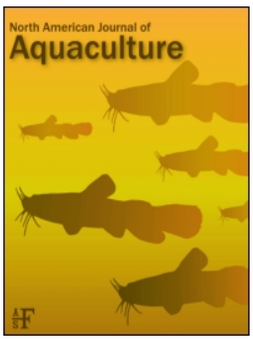

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ARTICLE

Vitrification as an Alternative Approach for Sperm Cryopreservation in Marine Fishes

Rafael Cuevas-Uribe*
Department of Fisheries Biology, Humboldt State University, One Harpst Street, Arcata, California 95521, USA

E. Hu
Center for Aquaculture Technologies, Inc., 8395 Camino Santa Fe, Suite E, San Diego, California 92121, USA

Harry Daniels and Adriane O. Gill
Department of Applied Ecology, North Carolina State University, Raleigh, North Carolina 27695, USA

Terrence R. Tiersch
Aquatic Germplasm and Genetic Resources Center, School of Renewable Natural Resources, Louisiana State University Agricultural Center, Baton Rouge, Louisiana 70820, USA

Abstract

The Southern Flounder *Paralichthys lethostigma* is a high-value species and a promising aquaculture candidate. Because sperm volume can be limited in this species (<500 µL), new sperm cryopreservation methods need to be evaluated. Vitrification is an alternative to conventional slow-rate freezing, whereby small volumes are cryopreserved at high cooling rates (>1,000°C/min). The goal of this work was to develop a standardized approach for vitrification of Southern Flounder sperm. The specific objectives were to (1) evaluate thawing methods and vitrification solutions, (2) evaluate the postthaw membrane integrity of sperm vitrified in different cryoprotectant solutions, (3) examine the relationship between membrane integrity and motility, and (4) evaluate the ability of vitrified sperm to fertilize eggs. From the vitrification solutions tested, the highest postthaw motility (28 ± 9% [mean ± SD]) and membrane integrity (11 ± 4%) was observed for 20% ethylene glycol plus 20% glycerol. There was no significant difference in postthaw motility of sperm thawed at 21°C or at 37°C. Fertilization from vitrified sperm in one trial yielded the same fertilization rate (50 ± 20%) as the fresh sperm control, while the sperm from the other two males yielded 3%. This is the first report of fertilization by vitrified sperm in a marine fish. Vitrification can be simple, fast, inexpensive, performed in the field, and, at least for small fishes, offers an alternative to conventional cryopreservation. Because of the minute volumes needed for ultrarapid cooling, vitrification is not presently suited as a production method for large fishes. Vitrification can be used to reconstitute lines from valuable culture species and biomedical models, conserve mutants for development of novel lines for ornamental aquaculture, and transport frozen sperm from the field to the repository to expand genetic resources.

Cryopreservation technology in aquatic species has enhanced hatchery and aquaculture operations by providing flexibility in spawning females, greater control in breeding programs, and the ability to store favorable genes for extended periods (Tiersch et al. 2007; Martinez-Paramo et al., in press). In addition, concern for native fish populations has resulted in examining sperm cryopreservation as a means to preserve genetic material and transfer genes between wild and hatchery populations (Tiersch 2011a). Problems typically associated with sperm cryopreservation for threatened and endangered fishes are the limited

*Corresponding author: aquaculture@humboldt.edu
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availability of captive broodstock and the lack of cryopreservation expertise (Tiersch et al. 2004). New and easy approaches to cryopreserve samples in the field are needed. Vitrification is an alternative approach to cryopreservation in which a liquid is solidified into a noncrystalline solid referred to as "glass." This amorphous solid retains a random molecular arrangement of a liquid (Fahy and Wowk 2015). To achieve this glass state, typically high concentrations of cryoprotectants (40–50%, v:v), and fast cooling and warming rates (>1,000°C/min) are needed (Fahy and Rall 2007; Fahy and Wowk 2015).

Although conventional cryopreservation is a proven method for long-term storage of genetic material, vitrification is an attractive alternative that has been used with mammals for the cryopreservation of spermatozoa, embryos, oocytes, stem cells, and organs (Tucker and Liebermann 2007). It offers a new approach to expand the maintenance of genetic resources, protect valuable stocks, reconstitute lines, and transport frozen sperm from the field to the repository. Studies in fish vitrification can be traced back to 1938 when Basile Luyet attempted to vitrify juvenile Goldfish *Carassius auratus* (40 mm SL) by plunging the fish into liquid air (−194°C) (Luyet 1938). Several attempts have been made to vitrify fish embryos with limited or no success (reviewed in Cuevas-Uribe and Tiersch 2011a).

