Gulf Research Reports, Vol. 9, No. 3, 183-188, 1996 Manuscript received April 20, 1995; accepted May 29, 1995

CRYOPRESERVATION OF SPERM OF SPOTTED SEATROUT (*CYNOSCION NEBULOSUS*)

William R. Wayman[1], R. Glenn Thomas[2], and Terrence R. Tiersch[1]*

[1] *School of Forestry, Wildlife, and Fisheries, Louisiana Agricultural Experiment Station, Louisiana State University Agricultural Center, Baton Rouge, Louisiana 70803, USA*

[2] *Louisiana Department of Wildlife and Fisheries, Lyle S. St. Amant Marine Biological Laboratory, P. O. Box 37, Grand Isle, Louisiana 70358, USA*

ABSTRACT Cryopreservation of fish sperm has applications in preserving genetic resources from stocks of endangered fishes, replenishing fisheries, reducing the number of males needed in hatchery situations, and allowing repeated spawning of specific males. As part of a larger study on artificial breeding of sciaenid fishes, we developed procedures for collection, handling, refrigerated storage, and cryopreservation of spotted seatrout sperm. Hanks' balanced salt solution (HBSS) was used as an extender for collection and storage of sperm. Sperm motility in relation to graded concentrations of HBSS was used to determine the osmolality at which sperm were activated. Based on these findings, HBSS was prepared at 201 mOsm/kg as an extender for sperm storage. To determine if ions present in HBSS were involved in sperm activation, separate activating solutions were prepared by the addition of NaCl, $CaCl_2$, KCl, Na_2HPO_4, or $MgSO_4$ to aliquots of a stock glucose solution (185 mOsm/kg). The chemicals were added at the concentration of each found in 1-x HBSS. Only the glucose solution containing 8 g/l NaCl (424 mOsm/kg) produced activation of sperm. We also evaluated four chemicals as cryoprotectants: methanol, glycerol, dimethyl sulfoxide (DMSO), and n,n-dimethyl acetamide. Two freezing rates were evaluated by placing samples at either of two heights within a nitrogen vapor shipping dewar. The highest post-thaw motilities were in 10% DMSO with an average retention of 60% of initial motility at the lower position in the dewar, and 37% at the upper position. A third freezing rate was produced using a computer-controlled freezer programmed for a rate of -45°C/min, yielding a retention of initial motility of 31%. Our freezing and transport of cryopreserved sperm in shipping dewars demonstrate the utility of this procedure for field applications.

INTRODUCTION

The family Sciaenidae contains several species important to recreational and commercial fisheries. The red drum (*Sciaenops ocellatus*), black drum (*Pogonias cromis*), and spotted seatrout (*Cynoscion nebulosus*) all have large commercial fisheries that were closed or restricted to prevent overfishing. The decline of these fisheries has stimulated interest in development of methods such as artificial spawning and the use of cryopreserved sperm to aid in restoration efforts. Cryopreservation of sperm can be used to preserve genetic resources from stocks of fishes that are endangered and to aid in replenishing fisheries. Cryopreserved sperm can be used to reduce the number of males maintained in the hatchery and allows repeated spawning of specific males when females are in spawning condition. Cryopreservation can be used to preserve gametes of improved stocks, to study hybridization and crossbreeding, and to accelerate genetic research.

The first studies of cryopreservation of fish sperm were performed in marine fishes to aid in hybridization of herring stocks that spawned at different times of the year (Blaxter 1953). Most subsequent studies, however, have been of freshwater species, especially salmonids (see reviews by Scott and Baynes 1980; Stoss 1983). Previous work in reproductive biology of sciaenids has addressed natural spawning (Saucier and Baltz 1993), induced spawning (Colura 1974; Thomas and Boyd 1988), and hybridization (Henderson-Arzapalo and Colura 1984; Henderson-Arzapalo et al. 1994). Cryopreservation of sperm from the Atlantic croaker (*Micropogonias undulatus*) was studied by Gwo et al. (1991) and represents the only report on cryopreservation of sperm from a sciaenid species.

