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Vitrification of sperm from marine fish: effect on motility and membrane integrity

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Abstract

Our goal was to develop a standardized approach for sperm vitrification of marine fish that can be applied generally in aquatic species. The objectives were to: (i) estimate acute toxicity of cryoprotectants over a range of concentrations; (ii) evaluate the properties of vitrification solutions (VS); (iii) evaluate different thawing solutions and (iv) evaluate sperm quality after thawing by examination of motility and membrane integrity. Sperm were collected from red snapper (*Lutjanus campechanus*), spotted seatrout (*Cynoscion nebulosus*) and red drum (*Sciaenops ocellatus*). A total of 29 combinations of cryoprotectants were evaluated for toxicity and glass formation. Samples were loaded onto 10-μL polystyrene loops and plunged into liquid nitrogen. There was a significant difference ($P < 0.05$) in post-thaw motility among VS and among species when using the same VS. The sperm in VS of 15% DMSO + 15% ethylene glycol + 10% glycerol + 1% X-1000™ + 1% Z-1000™ had an average post-thaw motility of 58% and membrane integrity of 19% for spotted seatrout, 38% and 9% for red snapper, and 30% and 19% for red drum. Adaptations by marine fish to higher osmotic pressures could explain the survival in the high cryoprotectant concentrations. Vitrification offers an alternative to conventional cryopreservation.

Keywords: sperm vitrification, cryopreservation, red snapper, spotted seatrout, red drum

Introduction

Cryopreservation has proven to be a useful tool for improvement, maintenance and distribution of genetic resources in aquatic species (Tiersch 2011a). There are two general procedures to attain cryopreservation: (i) slow equilibrium freezing, commonly referred to as 'slow freezing' and (ii) rapid non-equilibrium vitrification, often referred to as 'ultra-rapid cooling'. The main differences between these two procedures are the concentration of cryoprotectants, and the cooling and warming rates. The main purpose of these procedures is to avoid osmotic damage and intracellular ice formation, which have negative effects on gamete survival. While in equilibrium cooling these harmful effects are prevented by cellular dehydration (maintaining an osmotic equilibrium between intracellular and extracellular compartments), in vitrification ice crystal formation is avoided by converting the solution directly into a viscous glass that correspond to a viscosity of 10^{13} poise (Fahy & Rall 2007; Mazur, Leibo & Seidel 2008). Glass is formed when solutions reach the glass transition temperature (−130°C for water). This temperature can be raised by the addition of cryoprotectants, making it easier to attain. However, the high concentrations of cryoprotectants required for vitrification are near the maximum tolerated by cells (Fahy, MacFarlane, Angell & Meryman 1984). As such, the typical approach to vitrification is to reach the glass transition temperature as fast as possible through rapid cooling, and by increasing the concentration of cryoprotectants, thereby

bypassing or rapidly passing through the critical temperature range of ice formation (between −5 and −40°C) (Shaw & Jones 2003).

Vitrification has been applied to the cryopreservation of fish oocytes (e.g. Guan, Rawson & Zhang 2010), eggs (e.g. Urbanyi, Magyary, Horvath, Baranyai & Dinnyes 2011), embryos (e.g., Edashige, Valdez, Hara, Saida, Seki & Kasai 2006) and primordial germ cells (e.g., Kawakami, Saito, Fujimoto, Goto-Kazeto, Takahashi, Adachi, Arai & Yamaha 2012). Vitrification has been demonstrated to be a useful tool for the cryopreservation of primordial germ cells (Kawakami, Ishihara, Saito, Fujimoto, Adachi, Arai & Yamaha 2012). However, embryo vitrification has been inconsistent. There are reports of survival after embryo vitrification, but there has been a lack of reproducibility with these studies (e.g., Edashige *et al.* 2006; Hagedorn & Kleinhans 2011). Recently, sperm vitrification was applied successfully in freshwater fish, and offspring were produced from vitrified sperm samples of Russian sturgeon (*Acipenser gueldenstaedtii*, Brandt & Ratzeburg) (Andreev, Sadikova, Gakhova, Pashovkin & Tikhomirov 2009), channel catfish (*Ictalurus punctatus*, Rafinesque) (Cuevas-Uribe, Leibo, Daly & Tiersch 2011), green swordtail (*Xiphophorus hellerii*, Heckel) (Cuevas-Uribe, Yang, Daly, Savage, Walter & Tiersch 2011) and rainbow trout (*Oncorhynchus mykiss*, Walbaum) (Figueroa, Risopatron, Sanchez, Isachenko, Merino, Isachenko & Valdebenito 2013). Despite this, sperm vitrification remains unexplored in marine fish. It is generally recognized that sperm from marine fish have a higher quality following cryopreservation compared with freshwater species (Drokin, Stein & Bartscherer 1998; Suquet, Dreanno, Fauvel, Cosson & Billard 2000; Herraez, Cabrita & Robles 2012). This may be due to adaptations of marine fish sperm to deal with osmotic changes between body tissues and the external environment, or differences in sperm membrane composition (Kopeika & Kopeika 2008). Based on the feasibility of sperm vitrification in freshwater species, we decided to evaluate the utility of sperm vitrification when applied to marine fish.

Global marine fisheries are in crisis, with more than 30% of marine fish stocks monitored by FAO considered to be overexploited (FAO 2012). The decline of marine fisheries and the increasing consumer demand for seafood have increased intensive fish farming (Tal, Schreier, Sowers, Stubblefield, Place & Zohar 2009). As marine fish farming expands, there is an increasing need to apply sperm cryopreservation for repository development. Sperm cryopreservation can be used to preserve genetic resources from stocks of fish that are endangered and to aid in replenishing fisheries (Wayman, Thomas & Tiersch 1996). The spotted seatrout (*Cynoscion nebulosus*, Cuvier), red drum (*Sciaenops ocellatus*, Linnaeus) and red snapper (*Lutjanus campechanus*, Poey) are popular sport fish in the Gulf of Mexico, but chronic overfishing, habitat destruction and climate change have resulted in significant population declines in these species (Rummer 2007). Although sperm cryopreservation protocols have been developed for these species (Wayman *et al.* 1996; Wayman, Tiersch & Thomas 1998; Riley, Holladay, Chesney & Tiersch 2004), vitrification offers significant advantages over conventional cryopreservation due to its simplicity and speed. Furthermore, vitrification does not require specialized equipment, making it more user-friendly especially for on-farm procedures and field work in remote sites (Saragusty & Arav 2011). Although slow freezing has been proven effective, it requires more time and typically involves the use of expensive equipment (Moore & Bonilla 2006).

