Aquaculture Research, 2004, 35, 278–288

Transport and cryopreservation of sperm of the common snook, *Centropomus undecimalis* (Bloch)

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Abstract

Sperm were collected in Florida from wild common snook, *Centropomus undecimalis* (Bloch), and were shipped to Louisiana State University for analysis and cryopreservation. Threshold activation of sperm (10% motility) occurred at 370 mOsmol kg^{-1}, and complete activation occurred at 680 mOsmol kg^{-1}. These values were significantly different. Sperm samples stored at 1 °C in Hanks' balanced salt solution (HBSS) or in 0.6% NaCl solution at 200 mOsmol kg^{-1} retained motility for as long as 22 days. Mean motility remained above 50% for 9 days for sperm stored in HBSS and for 7 days for sperm stored in NaCl solution. Sperm exposed to 5% dimethyl acetamide (62 ± 10%; mean ± SD), 10% dimethyl sulphoxide (DMSO) (39 ± 16%), 5% glycerol (26 ± 5%) or 10% glycerol (6 ± 2%) for 30 min had significantly lower motility than did unexposed sperm (89 ± 9%). When used as a cryoprotectant, samples frozen with 5% or 10% DMSO or 5% methanol had significantly higher post-thaw motility than did samples frozen with other cryoprotectants. Sperm cryopreserved with 10% DMSO (38 ± 12%) had significantly higher post-thaw motility than did sperm cryopreserved with 15% DMSO (19 ± 10%) or 20% DMSO (4 ± 4%). There were no significant differences in hatch rates of eggs fertilized with fresh sperm (54 ± 29%) or cryopreserved sperm (41 ± 35%). Survival to first feeding was not different between fish produced with fresh sperm (37 ± 30%; range, 0–86%) or with thawed sperm (24 ± 29%; 0–77%). Transport of sperm to a cryopreservation laboratory and back to a hatchery for thawing and use enabled collaboration between groups with specific expertise and provides a model for the application of cryopreservation by transport of fresh and frozen samples.

Keywords: : germplasm, genetic resources, artificial spawning

Introduction

The common snook, *Centropomus undecimalis* (Bloch), is a prized sport and food fish of warm coastal waters. This fish is most common along continental shores, mangrove or salt marshes, estuaries, and brackish lagoons. It sometimes enters freshwater canals and rivers, and ranges from South Carolina to Texas to Southern Brazil (Robins, Ray, Douglass & Freund 1986; Taylor, Grier & Whittington 1998). The common snook is chiefly piscivorous but also eats crustaceans. They can attain sizes of 1.4 m and 24 kg, although they typically average 3–4 kg in the coastal waters of the southern United States. Commercial fishing is prohibited in Florida (Shipp 1986) where the common snook has been declared a 'species of special concern' and is protected by regulations enacted by the state legislature (Florida statutes, Chapter 46.21).

Artificial spawning has been studied in this species (Tucker 1987; Neidig, Skapura, Grier & Dennis 2000), but captive broodstock are not available, necessitating the collection of wild fish for spawning

purposes. Cryopreservation can assist in spawning by reducing the need to collect males at the same time as females. Cryopreservation can also increase the efficiency and control of genetic management (e.g., Cloud, Miller & Levanduski 1990; Van der Walt, Van der Bank & Steyn 1993). Refrigerated storage and cryopreservation of sperm have not been studied with common snook. This species can also serve as a model for cooperation between hatchery facilities and the laboratories practising cryopreservation.

The purpose of this study was to develop methods for the refrigerated storage and cryopreservation of common snook sperm. Our objectives were to: (1) characterize the relationship between osmotic conditions and sperm motility; (2) evaluate extenders for refrigerated storage of sperm; (3) develop methods for the cryopreservation of sperm; and (4) evaluate the ability of thawed sperm to fertilize eggs and produce viable larvae. We also sought to evaluate the potential for shipping cryopreserved sperm to the hatchery for evaluation involving eggs from fish of a different family.

Materials and methods

Collection and maintenance of broodstock

This study combined research at two locations more than 1000-km apart (Fig. 1). Hatchery work was performed at the Florida Marine Research Institute, Stock Enhancement Research Facility (SERF), Palmetto, FL, USA. Laboratory work was performed at the Louisiana State University Agricultural Center, Aquaculture Research Station (ARS), Baton Rouge, LA, USA. Common snook males were collected from southeast Tampa Bay, FL, USA during the summer of 1996 with a 150-m beach seine and were transported to the SERF. Male common snook were killed, and the testes were surgically removed. Sperm were collected into 3-mL syringes by gentle palpitation of the testes.