Vitrification offers little advantage over conventional cryopreservation for aquaculture production methods of large-bodied fishes due to the small volumes (<30 µL) used. However, due to its simplicity, speed, and utility for field applications with no equipment required, it offers a new approach to preserve genetic resources and to reconstitute strains or lines (Moore and Bonilla 2006; Saragusty and Arav 2011; Magnotti et al., in press). Vitrification is best suited for use in three areas: biomedical research fish models, genetically improved lines, and imperiled species. There are fish species that produce small sperm volumes, such as Zebrafish *Danio rerio* (<5 µL) (Jing et al. 2009) and Green Swordtail *Xiphophorus hellerii* (<9 µL) (Huang et al. 2004). In some larger-bodied species it is often difficult to collect large sperm volumes, such as in the endangered Apache Trout *Oncorhynchus apache* (<500 µL) (David et al. 2011). Furthermore, selection programs in fish could lead to negative genetic correlations such as decreased reproductive performance in some species. For example, small sperm volumes (<100 µL) were produced by broodstock of Atlantic Salmon *Salmo salar* from a selection program for fast growth and late maturation (Zohar 1996) compared with wild fish (>10 mL) (Kazakov 1981). Similarly, sex-reversed males from Dusky Grouper *Epinephelus marginatus* produce low sperm volumes (<400 µL) (Cabrita et al. 2009). Vitrification fits perfectly with the need to conserve germplasm from these important fishes.

Sperm vitrification has been applied in freshwater fishes, and offspring have been produced from vitrified sperm samples of Russian Sturgeon *Acipenser gueldenstaedtii* (Andreev et al. 2009), Channel Catfish *Ictalurus punctatus* (Cuevas-Uribe et al. 2011a), the live-bearing Green Swordtail (Cuevas-Uribe et al. 2011b), Rainbow Trout *O. mykiss* (Figueroa et al. 2013), Atlantic Salmon (Figueroa et al. 2015), and Eurasian Perch *Perca fluviatilis* (Kasa et al., in press). Our recent studies evaluated sperm vitrification in three marine species, Spotted Seatrout *Cynoscion nebulosus*, Red Drum *Sciaenops ocellatus*, and Red Snapper *Lutjanus campechanus*, in which sperm motility and membrane integrity were general indicators of gamete quality (Cuevas-Uribe et al. 2015). Most recently, sperm motility and sperm head morphometry were evaluated after vitrification of the marine European Eel *Anguilla anguilla* (Kasa et al., in press). However, these studies did not attempt fertilization or offspring production (Magnotti et al., in press).

The Southern Flounder *Paralichthys lethostigma* is a high-value species and a promising aquaculture candidate. Because females of Southern Flounder grow two to three times larger than males, all-female culture is desirable for commercial aquaculture (Morgan et al. 2006). Neomales with an XX genotype were produced by sex-reversal of gynogenetic progeny. The production of these neomales is tedious and labor intensive. This is due to the time required for progeny testing and the low survival of the gynogens (<2%) (Morgan et al. 2006). In addition, males produce a very small volume (<500 µL) of sperm (Daniels 2000). Vitrification could offer a new way to protect this investment and reconstitute these valuable sex-reversed males as was demonstrated for neomales of Rainbow Trout (Figueroa et al. 2013).

The goal of this work was to develop a standardized approach for vitrification of Southern Flounder sperm. The specific objectives in the present study were to (1) evaluate thawing methods and vitrification solutions, (2) evaluate the postthaw membrane integrity of sperm vitrified in different cryoprotectant solutions, (3) examine the relationship between membrane integrity and motility, and (4) evaluate the ability of vitrified sperm to fertilize eggs. Here we report the first fertilization by use of vitrified sperm in a marine fish. Vitrification is well suited for use in the field and offers a new option for conservation biology.

METHODS

Collection of sperm.—Adult Southern Flounder broodstock held at the North Carolina State University (NCSU) Lake Wheeler Field Laboratory Facilities, Raleigh, North Carolina, were induced to spawn by manipulating photoperiod and temperature (Daniels and Watanabe 2002; Watanabe et al. 2006) during April 2009 and March–April and August 2010. The fish were cultured in an artificial seawater (33 g/L) (Crystal Sea Marinemix, Marine Enterprises International, Baltimore, Maryland) system with a photoperiod of 9 h light : 15 h dark at 16°C and were fed every other day to satiation with BioBrood pellets (Bio-Oregon, Longview, Washington).

The fish used were 3-year-old, F3, sex-reversed males (XX neomales), which weighed 0.41 ± 0.23 kg (mean ± SD). Males were anesthetized with tricaine methanesulfonate (40 mg/L) (MS-222; Argent Chemical Laboratories, Redmond, Washington) and checked for spermiation by applying pressure to the gonadal area. Spermiating males were dried with towels, and sperm was aspirated into 1-mL pipette tips by applying slight pressure to the abdomen. Care was taken to avoid contamination of the samples with urine, feces, or water. Feed was withheld for 2 d before sperm collection to avoid the release of feces during handling. The sperm samples and a sample of the seawater from the system were secured in ZipLoc plastic bags and shipped overnight to the Aquaculture Research Station (ARS) of the Louisiana State University Agricultural Center in a styrofoam box containing two foam refrigerant blocks frozen to −20°C. A cardboard divider was placed on top of the refrigerant blocks to avoid direct contact with the samples (Tiersch 2011b). Therefore, all vitrification research was performed on samples 24–48 h after collection.