Based on previous cryopreservation studies done with spotted seatrout, red drum and red snapper, and on the availability of gametes from recreational fisheries, we decided to use these fish to evaluate the application of sperm vitrification for marine fish. The goal of this project was to develop a standardized approach for sperm vitrification of marine fish that can be applied generally in aquatic species. The specific objectives in this study of marine fish were to: (i) estimate acute toxicity of cryoprotectants over a range of concentrations; (ii) evaluate the properties of vitrification solutions (VS); (iii) evaluate different thawing solutions and (iv) evaluate sperm quality after thawing by examination of motility and membrane integrity. Here, we report the first successful sperm vitrification in marine fish. Vitrification offers an alternative to conventional cryopreservation and it is ideally suited to work with small volumes.

Materials and methods

Fish and sperm collection

Fish were collected by recreational anglers on charter boats off coastal Louisiana during June (spotted seatrout: $n = 80$, and red snapper: $n = 29$) and

October (red drum: $n = 6$) of 2009. After capture, the fish were placed in insulated coolers with ice and transported to either Coco Marina or Sportman's Paradise (Cocodrie, LA, USA). Testes were removed within 4–8 h of capture as in previous studies (Riley, Chesney & Tiersch 2008), before the fish were cleaned and filleted for customers. Testes were placed in 4-L Ziploc® freezer bags (S.C. Johnson and Son Inc., Racine, WI, USA) with calcium-free Hanks' balanced salt solution and adjusted to 200 mOsmol kg^{-1} with ultrapure water (C-F HBSS) (Riley *et al.* 2008). The osmolality was measured with a vapour pressure osmometre (Model 5520; Wescor Inc., Logan, UT, USA). The samples were placed on ice and transported to the Louisiana Universities Marine Consortium in Cocodrie (10 min) where the testes were removed from bags, blotted dry with paper towel and weighed. Because the testes of spotted seatrout are smaller than those of red drum and red snapper, they were treated differently. Sperm from spotted seatrout were released by crushing of testes in a quart Ziploc® freezer bag after addition of 1 mL (volume) of C-F HBSS per 1 g (weight) of testes. The sperm suspensions were filtered through a mesh series consisting of a 7.62-cm round mesh strainer (1-mm mesh), a 15.24-cm round mesh strainer (0.5-mm mesh) and a 200-μm mesh filter. Testes from red drum and red snapper were sliced to release sperm (Riley *et al.* 2004). Sperm were collected in 50-mL plastic centrifuge tubes (Corning, NY, USA) and diluted 1:3 (v:v) with C-F HBSS. Sperm concentration was estimated by use of a haemacytometre (Hausser Scientific, Horsham, PA, USA) and diluted to a final concentration of 2×10^9 sperm mL^{-1} with C-F HBSS. The sperm solutions were refrigerated at 4°C and stored (from 2 to 24 h) until used in cryopreservation experiments. Refrigeration storage was demonstrated to preserve sperm motility as long as 6 days (Riley 2002).

Estimation of sperm motility

The per cent motility in each sperm sample was estimated using dark-field microscopy at 200-x magnification. Motility was determined by visually comparing the percentage of sperm that were actively moving forward to non-motile sperm. Sperm that vibrated in place without forward movement were not considered to be motile. Activation of sperm from all three species was initiated by placing 2 μL of sperm onto a microscope slide and dilution it with 20 μL of filtered seawater collected from the Gulf of Mexico, at 970 mOsmol kg^{-1} (~30 ppt). Estimates of motility were based on an observation of three to five different fields within 10 s of adding the seawater. For consistency, motility was evaluated by a single skilled operator in a blind protocol (the examiner did not know the treatment). Sperm suspensions with at least 50% motility and sperm concentrations of greater than 2×10^9 sperm mL^{-1} were used for these experiments (based on these criteria, samples from seven spotted seatrout, four red snapper and four red drum were studied).

Cryoprotectant concentration study

Sperm from three independent samples of spotted seatrout and three red snapper males were collected and diluted to a final concentration of 2×10^9 sperm mL^{-1} with C-F HBSS (Riley 2002). Eight cryoprotectants at different concentrations (v/v) were evaluated for their effects on sperm motility over 15 min at 4°C: (i) ethylene glycol (EG; Mallinckrodt Baker, Paris, KY, USA) was used at 10%, 15%, 20%, 25% and 30%; (ii) dimethyl sulphoxide (DMSO; OmniSolv, Gibbstown, NJ, USA) was used at 15%, 20%, 25% and 30%; (iii) 1,2-propanediol (PROH; Sigma-Aldrich, St Louis, MO, USA) and (iv) glycerol (Gly; Mallinckrodt Baker) were used at 10%, 15% and 20%; (v) methanol (MeOH; Fisher Scientific, Fair Lawn, NJ, USA); (vi) methyl glycol (2-methoxyethanol, MG; Sigma-Aldrich) were used at 15% and 20%; (vii) polyethylene glycol (PEG MW 200; Sigma-Aldrich) and (viii) 2,3-butanediol (BD; Acros Organics, Fair Lawn, NJ, USA) were used at 10%. Cryoprotectant solutions were prepared in C-F HBSS at double the final concentration and kept cold (4°C) before being added at that temperature to the sperm suspension at a ratio of 1:1 (100 μL of cryoprotectant solution: 100 μL of sperm suspension). Motility was estimated immediately (within 10 s) and at 5, 10 and 15 min. Three replicates were produced for each treatment with the different fish sperm. The present study evaluated relatively high concentrations of cryoprotectants (as high as 30%) at regular exposure times (every 5 min). The acute toxicity experiments were performed in June and the only gametes available were from spotted seatrout and red snapper. No further attempts were made to estimate acute toxicity in red drum, which spawn from September to October in the Gulf of Mexico.

Acute toxicity of combined cryoprotectants

Sperm from three spotted seatrout (one male was used in the previous experiment) and three red snapper males (the same used in the previous experiment) were diluted to a final concentration of 2×10^9 sperm mL^{-1} with C-F HBSS. Twenty-nine different cryoprotectant combinations were tested (Table 1). For each, double-strength cryoprotectant solutions were prepared in C-F HBSS and mixed with sperm suspensions at a ratio of 1:1 at 4°C. Motility was estimated immediately (within 10 s) and at 5 and 10 min. Three replicates were produced for each treatment with different fish sperm.

