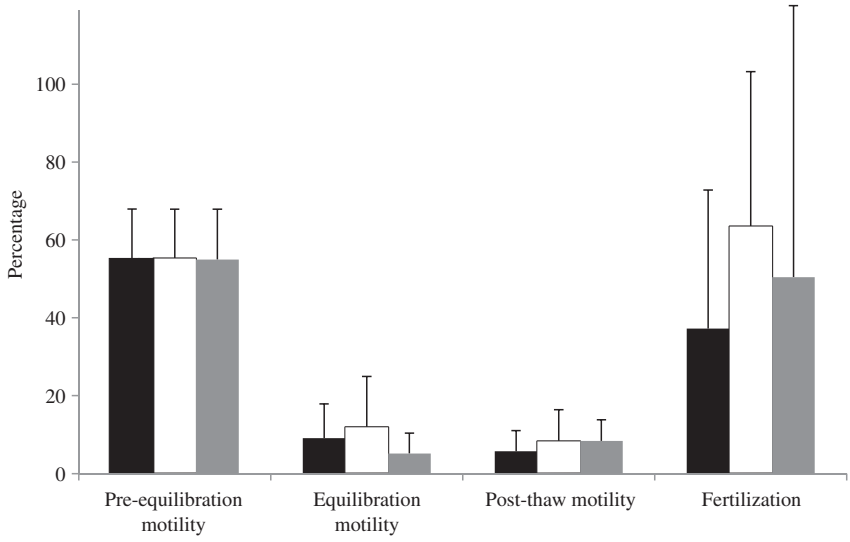

FIGURE 5. *Evaluation of cooling rates (black: 5 C/min, white: 20 C/min, and gray: 40 C/min) for sperm cryopreservation in the Southern flounder with 10% dimethyl sulfoxide as cryoprotectant.*

flounder sperm did not appear to be sensitive to specific ions. Balanced salt solutions could preserve fresh sperm of Southern flounder against activation before cryogenic storage or artificial fertilization (Glenn and Tiersch 2002).

Choice of Extender

In this study, different extender solutions were compared. HBSS has been commonly used in sperm cryopreservation of freshwater fishes (Christensen and Tiersch 1997; Yang et al. 2007), euryhaline species (Yang and Tiersch 2009), and marine species (Riley et al. 2004). The advantage of Mounib solution is its simple composition. This solution had protein base and was originally utilized for *Gadus morhua* L. (Mounib et al. 1968; Butts et al. 2010). HEPES buffer was applied in Salmonidae (*Oncorhynchus mykiss*, *Salmo trutta f. lacustris*, *Salvelinus fontinalis*, *Salvelinus alpinus*, *Salmo trutta f. fario*, *Hucho hucho*, *Coregonus lavaretus*, *Thymallus thymallus*) and pike, *Esox lucius* (Lahnsteiner 2000). The Southern flounder lives in marine and brackish water environments and experiences environment–physiology interaction similar to some salmon species. So those two protein-based extender solutions were listed as candidates along with HBSS. All extender solutions lengthened sperm storage time, but HEPES buffer provided the significantly longest time. Shipping of undiluted sperm proved more practical than mixing with extender solutions, because undiluted sperm required less space and avoided transport of large volume of liquid. However, if overnight shipping was not available or suspended sperm were required during experiments, sperm could be diluted with HEPES buffer at 1:3 ratio. Diluted sperm provided enough volume for high-throughput processing, and reduced the chance of having mechanical problems such as coagulates during packaging of the sperm suspension into straws. However, dilution can have negative effects. In this study, dilution at a 1:99 ratio disabled the preservation function of most extender solutions, providing less storage time than undiluted sperm.

Choice of Cryoprotectants for Sperm Cryopreservation

In large-scale cryopreservation, the freezing capacity usually is not a constraint. A longer equilibration time would yield a longer available packaging time. Therefore, a less toxic cryoprotectant would help increase the overall production efficiency by lengthening the time available for packaging and enabling freezing in

larger batches. The cryoprotectants DMSO and PG maintained sperm motility for at least 30 min without significant decrease, which provided a convenient minimum time for high-throughput processing of samples. Because the cryoprotectant PEG reduced motility rapidly, it was determined to be too toxic for sperm cryopreservation. However, other effects could affect the final quality of cryopreserved sperm. Fertilization tests indicated that a cryoprotectant with lower toxicity (10% DMSO) did not achieve a significantly higher fertilization rate, however, the post-thaw motility and fertilization were consistent. In a recent study in another paralichthys flounder, the summer flounder, 10% DMSO was used as a cryoprotectant with a short equilibration time (<3 min), but no assessment of acute toxicity of cryoprotectants was reported (Brown et al. 2013). For a commercial-scale application, a longer equilibration time is required for handling of samples, thus in the present study, an equilibration time of 30 min was defined and used.