Motility estimation and preparation of sperm samples.—Upon arrival at the ARS, samples were inverted to mix, and the motility of the sperm was estimated using darkfield microscopy (Optiphot-2, Nikon, Garden City, New York) at 200× magnification. A 1-μL sperm suspension was placed on a glass slide and was activated by mixing it with 20 μL of artificial seawater (995 mOsmol/kg). Motility was estimated by observing three to five different fields within 20 s after activation and expressed as the percentage of sperm swimming progressively forward within the sample. Sperm cells that vibrated in place were not considered to be motile.

Sperm concentration was estimated by measuring the absorbance of 2-μL aliquots at a wavelength of 601 nm using a microspectrophotometer (Nanodrop 1000, Thermo Scientific, Wilmington, Delaware) and using these absorbance values in the following calculation:

$$\text{Sperm concentration (cells/mL)} = \text{absorbance} \times 9.77 \times 10^8 - 7.68 \times 10^7.$$

This equation was generated from a standard curve between absorbance readings of serially diluted sperm suspensions and the sperm concentration ($r^2 = 0.987$) as determined by the use of a hemocytometer (Cuevas-Uribe and Tiersch 2011b).

Samples were diluted to a concentration of 2×10^9 cells/mL using sperm motility-inhibiting saline solution (SMIS) (Lahnsteiner 2000). The SMIS was composed of 600 mg NaCl, 315 mg KCl, 15 mg $CaCl_2 \cdot 2\ H_2O$, 20 mg $MgSO_4 \cdot 7H_2O$, and 470 mg HEPES in 100 mL of ultrapure water (pH 7.8), with 1.5 g bovine serum albumin and 0.5 g sucrose at 324 mOsmol/kg. The osmolality was measured with a vapor pressure osmometer (Model 5520, Wescor, Logan, Utah). The samples were placed on ice until use in vitrification experiments.

Sperm vitrification.—As indicated above, sperm were diluted to a concentration of 2×10^9 cells/mL with SMIS. Cryoprotectant solutions were prepared at double strength in SMIS. For vitrification sperm samples were mixed with double-strength cryoprotectants at a ratio of 1:1 (v/v). Samples were immediately loaded (within 15 s) into 10-μL, polystyrene loops (Nunc, Roskilde, Denmark) without equilibration and quickly immersed individually in liquid nitrogen within 1 min (~50 s) of the addition of the cryoprotectant solution. Loops were stored in goblets (three per goblet) in liquid nitrogen. After at least 12 h of storage in liquid nitrogen, the vitrified loop samples were thawed directly onto a microscope slide containing a 30-μL drop of seawater (~1,000 mOsmol/kg) at room temperature (24°C) or other temperatures as noted, and the motility of thawed sperm was estimated within 30 s.

Experiment 1: effect of thawing temperatures.—The cryoprotectants used were: dimethyl sulfoxide (DMSO; OmniSolv, France), ethylene glycol (EG; Mallinckrodt Baker, Paris, Kentucky), 1,2-propanediol (PROH; Sigma-Aldrich, St. Louis, Missouri), and glycerol (Gly; Mallinckrodt Baker). Six vitrification solutions were tested: (1) 20% DMSO + 20% EG, (2) 20% DMSO + 20% PROH, (3) 20% DMSO + 20% Gly, (4) 20% EG + 20% PROH, (5) 20% EG + 20% Gly, and (6) 20% PROH + 20% Gly. Double-strength cryoprotectant solutions were prepared in SMIS and diluted at 4°C with sperm suspension at a ratio of 1:1 (final sperm concentration, 1×10^9 cells/mL). Samples were immediately loaded (within 15 s) into 10-μL, polystyrene loops without equilibration and submerged in liquid nitrogen within 1 min after the addition of the vitrification solutions.

Glass formation was assessed by observing the appearance of the vitrified sample (a milky appearance indicated ice crystal formation) (Ali and Shelton 1993). Loops were thawed directly onto a microscope slide containing a 30-μL drop of seawater at two temperatures (21°C and 37°C). The motility of each sample was estimated immediately after thawing. Sperm from three males were used in this experiment, and one replicate was produced from individual males for each treatment.

