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Aquaculture 238 (2004) 183–194
www.elsevier.com/locate/aqua-online

Cryopreservation of sperm of red snapper (*Lutjanus campechanus*)

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Received 18 November 2003; received in revised form 9 April 2004; accepted 12 April 2004

Abstract

Interest in the culture of snappers (family Lutjanidae) has developed throughout the world because of declines in wild stocks combined with a consistent high demand and market value. Some snappers, such as the red snapper *Lutjanus campechanus* have proven to be difficult to spawn and culture in captivity. As part of a larger study to improve propagation techniques for red snapper, procedures were developed for the collection, handling, and commercial-scale cryopreservation of sperm. Utilization of cryopreserved sperm in spawning of red snapper allows efforts to be focused on maintaining female broodstock, monitoring ovarian development, and increasing efficiency during the strip-spawning process. Red snapper were collected during the 2000 and 2001 spawning seasons (May to August) off coastal Louisiana by hook and line. Testes were surgically removed and sliced to release sperm. Sperm were collected in 50-ml centrifuge tubes and diluted 1:3 (v:v) with calcium-free Hanks' balanced salt solution. Dimethyl acetamide, dimethyl sulfoxide, methanol, and glycerol were evaluated as cryoprotectants. Dimethyl sulfoxide produced the smallest reduction in motility of sperm cells in acute toxicity trials and produced the highest motilities after thawing ($71 \pm 16\%$) in initial cryopreservation trials. During the 2-year study, sperm samples from 20 red snapper were frozen using commercial-scale cryopreservation methods developed for dairy bulls. Although sperm motility after thawing was typically greater than 80%, comparison with fresh samples revealed a significant difference in motility ($P = 0.048$). Refrigerated and cryopreserved sperm samples with motilities above 80% were used in fertilization trials. Fertilization rates were variable among females (11–85%), although results with refrigerated (non-frozen) and cryopreserved sperm were highly

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0044-8486/$ - see front matter
doi:10.1016/j.aquaculture.2004.04.025

correlated ($r = 0.85$). These results demonstrate that refrigerated and cryopreserved sperm were each effective for the artificial fertilization of red snapper eggs.

Keywords: Cryopreservation; Sperm; Red snapper; *Lutjanus campechanus*

1. Introduction

The red snapper *Lutjanus campechanus* is the basis for an economically important sport and commercial fishery throughout its range. Fishery managers concerned with the status of red snapper stocks have established harvest quotas for the recreational and commercial fisheries in the US waters of the Southeastern Atlantic and Gulf of Mexico in an effort to rebuild wild stocks (Goodyear, 1995). Red snapper stocks in the Gulf of Mexico experience pressures not only from fishing, but also from other factors such as habitat degradation (i.e. hypoxia, pollution) and capture as bycatch in trawl fisheries (Rabalais et al., 1996; Chesney et al., 2000). The decline of wild stocks combined with a high global demand and high market value have stimulated interest in the development of red snapper aquaculture.

In recent years, culturists have begun to examine the practical applications and benefits of using cryopreserved sperm in the hatchery. Cryopreserved sperm can improve efficiency in artificial spawning and allows for long-term, repeated use. Additional benefits of cryopreserved sperm include: (1) genetic improvement through selective breeding; (2) production of reference stocks for culture or research; (3) production of hybrids; (4) reduction of the cost and labor of maintaining broodstocks; (5) elimination of the need for precise synchronization of males and females, and (6) genetic resource conservation and development of germplasm repositories (Chao and Liao, 2001).

Since the first attempts to cryopreserve sperm for hybridizing spring and autumn herring 50 years ago (Blaxter, 1953), cryopreservation protocols have been developed and published for more than 32 species of marine fish (Tiersch, 2000). The development of cryopreservation methods for fish sperm parallels techniques developed for the cryopreservation of bull sperm for the dairy industry (Chandler, 2000; Rana, 1995). While cryopreservation methods have been developed for semen from several species of domestic livestock (e.g. boar, ram, stallion), the dairy industry is the only worldwide industry that has incorporated cryopreservation of semen into commercial artificial insemination practices (Curry, 2000). These methods address protocols for freezing, long-term storage, and thawing of sperm samples.