As part of a larger study on artificial breeding of sciaenids, we developed procedures for collection, handling, refrigerated storage, and cryopreservation of spotted seatrout sperm. Our objectives were to: 1) determine the relationship of osmotic pressure and sperm activation to allow safe storage; 2) evaluate the effect on sperm activation of specific ions used in the extender solution; 3) evaluate the effectiveness of different cryoprotectants; and 4) evaluate the success of different freezing rates. Motility estimates were used as a measure of sperm viability. To our knowledge, this is the first report of the cryopreservation of sperm of spotted seatrout.

*Corresponding author

MATERIALS AND METHODS

Blood Plasma Osmolality and Extender Preparation

Blood samples were collected from 34 spotted seatrout caught from April-August, 1994 in Barataria Bay, LA (29°19' N, 89°56' W). Water in the Bay ranged in osmolality from 450-750 mOsm/kg during the collection period. The blood samples were allowed to clot, and 10 µl of plasma were used to determine osmolality with a vapor pressure osmometer (model 5500, Wescor Inc., Logan, UT). The osmolality of blood was 356.0 ± 18.4 mOsm/kg (mean ± SD). This value is similar to plasma values (350 mOsm/L) obtained for red drum (Crocker et al. 1983), another member of the family Sciaenidae. Sperm of marine species typically become motile in solutions of higher osmotic pressure than the blood plasma. Therefore, Hanks' balanced salt solution (HBSS) was prepared (Tiersch et al. 1994) using reagent grade chemicals (Sigma Chemical Corp., St. Louis, MO) at an osmotic pressure (300 mOsm/kg) below that of the blood plasma to ensure that sperm remained inactive when placed in the extender for storage.

Estimation of Sperm Motility

The percent motility of each sperm sample was estimated using darkfield microscopy at 100x immediately after addition of the activating solution. Percent motility was defined as the percentage of progressively motile sperm within each activated sample. The osmolality of the activated sperm mixture was determined by removing 10 µl of diluted sample directly from the microscope slide for analysis by a vapor pressure osmometer. The threshold activation point was defined as the osmotic pressure at which 10% of the sperm became motile. The complete activation point was defined as the lowest osmotic pressure that elicited the highest percentage of motile sperm.

Osmotic Analysis of Sperm Activation

Sperm samples were collected by manual stripping of three males caught in April 1994 in Barataria Bay, Louisiana. For this, fish were dried with a towel, and gentle pressure was applied to the abdomen. Sperm were collected in 75-µl hematocrit tubes, transferred to 1.8-ml centrifuge tubes, and diluted with 1 ml of HBSS (300 mOsm/kg). Sperm activation was evaluated according to Bates et al. (1996) by dilution of 2-µl aliquots of sperm with 20 µl of solutions ranging in osmotic pressure from deionized water (8 mOsm/kg) to double-strength HBSS prepared at 600 mOsm/kg. Because HBSS is highly ionic, solutions of mannitol (Sigma) were prepared at three concentrations (200, 300, and 350 mOsm/kg) and used to test activation of sperm in solutions deficient in ions.

Ionic Analysis of Sperm Activation

Because there was a persistent low level of spontaneous motility (1%) of sperm placed in the HBSS extender solutions, we tested storage of sperm in HBSS at osmotic pressures as low as 152 mOsm/kg. To determine if ions present in HBSS were involved in sperm activation, separate activating solutions were prepared by addition of NaCl, $CaCl_2$, KCl, Na_2HPO_4, or $MgSO_4$ to aliquots of a stock glucose solution (185 mOsm/kg). The chemicals were added at the concentration of each found in 1-x HBSS. Glucose and sucrose solutions prepared at higher osmolalities were used as control treatments to test effects on sperm activation (Table 1), and motility estimates were performed as described above. Artificial seawater (Forty Fathoms Bio-crystals Marinemix, Marine Enterprises International, Inc., Baltimore, MD) prepared at an osmolality of 628 mOsm/kg was used to establish the level of complete motility.