Experiment 2: evaluation of vitrification solutions.—Sperm samples from three males were used to evaluate three vitrification solutions: (1) 20% EG + 20% Gly, (2) 10% DMSO + 30% EG + 0.25 M trehalose dehydrate (Tre; Acros Organics, Fair Lawn, New Jersey), and (3) 15% DMSO + 15% EG + 10% Gly + 1% X-1000 (21st Century Medicine, Fontana, California) + 1% Z-1000 (DEGXZ) (21st Century Medicine). The general vitrification procedure was performed. Loops were thawed directly onto a microscope slide containing 30 μL of seawater at room temperature (24°C). The motility of each sample was estimated immediately after thawing. All trials were replicated a minimum of two times for each individual male.

Experiment 3: effect of vitrification solutions on membrane integrity.—Sperm samples from three males were vitrified using

three vitrification solutions: (1) 20% DMSO + 20% EG, (2) 20% DMSO + 20% Gly, and (3) 20% EG + 20% Gly. The samples were stored in liquid nitrogen for 13 d before flow cytometry analysis. To thaw the sperm, each loop was warmed directly in 495 µL of SMIS at room temperature (24°C) to yield a sperm concentration of around 5×10^6 cells/mL. One of the most common assays to assess plasma membrane integrity in fresh and postthaw sperm is the SYBR 14–propidium iodide stain combination, often referred to as a "Live/Dead" or "sperm viability" assay (Daly and Tiersch 2011). To evaluate membrane integrity, fresh and thawed sperm were filtered through 35-µm, nylon mesh and duplicate aliquots of 250 µL were stained with the fluorescent dyes SYBR-14 and propidium iodide (PI) (live–dead sperm viability kit, Molecular Probes, Eugene, Oregon). Final concentrations of the fluorescent dyes were 100 nM SYBR-14 and 12 µM PI, and samples were incubated in the dark for 10 min at room temperature before analysis. Flow cytometry was performed using an instrument (C6 Accuri Cytometers, Ann Arbor, Michigan) equipped with a 488-nm, 50-mW, solid-state laser. Flow cytometer performance was assessed using fluorescent validation beads (Spherotech, Accuri Cytometers) to ensure that CV (100·SD/mean) values were <3.0% (calculated based on full peak height) for the fluorescence detectors (FL1, FL2, FL3, and FL4). Each microcentrifuge tube was gently flicked with a finger three times before analysis to ensure suspension of the cells, and 10 µL of sample were analyzed at a flow rate of 35 µL/min using Cflow software (version 1.0.202.1, Accuri Cytometers). Green fluorescence (SYBR 14) was detected with a 530 ± 15-nm bandpass filter (FL1), and red fluorescence (PI) was detected with a >670-nm longpass filter (FL3). Events were viewed on forward-scatter (FSC) versus side-scatter (SSC) plots, and a gate (used to define target cells within the total event population) was drawn around the sperm population to exclude nonsperm events. Gated events were viewed on a scatter plot showing FL1 versus FL3, and fluorescence compensation was based on the computed median fluorescence values of the different populations to reduce spectral overlap. Sperm that stained with SYBR 14 alone were considered to have intact membranes, and those that stained with both SYBR 14 and PI or PI alone were considered to be membrane-compromised.

Experiment 4: fertilization trials.—Females (body weight, 1.09 ± 0.28 kg [mean ± SD]) were injected intramuscularly with 0.5 mL/kg of Ovaprim yielding 10 µg/kg salmon gonadotropin releasing hormone analog + 10 µg/kg of Domperidone (Syndel International, Vancouver, British Columbia) at the NCSU Lake Wheeler Field Laboratory Facilities. The hormones were administered in two injections. The first injection was 10% of the total dosage; the second injection, given 24 h later, was the remaining 90%. Eggs were collected approximately 48 h after the second injection. Aliquots of 0.1 mL of eggs (129 ± 35 eggs) were placed into 60-mL, plastic cups and held (<1 h) for the fertilization trials.

Based on the results of the previous experiments in April 2009, sperm samples from three males were vitrified at a final concentration of 5×10^8 cell/mL using 20% EG + 20% Gly in 10-µL polystyrene loops at the ARS and shipped overnight to NCSU for the fertilization trials. In addition to the vitrification solution, sperm samples from these three males were vitrified without the use of cryoprotectants (cryoprotectant-free vitrification) at a final concentration of 1×10^9 cells/mL. For artificial fertilization, three loops of vitrified samples from each individual male were thawed into 15-mL conical tubes (Corning Inc., Corning, New York) containing 5 mL of seawater at ~20°C. Loops contained in the tube were gently agitated for <10 s, and the suspensions were mixed with egg aliquots. The estimated sperm-to-egg ratio was 1×10^5 sperm per egg. Other aliquots of eggs were mixed with 30 µL of a pool of fresh sperm from at least two males and were used as a control to evaluate the egg quality. Fresh sperm was collected on the same day as the fertilization and held refrigerated until use. Eggs from three females were used for the fertilization trials during this year. The eggs were incubated at ~20°C. Fertilization rate was estimated by assessing embryo development to the 64–128 cell division stage (3–5 h after fertilization) by use of a dissecting microscope.

