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Refrigerated storage and cryopreservation of sperm of red drum, *Sciaenops ocellatus* L.

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Abstract

To aid in artificial spawning of sciaenid fishes, the present authors developed techniques to collect, handle and cryopreserve sperm from red drum, *Sciaenops ocellatus* L. Sperm were collected by removing and slicing the testis, and adding Hanks' balanced salt solution (HBSS) or NaCl solution (each at 200–400 mOsm kg^{-1}) as an extender. Sperm were activated with 800 mOsm kg^{-1} artificial sea water (ASW) to characterize motility. Sperm reached maximum motility (highest percentage motility observed for that sample) within 8 ± 1 s (mean ± SD) and remained at maximum motility for 33 ± 4 s. Sperm were exposed to graded osmotic pressures of ASW (8–800 mOsm kg^{-1}) to determine the range of osmolalities that elicited motility. Threshold activation (defined as ~10% motility) occurred at 351 ± 4 mOsm kg^{-1} and complete activation occurred at 539 ± 2 mOsm kg^{-1}. Sperm stored at 200 mOsm kg^{-1} retained motility for up to 13 days. Dimethyl sulphoxide (DMSO) was used as a cryoprotectant at concentrations ranging from 7.5% to 15% (v:v) in HBSS (200 mOsm kg^{-1}). There were no significant differences among post-thaw motilities of sperm cryopreserved at any concentration of DMSO. Sperm thawed on the benchtop at 21°C had lower post-thaw motility than did sperm thawed at 10, 20, 30, 40, 50 or 60°C in a water bath.

Introduction

The red drum, *Sciaenops ocellatus* L., is a member of the family Sciaenidae, ranging along the coasts of the Atlantic Ocean and Gulf of Mexico from New York State to northern Mexico (Overstreet 1983). In 1994, 5.9 million fish were landed recreationally in the Gulf of Mexico, accounting for 79% of the total red drum landings for the United States (U.S. Department of Commerce 1995). Red drum support a large recreational fishery throughout their range, but in 1985, the commercial fishery was closed in Louisiana to protect declining stocks. After closure of the commercial red drum fishery, aquaculture of this species began to receive interest.

Water quality requirements have been determined for juvenile red drum (e.g. Pursley & Wolters 1994) and for fingerlings in freshwater ponds (Thomas & Wolters 1992); however, low survival is a problem during cold winters. Miranda & Sonski (1985) determined the thermal minima (obtained by temperature reduction of 1°C per day until death) for red drum fingerlings in freshwater to be between 3.0 and 0.8°C. Possible solutions to the overwintering problem include selective breeding

(or crossbreeding) or hybridization of red drum with a more cold-tolerant species. The use of artificial spawning and cryopreserved sperm would aid selection programmes and allow hybridization when females of different species were in spawning condition.

As part of a larger study on reproduction of sciaenids, the present authors developed procedures for the collection, handling and cryopreservation of red drum sperm. The objectives of the present study were to: (1) determine the osmotic pressures that activate red drum sperm; (2) characterize the motility of sperm; (3) determine extenders for refrigerated storage of sperm; (4) evaluate the effect of dimethyl sulphoxide (DMSO) concentration on post-thaw motility; and (5) evaluate different thawing rates for cryopreserved sperm. To the present authors' knowledge, this is the first report of the cryopreservation of sperm of red drum.

Materials and methods

Fish and sperm collection

The Lyle S. St Amant Marine Biological Laboratory of the Louisiana Department of Wildlife and Fisheries was used as a field site for the collection of fish and sperm, and for initial cryopreservation experiments. Fish were collected by long-line, or rod and reel from Barataria Pass (29° 16' N, 89° 57' W), Pass Abel (29° 17' N, 89° 54' W) and Independence Island (29° 19' N, 89° 56' W) in Barataria Bay, Louisiana, USA, from October to December 1995.

As a preliminary study, blood samples were collected from 38 red drum and allowed to clot, and 10 µL of plasma were removed. Osmotic pressure of the blood plasma, determined by vapour pressure osmometry (model 5500, Wescor Inc., Logan, UT, USA), was 375 ± 28 mOsm kg^{-1} (mean ± SD). This value is higher than the plasma values (350 mOsm kg^{-1}) obtained previously for red drum (Crocker, Arnold, DeBoer & Holt 1983), values (356 ± 18) obtained for spotted seatrout, *Cynoscion nebulosus* (Cuvier), (Wayman, Thomas & Tiersch 1996), and values (342 ± 12 mOsm kg^{-1}) obtained for black drum, *Pogonias cromis* L., (Wayman, Thomas & Tiersch 1997).

