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REFRIGERATED STORAGE AND CRYOPRESERVATION OF BLACK DRUM (Pogonias cromis) SPERMATOZOA

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ABSTRACT

Procedures were developed for the collection, refrigerated storage and cryopreservation of black drum spermatozoa. Sperm samples were collected by removing and slicing the testis, and suspending the spermatozoa in Hanks' balanced salt solution (HBSS) at 200 mOsm/kg. Threshold activation (10%) of black drum spermatozoa occurred at 370 mOsm/kg, and complete activation occurred at 580 mOsm/kg in HBSS. Sperm cells activated in artificial seawater had higher motility than those activated in HBSS at osmolalities from 350 to 500 mOsm/kg. Spermatozoa stored at 4°C in HBSS or artificial seawater at osmolalities from 202 to 290 mOsm/kg retained motility longer than did those stored at other osmolalities. Dilution rate had no effect on sperm storage time at 4°C. Four chemicals were evaluated as cryoprotectants: dimethyl sulfoxide (DMSO), n,n-dimethyl acetamide (DMA), methanol, and glycerol. Glycerol and DMA at concentrations of 10% significantly reduced motility within 52 min. Spermatozoa were cryopreserved at 3 freezing rates (-27, -30, or -45°C/min) in a nitrogen vapor shipping dewar or a computer-controlled freezer. Spermatozoa frozen using 10% DMSO had the highest post-thaw motility at a freezing rate of -27 or -30°C/min. Spermatozoa frozen using 5% glycerol, 5% DMSO, or 10% DMSO had the highest post-thaw motility at a freezing rate of -45°C/min.

Key words black drum, Pogonias cromis, spermatozoa, cryopreservation, aquaculture

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INTRODUCTION

The black drum (Pogonias cromis) is the largest member of the family Sciaenidae in the southeastern United States (13). The black drum ranges from New York to Texas, with reports of as far north as Massachusetts (14), and supports recreational and commercial fisheries within the Gulf of Mexico. From 1979 to 1990 black drum recreational landings decreased from 1,535,000 to 347,000 kg in the Gulf of Mexico (18,20). Due to increasing fishing pressure, Louisiana restricted commercial and recreational fishing in 1990 (9), which stimulated interest in black drum aquaculture.

Hybridization of fishes has been a useful technique in aquaculture. Hybrids can exhibit characteristics such as growth and survival rates that are better than the parental species. Growth rates of red drum x black drum hybrids (female x male) are reported to be faster than either of the parental species (8). Red drum and black drum are hybridized by artificial fertilization, in which eggs and spermatozoa are stripped manually from fish and mixed to create appropriate crosses (7). Red drum and black drum spawn at different times of the year, but can be induced to spawn out of season by manipulation of temperature and photoperiod (7,8). Temperature and photoperiod manipulation can compress an annual cycle into a few months, allowing the fish to spawn when desired. Cryopreserved spermatozoa can be used in hybridization and allows for artificial spawning when eggs of either species are available.

As part of a larger study on the reproduction of sciaenid species, we developed procedures for the handling, refrigerated storage and cryopreservation of black drum spermatozoa. The objectives of the study were 1) to determine the relationship between osmolality and sperm activation; 2) to evaluate the effects of extender osmolality and sperm dilution on refrigerated storage; 3) to assess acute toxicity of cryoprotectants to spermatozoa; and 4) to evaluate different freezing rates for the cryopreservation of black drum spermatozoa. To our knowledge this is the first report on cryopreservation of sperm from this species.

MATERIALS AND METHODS

Fish and Sperm Collection

The Lyle S. St. Amant Marine Laboratory on Grand Terre Island was used as a field site for the collection of sperm and initial cryopreservation studies with nitrogen vapor shipping dewars. Fish were collected by angling or long line from Barataria Pass (29°16' N, 89°57' W) along the Louisiana coast during the 1995 spawning season (February to March). The long-line consisted of 20 size 14/0 circle hooks spaced 6 m apart and anchored at each end. The long line was baited with crabs and set out for between 3 and 24 h. Two black drum collected on February 19 from Barataria Pass provided spermatozoa for the osmotic analysis of sperm activation, osmolality storage study, and the dilution storage study. Four black drum caught in Barataria Pass on March 11 provided spermatozoa for the study of cryoprotectant toxicity and freezing rates.