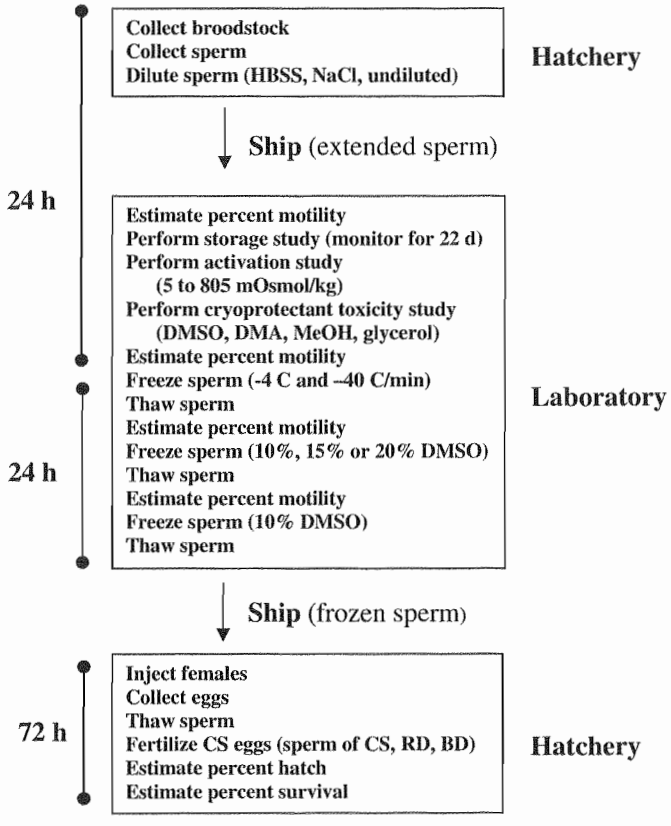

Figure 1 Overview and chronology of activities at the hatchery and laboratory for cryopreservation of sperm of the common snook, *Centropomus undecimalis* (Bloch). The cryoprotectants studied were dimethyl sulphoxide (DMSO), dimethyl acetamide (DMA), methanol (MeOH), and glycerol. Samples of cryopreserved sperm evaluated at the hatchery included common snook (CS), red drum (RD), *Sciaenops ocellatus* L., and black drum (BD), *Pogonias cromis* L.

Sperm were transferred to 50-mL centrifuge tubes (Fisher Scientific, St Louis, MO, USA #05-539-8) and diluted 1:1 (v:v) with Hanks' balanced salt solution (HBSS) (200 mOsmol kg^{-1}) (Wayman, Thomas & Tiersch 1998), 0.6% NaCl solution (200 mOsmol kg^{-1}) or left undiluted. The samples were shipped to Louisiana State University by overnight delivery.

Upon arrival, samples were inverted to mix and motility was tested. Motility was estimated using dark-field microscopy (Optiphot-2, Nikon, Garden City, NY, USA) at ×100 magnification. Sperm samples (2 μL) were activated with 20 μL of 800 mOsmol kg^{-1} artificial seawater (ASW) (Forty Fathoms Bio-crystals Marine Mix, Marine Enterprises International, Baltimore, MD, USA). The percentage of actively swimming sperm was estimated immediately after addition of ASW. Sperm cells that vibrated in place were not considered to be motile. For the determination of osmotic pressure of the extended samples, approximately 75 μL of semen were transferred into plain micro-haematocrit tubes (VWR Scientific, West Chester, PA, USA #15401-650) and were centrifuged for 10 min at 8000 rpm. Osmotic pressure of the supernatant was measured with a vapour pressure osmometer (model 550, Wescor, Logan, UT, USA). Seminal plasma was not obtained from the undiluted samples, because they were too viscous for plasma collection.

Effects of osmolality on sperm motility

To investigate the effect of osmotic pressure on sperm motility, samples of undiluted sperm ($n = 4$) were activated with a graded series of 12 ASW dilutions, which ranged from 805 to 5 mOsmol kg^{-1} (deionized water) in increments of ~80 mOsmol kg^{-1}. Motility was estimated immediately after mixing of 2 μL of sperm with 20 μL of an activating solution. To determine the osmotic pressure, 10 μL of the activated sample were removed from the slide and analysed with a vapour pressure osmometer. Threshold activation was defined as the osmotic pressure eliciting 10% motility. Complete activation was defined as the lowest pressure eliciting maximal motility for the sample (Bates, Wayman & Tiersch 1996).

Refrigerated storage study

To evaluate the potential for refrigerated storage, sperm samples ($n = 4$) diluted with HBSS, NaCl, or left undiluted were stored in a refrigerator at 1 °C in loosely capped 50-mL centrifuge tubes. Motility of each sample was estimated daily.