Evaluation of Cryoprotectant Toxicity

Initial cryopreservation studies were performed in the field at the Louisiana Department of Wildlife and Fisheries Lyle S. St. Amant Marine Biological Laboratory on Grand Terre Island. Sperm from two males caught on April 9 were collected by surgical removal and smashing of testis. The sperm were stored in HBSS (186 mOsm/kg) at 4°C. We evaluated four reagent-grade chemicals (Sigma) as cyroprotectants: methanol, glycerol, dimethyl sulfoxide (DMSO), and n,n-dimethyl acetamide (DMA). Each cryoprotectant was mixed at 50%:50% (v:v) with HBSS before addition to sperm mixtures. All cryoprotectants were used at concentrations of 5% and 10% except DMA, which was only used at a 5% concentration because of acute toxicity at higher concentrations (data not shown). The time between addition of cryoprotectant to the sperm and initiation of the freezing procedure was 15 min. Motility was estimated at the initiation of the freezing procedure to determine the acute toxicity of each cryoprotectant to spotted seatrout sperm. The sperm used in this study were subsamples of the samples used in the cryoprotectant toxicity and freezing rate study described below.

Evaluation of Cryoprotection and Freezing Rates

Sperm were cryopreserved in 0.5-ml straws (IMV International Corp., Minneapolis, MN) with two replicates per fish (n = 2) for each treatment. Straws were sealed using glass balls (Minitube of America, Madison, WI), and were placed into an RPE embryo freezer (Peter Elsden and Associates, Collierville, TN) designed for the cryopreservation of mammalian embryos. The RPE embryo freezer consisted of a metal cylinder, with holes drilled for sixteen 0.5-ml straws, designed to create a uniform freezing

rate when lowered into nitrogen vapor in a vapor shipping dewar (Model CP-35, Taylor-Wharton, Theodore, AL). Two freezing rates were accomplished by placing the freezer at either of two heights within the dewar. Placing the center of the RPE embryo freezer 220 mm from the bottom of the dewar yielded the fastest freezing rate; placement 320 mm from the bottom of the dewar yielded a slower freezing rate. To document the freezing rates, a straw filled with HBSS was inserted into the freezer and the temperature was recorded using a type-T thermocouple and a strip chart recorder. The recording was initiated at the time the straws were placed into the RPE embryo freezer and ended when the straws were removed (30 min after reaching -80°C). Straws were transferred immediately to a larger shipping dewar (Model CP-65, Taylor-Wharton) for storage. After 72 hours of storage, the straws were thawed for 7 sec in a water bath at 40°C. The straws were dried and the ends cut to release the sperm into 1.8-ml tubes. For estimation of sperm motility, a 2-µl aliquot of each sample was activated with 20 µl of HBSS (600 mOsm/kg) to obtain maximal motility.

In an experiment performed in the laboratory at Louisiana State University, a computer-controlled freezer (Kryo-10, Planer Products Ltd., England) was used to produce a third freezing rate (-45°C/min). Sperm from the two males used for the dewar studies were transported at 4°C and stored for 24 hr before analysis. Dimethyl sulfoxide at 10% was chosen as the cryoprotectant for this experiment, based on results of the experiments performed in the shipping dewar. Straws were frozen using a two-step procedure. The straws were first cooled to a temperature of 5°C for 5 min, and then frozen at a rate of -45°C/min until reaching -80°C. The straws were maintained at -80°C for 20 min, removed from the freezer, and plunged immediately into liquid nitrogen for storage. After 48 hr of storage, sperm samples were thawed as described above and motility estimated.

TABLE 1

Activation of spotted seatrout sperm. Sperm were stored at 4°C in Hanks' balanced salt solution (HBSS) at either 152 mOsm/kg or 201 mOsm/kg for 2 days prior to analysis. Two-µl aliquots of sperm were activated with 20 µl of activating solution. Motility was estimated under 100x dark-field microscopy. Sugar solutions and artificial seawater were used to establish control values. Solutions containing ionic components of HBSS were prepared in a glucose solution (185 mOsm/kg) to supplement osmotic pressure.