Fish sperm that has been removed from the testes must be diluted to an optimal concentration with extender solutions, which prolong cell viability by suppressing motility and preventing death of sperm cells from desiccation and hypoxia. Extender solutions are similar in ionic composition and osmotic pressure to the blood and seminal plasma of the candidate species (Morisawa and Suzuki, 1980; Bates et al., 1996). Hanks' balanced salt solution (HBSS) has been used successfully in the refrigerated storage and cryopreservation of several marine species (Wayman and Tiersch, 2000).

Prior to freezing, permeating cryoprotectants are typically added to help preserve the cellular integrity of sperm during freezing. Examples of commonly used permeating

cryoprotectants include glycerol, dimethyl sulfoxide, *n*,*n*-dimethyl acetamide, and methanol. Equilibration time is needed after the addition of permeating cryoprotectants to allow the chemicals to enter the cells. Caution must be used because the cryoprotectants are often toxic to sperm cells at concentrations as low as 5 or 10% (Wayman and Tiersch, 2000). Acute toxicity experiments with sperm solutions can help determine the appropriate concentration of cryoprotectant and equilibration period needed for freezing of samples.

Several cryopreservation techniques have been developed for the cooling and freezing of sperm solutions. Sperm solutions packaged in 0.25- or 0.50-ml French straws can be cooled in the laboratory utilizing a computer-controlled freezer or a nitrogen-vapor shipping dewar; however, each of these methods is time consuming and inefficient for the freezing of the large volumes of semen needed for commercial production (Wayman and Tiersch, 2000). The dairy industry has successfully implemented cryopreservation by the use of computerized straw labelers, automated straw-fillers, large freezing and storage chambers, and an organized database to manage collection, storage, and distribution of samples.

The goal of this study was to develop procedures for the collection, handling, and cryopreservation of red snapper sperm. Our objectives were to: (1) assess the acute toxicity of cryoprotectants to spermatozoa; (2) evaluate the long-term storage of cryopreserved sperm; (3) evaluate fertilization of red snapper eggs with cryopreserved sperm; (4) evaluate the effects of cryoprotectant concentration on the fertilization of red snapper eggs, and (5) evaluate techniques used commercially in the dairy industry for use with sperm from red snapper. To our knowledge this is the first published report on the successful production of red snapper with cryopreserved sperm.

2. Methods

2.1. Collection of red snapper

Red snapper broodstock (1.0 to 3.8 kg) were collected during the 2000 and 2001 spawning seasons (May to August) off coastal Louisiana by hook and line. Upon collection, swim bladders were deflated by puncture with a sterile 16-G needle. Ripe male red snapper were identified by an extended urogenital papilla and the presence of flowing milt upon palpation of the abdomen. Males were killed immediately after capture and placed on ice until used for the collection of fresh sperm. Female red snapper were placed into an onboard oxygenated live-well and were transported to the hatchery. Additional sperm were obtained from red snapper ($N=21$) caught on chartered recreational fishing boats based in Coco Marina in Cocodrie, LA, during the study period and during preliminary investigations in the summer of 1998. All fish were sampled within 6 h of capture.

2.2. Collection of sperm

After males were measured (total length) and blotted to avoid contamination of samples, testes were surgically removed and placed in 4-l Ziploc® freezer bags (S. C.

Johnson and Son, Racine, WI) with HBSS added to suspend the testes. The HBSS was prepared without calcium at 200 mosMol/kg (Wayman et al., 1996). The samples were placed on ice and transported to the laboratory (30 min) where the testes were removed from the bags, blotted, and weighed. The testes were sliced to release spermatozoa, which were collected in 50-ml plastic centrifuge tubes and diluted 1:3 (v:v) with HBSS. The sperm solutions were refrigerated at 4 °C until use in cryopreservation experiments.

2.3. Estimation of sperm motility

The percent motility of each sperm sample was estimated using darkfield microscopy at 200-× magnification. Activation of sperm was initiated by placing 2 μl of sperm onto a microscope slide and diluting it with 20 μl of activating solution. Artificial seawater (Marinemix®, Wiegandt, Krefeld, Germany) prepared at 870 mosMol kg^{-1} was used to activate sperm. Experienced personnel determined motility as the percentage of sperm actively moving forward.