Cryopreservation study

Sperm samples (1 mL) ($n = 4$) that were shipped undiluted were extended with 3 mL of 205 mOsmol kg^{-1} ASW. Methanol, glycerol, dimethyl acetamide (DMA) and dimethyl sulphoxide (DMSO) were used as cryoprotectants. Methanol, glycerol, and DMSO were used at concentrations of 5% and 10% (v:v). DMA was used at a concentration of 5%. Sperm were mixed with cryoprotectant and allowed to equilibrate for 30 min. Samples were placed into a controlled-rate freezer (Kryo-10, Planer Products, Middlesex, UK), and at this time percent motility was determined to evaluate the effects of acute cryoprotectant toxicity. Sperm samples were drawn into 0.5-mL French straws (IMV International, Minneapolis, MN, USA), placed in goblets on canes (five straws per goblet, two goblets per cane), and cooled in the freezer at a rate of −40 °C min^{-1}. The samples were held at −80 °C for 10 min and plunged into liquid nitrogen. Samples were stored in liquid nitrogen for 24 h and thawed in a 40 °C water bath for 7 s. Motility was estimated immediately after thawing.

DMSO concentration study

Because in the previous experiment DMSO resulted in the highest post-thaw motility, we conducted a study to determine if higher concentrations of DMSO could increase post-thaw motility. Samples ($n = 4$) were frozen using the same conditions as above except that DMSO was used at concentrations of 10%, 15%, and 20%. Samples were stored in liquid nitrogen for 11 days, and thawed at 40 °C for 7 s in a water bath. Motility was estimated immediately after thawing.

Hatchery evaluation of cryopreserved sperm of common snook

Based on the results of post-thaw motility observed in the studies above, we chose to evaluate the utility in the hatchery of sperm cryopreservation with 10% DMSO. Straws (0.5 mL) containing sperm from three additional males were cryopreserved using the procedures described above. The frozen samples were transported by overnight delivery in nitrogen vapour shipping dewars (Model CP-65, Taylor-Wharton,

Theodore, AL, USA) to the SERF for evaluation of hatching and survival to first feeding of common snook produced with cryopreserved sperm.

For collection of unfertilized eggs, female common snook were injected with human chorionic gonadotropin (HCG) (Chorulon Intervet, Millsboro, DE, USA) or were implanted with pellets that released gonadotropin-releasing hormone (GnRHa) (Sigma, St Louis, MO, USA) (Innovative Research of America, Sarasota, FL, USA), at a rate of 25 or 50 μg day^{-1} over 5 days.

Eggs were stripped by abdominal pressure at 24–34 h after injection or implantation. Males were killed just prior to anticipated ovulation, and milt was collected from the dissected testis and stored in a refrigerator at 4 °C. No treatment was made to standardize sperm concentrations or sperm-to-egg ratios among samples. Cryopreserved sperm were thawed by holding straws in tap water for 7 s at 35–40 °C. The ends of the straws were cut, and the sperm were released onto the eggs. Single straws (0.5 mL) or 0.1 mL of fresh sperm were used to fertilize ~5 mL of eggs (13 815 eggs). About 50 mL of filtered seawater was added to activate the gametes. After 1 min, 250 mL of seawater was added to the gamete mixture. The eggs were stirred, three replicate samples (1.25-mL aliquot) were taken from each trial, and percent fertilization and hatch were determined according to the procedures of Neidig *et al.* (2000). Each aliquot was placed in 750 mL of seawater in a 1-L container and placed in a water bath at 27–28 °C. Percent fertilization was determined at 2–3 h after fertilization at the blastula stage. After hatching (16 h at 28 °C) the samples were preserved by adding 2 mL of 37% formalin, which produced an opaque white coloration of the larvae. Samples were examined against a black background using a dissecting scope at ×40 magnification to determine the percent hatch. An *a priori* decision was made to not include in the data analysis those egg batches with fertilization rates below 20% using fresh sperm.

To determine the percentage of larvae that survived to first feeding, three replicates of 50 floating eggs were removed and counted from each trial. The eggs were placed in 250-mL beakers with Nitex (125 μm) mesh collars and were suspended in water at 27–29 °C (Neidig *et al.* 2000). The system consisted of an 1100-L rectangular tank equipped with a biofilter and ultraviolet light to provide a stable environment for maintaining larvae. At 60 h after fertilization, the percentages of live larvae were recorded.

Hatchery evaluation of cryopreserved sperm of red drum and black drum

We sought to make a preliminary appraisal of the feasibility of evaluating sperm quality in the hatchery by heterologous fertilization using cryopreserved sperm with eggs of other species. In previous studies we cryopreserved sperm of red drum, *Sciaenops ocellatus* L. (Wayman *et al.* 1998) and black drum, *Pogonias cromis* L. (Wayman, Thomas & Tiersch 1997). These samples were cryopreserved in March 1995 (black drum) and October 1995 (red drum) using 0.5-mL straws, 10% DMSO, cryoprotectant exposure times of 30–50 min before freezing, and a cooling rate of −40 °C min^{-1}. Post-thaw motility of these samples was 50–60%. The samples were transported in nitrogen-vapour shipping dewars to the SERF where they were thawed and mixed with eggs of common snook as described above. Percent fertilization and percent hatch were determined according to the methods of Neidig *et al.* (2000). Larvae were photographed after hatching and were compared with normal larvae of common snook.