Blood samples (n = 8) were collected and allowed to clot. Ten microliters of plasma were used to determine osmolality with a vapor-pressure osmometer (model 5500, Wescor Inc., Logan, UT). Blood plasma osmolality was 342.0 ± 12.2 mOsm/kg (mean ± SD). This value is similar to values reported for red drum (350 mOsm/kg) by Crocker et al. (4) and for spotted seatrout (356.0 ± 18.4 mOsm/kg) by Wayman et al. (20). Accordingly, Hanks' balanced salt solution (HBSS) prepared at 200 mOsm/kg was used as the sperm extender (20). Spermatozoa were collected by removing the testes, unless otherwise indicated. Fish were killed with an overdose of tricaine methanesulfonate (MS222; Argent Chemical Laboratories, Redmond, WA). The testes were removed and sliced to release spermatozoa which were then diluted in Hanks' balanced salt solution (~100 ml).

Estimation of Sperm Motility

The percentage of motility of each sperm sample was estimated using darkfield microscopy at x100 magnification immediately after addition of an activating solution. The activating solution was 800 mOsm/kg artificial seawater (Forty Fathoms, Marine Enterprises International, Inc., Baltimore, MD) for all experiments, except for the osmotic effect on sperm activation. The percentage of motility was defined as the percentage of progressively motile spermatozoa within each activated sample. Progressively motile spermatozoa were defined as actively swimming in a forward motion. Sperm cells that vibrated in place were not considered to be motile.

Osmotic Effect on Sperm Activation

Sperm activation was evaluated according to Bates et al. (1) by dilution of 2-µl aliquots of spermatozoa with 20-µl of activating solution. The activating solutions were HBSS or artificial seawater, prepared at osmotic pressures ranging from 112 to 764 mOsm/kg in increments of approximately 66 mOsm/kg. The osmolality of the activated sperm mixture was determined by removing 10 µl of diluted sample from the microscope slide for analysis by vapor pressure osmometer. The threshold activation point was defined as the osmotic pressure at which 10% of the spermatozoa became motile. The complete activation point was defined as the lowest osmotic pressure that elicited the highest percentage of motile spermatozoa.

Effect of Osmolality on Refrigerated Storage

Sperm samples (1 ml) were mixed with 19 ml of HBSS or ASW. Samples were prepared at osmolalities ranging from 112 to 564 mOsm/kg in increments of approximately 100 mOsm/kg. Spermatozoa were stored at 4°C in 50-ml disposable plastic beakers (#1543-D30, Thomas Scientific, Swedesboro, NJ). Motility was estimated daily for 4 d and once at 8 d.

Effect of Dilution on Refrigerated Storage

Spermatozoa were collected by slicing 10 g of testis and suspending the sperm cells in 50 ml of 203 mOsm/kg HBSS. Spermatozoa were aliquoted into 50-ml beakers and brought to the appropriate dilution ratio (g of testis/ml of HBSS) with 203 mOsm/kg HBSS, except for the undiluted spermatozoa. Undiluted spermatozoa were collected by crushing 1 g of testis and

straining the sperm cells through a tissue sieve (Collector®, E-C Apparatus Corp.) into a beaker. Motility was estimated daily for 19 d.

Effect of Cryoprotectant Toxicity

Four reagent grade chemicals (Sigma Chemical Corp., St. Louis, MO) were evaluated as cryoprotectants: methanol, glycerol, n,n-dimethyl acetamide (DMA), and dimethyl sulfoxide (DMSO). Each cryoprotectant was diluted 50:50 (v:v) with HBSS (196 mOsm/kg) prior to addition to sperm. All cryoprotectants were added at concentrations of 5% or 10% (v:v). The time between addition of cryoprotectant and initiation of freezing was 52 min. Motility was estimated at the time of freezing to determine the acute toxicity of each cryoprotectant.