Storage solution	n	Activating solution ingredients(osmolality)	Final osmolality[1]	Percent motility
152	2	Artificial sea water (628 mOsm/kg)	585	18
201	2		597	35
152	2	Sucrose (757 mOsm/kg)	724	18
201	2		713	35
152	1	Glucose (321 mOsm/kg)	318	5
152	1	Glucose (258 mOsm/kg)	258	1
152	2	Glucose (207 mOsm/kg)	211	0
201	2		214	0
152	1	Glucose + 8g/L NaCl	424	10
201	1	Glucose + 0.16g/L CaCl	189	0
201	1	Glucose + 0.4 g/L KCl	199	0
201	1	Glucose + 0.06 g/L Na_2HPO_4	183	0

[1] mOsm/ Kg

Statistical Analysis

All percent motility values were arcsine-squareroot transformed prior to statistical analysis. Motility data derived from the osmotic analysis of sperm activation were compared using a paired Student's t-test (Microsoft Excel 5.0, Microsoft Corp.). In the cryoprotectant toxicity study, differences in pre-freezing motility were determined using a one-factor analysis of variance (SAS 6.08, SAS Institute Inc., Cary, NC). In the study evaluating post-thaw motility as a function of cryoprotectant and freezing rate, differences were determined using a two-factor analysis of variance (SAS 6.08). Means were separated using Duncan's multiple range test, and were considered significant when $P \leq 0.05$.

RESULTS

Osmotic Analysis of Sperm Activation

The motility of spotted seatrout sperm increased with increased osmolality of the HBSS or mannitol activating solutions, with maximum motility (90%) observed at ~375 mOsm/kg and above (Figure 1). Motility of sperm activated in mannitol solutions was not significantly different ($P > 0.27$) from motility of sperm activated in HBSS. In general, ~5% of the sperm (subthreshold) became motile at 242 mOsm/kg. The threshold activation point (10% motility) was 262 mOsm/kg, and the complete activation point (90% motility) was 370 mOsm/kg.

Ionic Analysis of Sperm Activation

To evaluate the activating effect of the various ions contained in HBSS, individual chemical components of HBSS (at the concentration used in 1-x HBSS) were dissolved in glucose solutions (prepared at 185 mOsm/kg to supplement the osmotic pressure) and used as activating solutions for spotted seatrout sperm. Only the glucose solution containing 8 g/l NaCl produced sperm activation. The osmolality of this solution was 424 mOsm/kg (Table 1), above the complete activation point identified in Figure 1. Sperm activated by sucrose solutions at osmolalities of 724 mOsm/kg produced motility equal to that of sperm activated by artificial seawater at 628 mOsm/kg (Table 1). Spotted seatrout sperm stored at 152 mOsm/kg demonstrated reduced motility of as much as 50% compared to sperm stored at 201 mOsm/kg.

Evaluation of Cryoprotectant Toxicity

The average initial motility of sperm samples was 75% at the time of addition of cryoprotectants. Sperm motility at the time of freezing was reduced significantly ($P = 0.0001$) by exposure to glycerol and DMA (Table 2). This loss of motility was likely due to acute toxic effects of the chemicals on sperm. Of the four cryoprotectants, exposure to glycerol reduced pre-freeze motility the most (to ~1%). Pre-freeze motility of sperm exposed to methanol or DMSO was not significantly different ($P > 0.05$) from motility of control sperm not exposed to cryoprotectants.