Statistical analysis

All percent motility values were arc-sine square root transformed prior to statistical analysis. For analysis of the effect of osmolality on sperm activation, osmolality values for threshold and complete activation were compared using a Student's *t*-test (Microsoft Excel 2000, Microsoft Corporation, Redmond, WA, USA). In the refrigerated storage study, sperm motility

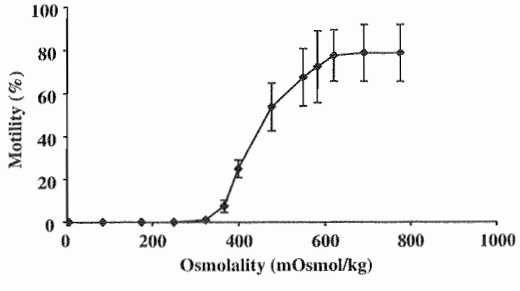

Figure 2 Motility of sperm of the common snook, *Centropomus undecimalis* (Bloch), in response to dilution in artificial seawater of various osmotic pressures. Threshold activation (defined as 10% motility) occurred at 370 mOsmol kg^{-1} and complete activation (highest motility observed) occurred at 690 mOsmol kg^{-1}. Each point represents the mean+SD of samples from four fish.

was evaluated over time using a repeated measures analysis of variance to determine the effects of extender and duration of storage (NCSS 2000, Number Cruncher Statistical Systems, Kaysville, UT, USA). The effects of three cryoprotectants on equilibration motility and post-thaw motility in the cryopreservation study and the effects of three concentrations in the DMSO concentration study were analysed using a one-factor analysis of variance (NCSS 2000). The effects of fresh or cryopreserved sperm on the hatching percentages of eggs in the studies on hatchery evaluation of cryopreserved sperm of common snook or red drum and black drum were analysed using a Student's *t*-test (Microsoft Excel 2000). Means were separated by Duncan's multiple range test, and were considered significantly different at $P < 0.05$.

Results

Sperm collection

The sample osmotic pressure was 222 ± 8 mOsmol kg^{-1} for sperm diluted in HBSS and 229 ± 7 mOsmol mOsmol kg^{-1} for sperm diluted with NaCl. These values were not significantly different ($P < 0.2471$).

Effect of osmolality on sperm motility

The motility of common snook sperm increased as the osmotic pressure of ASW was increased (Fig. 2). Threshold activation (10% motility) occurred at 370 mOsmol kg^{-1}, and complete activation (78% motility) occurred at 680 mOsmol kg^{-1}. These values were significantly different ($P < 0.0001$).

Refrigerated storage study

Sperm motility decreased during storage in each extender and was lost completely within 2 days in undiluted samples (Fig. 3). Sperm samples stored in HBSS or NaCl solution retained motility of ≥ 10% for 16 days, and motility ≥ 1% for as long as 22 days. Mean motility remained above 50% for 9 days for sperm stored in HBSS and for 7 days for sperm stored in NaCl solution. There were significant differences among motilities of sperm stored in extenders as compared with undiluted samples ($P < 0.0001$). However when analysed without the undiluted samples, the HBSS and NaCl extenders had similar motilities over time ($P = 0.3699$).

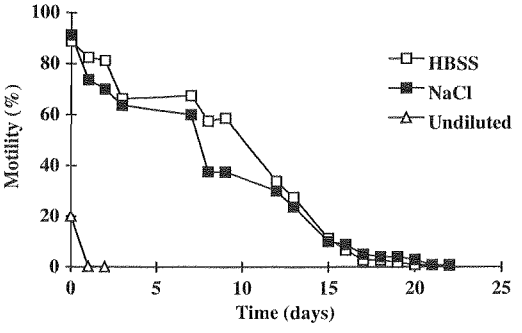

Figure 3 Sperm of the common snook, *Centropomus undecimalis* (Bloch), were stored at 1 °C diluted in Hanks' balanced salt solution (HBSS) (200 mOsmol kg^{-1}), 0.6% NaCl (200 mOsmol kg^{-1}), or left undiluted. Samples left undiluted lost all motility within 2 days. Each point represents the mean of samples from four fish.

Cryopreservation study

The average initial motility of common snook sperm samples was 89% prior to the addition of cryoprotectants. Sperm exposed to 5% DMA, 10% DMSO, or 5% or 10% glycerol for 30 min had significantly lower ($P < 0.0001$) motility than did unexposed sperm (Table 1). When used as a cryoprotectant, samples frozen with DMSO had significantly higher ($P < 0.0001$) post-thaw motility than did samples frozen with other cryoprotectants, except with 5% methanol (Table 1).

DMSO concentration study

Sperm exposed to 10% DMSO (56 ± 21%) for 30 min had significantly higher ($P = 0.0060$) motility than did sperm exposed to 15% (30 ± 11%) or 20% DMSO (12 ± 10%). Common snook sperm cryopreserved with 10% DMSO had significantly higher ($P = 0.0010$) post-thaw motility than did sperm cryopreserved with 15% or 20% DMSO. Post-thaw motility of cryopreserved sperm was 38 ± 12% with 10% DMSO, 19 ± 10% with 15% DMSO, and 4 ± 4% with 20% DMSO.