Effect of Cooling Rates on Sperm Cryopreservation

The different cooling rates did not show significant differences; however, 20 C/min can be recommended based on its consistent performance. Because sperm of marine species generally have a strong resistance to osmotic effects and cryogenic temperatures (Cuevas-Uribe 2011), they can likely survive a wide range of cooling rates. Marine species generally have smaller sperm than freshwater species (Alavi et al. 2008), which reduces the probability of intracellular ice crystal damage. In a previous study, post-thaw sperm viability of summer flounder cryopreserved at 5 C/min yielded the same membrane integrity as did fresh sperm (Brown et al. 2013). With respect to high-throughput, although 40 C/min required less time and shortened the production cycle, quality was inconsistent. Therefore, 20 C/min became the preferred choice. Future research could compare fertilization under narrower ranges of cooling rates.

Beyond Protocol Development

This study developed and verified a sperm cryopreservation protocol suitable for high-throughput application for the Southern flounder; however this is not the only potential solution to the problem of germplasm preservation in this species. The strategies behind protocol development should be tailored to specific applications. For example, in this study, the goal was cryopreserving sperm from high-value brood fish ("neomales") for future breeding purposes. The practical constraints were small volumes of sperm and a high requirement for biosecurity and labeling. The neomale has high value because the success rate of gynogenesis is very low (Luckenbach et al. 2003; Morgan et al. 2006), and each male can only provide less than 500 μL of undiluted sperm. Also, there will always be male-to-male variations that can influence the success of cryopreservation (Butts et al. 2011). Careful designs are needed for successful cryopreservation and efficient use of thawed sperm. Further, because neomales have an exclusive XX genotype, all sperm are of the X genotype. In a cryogenic storage dewar, sperm from other individuals as well as other strains of Southern flounder can be submerged in liquid nitrogen. It is important to avoid cross-contamination that might mix Y genotype sperm with neomale sperm.

These aspects justified the strategy in this project: adopting high-throughput cryopreservation with high-security CBS straws that can be sealed at both ends. There was a previous study with larger volume packaging (5 mL) of sperm (Liu et al. 2015) for summer flounder. If the purpose was to store sperm for a large breeding program, large volume packaging and high-throughput systems could each meet the requirements. However, the disadvantage of large-volume packaging is increased risk of losing or wasting samples. The disadvantage of high-throughput processing is a large quantity of containers that must be stored and inventoried. As such, specific applications for germplasm preservation should be balanced across goals and limitations in materials and resources. However, all protocols should be verified by fertilization trials, including the standardization

and quality control (QC) and quality assurance (QA) approaches that have been described for high-throughput systems (Hu et al. 2013). A simple way to integrate various, individual protocols, is to establish guidelines for minimum standards (e.g., www.NAAB-CSS.org). To accelerate acceptance by public, a standardized platform for future protocol development can play an essential role. Eventually, all results from such a platform could be easily shared and compared in a form of network.

Based on the results of this study, a protocol suitable for high-throughput application for sperm cryopreservation in the Southern flounder was concluded as: (1) avoiding all contamination during sperm collection and holding of samples at 4 C during short-term storage and transportation, (2) suspending sperm samples with HEPES buffer at 300 mOsmol/kg and adjusting the concentration to 2×10^8 cells/mL, (3) mixing with 20% DMSO in HEPES at a ratio of 1:1 (v:v) to yield a final 10% of DMSO and 1×10^8 sperm/mL, (4) equilibrating from 15 to 30 min, (5) cooling at a rate of 20 C/min from 5 to −80 C and maintaining at −80 C for 5 min, and (6) plunging the sorted frozen samples in liquid nitrogen for long-term storage. This protocol did not place sole focus on survival of cryopreserved sperm, but also included economic feasibility and minimization of variation in quality. This protocol was tested using existing commercially available high-throughput systems and could be expanded to large-scale production. With cryopreserved sperm, hatcheries and researchers can preserve valuable lines or obtain access to desired material for genetic improvement. With aquaculture of the Southern flounder becoming more popular, cryopreservation can provide genetic improvement and sustainability that can ensure long-term profitability and genetic security.

Acknowledgments

We thank C. Staudermann, D. Kuenz, J. Christensen, N. Novelo, and S. Harris for technical assistance and suggestions. This work was supported in part by funding from the Louisiana Sea Grant College Program. This manuscript has been approved for publication by the Director of the Louisiana Agricultural Experiment Station as number 2015-241-22737. This research was supported in part by the National Institutes of Health grant R24RR023998.

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