Evaluation of Cryoprotection and Freezing Rate

Samples were frozen at 2 rates in a nitrogen-vapor shipping dewar by placing the samples in 10-mm goblets (IMV International Corp., Minneapolis, MN) at the bottom (-30°C/min) or top (-27°C/min) of 29-cm aluminum canes (Southland Cryogenics Inc., Carrollton, TX). Samples were frozen in 0.5-ml straws (IMV International Corp.) with 2 replicates per fish for each treatment. A type-T thermocouple (#TMTSS-040G-12, Omega Engineering, Inc., Stamford, CT) and data-logger (OM-550, Omega Engineering) were used to determine when samples were frozen by measuring the temperature inside a straw containing buffer and cryoprotectant. The straws reached -105°C within 5 min. Straws were transferred to liquid nitrogen storage dewars after 24 h. After 48 h of storage, spermatozoa were thawed in a 40°C water bath for 7 sec. The straws were dried and the ends cut to release the spermatozoa into 1.5-ml tubes.

A third freezing rate (-45°C/min) was produced using a computer-controlled freezer (Kryo-10; Planer Products Ltd., Sunbury-on-Thames, England). Sperm samples used in the computer-controlled freezer were subsamples from the previous study and were stored at 4°C for 24 h prior to freezing. Cryoprotectants were added at the same concentrations as in the shipping dewar study, but the time between addition of cryoprotectants and the initiation of the freezing protocol was 35 min. Samples were placed in the freezer and held at 5°C for 10 min. The temperature was lowered to -80°C at a rate of -45°C/min. Samples were transferred to liquid nitrogen storage dewars 20 min after the samples reached -80°C. After 1 mo of storage, the samples were thawed in a 40°C water bath for 7 sec. For comparison of freezing rates, motility reduction was calculated by the following formula:

100 x ((initial motility - post-thaw motility) / initial motility) = percentage of initial motility

and was used instead of absolute values because of possible differences in the initial sperm motility among samples.

Statistical Analyses

All percentage motility values were arcsine-square root transformed prior to statistical analysis. In the osmotic effect on sperm activation, osmolalities at threshold and complete activation were compared using a paired Student's t-test (Microsoft Excel 5.0, Microsoft Corp.), and the regression coefficients for motility curves of HBSS and artificial seawater were compared using a **Student's t-test**

(21). In the osmotic and dilution storage studies, the percentage of motility was analyzed using a repeated measures 1-factor ANOVA (SAS 6.08) to test the effects of osmolality or sperm dilution on storage through time. In the cryoprotectant toxicity study, differences in pre-freezing motility were determined using a 1-factor ANOVA (SAS 6.08) to test the effect of cryoprotectants (methanol, glycerol, DMA, and DMSO) on motility. In the freezing rate study, the percentage of reduction of initial motility was analyzed using a 2-factor ANOVA (SAS 6.08) to examine the main effects and interactions of cryoprotectants and freezing rates. A 1-factor ANOVA (SAS 6.08) was used to determine differences in post-thaw motility within freezing rates. Means were separated using Duncan's multiple range test, and were considered to be significant at $\mathbf{P} \leq 0.05$.

RESULTS

Osmotic Effect on Sperm Activation

Black drum spermatozoa were immotile at osmolalities less than 200 mOsm/kg. Sperm cells became motile with increased osmolality of the activating solutions (Figure 1). Motility of spermatozoa activated in artificial seawater was significantly higher ($\mathbf{P} < 0.001$) than that of spermatozoa activated with HBSS at osmolalities from 350 mOsm/kg to 500 mOsm/kg. Threshold activation (10% motility) occurred at 370 mOsm/kg, and complete activation (>75% motility) occurred at 580 mOsm/kg. In artificial seawater, threshold activation occurred at 350 mOsm/kg, and complete activation occurred at 482 mOsm/kg.