Hatchery evaluation of cryopreserved sperm of common snook

The post-thaw motility of sperm samples was 30 ± 7%. For 13 trials with eggs collected from 11 females, 61 ± 24% of the common snook eggs fertilized with fresh sperm developed to hatching, while 47 ± 34% of the eggs fertilized with thawed sperm hatched (Table 2). These values were not significantly

Table 1 Motility of common snook sperm (mean ± SD) after 30-min exposure to cryoprotectants before freezing (equilibration motility) and after thawing ($n = 4$)

Cryoprotectant	Concentration (%)	Equilibration motility (%)*	Post-thaw motility (%)*
Dimethyl acetamide	5	62 ± 10^{c}	25 ± 11^{bc}
Dimethyl sulphoxide	5	81 ± 8^{d}	35 ± 14^{ab}
	10	39 ± 16^{b}	44 ± 5^{a}
Glycerol	5	26 ± 5^{b}	12 ± 3^{c}
	10	6 ± 2^{a}	11 ± 2^{c}
Methanol	5	76 ± 18^{cd}	29 ± 8^{ab}
	10	84 ± 8^{d}	1 ± 0^{d}
Control†	–	89 ± 9^{d}	–

*Values sharing letters within columns were not significantly different.
†Control represents motility of sperm at the time of addition of cryoprotectants.
Sperm were cooled at a rate of –40 °C min^{-1}. After 24 h of storage in liquid nitrogen, samples were thawed in a water bath (40 °C).

Table 2 Percent fertilization, percent hatching and percent survival to first feeding for common snook (CS) eggs fertilized with fresh or thawed sperm of common snook or thawed sperm of red drum (RD) or black drum (BD)

	Percent fertilization				Percent hatching				Survival to feeding	
	Fresh	Thawed			Fresh	Thawed			Fresh	Thawed
Date of female collection and hormone used*	CS (%)	CS (%)	RD (%)	BD (%)	CS (%)	CS (%)	RD (%)	BD (%)	CS (%)	CS (%)
Collected 17 June 1996										
HCG 1000 IU	98	–	–	–	31	88	0	0	86	–
HCG 2000 IU	47	–	–	–	29	8	0	0	61	–
Collected 24 June 1996										
HCG 2000 IU	79	–	–	–	59	63	0	0	43	10
HCG 2000 IU	87	65	–	–	37	3	0	3	13	8
HCG 2000 IU	32	–	–	–	28	7	0	0	6	0
HCG 500 IU	–	–	–	–	37	20	6	0	36	66
Collected 8 July 1996										
HCG 500 IU	88	87	66	71	76	73	–	–	29	45
HCG 500 IU	100	–	83	14	91	89	–	–	54	35
HCG 500 IU	93	20	51	71	82	87	–	–	45	77
HCG 500 IU	88	–	–	–	87	85	–	–	39	76
Collected 13 August 1996										
GnRH 10 μg	89	34	–	–	87	32	–	–	62	40
GnRH 10 μg	12	10	–	–	65	24	–	–	67	21
HCG 500 IU	71	57	–	–	83	30	–	–	78	9

*HCG, human chorionic gonadotropin; GnRH, gonadotropin releasing hormone.

different ($P = 0.1146$). Survival to first feeding was not different ($P = 0.4721$) between fish produced with fresh sperm (43 ± 22%; range 6–78%) or fish produced with thawed sperm (35 ± 28%; 0–77%).

Hatchery evaluation of cryopreserved sperm of red drum and black drum

Percent hatch of common snook eggs fertilized with sperm of red drum or black drum sperm (Table 2) was significantly lower ($P = 0.0010$) than that with fresh sperm of common snook. Deformities in the yolk-sac, fin-fold, optic vesicles, and trunk were common among the larvae (Figs 4 and 5), and none of the larvae survived to feeding.

Discussion

The dependence of various species of snook on habitats such as mangroves, salt marshes, and estuaries makes them especially vulnerable to habitat destruction due to development or pollution. Based on this