The general procedure for vitrification of sperm and glass formation

Sperm were diluted to a concentration of 2×10^9 sperm mL^{-1} with C-F HBSS. Double-strength cryoprotectant solutions were prepared in C-F HBSS, and mixed with sperm suspensions at a ratio of 1:1. Samples were loaded within 15 s into 10-μL polystyrene loops (Nunc™, Roskilde, Denmark) without equilibration, and individually submerged in liquid nitrogen within 1 min (~50 s) of the addition of the cryoprotectant solution. Glass formation was assessed by observing the appearance of the vitrified sample. A milky appearance indicated ice crystal formation, and a clear transparent appearance indicated glass formation (Ali & Shelton 1993). Loops were stored in goblets (three per goblet) in liquid nitrogen. After at least 12 h (range: 12–48 h) of storage in liquid nitrogen, the vitrified loop samples were thawed directly onto a microscope slide containing a 30 μL drop of filter seawater (~1000 mOsmol kg^{-1}) at room temperature (24°C), and the motility of thawed sperm was estimated within 10 s.

Evaluation of vitrification and thawing solutions

Based on the toxicity of combined cryoprotectants in previous research (Wayman *et al.* 1996, 1998; Riley 2002; Ali & Shelton 2007), and on the vitrification properties found in this study, eight VS were selected to vitrify sperm of red snapper from three males: 35ET, 40ET, 20E20G, 30E10B, 10D30ET, 40% EG, 35EXZ and DEGXZ (Table 1). In addition to these VS, sperm were also vitrified without the use of cryoprotectants (cryoprotectant-free vitrification). The VS tested for sperm from three spotted seatrout were: 40ET, 10D30ET and DEGXZ (Table 1). The vitrification procedure was performed as described above. Loops were thawed directly onto a microscope slide containing 30 μL (this volume was chosen because of reliability of thawing compared with 20 μL) of two thawing

Table 1 Combined cryoprotectants used for the acute toxicity experiment. Glass formation was assessed by plunging samples into liquid nitrogen. Appearance of glass (clear, transparent) or ice crystals (opaque, milky) was observed

Combined cryoprotectants	Abbreviation	Glass (%)
20% EG + 15% Ace	20EA	0
25% DMSO + 0.25 M Tre	25DT	0
30% DMSO + 0.25 M Tre	30DT	0
30% EG + 0.25 M Tre	30ET	10
35% DMSO + 0.25 M Tre	35DT	10
35% EG + 0.25 M Tre	35ET	60
35% PROH + 0.25 M Tre	35PT	80
35% PROH + 3% PVA	35PP	70
40% DMSO + 0.25 M Tre	40DT	100
40% EG + 0.25M Tre	40ET	100
20% DMSO + 20% EG	20D20E	90
20% DMSO + 20% MG	20D20M	90
20% DMSO + 20% PROH	20D20P	100
20% DMSO + 20%Gly	20D20G	95
20% EG + 20% Gly	20E20G	50
20% EG + 20% MG	20E20M	90
20% MeOH + 20% MG	20Me20M	100
25% DMSO + 15% EG	25D15E	90
30% DMSO + 10% BD	30D10B	100
30% EG + 10% BD	30E10B	100
30% EG + 10% MeOH	30E10Me	100
10% DMSO + 30% EG + 0.25 M Tre	10D30ET	95
15% DMSO + 15% EG + 10% Gly	15D15E10G	50
20% DMSO + 15% EG + 0.25 M Tre	20D15ET	0
20% DMSO + 10% EG + 10% PROH	20D10E10P	100
20% DMSO + 10% MG + 10% PROH	20D10M10P	100
20% DMSO + 10% PROH + 6% PEG + 15% Ace	DPPA	30
25% DMSO + 10% BD + 15% Ace	25D10BA	100
25% DMSO + 15% EG + 15% Ace	25D15EA	95
35% EG + 1% X + 1% Z*	35EXZ	80
15% DMSO + 15% EG + 10% Gly + 1% X + 1% Z	DEGXZ	100

The cryoprotectants were: ethylene glycol (EG), acetamide (Ace), dimethyl sulphoxide (DMSO), trehalose (Tre), propanediol (PROH), polyvinyl alcohol (PVA), methyl glycol (MG), glycerol (Gly), methanol (MeOH), butanediol (BD), polyethylene glycol (PEG), X-1000™ (X) and Z-1000™ (Z).
*Not used in cryoprotectant toxicity experiment.

solutions: filtered seawater (~1000 mOsmol kg^{-1}) or 10% DMSO in seawater (~2000 mOsmol kg^{-1}) at room temperature (24°C). The motility of each sample was estimated immediately after thawing. All trials were replicated for each individual male.

Membrane integrity assessment

Sperm samples from three spotted seatrout, three red snapper and four red drum were used in this experiment. Sperm samples were vitrified using three treatments (10D30ET, DEGXZ and 40ET; Table 1) for spotted seatrout and red snapper, and two treatments (10D30ET and DEGXZ) for red drum. Sperm membrane integrity was evaluated with the fluorescent dyes SYBR-14 and propidium iodide (PI) (live/dead sperm viability kit; Molecular Probes, Eugene, OR, USA). Each loop was thawed directly in 495 μL of C-F HBSS at room temperature (24°C), and duplicate aliquots of 250 μL of sperm sample at a concentration of ~5×10^6 cells mL^{-1} were stained with 100 nm SYBR-14 and 12 μM PI for 10 min. Membrane integrity was assessed by analysing 10 μL of sperm sample at a flow rate of 35 μL min^{-1} using a BD Accuri C6 flow cytometre equipped with a 488 nm, 50 mW solid-state laser (BD Accuri; BD Biosciences, San Jose, CA, USA), and CFlow® Plus software (BD Accuri; BD Biosciences). 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 with gating to exclude non-sperm events. Gated events were viewed on a scatter plot showing FL1 versus FL3 with fluorescence compensation to reduce spectral overlap. The proportion of intact sperm was expressed as a percentage of the fluorescent population (i.e. sperm stained with SYBR 14, PI, or both) to exclude non-sperm particles from calculations (Daly & Tiersch 2011).