Sperm were collected from red drum by surgically removing the testis. The testis was placed in a plastic bag and sliced to release sperm. Hanks' balanced salt solution (HBSS) (Wayman *et al.* 1996) was used at 200 mOsm kg^{-1} as an extender for all experiments except for the refrigerated storage experiment. Sperm samples were stored on ice and transported to Louisiana State University within 48 h. All chemicals used in the present study, unless otherwise noted, were of reagent grade (Sigma Chemical Corp., St Louis, MO, USA). Deionized water (18 MΩ-cm) was used for all solutions.

Estimation of sperm motility

The percentage motility of each sperm sample ($n = 3$) was estimated subjectively using darkfield microscopy (at 100× magnification) immediately after addition of an activating solution. The activating solutions consisted of 20-µL aliquots of artificial sea water (ASW) (Forty Fathoms, Marine Enterprises International, Inc., Baltimore, MD, USA) or deionized water which were added to 2 µL of sperm. In preliminary experiments, the present authors determined that a 1:10 dilution was optimal for the studies of motility (data not shown). Per cent motility was defined as the percentage of progressively motile sperm within each activated sample. Sperm that vibrated in place without forward movement were not considered to be motile. Estimates were made within 3 s of adding activating solutions.

Motility characterization

Motility was characterized by activating sperm samples ($n = 3$) with 800 mOsm kg^{-1} ASW, which allowed classification into three periods: (1) the time to reach maximum motility; (2) duration of maximum motility; and (3) the time until all motility in the sample ceased. Maximum motility was defined as the highest percentage of motility observed for a sperm sample.

Osmotic analysis of sperm activation

Samples ($n = 3$) used in this experiment were subsamples of the sperm used in the motility characterization experiment. Sperm activation was evaluated according to Bates, Wayman & Tiersch (1996) by dilution of 2-µL aliquots of sperm with 20 µL of a graded series of solutions ranging in osmotic pressure from deionized water (8 mOsm

kg^{-1}) to ASW prepared at 800 mOsm kg^{-1}. The osmolality of each sperm mixture was determined by removing 10 µL of diluted sample from the microscope slide for analysis by vapour pressure osmometer. Threshold activation was defined as the osmotic pressure that induced motility in ~10% of sperm cells in a sample. The complete activation point was defined as the lowest osmotic pressure that elicited the highest percentage of motile sperm.

Evaluation of osmolality and refrigerated storage

Slices of testis (~3 g) from each of six fish were crushed to release sperm, and 20 mL of a NaCl solution or HBSS at osmolalities of 200, 300 or 400 mOsm kg^{-1} were added as extenders. Osmotic effects on refrigerated storage were evaluated by daily estimation of percentage motility for each sample until no motile sperm were observed upon addition of ASW. Sperm were stored at 1°C in loosely capped 50-mL tubes (#25330–50, Corning Inc., Corning, New York, NY, USA).

Evaluation of cryoprotection and DMSO concentration

Sperm samples ($n = 6$) used in this study were collected by adding 100 mL of 200 mOsm kg^{-1} HBSS to surgically removed testis from six males. Dimethyl sulphoxide was used as a cryoprotectant at 7.5%, 10%, 12.5% and 15% with a pre-freezing exposure time of 58 min. Samples were frozen in 0.5-mL straws (IMV International Corp., Mineapolis, MN, USA) in a computer-controlled freezer (Kryo-10, Planer Products Ltd, Middlesex, UK) at a rate of –45°C min^{-1}. Samples were transferred to a liquid nitrogen storage dewar 20 min after the temperature reached –80°C. After 2 months of storage, samples were thawed for 7 s in a water bath (Model 1141, VWR Scientific, Niles, IL, USA) at 40°C. Immediately after thawing, the motility of each sample was estimated as described above.