In March 2011, sperm samples from three males were vitrified at a final concentration of 1×10^9 cell/mL using 20% EG + 20% Gly in 10-µL, polystyrene loops at the ARS and shipped overnight to NCSU for fertilization trials done in February–March 2011, as described above. Eggs from eight females were used for the fertilization trials during that year. The estimated sperm-to-egg ratio was 3×10^5 thawed sperm per egg.

Statistical analysis.—Data were analyzed using SAS software (Statistical Analysis System, version 9.1; SAS institute, Cary, North Carolina). Analyses were conducted using a mixed ANOVA procedure for all interactions. For the thawing experiment, the fixed treatments were temperature and vitrification solution, and the dependent variable was postthaw motility. Membrane integrity data were analyzed by means of a mixed ANOVA procedure using cryoprotectants as fixed treatments and membrane intact as a dependent variable. Percentage data were arcsine-square-root transformed for normalization before analysis, and the post hoc Tukey's test was used to locate differences. The significance level was set at $P < 0.05$.

RESULTS

Experiment 1: Thawing Temperatures

The motility of fresh sperm before vitrification was 50 ± 10% (mean ± SD). There were no significant differences ($P = 0.697$) in postthaw motility of sperm thawed at 21°C or at 37°C in all the treatments (Table 1). The highest postthaw motility was 35% for 20% EG + 20% Gly and 20% DMSO + 20% Gly, and these were not significantly different ($P = 0.606$). The

motility (14 ± 10%) in the vitrification solution of 20% PROH + 20% Gly was not significantly different from motility (21 ± 9%) in 20% DMSO + 20% Gly (P = 0.059), but it was significantly different from motility (22 ± 7%) in 20% EG + 20% Gly (P = 0.018). The motilities in 20% DMSO + 20% EG (2 ± 2%), 20% DMSO + 20% PROH (2 ± 1%), and 20% EG + 20% PROH (3 ± 3%) were not significantly different (P > 0.446). Total glass formation was observed in all vitrification solutions, except 20% DMSO + 20% Gly, which formed around 80–90% glass (<20% had a milky appearance).

Experiment 2: Vitrification Solutions

The motility of fresh sperm before vitrification was 60 ± 10%. The highest postthaw motility was 40% for 20% EG + 20% Gly, followed by 30% for 15% DMSO + 15% EG + 10% Gly + 1% X-1000 + 1% Z-1000 (DEGXZ). These two treatments were significantly different in motility (P = 0.039) (Table 1). Motility (7 ± 3%) in 10% DMSO + 30% EG + 0.25 M Tre was not significantly different from motility (14 ± 10%) in DEGXZ (P = 0.114), but it was significantly different from 20% EG + 20% Gly (28 ± 9%) (P = 0.008).

Experiment 3: Membrane Integrity

Fresh sperm motility (57 ± 9%) was positively correlated (r = 0.80) with membrane-intact cells (89 ± 1%), but there was a significant difference between them (P < 0.001) (Table 2). There was no correlation between motility of thawed sperm and membrane integrity in all treatments (Table 2). The highest percentage of membrane-intact sperm after vitrification (17%) was for 20% EG + 20% Gly, and there was no significant difference in motility (P = 0.252). The vitrification solution 20% EG + 20% Gly was not significantly different in motility (P = 0.076) from 20% DMSO + 20% Gly, but it was significantly different in membrane integrity (P = 0.037). The vitrification solution 20% DMSO + 20% EG was significantly different in membrane integrity from 20% EG + 20% Gly (P = 0.004) and from 20% DMSO + 20% Gly (P = 0.045).

Experiment 4: Fertilization Trials

Of the three females used in 2009, only one yielded usable eggs (>20% fertilization in control). Fertilization from vitrified sperm of one male yielded the same fertilization as the fresh sperm control (Table 3). But the vitrified sperm from the other two males yielded low fertilization (<5%). Cryoprotectant-free vitrification did not yield fertilization.

From the eight females used in 2011, data from four females with control fertilization of >9% were used. The same males were used for females 1, 2, and 3 (Table 3). Due to logistic problems no fertilization was attempted for males 1 and 3 with female 3 (Table 3). There was male-to-male variation or variation within egg batches in the fertilization trials. For example, male 2 yielded 23% fertilization with female 1, while male 3 yielded 6% with the same female. For female 2, the fertilization from vitrified sperm yielded the same fertilization rate as the fresh control. Fertilization for female 4 yielded low levels of fertilization (<10% versus 20% for the control) (Table 3).