Statistical analysis

Data were analysed as a factorial randomized block design. Analyses were conducted using a mixed ANOVA procedure for all interactions. For acute toxicity experiments, the fixed treatment variables were: cryoprotectant, concentration and incubation time. The males were grouped in a block to remove variation among individual motility from the error term. The dependent variable was sperm motility (per cent). The control (fresh sperm) was excluded from the model, but was used as a reference to ensure that the sperm were still motile. For the thawing experiment, the fixed treatments were VS and thawing solution, and the dependent variable was post-thaw motility (per cent). For membrane integrity experiments, the fixed treatments were cryoprotectants, and the dependent variable was membrane intact (per cent). Correlation between membrane-intact and post-thaw motility was estimated. Statistical differences were determined at an $\alpha = 0.05$ level using Tukey's adjustment. Statistical analyses were performed using SAS software (Statistical Analysis System Inc., version 9.1; SAS institute, Cary, NC, USA).

Results

Collection of fish, testes and sperm

In total, 80 spotted seatrout, 29 red snapper and six red drum males were collected from the recreational fishery. The total body length (TL; mean ± SD) was 40.6 ± 5.9 cm for spotted seatrout, 63.0 ± 6.6 cm for red snapper and 90.4 ± 13.4 cm for red drum. Testes weight (mean ± SD) was 9.6 ± 5.0 g for spotted seatrout, 35.5 ± 27.4 g for red snapper and 95.8 ± 79.5 g for red drum. Testes size and development was noticeably variable among similar-sized fish.

Cryoprotectants concentration study

Spotted seatrout

The motility of fresh sperm before incubation with cryoprotectant was 70 ± 17% (mean ± SD). The highest cryoprotectant concentration without significantly different motility was 20% for EG, DMSO and MG and 15% for PROH (Table 2). DMSO at 20% was not different in motility from EG at 20% ($P = 0.066$), or from MG at 20% ($P = 0.665$).

Red snapper

The motility of fresh sperm before incubation with cryoprotectant was 77 ± 6% (mean ± SD). The highest cryoprotectant concentration that did not significantly reduce motility was 25% for EG, 20% for DMSO, PROH, MeOH and MG (Table 3). DMSO at 20% was not different from EG at 25% ($P = 0.137$) or from MG at 20% ($P = 0.201$) or PROH at 20% ($P = 0.667$).

Table 2 Motility (%) over time of spotted seatrout sperm exposed to various cryoprotectants. Values presented are mean ± SD (n = 3)

		Exposure time (min)			
Cryoprotectant	**Concentration**	**<1**	**5**	**10**	**15**
None (Control)	–	61 ± 3	64 ± 2	64 ± 2	63 ± 3
Ethylene glycol	10%	53 ± 15	67 ± 15	63 ± 15	63 ± 15
	15%	63 ± 15	55 ± 22	57 ± 20	57 ± 20
	20%	58 ± 19	57 ± 15	58 ± 12	53 ± 15
	25%	53 ± 15	45 ± 9	45 ± 13	37 ± 28
	30%	47 ± 30	38 ± 30	18 ± 18	7 ± 3
Dimethyl sulphoxide	15%	63 ± 15	67 ± 15	67 ± 11	52 ± 3
	20%	60 ± 10	60 ± 17	63 ± 20	48 ± 22
	25%	67 ± 15	53 ± 28	38 ± 29	18 ± 13
	30%	33 ± 17	2 ± 1	0 ± 0	0 ± 0
Propanediol	10%	60 ± 20	63 ± 11	50 ± 0	48 ± 19
	15%	67 ± 15	57 ± 11	37 ± 27	10 ± 8
	20%	63 ± 21	50 ± 30	22 ± 24	1 ± 1
Glycerol	10%	57 ± 20	38 ± 20	12 ± 12	9 ± 13
	15%	50 ± 20	32 ± 27	12 ± 16	13 ± 16
	20%	63 ± 12	10 ± 17	2 ± 2	2 ± 2
Methanol	15%	67 ± 15	53 ± 15	40 ± 10	37 ± 15
	20%	67 ± 15	47 ± 11	12 ± 11	1 ± 1
Methyl glycol	15%	70 ± 10	63 ± 15	67 ± 11	57 ± 20
	20%	67 ± 15	60 ± 20	57 ± 25	37 ± 25
Polyethylene glycol	10%	60 ± 17	35 ± 27	2 ± 2	0 ± 0
Butanediol	10%	63 ± 15	60 ± 10	53 ± 15	57 ± 15

Acute toxicity of combined cryoprotectants

Spotted seatrout

The motility of fresh sperm before incubation with combined cryoprotectants was 73 ± 21%. Time was a critical factor in the toxicity of combined cryoprotectants, which could be separated into two groups at <1 min. The first group of motility estimates were close to zero at <1 min and remained the same afterward (Table 4). In the second group, there was a significant difference in motility between <1 min and at 5 min. From the 29 combined cryoprotectants solutions that were evaluated for toxicity and glass formation, 18 solutions vitrified and formed a transparent glass (>70%; Table 1). This partial vitrification value was chosen because the size of the ice crystals could remain relatively small after warming and therefore, be less damaging to the cell.

Red snapper

The motility of fresh sperm before incubation with combined cryoprotectants was 77 ± 6%. Time was a critical factor in the toxicity of combined cryoprotectants, which could be separated into three groups. The first group of motility estimates were close to zero at <1 min and remained the same afterward (Table 5). In the second group there was no significant difference in motility between <1 min and at 5 min. In the third group there was a significant difference in motility between <1 min and at 5 min.

Effect of thawing solutions

Spotted seatrout

The motility of fresh sperm before vitrification was 77 ± 11%. There was no difference in motility between thawing in seawater or in seawater containing 10% DMSO (P = 0.709). The highest post-thaw motility was 70% for DEGXZ, followed by 60% for 10D30ET (Table 6). There was a significant difference in motility among treatments (P < 0.001).