Evaluation of thawing rates

The sperm samples ($n = 6$) used in the present study were subsamples of the sperm used in the study above. Samples were frozen using the same protocol as in the previous experiment, except with 10% DMSO as a cryoprotectant. Rates were determined by thawing straws containing a test solution of HBSS (200 mOsm kg^{-1}) and 10% DMSO. A type-T thermocouple (#TMTSS-040G-12, Omega Engineering, Inc., CT, USA) and datalogger (OM-550, Omega Engineering, Inc., Stamford, USA) were used to determine the average time for the temperature of the contents of each straw to rise above 0°C, and to calculate thawing rates. A water bath was used to thaw the straws, except for a group of straws that were thawed on the bench at room temperature (21°C). The temperatures studied in the water bath were 10, 20, 30, 40, 50 and 60°C. Motility was estimated immediately after thawing.

Statistical analyses

All percentage motility values were arc-sine square root transformed prior to statistical analysis. In the osmotic analysis of sperm activation, osmolality values for threshold and complete activation were compared using Student's *t*-test. In the storage study, sperm motility was evaluated over time using a one-factor repeated measures analysis of variance (ANOVA) to determine the effects of osmolality on refrigerated storage. The effects of four concentrations of DMSO on post-thaw motility in the cryopreservation study and the effects of thawing temperatures in the thawing study were analysed using a one-factor ANOVA. Means were separated by Duncan's multiple range test and were considered significantly different at $P \leq 0.05$.

Results

Motility characterization

Sperm began swimming vigorously when activated with 800 mOsm kg^{-1} ASW. Motility was characterized in three periods (Fig. 1). The time to reach maximum motility was 8 ± 1 s (mean ± SD) after the addition of ASW. Maximum motility was maintained for a period of 33 ± 4 s and the time until cessation of all motility was 335 ± 74 s after the addition of ASW.

Osmotic analysis of sperm activation

Sperm were not activated by ASW at concentrations of lower than 240 mOsm kg^{-1}. Motility increased as the osmolality of ASW increased (Fig. 2). The osmolality (539 ± 2 mOsm kg^{-1}) that caused

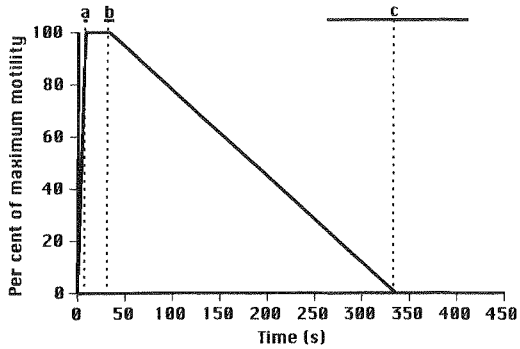

Figure 1 Motility characterization of red drum sperm activated with 800 mOsm kg^{-1} artificial sea water. Motility was characterized in three periods: (a) time to reach maximum motility; (b) duration of maximum motility (a–b interval); and (c) the time until all motility ceased. Each point represents the mean value of three fish. Error bars (shown as horizontal lines above graph) represent ± 1 SD.

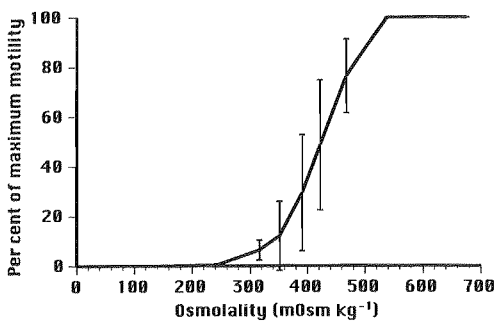

Figure 2 Per cent motility of red drum sperm activated with artificial sea water at various osmotic pressures. Threshold activation occurred at 351 ± 4 mOsm kg^{-1} (mean ± SD), and complete activation occurred at 539 ± 2 mOsm kg^{-1}. Each point represents the mean value of three fish. Error bars represent ± 1 SD.

complete activation was significantly higher ($P < 0.0001$) than the osmolality that caused threshold activation (351 ± 4 mOsm kg^{-1}).