DISCUSSION

Vitrification is an alternative approach to cryopreservation and offers a new way for the conservation of genetic material (Tucker and Liebermann 2007). Recently vitrification has been applied for sperm cryopreservation of marine fishes (Cuevas-Uribe et al. 2015; Kasa et al., in press). Vitrified sperm samples from Spotted Seatrout, Red Snapper, and Red Drum yielded motility as high as 60% and membrane integrity as high as 23%. Another study with the marine European Eel yielded motilities of 5% after vitrification using Cryotop, a commercially available device designed for use with human oocytes and embryos (Kitazato BioPharma, Shizuoka, Japan) designed for 2 μL of solution (Kasa et al., in press). However, these studies did not attempt fertilization or offspring production. One of the most important assessment methods for sperm quality of cryopreserved sperm is the ability to fertilize eggs

TABLE 1. Percent sperm motility (mean ± SD) of Southern Flounder after thawing at different temperatures and the osmolality for different vitrification solutions. DMSO: dimethyl sulfoxide; EG: ethylene glycol; PROH: propanediol; Gly: glycerol; Tre: trehalose; X: X-1000; Z: Z-1000. Mean values with different letters within a row are significantly different (P < 0.05).

Vitrification solution	Thawing temperature			Osmolality (mOsmol/kg)
	21°C	24°C	37°C	
20% DMSO + 20% EG	2 ± 2 z		2 ± 2 z	5,988
20% DMSO + 20% PROH	2 ± 2 z		1 ± 1 z	4,906
20% DMSO + 20% Gly	20 ± 12 z		21 ± 6 z	5,542
20% EG + 20% PROH	3 ± 4 z		3 ± 3 z	5,150
20% PROH + 20% Gly	15 ± 12 z		12 ± 8 z	4,680
20% EG + 20% Gly	19 ± 5 z	28 ± 9 y	26 ± 8 zy	7,594
10% DMSO + 30% EG + 0.25 M Tre		7 ± 3		6,104
15% DMSO + 15% EG + 10%Gly + 1% X + 1% Z		14 ± 10		7,510

TABLE 2. Postthaw motility (percent [mean ± SD]), membrane-intact cells (percent intact [mean ± SD]), and their correlation of Southern Flounder sperm vitrified with different vitrification solutions. DMSO: dimethyl sulfoxide; EG: ethylene glycol; Gly: glycerol. Motility and membrane integrity by fresh sperm are included for comparison. Mean values with different letters within rows are significantly different ($P < 0.05$).

Treatment	% Motility	% Intact	Correlation coefficient (r)
Fresh	57 ± 9 z	89 ± 1 y	0.80
20% DMSO + 20% EG	0 ± 1 z	2 ± 1 y	−0.26
20% DMSO + 20% Gly	7 ± 6 z	6 ± 4 z	−0.33
20% EG + 20% Gly	13 ± 6 z	11 ± 4 z	0.18

and produce offspring. The purpose of the present study was to evaluate the fertilization ability of vitrified sperm.

The greatest challenge in applying the vitrification approach is to formulate an appropriate vitrification solution and develop equilibration and dilution procedures that minimize osmotic and toxic injury (Rall 1991). In the present study, we used vitrification solutions developed from previous studies with marine fish (Cuevas-Uribe et al. 2015) and the freshwater Green Swordtail (Cuevas-Uribe et al. 2011b). Also, cryoprotectants were selected to formulate the vitrification solutions based on our previous study of high-throughput sperm cryopreservation in Southern Flounder (Hu et al. 2016) and from other studies on paralichthid flounders. For example, DMSO and Gly were used for conventional cryopreservation of sperm from Olive Flounder *Paralichthys olivaceus* (Zhang et al. 2003), Brazilian Flounder *P. orbignyanus* (Lanes et al. 2008), and Summer Flounder *P. dentatus* (Brown et al. 2013), while PROH and EG were used to cryopreserve sperm from Summer Flounder (Liu et al. 2015). All of these previous studies used comparatively low concentrations of cryoprotectants (<20%). The high concentrations of cryoprotectants required for vitrification (>40%) are close to the maximum tolerated by cells (Mazur et al. 2008). Thus, to decrease the toxicity of individual cryoprotectants, a mixture was used in order to combine the cumulative beneficial properties of each cryoprotectant such as permeability and glass formation (Weiss et al. 2010).

In experiment 1 of the present study, four cryoprotectants were chosen, and eight vitrification solutions were tested. The solutions that contained Gly yielded the highest postthaw motilities. There was with no relationship between osmolality of the vitrification solutions and postthaw motility. This result was different from previous studies in mammals that suggested the molarity be lowered to reduce the toxicity of the vitrification solutions (Ali and Shelton 2007). The highest postthaw motility was for the combination of Gly and EG. A previous study in Summer Flounder that used EG for conventional cryopreservation at 20% reported postthaw motilities of 70% (Liu et al. 2015). In a vitrification study that used 20% EG + 20% Gly to vitrify sperm from Red Snapper, the average postthaw motility was 23% (Cuevas-Uribe et al. 2015), which was similar to the motility yielded of this study (26%). In a vitrification study with the freshwater Green Swordtail, the same vitrification solutions yielded postthaw motilities of ~10% (Cuevas-Uribe et al. 2011b).