2.4. Cryoprotectant toxicity study

Sperm samples with motility greater than 95% were selected for use in a cryoprotectant toxicity study. Reagent grade dimethyl sulfoxide (DMSO), glycerol, methanol (MeOH), and *n*,*n*-dimethyl acetamide (DMA) (Sigma, St. Louis, MO) were evaluated for their effects on sperm motility over 60 min at 4 °C. Each cryoprotectant was diluted 1:1 (v:v) with HBSS (200 mosMol kg^{-1}) and refrigerated (4 °C) before addition to sperm solutions. The motility of sperm solutions containing final concentrations of 0%, 5%, 10%, 15%, 20%, or 25% of cryoprotectant were estimated every 15 min.

2.5. Cryopreservation of sperm

In order to thoroughly examine the effectiveness of DMA, DMSO, and MeOH as cryoprotectants, sperm were frozen with final concentrations of 5% or 10% of each chemical. All sperm samples were cryopreserved at Genex Custom Collection Inc. located at the Louisiana State University T. E. Patrick Dairy Improvement Center in Baton Rouge. Sperm were prepared for freezing in a walk-in cooler held at 5 °C. Sperm solutions were mixed with cryoprotectants and allowed 20 min to equilibrate before beginning the freezing process. Using an automated straw filler (model MRS 1, IMV Int., Minneapolis, MN), 0.5-ml French cryopreservation straws were filled with sperm solutions. The straws were placed on horizontal racks with enough water-filled straws added to standardize the heat load within the freezing chamber (660 total straws) and were placed in the freezing chamber (− 140 °C). During the first 3 min of the freezing process, the chamber was allowed to warm from − 140 to − 60 °C as a result of the heat load of the samples. Liquid nitrogen was added to the chamber to cool it at a rate of 16 °C min^{-1} until the chamber returned to − 140 °C (Fig. 1) (Chandler et al., 1984). Once frozen, the samples were removed and placed in a liquid nitrogen storage container for sorting and preparation for storage.

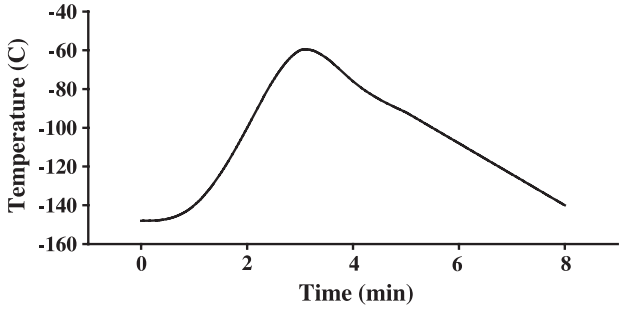

Fig. 1. Cooling profile of the dairy method of cryopreserving sperm used at the T. E. Patrick Dairy Improvement Center of the Louisiana State University Agricultural Center. After the freezing chamber warmed from the heat load of the samples, the chamber was cooled at a rate of 16 °C min^{-1}. Samples were plunged into liquid nitrogen (− 196 °C) after 8 min.

2.6. Thawing of samples

Two straws from each male were thawed to estimate the post-thaw motility of sperm. Samples were thawed in a 40 °C water bath for 7 s. The rest of the cryopreserved sperm samples were held in nitrogen-vapor shipping dewars for use in hatchery experiments and bulk samples were placed into long-term cryogenic storage at the Dairy Improvement Center.

2.7. Hormone injection, spawning, and fertilization

Females were injected intramuscularly with a 500 IU kg^{-1} priming dose of human chorionic gonadotropin (HCG) (Chorulon, Intervet, Millsboro, DE), tagged with a colored anchor tag (Floy Tag, Seattle, WA), and placed in a recirculating culture system. Female red snapper were injected with a 1000 IU kg^{-1} resolving dose of HCG approximately 16 h later. The females were monitored for oocyte maturation and were stripped after ovulation (Riley, 2002). In a series of 2×2 trials, aliquots of 200 eggs were fertilized with 1 ml of refrigerated or cryopreserved sperm. No attempts were made to standardize the sperm concentrations of refrigerated or cryopreserved sperm samples, although the concentrations were approximately 1.0×10^9 cells ml^{-1}. Filtered (5 μ) ultraviolet-sterilized seawater (35‰) (FSW) was added to activate sperm and initiate fertilization. Sperm and eggs were mixed with 1 l of FSW and transferred to 4-l Ziploc® freezer bags and incubated at 30 °C (Carmichael et al., 1996). All samples were preserved in a solution of 5% formalin in buffered seawater after 14 h. Fertilization rate was estimated by assessing embryo development to neurulation (14 h after fertilization) through use of a dissecting microscope.