Evaluation of osmolality and refrigerated storage

Motility retention of sperm stored in HBSS was not significantly different from that of sperm stored in NaCl at similar osmolalities ($P = 0.1527$). Sperm stored for 3 days at osmolalities of 200 or 300 mOsm kg^{-1} retained significantly higher motility ($P = 0.0001$) than sperm stored at 400 mOsm kg^{-1} (Figs 3 & 4). Sperm extended at 200 mOsm kg^{-1} remained motile for as long as 13 days. All sperm samples stored at 300 or 400 mOsm kg^{-1} were non-motile after 11 days.

Evaluation of cryoprotection and DMSO concentration

Post-thaw motilities were not different ($P = 0.4747$) for sperm frozen at any concentration of DMSO (Table 1). Mean post-thaw motility was 52 ± 16%.

Evaluation of thawing rates

Thawing times were measured for each thawing temperature (Table 2) and rates were calculated. There was no significant difference ($P = 0.8116$) among motilities of sperm thawed in the water bath at rates of from 175 to 838°C min^{-1}. Overall, motility of sperm thawed on the benchtop at room temperature (21°C) was significantly lower ($P = 0.0138$) than motility of sperm thawed in the water bath (10–60°C).

Discussion

The blood plasma osmolality of red drum observed in the present study was higher than plasma osmolalities observed previously for sciaenids (Crocker *et al.* 1983; Wayman *et al.* 1996; Wayman *et al.* 1997). This could have been caused by high salinity (560–754 mOsm kg^{-1}) within the bay during the spawning season (from October to December 1995). The majority of red drum used in this study were captured by long-line and blood osmolalities could have been elevated because of stress. Weirich & Tomasso (1991) reported that stress can cause osmoregulatory changes in red drum juveniles exposed to high- (36‰) or low-salinity (4‰) waters, allowing osmotic pressure of blood plasma to conform to the salinity of the external environment. Hanks' balanced salt solution was used as an extender for red drum sperm and was formulated at 200 mOsm kg^{-1}, based on previous studies (Wayman *et al.* 1996), to ensure that sperm would not be activated when diluted.

The motility of red drum sperm increased as the osmolality of the activating solution increased. Sperm were activated at an osmolality (351 mOsm kg^{-1}) similar to that which activated black drum sperm (350 mOsm kg^{-1}) (Wayman *et al.* 1997). Spotted sea-

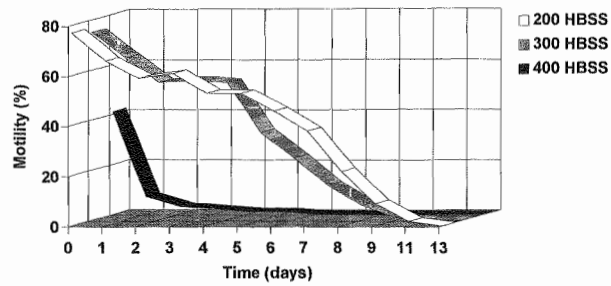

Figure 3 Per cent motility of red drum sperm stored at 4°C in different osmolalities of Hanks' balanced salt solution. Sperm stored for 3 days at 200 or 300 mOsm kg^{-1} retained significantly higher motility ($P = 0.0001$) than sperm stored at 400 mOsm kg^{-1}. Each point represents the mean value of six fish.

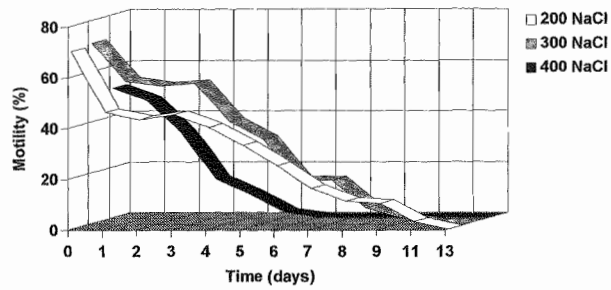

Figure 4 Per cent motility of red drum sperm stored at 4°C in different osmolalities of NaCl solution. Sperm stored for 3 days at 200 or 300 mOsm kg^{-1} retained significantly higher motility ($P = 0.0001$) than sperm stored at 400 mOsm kg^{-1}. Each point represents the mean value of six fish.

Table 1 Mean post-thaw motility (± SEM) of red drum sperm frozen at four concentrations of dimethyl sulphoxide (DMSO). Motility was not different ($P = 0.4747$) among the concentrations of DMSO

DMSO (%)	n^a	Post-thaw motility (%)
7.5	11	52 ± 5
10.0	10	58 ± 5
12.5	9	49 ± 5
15.0	10	51 ± 4

[a]n = number of replicates per concentration.