In this study, the two temperatures (21°C and 37°C) used to thaw samples were not significantly different in their effect on

TABLE 3. Percent egg fertilization (mean ± SD) achieved by Southern Flounder sperm vitrified with 20% ethylene glycol (EG) + 20% glycerol (Gly) in 10-µL polystyrene loops. Fertilization capability was assessed at the 64–128 cell division stage. The same males were used for females 1, 2, and 3 in 2011. Fertilization rates by fresh sperm are included for comparison of egg quality.

		Male				
Female	Year	1	2	3	Average	Control
1[a]	2009	50 ± 20	3 ± 2	3 ± 1	19 ± 25	50 ± 0
1[b]	2011	12	23	6	14 ± 9	54 ± 5
2[b]	2011	8	7	8	8 ± 1	9 ± 2
3[b]	2011		13		13 ± 0	27 ± 6
4[c]	2011	1 ± 0	0 ± 0	9 ± 2	3 ± 4	20 ± 4

[a] Mean ± SD of three replicates of egg batches.

[b] No replicates for individual males.

[c] Mean ± SD of two replicates of egg batches.

motility. This corresponded with a previous study with the Green Swordtail where no difference in motility was found between thawing at 24°C or 37°C (Cuevas-Uribe et al. 2011b). Because small volumes of vitrification solutions were used, both temperatures yielded warming rates that were sufficient to avoid ice crystal formation (devitrification). Katkov et al. (2003) estimated the warming rate for a similar loop at 37°C could be as fast as 200,000°C/min. This result agrees with Mazur and Seki (2011) who found that a fast warming rate (118,000°C/min) was more critical than cooling rates (95–69,250°C/min) in the survival of vitrified oocytes and embryos in mammals.

In experiment 2, two vitrification solutions developed for use with marine fishes that yielded high motility (>20%) (Red Snapper, Spotted Seatrout, and Red Drum) were evaluated (Cuevas-Uribe et al. 2015). To enhance glass formation, trehalose or the proprietary polymers X-1000 and Z-1000 were added to cryoprotectant mixtures. Trehalose can have a protective benefit in conventional cryopreservation for marine fishes such as in sex-reversed Orange-spotted Grouper *Epinephelus coioides* (Peatpisut and Bart 2010) and Longtooth Grouper *E. bruneus* (Miyaki et al. 2005). In comparison, the addition of trehalose in the present study did not increase the survival of Southern Flounder sperm after vitrification. Our result is similar to a study that used the same vitrification solution that resulted in low postthaw motility (~4%) in the Green Swordtail (Cuevas-Uribe et al. 2011b).

The other macromolecules used in the present study were the commercial polymers X-1000 and Z-1000. In addition to reducing the cryoprotectant concentration needed to vitrify, these polymers can act as "ice blockers" that inhibit ice nucleation and growth (Wowk and Fahy 2002). The vitrification solution DEGXZ was used with sperm from marine fishes, yielding high postthaw motilities in Red Snapper (~40%), Spotted Seatrout (~60%), and Red Drum (~30%) (Cuevas-Uribe et al. 2015). Our results yielded postthaw motility of ~14% with this vitrification solution.

The highest postthaw motilities were observed in experiments 1 and 2 that used 20% EG + 20% Gly. These cryoprotectants have low toxicity (Shaw and Jones 2003), but Gly is a less-effective glass former (forms glass at 46% concentration) compared with EG (forms glass at 40% concentration), and EG permeates cells faster than Gly (Shaw and Jones 2003). The combination of Gly and EG is one the most common used for vitrification (Ali and Shelton 2007). This mixture between a poor glass former and a fast permeable cryoprotectant was advantageous for Southern Flounder (postthaw motility, 20–30%).

Sperm motility alone is not necessarily a reliable predictor of fertilization (Kopeika and Kopeika 2008). The high concentrations of cryoprotectants (>40%) and the high osmotic pressures (>4,500 mOsmol/kg) from the vitrification solutions could damage sperm by chemical toxicity or osmotic effects that include changes in plasma membrane integrity. If the plasma membrane is not functionally intact the sperm would be compromised in viability and its capability to fertilize (Silva and Gadella 2006).

For the analysis of membrane integrity, "viable sperm" were defined as cells that possess an intact plasma membrane. In a previous study with sperm vitrification in Channel Catfish, the percentage of membrane-intact cells increased as the chosen cryoprotectants yielded more glass formation (Cuevas-Uribe et al. 2011a). In another study with Green Swordtail sperm, membrane integrity was evaluated after the addition of the vitrification solutions just before and after vitrification. Before vitrification, membrane-intact cells were ~70%, and around 10% viable sperm were recorded after vitrification (Cuevas-Uribe et al. 2011b). In this study the highest percentage of membrane-intact cells was for the vitrification solution 20% EG + 20% Gly, which corresponded with the treatment with the highest postthaw motility. This vitrification solution did not contain DMSO.