Effect of Osmolality on Refrigerated Storage

Sperm motility decreased in all solutions over time. Spermatozoa stored in artificial seawater (205 and 290 mOsm/kg) and HBSS (202 and 286 mOsm/kg) retained motility significantly longer ($\mathbf{P} = 0.001$) than spermatozoa stored in other solutions (Figures 2 and 3). Spermatozoa stored in 205 or 290 mOsm/kg artificial seawater retained ≥ 5% motility after 4 d. Spermatozoa stored in 202 or 286 mOsm/kg HBSS retained ≥ 8% motility after 8 d.

Effect of Dilution on Refrigerated Storage

Motility of undiluted spermatozoa was significantly lower ($\mathbf{P} < 0.0029$) than that of diluted sperm samples at the start of the experiment. Motility of diluted spermatozoa was not different ($\mathbf{P} > 0.11$) throughout the duration of the study (Figure 4). Length of storage time until no motile spermatozoa were present was not different ($\mathbf{P} = 0.39$) for all the dilutions tested.

Evaluation of Cryoprotectant Toxicity

The average initial motility of sperm samples was 92% at the time of addition of the cryoprotectants. Motility at the time of freezing was reduced significantly ($\mathbf{P} < 0.0001$) by 10% glycerol and 10% DMA but was not by the other cryoprotectants (Table 1).

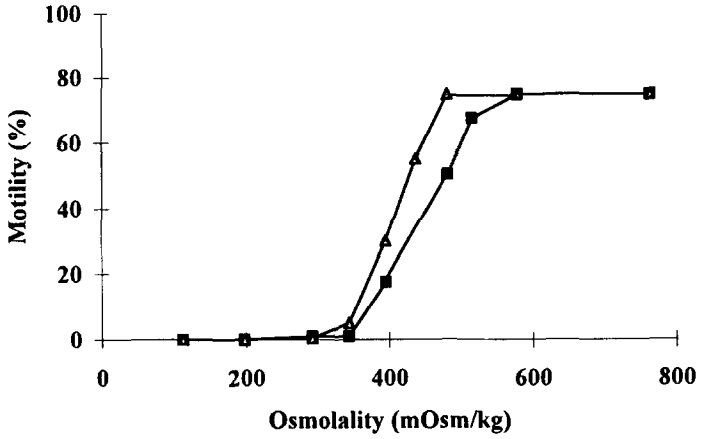

Figure 1. Percentage of motility of black drum spermatozoa activated with Hanks' balanced salt solution (HBSS; squares) or artificial seawater (ASW; triangles) at various osmolalities. Each point represents the mean of sperm from two fish. Motility of spermatozoa activated with ASW was significantly higher ($P < 0.001$) than that of spermatozoa activated with HBSS at osmolalities between 350 and 500 mOsm/kg.

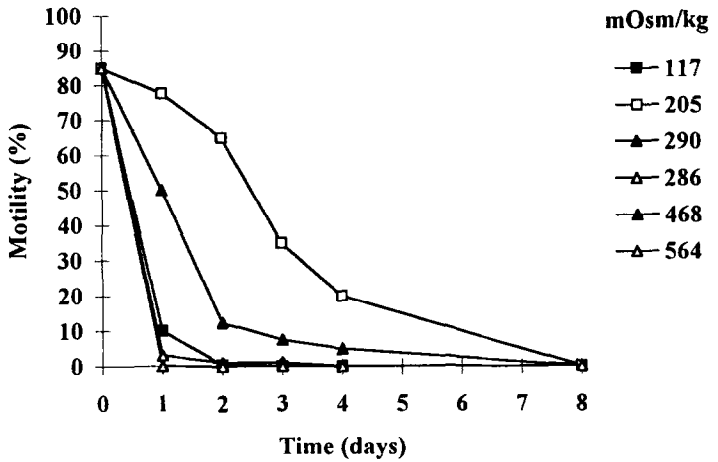

Figure 2. Black drum sperm stored at 4°C in artificial seawater (ASW) at osmolalities ranging from 117 to 564 mOsm/kg. Each point represents the mean of 2 fish. Motility of spermatozoa stored in ASW at 205 mOsm/kg was significantly higher ($P < 0.05$) than that of spermatozoa stored at other osmolalities.