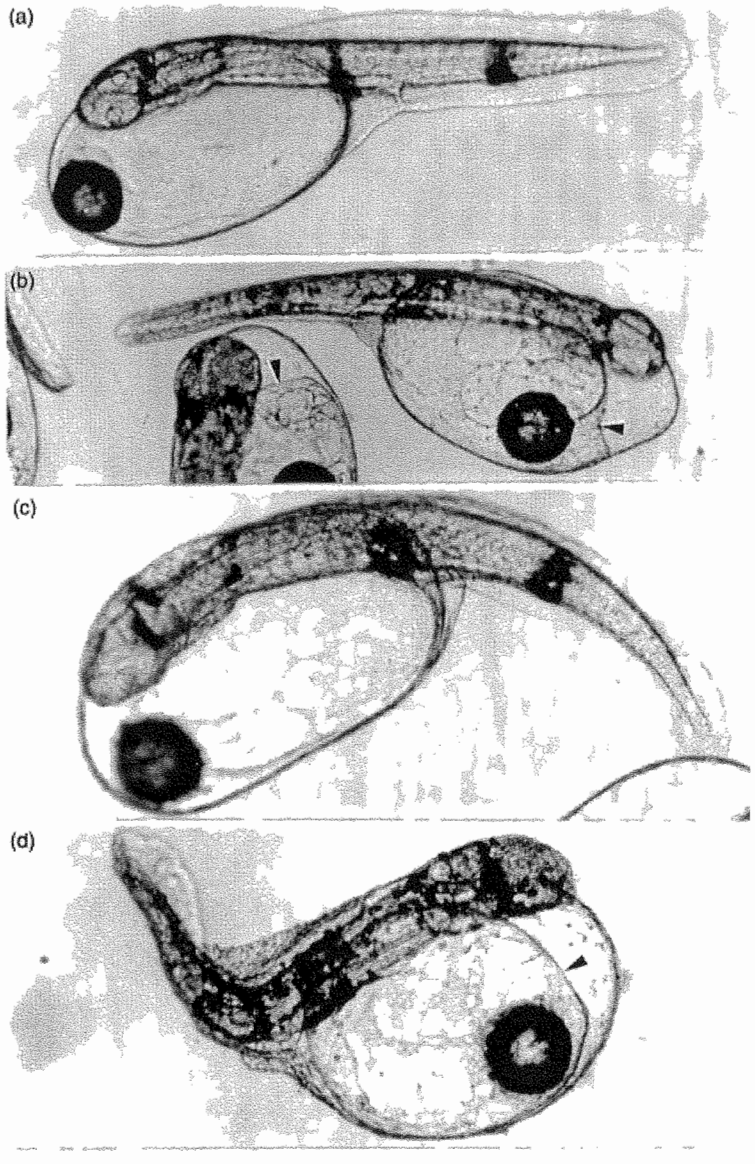

Figure 4 Newly hatched larvae of the common snook, *Centropomus undecimalis* (Bloch) (a), common snook eggs fertilized with thawed sperm of red drum, *Sciaenops ocellatus* L. (b, d) and common snook eggs fertilized with thawed sperm of black drum, *Pogonias cromis* L. (c). Deformities in the yolk sac, fin-fold, optic vesicles, and trunk were common in the larvae produced with heterologous sperm, and these larvae typically died within 24 h after hatching. In common snook (a), there were distinctive pigment bands, the oil droplet was anterior, and yolk filled the yolk sac. In hybrids, the pigment bands were disrupted, the yolk sac was not full of yolk (arrowheads in b and d), and there were ventral flexures (b) or curvatures (c) of the trunk. Muscle contractions propelled these larvae in circles. Also in hybrids, the optic vesicles and circulatory system were poorly developed.

study, the use of cryopreserved sperm appears to be straightforward for producing common snook, and with appropriate genetic management could serve as a tool in hatchery programmes for stock enhancement. The only other member of this family (Centropomidae) in which cryopreservation has been studied is the baramundi, *Lates calcarifer* (Leung 1987). In that preliminary study, 5% DMSO in combination with 15% milk powder or 20% egg yolk yielded the highest post-thaw motility (70–100%) for the pooled sperm of two males. The baramundi is reported to be hermaphroditic with protandrous sequential