Red snapper

The motility of fresh sperm before vitrification was 77 ± 6%. There was no difference in motility between thawing in seawater or in seawater containing 10% DMSO (P = 0.708). The highest

Table 3 Motility (%) over time of red snapper sperm exposed to various cryoprotectants. Values presented are mean ± SD (n = 3)

		Exposure time (min)			
Cryoprotectant	**Concentration**	**<1**	**5**	**10**	**15**
None (Control)	–	77 ± 3	77 ± 1	75 ± 3	74 ± 3
Ethylene glycol	10%	73 ± 6	77 ± 6	80 ± 0	77 ± 6
	15%	80 ± 0	77 ± 6	72 ± 3	77 ± 6
	20%	73 ± 11	73 ± 6	73 ± 6	73 ± 6
	25%	77 ± 6	77 ± 6	70 ± 0	70 ± 10
	30%	73 ± 6	57 ± 11	57 ± 11	55 ± 13
Dimethyl sulphoxide	15%	73 ± 6	77 ± 6	75 ± 9	73 ± 6
	20%	80 ± 0	80 ± 0	80 ± 0	77 ± 6
	25%	80 ± 0	63 ± 11	60 ± 8	30 ± 21
	30%	73 ± 6	32 ± 14	3 ± 2	0 ± 0
Propanediol	10%	77 ± 6	72 ± 8	73 ± 6	70 ± 5
	15%	80 ± 0	65 ± 21	77 ± 6	73 ± 11
	20%	80 ± 0	80 ± 0	80 ± 0	70 ± 0
Glycerol	10%	80 ± 0	3 ± 3	3 ± 2	2 ± 2
	15%	77 ± 6	2 ± 2	1 ± 1	1 ± 1
	20%	67 ± 15	0 ± 0	0 ± 0	0 ± 0
Methanol	15%	82 ± 3	77 ± 6	77 ± 6	72 ± 3
	20%	75 ± 5	68 ± 7	65 ± 5	65 ± 5
Methyl glycol	15%	78 ± 3	80 ± 0	70 ± 17	63 ± 20
	20%	77 ± 6	77 ± 6	77 ± 6	67 ± 15
Polyethylene glycol	10%	80 ± 0	77 ± 6	63 ± 11	63 ± 11
Butanediol	10%	72 ± 3	63 ± 6	67 ± 6	65 ± 5

Table 4 Sperm motility (mean ± SD; n = 3) of spotted seatrout (*Cynoscion nebulosus*) in relation to combined cryoprotectant solutions were compared at two exposure times. Treatments with a significant difference in motility between the two times were grouped on the left side while treatments without difference in motility were grouped on the right side. Fresh sperm motility before exposure was 70 ± 17%

	Motility (%)				Motility (%)		
Treatment	**<1 min**	**5 min**	***P*-value**	**Treatment**	**<1 min**	**5 min**	***P*-value**
30DT	63 ± 15	3 ± 2	<0.001	20Me20M	0 ± 0	0 ± 0	1.000
15D15E10G	63 ± 15	1 ± 1	<0.001	20E20M	0 ± 0	0 ± 0	1.000
20D15ET	62 ± 28	37 ± 27	<0.001	30D10B	0 ± 0	0 ± 0	1.000
25DT	60 ± 26	28 ± 36	<0.001	40DT	0 ± 0	0 ± 0	1.000
20E20G	53 ± 23	0 ± 0	<0.001	20D10M10P	1 ± 1	0 ± 0	1.000
35PP	53 ± 23	3 ± 2	<0.001	20D20M	2 ± 2	0 ± 0	0.833
20EA	52 ± 8	17 ± 20	<0.001	30E10B	5 ± 4	2 ± 3	0.527
35PT	52 ± 10	0 ± 0	<0.001	25D15E	7 ± 6	0 ± 0	0.423
35ET	50 ± 26	22 ± 24	0.001	25D10BA	7 ± 11	0 ± 0	0.399
35DT	42 ± 35	0 ± 0	<0.001	25D15EA	10 ± 17	0 ± 0	0.207
10D30ET	38 ± 10	7 ± 7	<0.001	20D20P	12 ± 16	0 ± 0	0.178
40ET	33 ± 32	4 ± 1	<0.001	20D20G	15 ± 9	0 ± 0	0.065
DPPA	25 ± 32	0 ± 0	0.002	20D10E10P	15 ± 13	0 ± 0	0.059
30E10Me	18 ± 27	0 ± 0	0.022	30ET	63 ± 25	57 ± 25	0.399
20D20E	17 ± 28	0 ± 0	0.033				

post-thaw motility was 60% for 10D30ET but this was not different from DEGXZ and 40ET (Table 6). Motility in the 35ET treatment was not different from 40% EG (P = 0.051) and 20E20G (P = 0.284). Post-thaw motility was not observed with cryoprotectant-free vitrification. Motility in the 30E10B treatment was not different from motility in the 35EXZ treatment (P = 0.489).

Table 5 Sperm motility (mean ± SD; $n = 3$) of red snapper (*Lutjanus campechanus*) in relation to combined cryoprotectant solutions were compared at two exposure times. Treatments with a significant difference in motility between the two times were grouped on the left side while treatments without differences in motility were grouped on the right side. Fresh sperm motility before exposure was 77 ± 6%

	Motility (%)				Motility (%)		
Treatment	**<1 min**	**5 min**	***P*-value**	**Treatment**	**<1 min**	**5 min**	***P*-value**
35PT	77 ± 6	2 ± 1	<0.001				
20D15ET	77 ± 6	20 ± 13	<0.001	No difference between <1 min and 10 min			
40ET	77 ± 6	53 ± 25	0.004				
35PP	75 ± 5	22 ± 32	<0.001	20D20P	12 ± 16	0 ± 0	0.153
10D30ET	75 ± 5	47 ± 15	0.001	20D10M10P	7 ± 11	1 ± 1	0.474
25DT	73 ± 6	38 ± 29	<0.001	20E20M	5 ± 5	0 ± 0	0.527
15D15E10G	73 ± 12	10 ± 8	<0.001	40DT	3 ± 2	0 ± 0	0.736
30DT	70 ± 10	14 ± 22	<0.001	25D15EA	2 ± 2	0 ± 0	0.800
25D15E	63 ± 6	0 ± 0	<0.001	30D10B	0 ± 0	0 ± 0	1.000
20D20E	63 ± 12	5 ± 8	<0.001	25D10BA	0 ± 0	0 ± 0	1.000
20D10E10P	57 ± 6	0 ± 0	<0.001	20D20M	0 ± 0	0 ± 0	1.000
20E20G	57 ± 21	0 ± 0	<0.001	20Me20M	0 ± 0	0 ± 0	1.000
30E10B	47 ± 32	27 ± 10	0.013				
30E10Me	43 ± 6	0 ± 0	<0.001	No difference between <1 min and 5 min			
35DT	35 ± 5	0 ± 0	<0.001	35ET	78 ± 3	63 ± 15	0.060
DPPA	27 ± 23	0 ± 0	0.001	30ET	77 ± 6	70 ± 10	0.400
20D20G	17 ± 20	0 ± 0	0.037	20EA	75 ± 5	63 ± 11	0.142