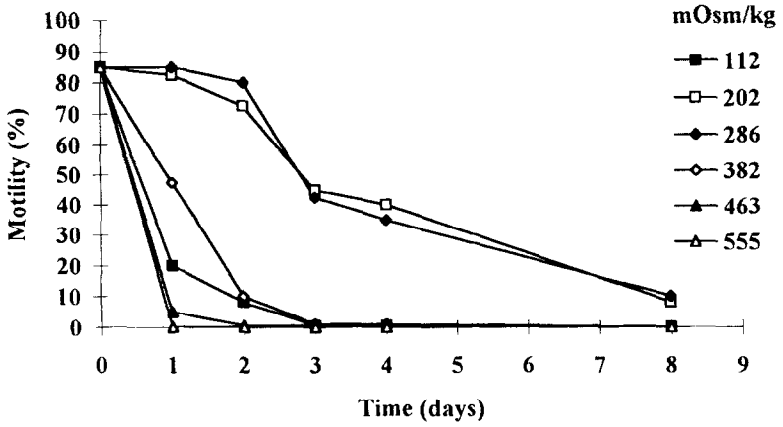

Figure 3. Black drum spermatozoa stored at 4°C in Hanks' balanced salt solution (HBSS) at osmolalities ranging from 112 to 555 mOsm/kg. Each point represents the mean of 2 fish. Motility of spermatozoa stored in HBSS at 202 mOsm/kg and 286 mOsm/kg was significantly higher ($P < 0.05$) than that of spermatozoa stored at other osmolalities.

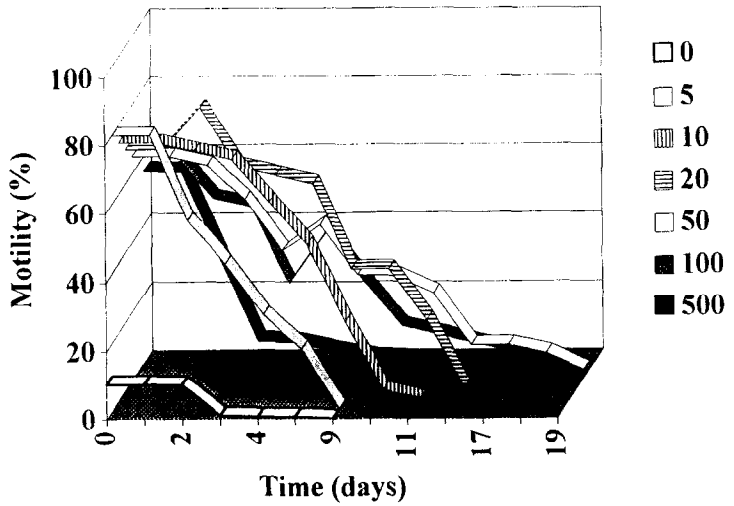

Figure 4. Black drum sperm stored at 4°C in Hanks' balanced salt solution (HBSS) at dilution ratios ranging from undiluted to 500:1 (ml HBSS:g of testis). Each point represents the mean of 2 fish. Motility at Time 0 was estimated within1 hour after dilution of sperm samples. Motility of spermatozoa was not significantly different at any dilution ratio except for undiluted spermatozoa ($P < 0.284$). Initial motility of undiluted spermatozoa was significantly lower than motility in other dilution ratios ($P < 0.003$).

Table 1. Mean motility (± SD) of black drum spermatozoa[a] before freezing and after thawing (n = 4). Cryopreservation was studied using 4 cryoprotectants at 2 concentrations and 3 freezing rates. Spermatozoa were frozen at either of 2 heights within a vapor shipping dewar: lower (-30°C/min) and upper (-27°C/min) or in a computer-controlled freezer (-45°C/min). Sperm cells frozen at the lower position within the dewar retained significantly higher ($P < 0.024$) post-thaw motility than did sperm cells frozen at the upper position. Using the dewar, spermatozoa frozen in 10% dimethyl sulfoxide (DMSO) retained significantly higher ($P < 0.0001$) post-thaw motility than those in the other cryoprotectants. Using the computer-controlled freezer, spermatozoa frozen in 5% glycerol and 5% and 10% DMSO retained significantly higher ($P < 0.0001$) post-thaw motility than spermatozoa frozen in the other cryoprotectants.