The postthaw sperm motilities in the membrane integrity experiment (experiment 3) were lower than in the previous experiments. For example, the postthaw motility in this experiment for the treatment 20% DMSO + 20% Gly (~7%) was different from experiment 1 (~20%). In a similar manner the postthaw motility for 20% EG + 20% Gly in this experiment (~13%) was different from experiment 1 (19–26%) and experiment 2 (~28%). These differences could be due to male-to-male variation or because of incomplete recrudescence. The sperm collected from experiment 2 were stripped in March, while the sperm used for the flow cytometry experiment were collected 5 months later. Although these males, from which the sperm were taken, were held in environmentally controlled recirculating systems, a minimum of 5 months is required for recrudescence to regain the requisite energy and storage depots such as lipids (Watanabe et al. 2006).

The highest postthaw motility and membrane integrity was observed for 20% EG + 20% Gly. This vitrification solution was subsequently used for the fertilization trials. The criterion for fertilization was determined by the examination of the incubated eggs for early stages of embryonic cleavage. The fertilization rate was determined at 3–6 h postfertilization. At this time the embryos are in a multicellular stage (64–128 cell division stage) that is easily identified (Daniels 2000). This fertilization criterion had been used in previous reproductive studies in Southern Flounder (Berlinsky et al. 1996; Hu et al. 2016). In the present study, fertilization percentage varied among females and males. Vitrified sperm yielded fertilization rates as high as 70% (fresh sperm control, 50%). On average, fertilization with vitrified sperm ranged from 10% to 20%. There were times that fertilization was low; this could be due to the loop-to-loop variation or to the difference among egg batches from the same female. Berlinsky et al. (1996) noticed that fertilization rates varied considerably between females and between spawns from individual fish. This could be due to the reproductive characteristics of Southern Flounder,

which is a serial spawner (i.e., multiple clutch, group synchronous) and produces multiple batches of eggs during the spawning season in intervals of 3–4 d (Watanabe and Daniels 2010). Each batch could have oocytes at different stages (Berlinsky et al. 1996), and not all the viable eggs that float are fertilized (Daniels et al. 2010). There is also variance between batches when the first spawn will have low fertility (<10%), but the fertility will improve (>50%) within a week and remain high for 1 month. But after that the egg production and fertility will start to decline (Daniels and Watanabe 2002).

The sperm samples vitrified without cryoprotectants (cryoprotectant-free vitrification) did not yield fertilization. This contrasts with results in Rainbow Trout that reported motility of ~80% after vitrification without cryoprotectants (Merino et al. 2011). No fertilization trials were done in the Rainbow Trout study, and there was no clear description of motility assessment. In another attempt of cryoprotectant-free vitrification, done in Red Drum, no motility was observed (Cuevas-Uribe et al. 2015). For estimation of percent motility, only sperm that are actively swimming in a forward motion should be included (Tiersch 2011c). In addition, the Rainbow Trout study reported mitochondrial membrane potentials of ~50% when bovine serum albumin was used. In the present study, the extender used (SMIS) contained bovine serum albumin and no motility was observed. Cryoprotectant-free vitrification has proved to be a useful tool in mammals. For example, in humans cryoprotectant-free vitrification yielded fertilization equal to slow cooling (Isachenko et al. 2004). In fish, cryoprotectant-free vitrification yielded limited success in Channel Catfish (<2% fertilization in 2 of 16 trials) (Cuevas-Uribe et al. 2011a) and in Persian Sturgeon *Acipenser persicus* (6% motility) (Abed-Elmdoust et al. 2015). More research needs to be done to evaluate the fertilization ability of cryoprotectant-free sperm vitrification in other fishes.

As more marine fish become imperiled directly from anthropogenic disturbances such as exploitation and habitat loss and degradation, or indirectly through the effects of climate change, there is an urgent need for immediate and decisive conservation action. Cryopreservation can contribute to conservation programs, but conventional techniques require specialized equipment unsuitable for use in field environments. New approaches that can be easily used in the field to cryopreserve samples and be applied to assist in conservation programs are urgently needed. In the present study a technique has been offered that could be used in the field and yields acceptable fertilization of 10–20% and is similar to the 20–30% of conventional cryopreservation (Hu et al. 2016). The urgent conservation situation of some species demands attempts for germplasm storage even in face of low expectation of success (Holt et al. 2003). Protection of genetic diversity by sperm vitrification offers new approaches for conservation biology, biomedical models, wild fisheries, and culture species.

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