Table 6 Sperm motility and membrane integrity (mean ± SD) after thawing for different vitrification solutions from three males of spotted seatrout and red snapper (fresh motility ~77%). Two thawing solutions were compared: seawater (1000 mOsmol kg^{-1}) and 10% DMSO in seawater (2000 mOsmol kg^{-1}). There was no difference in motility between the thawing solutions

		Post-thaw motility (%)		
Species	**Treatment**	**Seawater**	**10% DMSO**	**Membrane-intact (%)**
Spotted seatrout	DEGXZ	58 ± 9	62 ± 8	19 ± 3
	10D30ET	44 ± 12	42 ± 16	22 ± 2
	40ET	13 ± 9	15 ± 9	23 ± 3
Red snapper	DEGXZ	38 ± 10	37 ± 9	9 ± 2
	10D30ET	43 ± 14	38 ± 13	22 ± 6
	40ET	40 ± 7	33 ± 5	12 ± 4
	20E20G	23 ± 4	22 ± 7	–
	35ET	23 ± 11	21 ± 10	–
	40% EG	10 ± 8	21 ± 11	–
	35EXZ	7 ± 3	8 ± 3	–
	30E10B	2 ± 2	8 ± 4	–
	No CPA*	0 ± 0	0 ± 0	–

*Cryoprotectant-free vitrification.

Effect of vitrification on sperm membrane integrity

Spotted seatrout

There was a weak correlation between post-thaw motility and membrane integrity ($r = 0.241$). The treatment with the highest percentage of membrane-intact sperm did not correlate with the treatment with the highest motility (Table 6). There was no difference in the percentage of membrane-intact sperm among all the treatments ($P > 0.279$).

Red snapper

There was a low correlation between post-thaw motility and membrane integrity ($r = 0.411$). The highest percentage of membrane-intact sperm was

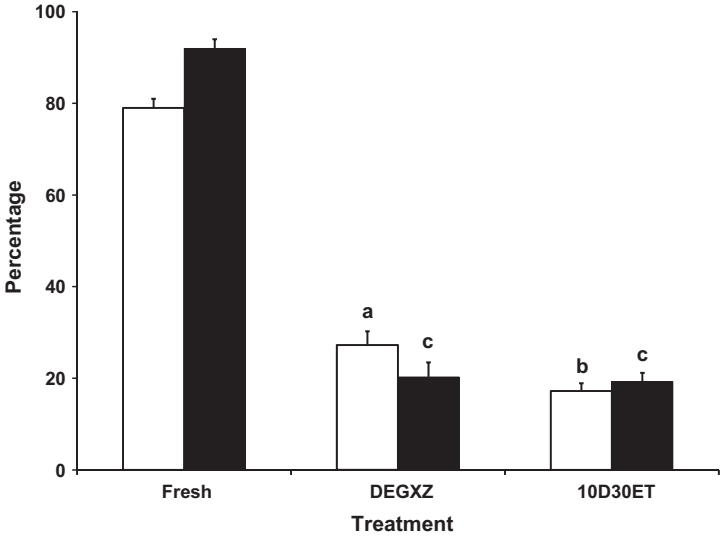

Figure 1 Per cent of sperm motility (white bars) and membrane integrity (black bars) of red drum (*Sciaenops ocellatus*; mean ± SE; $n = 9$). Sperm were vitrified with 15% DMSO + 15% EG + 10% Gly + 1% X-1000™ + 1% Z-1000™ (DEGXZ), and 10% DMSO + 30% EG + 0.25 M Trehalose (10D30ET). Post-thaw motility was significantly higher for the DEGXZ treatment than for the 10D30ET treatment. The percentage of membrane-intact sperm cells was not significantly different between treatments ($P = 0.833$). Different letters on the bars indicate significant difference ($P < 0.05$).

31% for 10D30ET, but there was no difference in membrane integrity among treatments ($P > 0.061$; Table 6).

Red drum

Fresh sperm motility (79 ± 6%) was positively correlated ($r = 0.83$) with membrane-intact cells (92 ± 6%), but there was a low correlation between motility and membrane integrity of thawed sperm ($r = 0.322$). Although the post-thaw motility was different between DEGXZ (as high as 40%) and 10D30ET (as high as 25%) ($P = 0.027$), the percentage of membrane-intact sperm was not different between these two treatments ($P = 0.833$; Fig. 1).

Discussion

Currently, marine biodiversity is threatened and it is estimated that 63% of assessed fishery stocks worldwide require rebuilding, and some form of protection (Worm, Hilborn, Baum, Branch, Collie, Costello, Fogarty, Fulton, Hutchings, Jennings, Jensen, Lotze, Mace, McClanahan, Minto, Palumbi, Parma, Ricard, Rosenberg, Watson & Zeller 2009). Cryopreservation can contribute to conservation programs, but conventional techniques often require specialized equipment that is unsuitable for use in field environments. New approaches that can be easily used in the field to cryopreserve samples, process small volumes and be applied to assist in conservation programs are urgently needed. Vitrification is well suited for use in the field, does not require expensive equipment, is simple, fast, inexpensive and offers a new option for conservation biology (Saragusty & Arav 2011). Vitrification has been applied successfully in the cryopreservation of mammalian embryos (Liebermann 2012), but in fish embryos, vitrification has yielded inconsistent results (Hagedorn & Kleinhans 2011). Sperm vitrification in freshwater fish has had limited success, largely because vitrification requires high concentrations of cryoprotectants (40–60%) which translates into high osmotic pressures (>2000 mOsmol kg^{-1}). Exposure to these conditions can damage sperm by chemical toxicity and osmotic effects including changes in plasma membrane integrity (Dzuba & Kopeika 2002). Sperm from freshwater fish are not generally adapted to deal with high osmotic pressures, as they typically become motile in response to reduced osmotic pressure. Sperm of marine species respond in the opposite manner, with motility activated by increased osmotic pressure (>1000 mOsmol kg^{-1};

Morisawa 2008). Because marine fish are adapted to higher osmotic pressures, we hypothesize that the application of vitrification to preserving sperm of marine fish would have greater success than in freshwater fish. The goal of the present project with marine fish was to develop a standardized approach for vitrification and evaluate the potential to apply it to marine species germplasm.