2.8. Long-term storage of sperm

The long-term storage and fertilizing capacity of red snapper sperm was evaluated by comparing fertilization rates of sperm samples cryopreserved in 1998 and 2000. The post-

thaw motility of all samples used in fertilization trials were greater than 90%. Replicate subsamples of 200 eggs from two ripe females were fertilized with 1 ml of fresh or cryopreserved sperm in each trial. Ultraviolet-sterilized seawater was added to activate sperm and initiate fertilization. Sperm and eggs were mixed with 1 l of FSW and transferred to 4-l Ziploc® freezer bags and incubated at 30 °C. Fertilization was assessed as neurulation (14 h).

2.9. Cryoprotectant effects on fertilization

In order to evaluate the effects of cryoprotectant concentration on fertilization success, eggs stripped from two female red snapper were evaluated in trials with fresh sperm mixed with DMSO at concentrations of 0%, 10%, 20%, and 50%. The average motility of the fresh sperm with no chemical treatment was 95%. No attempts were made to standardize the sperm concentrations. Sperm solutions were refrigerated for 15 min prior to use in fertilization trials. Aliquots of 200 eggs were fertilized with 1 ml sperm and cryoprotectant. Ultraviolet-sterilized seawater was added to activate sperm and initiate fertilization. Sperm and eggs were mixed with 1 l of FSW and transferred to 4-l Ziploc® freezer bags and incubated at 30 °C. Fertilization was assessed at neurulation.

2.10. Statistical analysis

All percent motility and fertilization values were arcsine-square root transformed prior to statistical analysis. A one-factor analysis of variance (ANOVA) (SAS 8.0; SAS Institute, Cary, NC) was used to compare the effect of cryoprotectants (DMSO, DMA, MeOH, glycerol) on sperm motility over time. Differences in the motility of red snapper sperm before freezing and after thawing were analyzed using a one-factor ANOVA. Differences were considered significant at $P \leq 0.05$. Duncan's multiple range test was used to determine if significant differences existed among treatment means. Linear regression analysis (Microsoft Excel 2000, Microsoft, Redmond, WA) was used to determine the correlation between fertilization rates with refrigerated and cryopreserved sperm. Variables were considered correlated when $r \geq 0.50$.

3. Results

3.1. Cryoprotectant toxicity study

Sperm samples from five males collected during the 2000 spawning season were used to evaluate the acute toxicity of the cryoprotectants. The average motility of the samples prior to exposure to cryoprotectants was 95% (Fig. 2). Within 30 min, the motility of samples diluted with 20% and 25% concentrations of all cryoprotectants were reduced below 50%. Glycerol was the most toxic chemical tested with all samples exhibiting significantly reduced motilities ($P < 0.0001$). At concentrations of 10%, 15%, 20%, and 25%, glycerol reduced sperm motilities to zero in less than 30 min. Dimethyl acetamide, DMSO, and MeOH prepared at 5% and 10% concentrations were least toxic to sperm