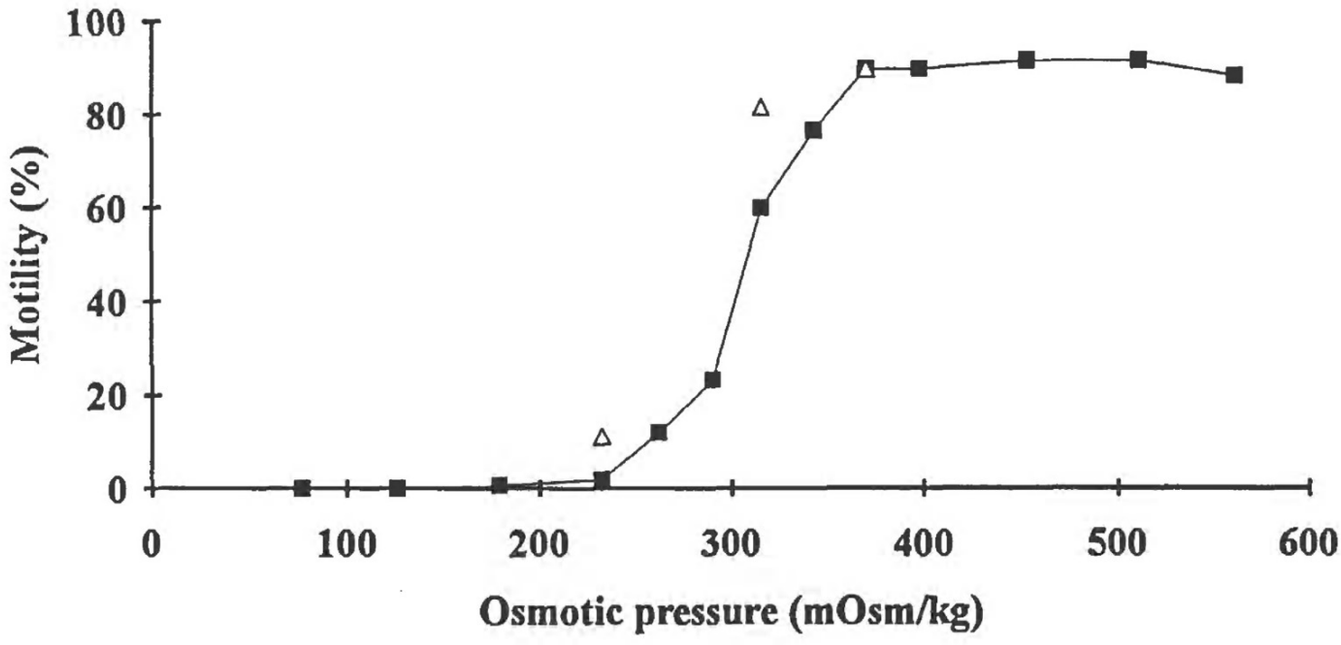

Figure 1. Percent of motility of spotted seatrout sperm activated with solutions of Hanks' balanced salt solution (squares) or mannitol (triangles) spanning a range of osmotic pressures. Each point represents the mean of sperm from three fish. Motility of sperm activated with mannitol was not significantly different ($P > 0.05$) from motility of sperm activated with HBSS at corresponding osmotic pressures. All standard errors were less than 10%.

TABLE 2

Mean motility[1] (± SD) before freezing and after thawing of sperm of spotted seatrout (n=2). Sperm was frozen at one of two positions in a nitrogen vapor shipping dewar: lower (-3.5 °C/min) and upper (-2.5 °C/min); or in a computer-controlled freezer (-45.0 °C/min). Sperm frozen in 10% DMSO had significantly higher (P=0.001) post-thaw motility than sperm frozen in other cryoprotectants. Position within the dewar had no significant effect on post-thaw motility (P=0.15). Pre-freeze motility values sharing a letter were not significantly different.

Cryoprotectant	Concentration	Pre-freeze motility[2](%)	Post-thaw motility (%)		
			Lower	Upper	Freezer
Control[3]	--	70 ± 0[a]	0 ± 0	0 ± 0	--
Methanol	5%	75 ± 6[a]	1 ± 1	0 ± 0	--
	10%	75 ± 6[a]	0 ± 0	0 ± 0	--
DMSO	5%	65 ± 6[ab]	13 ± 4	13 ± 11	--
	10%	63 ± 3[ab]	45 ± 21	28 ± 11	--
	10%	72 ± 4	--	--	22 ± 18
DMA	5%	49 ± 25[b]	3 ± 3	1 ± 0	--
Glycerol	5%	1 ± 1[c]	1 ± 0	1 ± 0	--
	10%	1 ± 1[c]	1 ± 0	1 ± 0	--

[1] Initial motility at time of collection was >75%.
[2] Motility estimated 15 min after the addition of cryoprotectant.
[3] Hanks' balanced salt solution without cryoprotectant.