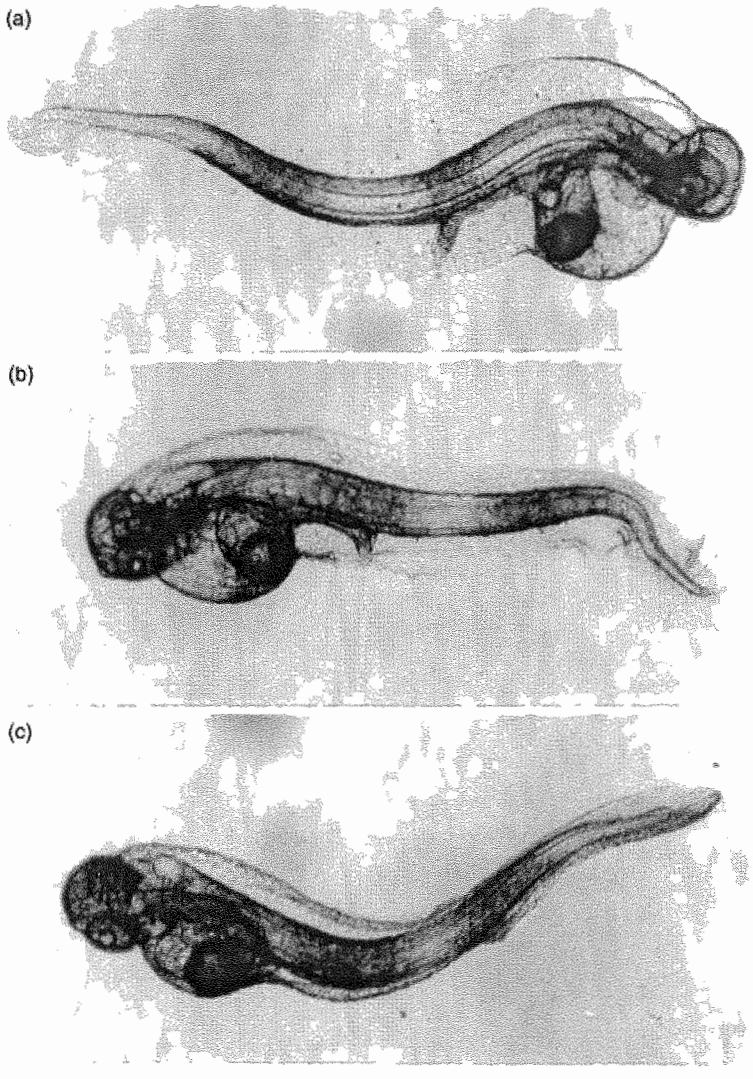

Figure 5 At 24 h after hatching trunk deformities were abundant in the common snook × red drum crosses (the common snook × black drum larvae were dead within 24 h). Of the 24 larvae examined, all had slight to extreme trunk deformities including graceful flexures (a), ventral (b), and dorsal (c) curvatures.

sex change (Guiguen, Cauty, Fostier, Fuchs & Jalabert 1994). Histological analysis of gonads of common snook suggests protandrous sex change in this species as well (Taylor, Whittington, Grier & Crabtree 2000). This suggests the possibility of producing highly inbred lines for research and culture by fertilizing eggs with cryopreserved sperm collected from the same fish.

At the level of the whole animal, common snook are especially sensitive to cold, not tolerating temperatures below 15 °C (Hoese & Moore 1977). We found no evidence of cold injury in sperm, even with extended storage of sperm at 1 °C. The ability to store sperm after dilution in extender solutions allows for shipping of samples from the hatchery to the laboratory for cryopreservation (Stoss 1983). Because it is difficult to collect stripped sperm samples that are free of urine or other contamination, it is useful to dilute the samples at the time of collection in an appropriate solution to increase storage time (Rana 1995; Tiersch, Wayman, Fiegel, Gorman, Williamson & Carmichael 1997).

This study provides a model for the application of cryopreservation to fish sperm. Samples were collected at a working hatchery and shipped overnight more than 1000 km to a laboratory for cryopreservation. One year later, the samples were sent back to the hatchery to fertilize eggs. It should also be noted that the shipping and cryopreservation conditions were

unknown prior to the study and were developed as part of this work. Thus, the facilities and expertise required for cryopreservation were available at a specialized laboratory and the facilities and expertise required for artificial spawning of fish remained at the hatchery. This is not a new approach (Stoss 1983), but it has received little application. In fact, despite 40 years of work in more than 90 species and more than 200 published reports (Rana 1995; Tiersch 2000), cryopreservation of fish sperm remains essentially a research activity with little commercial application (Tiersch 2001). Barriers to application include a diverse and widely distributed literature base (Tiersch 2000), technical problems, the use of small volumes of sperm (Wheeler & Thorgaard 1991), variable results (Rana & McAndrew 1989), and a general lack of access to the technology. Cooperative approaches for hatchery application of cryopreservation have been used with the endangered razorback sucker, *Xyrauchen texanus* (Abbot), (Tiersch, Figiel, Wayman, Williamson, Carmichael & Gorman 1998) and the red snapper, *Lutjanus campechanus* (Poey) (Roppolo 2000), and were used after techniques were developed for other endangered species such as the Mekong giant catfish, *Pangasias gigas* (Mongkonpunya, Chairak, Pupipat & Tiersch 1995), Colorado pikeminnow, *Ptychocheilus lucius* (Girard) (Tiersch, Figiel, Wayman, Williamson, Gorman & Carmichael 2004), and sperm and larvae of the eastern oyster, *Crassostrea virginica* (Paniagua-Chavez, Supan, Buchanan & Tiersch 1998).

A number of benefits could be derived from the approach of shipping milt from a hatchery to a well-equipped cryopreservation laboratory. Hatcheries could take advantage of the efficiency and genetic management offered by cryopreservation (Cloud *et al.* 1990), would not need to develop their own cryopreservation programmes, and could utilize sperm that would otherwise be wasted. This would be especially useful for production of hybrids between species that do not spawn at the same time. The cryopreservation laboratory would benefit from the availability of valuable or difficult to obtain samples, could coordinate activities with existing sampling or spawning programmes (Tiersch *et al.* 1997), and could obtain evaluation of thawed samples in a commercial hatchery environment (Paniagua-Chavez *et al.* 1998). Because of such cooperation, this study was able to use percent fertilization, hatch, and survival to first feeding (Neidig *et al.* 2000) rather than just percent motility to evaluate sperm quality. Most studies of cryopreservation of fish sperm are limited to percent motility after thawing or assessment of percent fertilization. Clearly, for application of this technology, studies also should include growth of fish produced with cryopreserved sperm (Van der Walt *et al.* 1993; Tiersch, Goudie & Carmichael 1994) through harvest (Kerby, Bayless & Harrell 1985) and spawning.