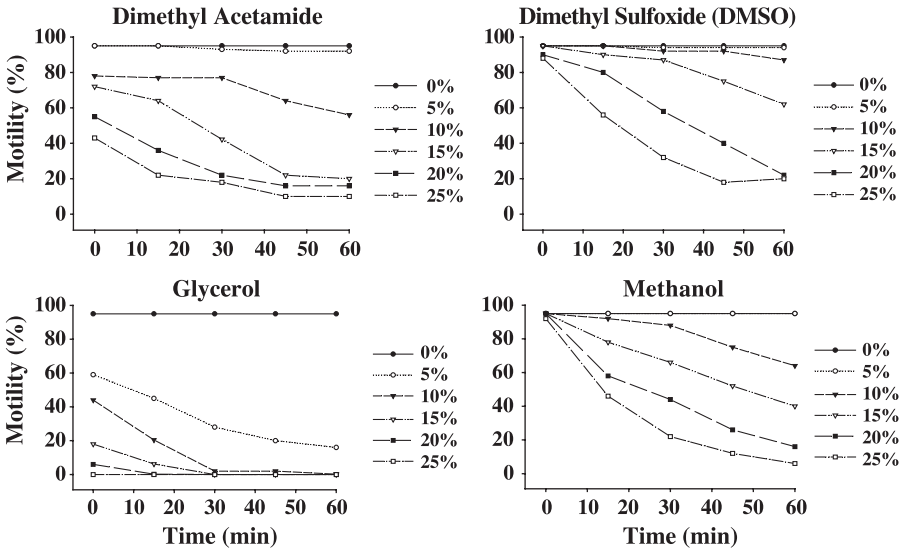

Fig. 2. Acute toxicity of various concentrations of four cryoprotectants to sperm of red snapper *L. campechanus*. The average motility of the samples ($N=5$) prior to exposure to cryoprotectants was 95%. While glycerol prepared at all concentrations was most toxic to sperm samples and reduced motilities within minutes after addition, DMA, DMSO, and MeOH prepared at 5% and 10% concentrations were less toxic throughout the 60-min experiment.

samples. These chemicals and respective concentrations were subsequently used in cryopreservation trials.

3.2. Cryopreservation of sperm

Sperm from six males were cryopreserved using 5% or 10% DMA, DMSO, or MeOH. The motility of sperm before addition of chemicals was $93 \pm 3\%$. Ten percent DMSO produced the highest post-thaw sperm motility (71%) of the cryoprotectants studied ($P<0.0001$) (Fig. 3). All other chemical treatments except 5% DMSO and 10% DMA yielded post-thaw sperm motilities below 50%.

During the 2-year study period, sperm samples from 20 red snapper were frozen using the commercial-scale cryopreservation methods employed at T. E. Patrick Dairy Improvement Center. The motility of samples before freezing was $90 \pm 6\%$ while the motility of samples after thawing was $80 \pm 23\%$. While most samples retained motility above 80% after thawing, comparison with fresh samples revealed a significant difference in motility ($P=0.048$).

3.3. Hormone injection, spawning, and fertilization

Fertilization rates with refrigerated and cryopreserved sperm were evaluated in experiments with eggs collected from eight female red snapper in 2000 and three female red

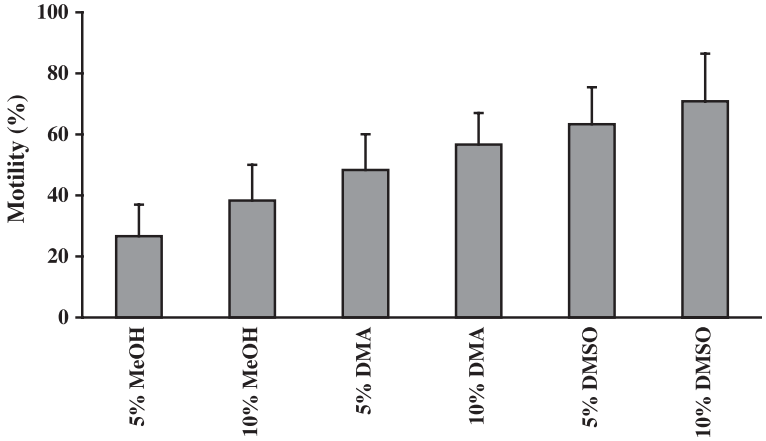

Fig. 3. Motility after thawing (mean ± S.D.) of red snapper sperm. Samples from six males were frozen in 5% and 10% solutions of methanol (MeOH), dimethyl acetamide (DMA), and dimethyl sulfoxide (DMSO). The motility of sperm before addition of chemicals was 93 ± 3%.

snapper in 2001. Fertilization rates were highly variable among females (11–85%), although results with refrigerated and cryopreserved sperm were highly correlated ($r = 0.85$) (Table 1).

3.4. Long-term storage of sperm

Eggs from two ripe females were fertilized with fresh sperm and cryopreserved sperm from males collected in 1998 and 2000. Fertilization rates for female 1 averaged 19 ± 1% while fertilization rates for female 2 averaged 66 ± 17% (Fig. 4). Although no statistical analysis was performed on these data due to the small sample

Table 1
Comparison of fertilization of red snapper eggs with fresh and cryopreserved sperm

Year	Female	Refrigerated	Cryopreserved
2000	1	81 ± 0	85 ± 6
	2	68 ± 15	46 ± 10
	3	29 ± 6	19 ± 2
	4	39 ± 3	37 ± 0
	5	17 ± 11	19 ± 1
	6	50 ± 5	54 ± 6
	7	33 ± 9	41 + 7
	8	81 ± 3	69 ± 1
2001	9	56 ± 8	38 ± 6
	10	23 ± 3	17 ± 2
	11	20 ± 0	11 ± 6

No attempts were made to standardize the concentrations of refrigerated and cryopreserved sperm samples. Fertilization rates were variable among females, although results with refrigerated and cryopreserved sperm were highly correlated ($r = 0.85$). The lower rates of fertilization for some spawns were due to poor egg quality.