Table 2 Mean post-thaw motility (± SEM) of red drum sperm thawed at various temperatures. Thawing time was determined by thawing straws filled with 10% DMSO in 200 mOsm kg^{-1} HBSS until the temperature exceeded 0°C. Sperm thawed in the water bath had significantly higher post-thaw motility ($P < 0.014$) than sperm thawed on the benchtop, but post-thaw motilities among sperm samples thawed in the water bath were not significantly different ($P = 0.812$). Each motility value represents the mean (± SEM) of five fish

Thawing temperature (°C)	Thawing time (s)	Thawing rate (°C min^{-1})	Post-thaw motility (%)
Benchtop			
21	240	26 ± 2	30 ± 4
Water bath			
10	25	175 ± 30	43 ± 6
20	13	291 ± 65	49 ± 6
30	9	494 ± 127	49 ± 7
40	7	719 ± 167	52 ± 8
50	7	722 ± 105	48 ± 6
60	6	838 ± 122	52 ± 5

trout sperm were activated at a lower osmolality (262 mOsm kg^{-1}), but sperm motility increased for all species as osmolality increased (Wayman *et al.* 1996). Sperm from other marine fishes activate at similar osmolalities. For example, sperm of the yellowfin bream, *Acanthopagrus australis* (Günther), were not activated at seawater concentrations of below 300 mOsM (Thorogood & Blackshaw 1992).

The osmolality of the extender solution had a significant effect on the refrigerated storage time of red drum sperm. Sperm stored at osmolalities below the blood plasma osmolality (200 and 300 mOsm kg^{-1}) retained motility longer than sperm stored at 400 mOsm kg^{-1}. These data, in combination with the data on activation, indicate that an extender for storage of red drum sperm should be lower in osmolality than 350 mOsm kg^{-1}.

Dimethyl sulphoxide has been used at various concentrations as a cryoprotectant for fish sperm. Gwo, Strawn, Longnecker & Arnold (1991) reported that

Atlantic croaker, *Micropogonias undulatus* (L.), sperm cryopreserved with 20% DMSO yielded higher fertilization rates than sperm cryopreserved with 5, 10 or 30% DMSO, but in another study, rainbow trout, *Oncorhynchus mykiss* (Walbaum), sperm cryopreserved with 10% DMSO yielded the highest fertilization rate (Stoss & Holtz 1983). Although previous reports have indicated that DMSO concentration can significantly affect fertilization rates for other species, DMSO concentrations of between 7.5 and 15% yielded the same post-thaw motility (~50%) for red drum sperm.

Thawing rate can be an important factor in the success of cryopreservation procedures. If the rate is too slow, recrystalization can damage organelles and membranes (Stoss 1983). A thawing temperature of 50°C in a water bath yielded higher motility than did temperatures of 30 or 40°C for sperm of channel catfish, *Ictalurus punctatus* (Rafinesque), (Christensen 1994). However, thawing temperature in a water bath had no effect on post-thaw motility of red drum sperm for thawing temperatures of 10–60°C. Sperm thawed on the benchtop (21°C) had significantly lower post-thaw motility. The thawing rate of straws on the benchtop was about ten times slower than at the same temperature in the water bath. This could have been caused by ice crystal formation on the outside surface of the straw (insulating the straw) or by greater convection in the water bath. Atlantic croaker sperm thawed at 25 or 50°C had similar fertility (45%), but sperm thawed at 0°C had reduced fertility (~30%) (Gwo *et al.* 1991). Optimal thawing temperatures may need to be determined on a species-by-species basis, but red drum sperm seem to tolerate a wide range of thawing temperatures without a significant effect on motility.

The present study indicates that red drum sperm can be extended with 200 or 300 mOsm kg^{-1} HBSS and stored for up to 3 days with a minimal loss (< 30%) of motility. Red drum sperm can be cryopreserved with DMSO concentrations of 7.5–15% (v:v) and thawed at temperatures of between 10 and 60°C. Further research on fertilization of eggs with cryopreserved sperm is necessary to improve procedures for the use of cryopreserved sperm in artificial spawning of red drum.

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