Acute toxicity of individual cryoprotectants

The key to successful vitrification is to achieve rapid cooling and warming rates and a high but tolerable (subtoxic) concentration of cryoprotectants. Previous cryopreservation studies in spotted seatrout and red drum did not evaluate the acute toxicity of cryoprotectants (Wayman *et al.* 1996, 1998). In the case of spotted seatrout a single equilibration time of 15 min was evaluated with four cryoprotectants (MeOH, Gly, DMSO and n,n-dimethyl acetamide: DMA) at two concentrations (5 and 10%). Equilibration motility at 15 min in this previous study was high for all treatments (>50% vs. control 70%), except when Gly was used (1%) (Wayman *et al.* 1996). In the case of red snapper, acute toxicity of four cryoprotectants (DMSO, Gly, MeOH and DMA) at five concentrations (as high as 25%) was evaluated in a previous study (Riley *et al.* 2004). Motility was estimated after the addition of cryoprotectant and every 15 min for as long as 60 min. This study indicated that the sperm from red snapper could tolerate high concentrations of cryoprotectants. Motilities after 15 min of exposure to cryoprotectants were 50% for 25% DMSO, 40% for 25% MeOH and 40% for 25% DMA (control 95%). Although Gly was toxic at higher concentrations, at 10% the motility was 40% immediately after addition but dropped to 20% at 15 min. To minimize the toxicity of cryoprotectants at higher concentrations, the exposure time can be shortened (Tiersch 2011b).

The least toxic cryoprotectant at higher concentrations in the present study was EG, followed by DMSO. Comparing these results with previous studies, DMSO was the least toxic and Gly was among the most toxic (Wayman *et al.* 1996; Riley *et al.* 2004). Dimethyl sulphoxide is the most common cryoprotectant used for sperm cryopreservation of marine fish (Gwo 2011). One of the reasons that DMSO was a better cryoprotectant than Gly is probably because of the faster cell permeability of DMSO, a process that is less dependent on temperature for DMSO than for Gly (Gwo, Jamieson & Leung 2009). The acute toxicity experiment indicated which of the cryoprotectants were relatively non-toxic at different concentrations and times, and those meeting these criteria were selected for the combined cryoprotectant study.

Acute toxicity of combined cryoprotectants

Most cryoprotectants tend to have toxic and hypertonic effects when used at concentrations that are effective for successful vitrification (Yavin & Arav 2007). Two common approaches to decrease this toxicity are to combine cryoprotectants, and to reduce the exposure time to a minimum. In general, a mixture of cryoprotectants has a lower aggregate toxicity to cells because it vitrifies at lower concentrations, and it combines the additive properties (such as permeability and glass formation) of each cryoprotectant (Ali & Shelton 2007). In addition, the concentration needed to vitrify can be reduced by adding small quantities of ice-blockers, such as polymers that directly inhibit ice nucleation and growth (Wowk 2010). Furthermore, the addition of sugars, especially disaccharides such as trehalose, can enhance glass formation and reduce the concentrations of cryoprotectants required for vitrification (Fuller 2004). Another approach to reduce the toxicity of cryoprotectants is to use 'toxicity neutralizers'. Acetamide, for example, has been recommended to block the damaging effect of DMSO (Fahy 1984). However, a recent study demonstrated that the benefits of adding acetamide were limited, and that other amides such as formamide work better (Fahy 2010).

Combinations of cryoprotectants that macroscopically appear to form a transparent glass can contain some ice nuclei and ice crystals, because the crystals only become optically detectable when they become larger than the wavelengths of visible light (Shaw & Jones 2003). In the present study, the combinations of cryoprotectants were highly toxic, especially the vitrifying solutions (those that formed glass). Acetamide is believed to neutralize the toxicity of DMSO (Fahy 1984), and has been used in the vitrification of fish embryos with little success (Ding, Xiao & Li 2007). And in the present study acetamide did not decrease the toxicity of cryoprotectants. The concentration of acetamide tested in this study could have been itself toxic to

the sperm of marine fish. The toxicity of acetamide is linked to the denaturation of proteins, and excessive total solution concentration (Fahy 2010). The addition of trehalose improved sperm survival in spotted seatrout. For example, 30% EG at 10 min yielded 18% motility, while 30% EG plus trehalose yielded 45% motility at 10 min. Trehalose was effective in enhancing glass formation, reducing the concentrations of cryoprotectants required for vitrification, and showed some protective benefits during vitrification. These benefits probably were due to the cell biostabilization properties of trehalose (Fuller 2004). Solutions that vitrified and proved to be of limited toxicity to sperm were tested further for their effects on sperm survival after vitrification.

Effect of thawing solutions

A previous study of sperm vitrification using loops compared two warming temperatures (24 and 37°C) in the green swordtail, and concluded that there was no significant difference in post-thaw motility between temperatures (Cuevas-Uribe, Yang *et al.* 2011). Using either of these temperatures yielded warming rates that were fast enough to avoid ice formation during warming (devitrification). Consequently, the present study used a single warming temperature (24°C).

In the present study, eight VS were used with sperm of red snapper. The addition of trehalose, as previously observed in the toxicity experiment, improved the post-thaw motility. For example, 40% EG alone yielded a post-thaw motility of 10%, while 40% EG plus trehalose yielded 40% motility. Trehalose has been used in previous conventional cryopreservation studies (not targeting vitrification), and high fertilization rates (95%) were obtained with thawed sperm of longtooth grouper (*Epinephelus bruneus* Bloch, formerly *E. moara*) using trehalose as the sole cryoprotectant (Miyaki, Nakano, Ohta & Kurokura 2005). In a similar study in orange-spotted grouper (*E. coloides*, Hamilton) trehalose was used as a cryoprotectant, and high fertilization rates (82%) were obtained with thawed sperm (Peatpisut & Bart 2010). Although in the present study trehalose was not used alone, trehalose was used to enhance glass formation in combination with other cryoprotectants. Other vitrification enhancers include synthetic ice-blocking agents such as the polymers X-1000™ and Z-1000™ (Wowk & Fahy 2002). In the present study, the combination of 15% DMSO + 15% EG + 10% Gly did not form a complete glass (~50% glass), but when the 'ice blocker' polymers were added, 100% glass formation was observed. When this combination (DEGXZ) was used to vitrify sperm, average post-thaw motility was 58% for spotted seatrout, 38% for red snapper and 30% for red drum.