The demonstration in this study of fertilization of eggs with thawed sperm from fish of another family is an extreme example suggesting other benefits that could be derived from cooperative arrangements between hatcheries and laboratories. The hatcheries would derive an efficient method to produce hybrid fishes (presumably between species within a genus or family), which could be useful for research or restricted culture (it should be emphasized that for responsible application of hybrids, they should pose little or no risk to natural environments and should be reproductively sterile and isolated from natural populations if possible). The cryopreservation laboratory would receive access to a method for evaluating the fertilizing capability of sperm, without the necessity of developing the capabilities for artificial spawning of that species. Although not a perfect solution to the problem of evaluating the fertilizing ability of thawed sperm, this would be useful if the laboratory worked with numerous species from different habitats. The alternative would be to rely on post-thaw motility of sperm (Terner 1986), or *in vitro* assays of viability, membrane integrity (Cabrita, Martinez, Real, Alvarez & Herraez 2001), mitochondrial function (Segovia, Jenkins, Paniagua-Chavez & Tiersch 2000), or other estimators of sperm quality (Gallant & McNiven 1991; Lahnsteiner, Berger & Patzner 1996).

The economic costs have been calculated for integration of cryopreservation into existing fish hatcheries (Caffey & Tiersch 2000). Even with expenses of less than $US10 000 for capital outlay, it is unlikely that many hatcheries would be able to develop their own on-site cryopreservation programmes. Access to established facilities that perform cryopreservation would make this technology more available and would improve the potential for success. Given that transport of broodstock is problematic, especially of large, sensitive animals such as common snook, the shipping of sperm is a practical method for enabling off-site cryopreservation. There are a number of requirements and constraints that must be addressed if cooperative relationships are to be developed for the use of cryopreserved sperm in fish hatcheries (Table 3). Such arrangements would involve resolution of issues in areas including scientific and technical

Table 3 Considerations for development of programs for off-site cryopreservation of fish sperm

Category	Requirements and constraints
Scientific and technical	Development of sperm storage solutions (extenders)
	Development of protocols for refrigerated storage (antibiotics, aeration, temperature control, etc.)
	Development of cryopreservation protocols (cryoprotectants, freezing and thawing rates, etc.)
	Development of methods for genetic characterization (DNA techniques, marker-assisted selection, etc.)
Economic	Setting of prices and valuation of materials
	Capital outlays, salaries, supplies, cash flow
	Cost analyses, budgets, economic models
Physical	Laboratory space
	Storage facilities for frozen samples
	Equipment for straw labeling, filling and freezing
Legal and ethical	Prevention or control of disease transfer
	Record keeping, chain-of-custody procedures
	Rules for use and disposal of samples
	Program of quality control and quality assurance
	Transfer agreements between parties
	Treaty and border agreements between countries

feasibility, economics, physical facilities and space, disease transmission (Tiersch & Jenkins 2003), and legal and ethical concerns (Wachtel & Tiersch 2000). These requirements and constraints exist within the private and public sectors (e.g., the USDA National Animal Gerplasm Program; www.ars-grin.gov/nag/), and would be influenced by the particular species being addressed. For example, cryopreservation of samples obtained from declining wild populations (Van der Walt *et al.* 1993) would involve different considerations than would cryopreservation of samples collected from selected lines of cultured species. This model could also allow development of commercial cryopreservation facilities that could provide services for customers that send sperm for freezing. Such facilities exist for livestock species such as cattle, with a long-established federation operating under self-imposed standards of performance (National Association of Animal Breeders and Certified Semen Services; website, www.naab-css.org). Livestock facilities could serve as examples for commercial cryopreservation in aquatic species or provide technology that can be transferred for application in fish and shellfish. Indeed, protocols for aquatic species could be adapted for use at livestock facilities, an approach that would take advantage of the existing infrastructure used for commercial-scale sperm cryopreservation (Lang, Riley, Chandler & Tiersch 2003).

Acknowledgments

This work was supported in part by funding from the Louisiana Sea Grant College Program, the United States Department of Agriculture, and the Marine Resources Conservation Trust Fund from the State of Florida. We thank B Dennis, B Falls, B Miller, and G Vermeer for hatchery assistance. We thank R Caffey, C Paniagua, and G Roppolo (LSU). This manuscript was approved by the Director of the Louisiana Agricultural Experiment Station as number 01-66-0720.

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