			Post-thaw motility (%)		
Cryoprotectant	Concentration	Pre-freeze motility (%)[b]	Lower level	Upper level	Freezer
n,n-dimethyl acetamide	5%	81 ± 21^{c}	15 ± 10^{f}	12 ± 8^{f}	17 ± 11^{de}
	10%	51 ± 35^{d}	20 ± 10^{ef}	19 ± 12^{f}	13 ± 8^{e}
DMSO	5%	90 ± 7^{c}	69 ± 18^{cd}	55 ± 18^{d}	61 ± 24^{c}
	10%	85 ± 17^{c}	76 ± 10^{c}	73 ± 5^{c}	65 ± 18^{c}
Glycerol	5%	71 ± 8^{cd}	61 ± 19^{d}	58 ± 21^{d}	67 ± 19^{c}
	10%	11 ± 3^{e}	37 ± 16^{e}	40 ± 18^{e}	27 ± 17^{d}
Methanol	5%	93 ± 3^{c}	16 ± 16^{f}	3 ± 2^{g}	2 ± 2^{f}
	10%	73 ± 18^{cd}	11 ± 5^{f}	20 ± 10^{f}	3 ± 3^{f}

[a] Initial motility at time of collection was ≥90%.
[b] Motility estimated 52 min after the addition of cryoprotectant.
[c-g] Motility estimates sharing letters within columns were not significantly different ($P > 0.05$).

Evaluation of Cryoprotection and Freezing Rate

Using the shipping dewar, spermatozoa frozen with 10% DMSO retained significantly higher ($P < 0.0001$) post-thaw motility than did spermatozoa frozen with other cryoprotectants. Using the computer-controlled freezer, spermatozoa frozen with 5% glycerol, 5% DMSO or 10%

DMSO retained significantly higher ($P < 0.0001$) post-thaw motility than spermatozoa frozen with other cryoprotectants (Table 1).

Spermatozoa frozen at the lower position within the dewar retained significantly higher ($P < 0.024$) motility than spermatozoa frozen at the upper position. Comparison of freezing rates between sperm cells frozen in the dewar and those frozen in the computer-controlled freezer (-45°C/min) was based on post-thaw percentages of initial motility. Freezing rate had a significant effect on post-thaw reduction of initial motility when methanol was used as a cryoprotectant ($P < 0.0027$). At a concentration of 5% methanol, post-thaw reduction of motility was lowest (84%) when sperm cells were frozen at the lower position within the dewar. At a concentration of 10% methanol, post-thaw reduction of motility was lowest (81%) when spermatozoa were frozen at the upper position within the dewar and highest (97%) when frozen in the computer-controlled freezer. Post-thaw reductions of initial motility were similar at all freezing rates for the other cryoprotectants tested.

DISCUSSION

The osmolality of blood plasma was used as an estimator of seminal plasma to allow for the development of an extender for sperm storage. The osmolality of black drum blood plasma (342 mOsm/kg) was similar to blood plasma osmolality values of other sciaenid species (4,20), and thus an extender developed previously for spotted seatrout sperm (20) was used for black drum sperm.

In marine fishes, sperm activation is usually associated with an increase in osmotic pressure (2,15). In the present study, an activation curve was used to determine the osmolality at which spermatozoa became motile in HBSS or artificial seawater. Motility of sperm cells was higher in artificial seawater than in HBSS at osmolalities between 350 and 500 mOsm/kg. The increased motility in artificial seawater may indicate that factors other than osmotic pressure may influence activation of black drum spermatozoa. Spermatozoa of some species of salmonids (**e.g.**, Oncorhynchus mykiss) are activated by a decrease in potassium ion concentration rather than by a decrease in osmotic pressure (11). Further research into the mechanisms of sperm activation is necessary to determine if ion concentration has an effect on black drum spermatozoa as well.