Evaluation of Cryoprotection and Freezing Rate

Ten percent DMSO produced the highest post-thaw motility of the four cryoprotectants studied (P = 0.001). Sperm frozen in other cryoprotectants yielded motilities of between 0% and 3%. Samples frozen without any cryoprotectant contained no motile sperm after thawing.

The average rates of freezing for the shipping dewar were -3.5°C/min for the lower position and -2.5°C/min for the upper position. The highest post-thaw motilities were in 10% DMSO with an average retention of 60% of initial motility when frozen at the faster rate, and 37% at the slower rate (Table 2). Freezing rate in the shipping dewar had no significant effect on the post-thaw motility (P = 0.15). Sperm frozen in the computer-controlled freezer(-45°C/min) retained an average of 31% of initial motility.

DISCUSSION

The osmolality of blood plasma (~350 mOsm/kg) was used as an estimator of the osmolality of seminal plasma to allow development of preliminary extender solutions for sperm storage. Our preliminary extender allowed storage for 24 h, but did not completely inhibit sperm activation. In a study on the cryopreservation of Atlantic croaker sperm, Gwo et al. (1991) used various extenders to freeze sperm. Although motility in the extenders was not mentioned, 1% unbuffered NaCl yielded the highest post-thaw fertilization rate. A study on cryopreservation of gilthead seabream (*Sparus aurata*) (Chambeyron and Zohar 1990) used two extenders developed by Billard (1984) in previous work with gilthead seabream. In these extenders, sperm motility decreased as the osmolarity of the extender neared the osmolarity of the blood (364 mOsm/l), but motility was observed in all samples after 1-2 min at osmolarities as low as 303 mOsm/l.

In the present study, an activation curve was used to determine the osmolality at which sperm became motile in HBSS. In general, sperm increased in motility as the activating solution increased in osmotic pressure. Other studies on marine fish sperm show the same relationship between sperm activation and increased osmotic pressure. For example, sperm from the pikey bream (*Acanthopagrus berda*) showed no activation in 0 ‰ to 5 ‰ seawater and highest motility in 35 ‰ seawater (Palmer et al. 1994). We used mannitol solutions to determine if a change in ionic concentration was involved in sperm activation. The curve generated for mannitol was not significantly different from that for HBSS (over the tested range of 232-370 mOsm/kg). These studies prompted us to reformulate the extender solution at an osmolality (200 mOsm/kg) that was below the threshold activation point. This extender did not completely prevent activation of sperm, but did reduce the level of spontaneous motility to ~1% and allowed storage of sperm for 3 d at 4°C. Sperm storage in solutions of HBSS with osmolality as low as 152 mOsm/kg did not prevent spontaneous activation and reduced sperm motility after storage at 4°C by as much as 50%, indicating that storage in buffers of low osmotic pressure may be detrimental to sperm.

Activation experiments were also performed with solutions of sucrose, glucose, and glucose supplemented with individual components of HBSS. Activation of sperm in glucose supplemented with ionic components of HBSS was not observed at osmotic pressures below 200 mOsm/kg, suggesting that the ions in 1-x HBSS were not the cause of the spontaneous activation observed during storage. Activation of spotted seatrout sperm may be triggered by changes in concentration of particular ions, osmotic pressure, or a combination of these factors. Further elucidation of the factors involved in the activation of spotted seatrout sperm would be useful for improvement of extender solutions.

Sperm from spotted seatrout were frozen in four cryoprotectants at two concentrations and at two freezing rates. The cryoprotectant yielding highest post-thaw motility was DMSO at 10% when a freezing rate of -3.5°C/min was used, although faster rates of freezing have been shown to be successful in the cryopreservation of Atlantic croaker sperm (Gwo et al. 1991). Sperm frozen in the computer-controlled freezer at -45°C/min retained a lower post-thaw motility than sperm frozen at either dewar freezing rate. Perhaps a freezing rate between -3.5°C/min and -45°C/min could increase the post-thaw motility of spotted seatrout sperm. A faster freezing rate in the shipping dewar could be obtained by freezing the straws in a suspended canister without the use of the RPE embryo freezer. Our freezing and transport of cryopreserved sperm in shipping dewars demonstrates the utility of this procedure for application to work outside of the laboratory.