The combination of DMSO and EG is one of the most popular combinations used in the vitrification of mammalian gametes (Quinn 2010). DMSO is a good glass former while EG is a weak glass former (Quinn 2010), but EG is less toxic and permeates faster than DMSO (Gilmore, McGann, Liu, Gao, Peter, Kleinhans & Critser 1995). The DMSO and EG combination decreases the total cryoprotectant concentration necessary to form glass and can improve viability. In the present study, trehalose was added to the combination of DMSO and EG (10D30ET), and average post-thaw motility was 44% for spotted seatrout, 43% for red snapper and 20% for red drum. It should be noted that the lower the concentration of cryoprotectant, the faster the cooling rate that was needed to avoid ice formation. The cooling rate calculated for loops similar to those used in the present study was as fast as 720 000°C min^{-1} (Isachenko, Isachenko, Katkov, Dessole & Nawroth 2003). This high cooling rate was due to the minimal volume and high ratio of surface area to volume used. The disadvantage of using small-volume samples is their susceptibility to accidental warming (Vajta, Nagy, Cobo, Conceicao & Yovich 2009). Devitrification (ice formation during warming) happens faster during thawing than during cooling because ice nucleation occurs at lower temperatures than ice growth (Wowk 2010). That is one of the reasons for application of high warming rates to obtain high survival of vitrified oocytes and embryos (Kuwayama & Leibo 2010).

By use of ultra-rapid cooling and warming rates, and small sample volumes (20 μL in cryoloops), human sperm has been vitrified without the use of cryoprotectants (Nawroth, Isachenko, Dessole, Rahimi, Farina, Vargiu, Mallmann, Dattena, Capobianco, Peters, Orth & Isachenko 2002). A similar technique was used to vitrify sperm of channel catfish without cryoprotectants, and yielded fertilization (<2%) in two of 16 trials (Cuevas-Uribe, Leibo *et al.* 2011). In a study using the microdrop method (20 μL dropped directly into liquid nitrogen), sperm from rainbow trout were vitrified

without addition of cryoprotectant, and yielded post-thaw motilities as high as 86% (Merino, Risopatrón, Sánchez, Isachenko, Figueroa, Valdebenito & Isachenko 2011). In the present study, sperm from red snapper were vitrified without cryoprotectant using 10 μL polystyrene loops but no post-thaw motility was observed. There are several differences between these studies, including sperm characteristics (such as cell size and concentration, and properties and composition of the cell membranes), and the methodology used (such as extender type, sperm centrifugation, apparatus used and volume). Due to these differences it is problematic to directly compare these studies without further research.

Effect of vitrification on sperm membrane integrity

Cryopreservation can alter the cellular structures and physiology of sperm. There are no routinely accepted criteria for estimating sperm quality of aquatic species, especially in comparison to that available for humans (WHO 2010) and livestock (Chenoweth, Spitzer & Hopkins 1992). The quality of cryopreserved aquatic sperm is often estimated by post-thaw motility but this is not always correlated with fertilization (Warnecke & Pluta 2003; He & Woods 2004). Damage induced by cryopreservation can occur to specific structures that would not be detectable by studying a single assay (Tiersch 2011b). The plasma membrane of spermatozoa is one of the main structures affected by cryopreservation, and it is an important component in the maintenance of sperm viability and the stability of its semi-permeable features (functional ion pumps; Jenkins 2011). One of the most common tests for plasma membrane integrity in aquatic species is the SYBR 14/PI assay, often referred to as a 'sperm viability' assay (Daly & Tiersch 2011). This assay was used in previous cryopreservation studies of red snapper (Riley 2002). Viable and non-viable thawed sperm were successfully stained with SYBR-14 and PI. As in the present study, no correlation was found between post-thaw sperm motility and membrane integrity, and motility estimates were higher than the estimated percentage of membrane-intact sperm. Also, in the present study there was no difference in the percentage of membrane-intact sperm among the treatments.

The weak correlation between membrane integrity with motility could be due to the time spent during staining (10 min) or differences in dilution. Time after thawing vitrified sperm is one of the most important factors, and motility decreased within seconds after activation. In addition, excessive dilution of thawed sperm samples could affect the osmotic balance of the cell. There have been other studies where the percentage of motile sperm had a weak correlation with the percentage of sperm with intact plasma membranes (Ogier de Baulny, Labbe & Maisse 1999; Dziewulska, Rzemieniecki, Czerniawski & Domagala 2011). In addition, a low percentage of membrane-intact sperm has produced high fertilization in other studies (Linhart, Rodina, Flajshans, Gela & Kocour 2005; Horvath, Wayman, Dean, Urbanyi, Tiersch, Mims, Johnson & Jenkins 2008). Having an intact membrane does not necessarily mean that the sperm preserves full fertilization capacity; other factors such as intracellular structures and ion composition are also important (Kopeika & Kopeika 2008). These parameters were beyond the scope of this work and were not tested. Comparison of flow cytometric analyses should be done carefully due to the variations in application, analysis and reporting (Daly & Tiersch 2012). More research needs to be done to evaluate if the integrity of the membranes from vitrified sperm changes over time, especially immediately after thawing.

Conclusions

In summary, the present study demonstrated the feasibility of using vitrification to cryopreserve sperm from marine fish. Based on the acute toxicity of cryoprotectants, VS were developed and applied for sperm from spotted seatrout, red snapper and red drum. Compared with previous vitrification studies in freshwater species, marine fish sperm had higher survival after vitrification, perhaps because they are adapted to deal with higher osmotic pressures. One important component of some of the VS used in the present study was the disaccharide sugar trehalose, which has previously been used for equilibrium (slow) cooling of sperm of marine fish. Future studies should systematically evaluate trehalose in relation with the different functions that it could provide in the cryoprotectant solution (e.g., as an energy substrate or biostabilization of the cell).

In this study, sperm were collected from marine fish captured from the recreational fishery. Sperm

vitrification from marine fish proved to be reliable in salvaging genetic material in the field from dead fish (Riley *et al.* 2008). There were differences in post-thaw motility among marine fish using the same VS. This could be due to the differences in sperm characteristics such as properties and composition of the cell membrane.

This is the first report on sperm vitrification in marine fish. This procedure should offer an alternative approach to conventional cryopreservation for conservation of valuable genetic lineages, such as endangered species, 'model' strains used in research, and improved farmed strains. Furthermore, sperm vitrification can be used to transport cryopreserved sperm from the field to the laboratory to expand the genetic resources available for germplasm repositories. Due to the small volumes used, this technique could be utilized to reconstitute lines, but it would require improvement before being useful as a mass production method. Fertilization will provide an ultimate assessment to further evaluate the efficiency of the technique. Because vitrification can be performed in the field, this work could be extended to management programs of endangered species, especially those with body sizes of less than 10 cm (Cuevas-Uribe *et al.* 2011).

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