Osmolality and sperm activation are also important factors in sperm storage. In our study, spermatozoa stored at osmolalities between 200 and 286 mOsm/kg retained motility for significantly longer periods than did sperm samples stored at other osmolalities. Spotted seatrout spermatozoa stored in HBSS at 152 mOsm/kg had a reduced storage time compared with that at 200 mOsm/kg (20). Reduced storage times for solutions above 286 mOsm/kg could be caused by activation of sperm cells during storage.

In the present study, sperm dilution with HBSS or artificial seawater was evaluated for effects on storage time. All dilution ratios tested allowed refrigerated storage of sperm for at least 5 d. In previous studies, channel catfish (Ictalurus punctatus) spermatozoa were stored for up to 19 d at a dilution ratio of 1 g of testis per 20 ml of HBSS (3). Sperm concentration was also an important factor in the fertilization of Atlantic croaker eggs (6). Stoss and Holtz (16) reported

that a 15-fold increase in thawed sperm-to-eggs ratio was required to equal the fertilization success of fresh spermatozoa of rainbow trout (Salmo gairdneri). Although all ratios allowed for storage of black drum sperm for up to 5 d, dilution ratios between 10:1 and 50:1 (ml of HBSS:g of testis) were best suited for estimating motility.

Exposure of spermatozoa to cryoprotectants can reduce sperm motility within a short period of time. Of the cryoprotectants studied, only glycerol and DMA (at a concentration of 10%) reduced black drum sperm motility. Glycerol and DMA toxicity have been reported for spermatozoa of other species. Spermatozoa of chinook salmon (Oncorhynchus tshawytscha) and brown trout (Salmo trutta fario) had reduced fertilizing ability after exposure for 20 min to 7% glycerol (5). Dimethyl acetamide has been reported as a cryoprotectant for rainbow trout (Oncorhynchus mykiss) spermatozoa, although the equilibration time was not reported (10), while DMSO at concentrations from 3.6 to 12.5% (v:v) has been shown to reduce fertility of cryopreserved rainbow trout spermatozoa after 1 min of equilibration time (17).

Of the 4 cryoprotectants tested, spermatozoa cryopreserved in 10% DMSO retained the highest post-thaw motility at all freezing rates. Using the computer-controlled freezer, spermatozoa cryopreserved in 5% glycerol or 5% DMSO retained post-thaw motility similar to that in 10% DMSO. Glycerol and DMSO have been used to cryopreserve the spermatozoa of at least 55 species of fishes (CR Figiel, **personal** communication), and 10% DMSO has been used to cryopreserve the spermatozoa of other sciaenid species (6,20). Pillai et al. (12) found that 15% DMSO and direct immersion into liquid nitrogen was the only protocol which resulted in the maintaining of most of the Pacific herring spermatozoa (Clupea pallasi) intact and viable.

When using methanol as a cryoprotectant, the freezing rate had a significant effect on post-thaw percentage of motility. Christensen (3) found that the freezing rate had a significant effect on post-thaw motility of channel catfish spermatozoa frozen with 5% methanol as the cryoprotectant. However, the freezing rate had no significant effect on the post-thaw percentage of initial motility when spermatozoa were frozen using other cryoprotectants. Gwo et al. (6) found no difference in fertilization rates using Atlantic croaker spermatozoa frozen over a range of rates from -10°C/min to -150°C/min with 10% DMSO as the cryoprotectant. Although black drum eggs were not available for fertilization trials, in a subsequent study (H. Grier, **personal** communication), cyropreserved black drum spermatozoa were transported to the Florida Marine Research Institute and used to fertilize eggs of snook (Centropomus undecimalis). Further research into the fertilization of eggs with cryopreserved spermatozoa is necessary before cryopreservation techniques can be optimized; however, a freezing rate between -27°C/min and -45°C/min with 10% DMSO as the cryoprotectant appears to be efficacious for the cryopreservation of black drum spermatozoa.

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