ACKNOWLEDGMENTS

This research was supported by the Louisiana Sea Grant College Program, a part of the National Sea Grant College Program, maintained by NOAA, U. S. Dept. of Commerce, Project 732. We thank R. Becker of Louisiana Sea Grant and C. Boudreaux and M. Schexnayder of the Louisiana Department of Wildlife and Fisheries for administrative support. We also thank J. Buchanan, J. Lighter, M. Mayeaux, B. McNamara, D. Suggs, and R. Talbot for assistance in collection of samples.

LITERATURE CITED

Bates, M.C., W.R. Wayman, and T.R. Tiersch. 1996. Effect of osmotic pressure on the activation and storage of channel catfish sperm. Trans Amer Fish Soc 125: in press.

Billard, R. 1984. La conservation des gamètes et l'insémination artificielle chez le bar et la daurade. In: G. Barnabé and R. Billard, (eds.). *L'Aquaculture du Bar et des Sparidés*. p 227-231. INRA Publications, Paris.

Blaxter, J.H.S. 1953. Spring storage and cross-fertilization of spring and autumn spawning herring. Nature 172:1189-1190.

Chambeyron, F. and Y. Zonar. 1990. A diluent for sperm cryopreservation of gilthead seabream, *Sparus aurata*. Aquaculture 90:345-352.

Colura, R. L. 1974. Induced spawning of the spotted seatrout, *Cynoscion nebulosus* (Cuvier). Proc Fifth Ann Workshop World Mar Soc, p 319-330.

Crocker, P.A., C.R. Arnold, J.A. DeBoer, and G.J. Holt. 1983. Blood osmolality shift in juvenile red drum, *Sciaenops ocellatus L.*, exposed to fresh water. J Fish Biol 23:315-319.

Gwo, J., K. Strawn, M.T. Longnecker, and C.R. Arnold. 1991. Cryopreservation of Atlantic croaker spermatozoa. Aquaculture 94:355-375.

Henderson-Arzapalo, A. and R.L. Colura. 1984. Black drum x red drum hybridization and growth. J World Mar Soc 15: 412-420.

Henderson-Arzapalo, A., R.L. Colura, and A.F. Maciorowski. 1994. A comparison of black drum, red drum, and their hybrid in saltwater pond culture. J World Aquacult Soc 25: 289-296.

Palmer, P.J., A.E. Hogan, and C.G. Barlow. 1994. Chilled storage of pikey bream (*Acanthopagrus berda*) sperm and activation in different salinities. Asian Fish Sci 7:35-40.

Saucier, M. C. and D. M. Baltz. 1993. Spawning site selection by spotted seatrout, *Cynoscion nebulosus*, and black drum, *Pogonias cromis*, in Louisiana. Environ Biol Fish 36:257-272.

Scott, A.P. and S.M. Baynes. 1980. A review of the biology, handling and storage of salmonid spermatozoa. J Fish Biol 17:707-739.

Stoss, J. 1983. Fish gamete preservation and spermatozoan physiology. In: W.S. Hoar, D.J. Randall and E.M. Donaldson, (eds.), Fish physiology, Vol. 9B, p 305-351. Academic Press, New York.

Thomas, P. and N. Boyd. 1988. Induced spawning of spotted seatrout, red drum, and orangemouth corvina (Family: Sciaenidae) with luteinizing hormone-releasing hormone analog injection. Contrib Mar Sci 30:43-48.

Tiersch, T.R., C.A Goudie, and G.J. Carmichael. 1994. Cryopreservation of channel catfish sperm: storage in cryoprotectants, fertilization trials, and production of channel catfish with cryopreserved sperm. Trans Amer Fish Soc 123:580-586.