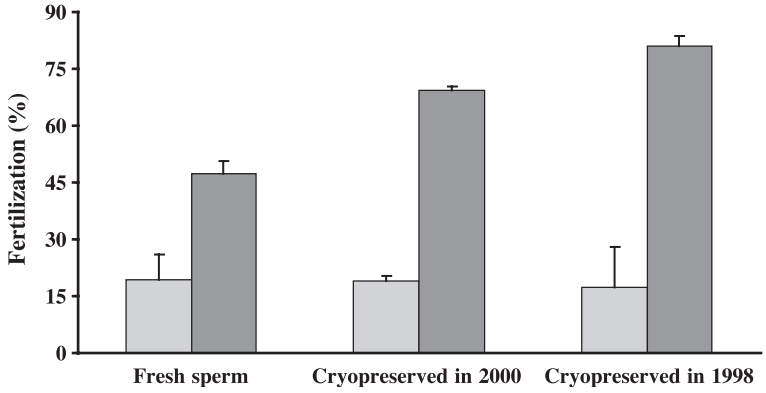

Fig. 4. Fertilization rates (mean ± S.D.) with fresh and cryopreserved sperm of eggs from two red snapper females (female 1, light bars; female 2, dark bars) spawned in 2001. No statistical comparisons were made because of the small sample sizes.

size, cryopreserved sperm yielded fertilization rates comparable of those with fresh sperm.

3.5. Cryoprotectant effects on fertilization

Eggs fertilized with sperm solutions containing 20% or 50% DMSO resulted in significantly lower rates of fertilization than did sperm solutions containing 0% or 10% DMSO ($P < 0.001$). Although sperm solutions containing 10% DMSO resulted in lower fertilization rates than did sperm solutions without DMSO, the difference was not statistically significant (Fig. 5).

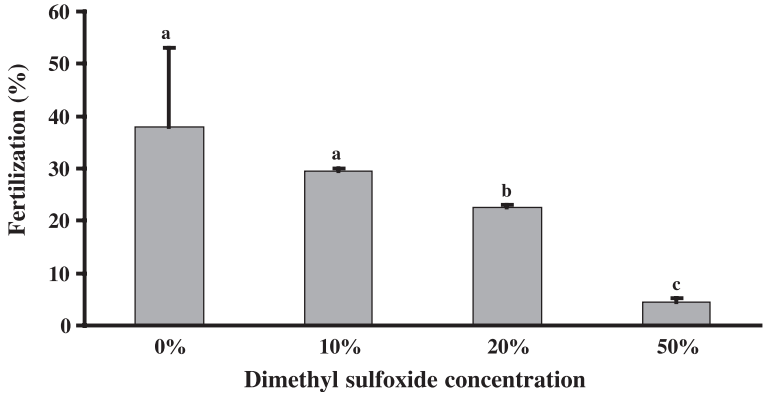

Fig. 5. Fertilization rates (mean ± S.D.) of red snapper eggs presented with fresh sperm containing various concentrations of DMSO. Although sperm solutions containing 10% DMSO resulted in lower rates of fertilization than did sperm solutions without DMSO, this difference was not statistically significant. Eggs fertilized with sperm containing 20% and 50% DMSO had significantly lower rates of fertilization ($P < 0.001$).

4. Discussion

A goal of this study was to develop procedures for the collection, handling, and cryopreservation of red snapper sperm. These procedures were developed to aid in artificial spawning. As seen in previous studies, fishing tournaments and recreational anglers can serve as a source of fish for collection of testes and viable sperm (Caylor et al., 1994; Roppolo, 1999). It has been our experience that red snapper males as well as males of other estuarine and marine species are available from marinas and fishing tournaments along coastal Louisiana. Testes and sperm can easily be collected and sperm quality remains high when fish and sperm samples are refrigerated or iced properly. The advantage of this approach is that sampling of fish from coastal marinas and fishing tournaments can provide large volumes of sperm from a diversity of species or individuals at minimal cost.

Red snapper sperm were evaluated in a series of acute toxicity tests with four cryoprotectants at five concentrations. Because DMA, DMSO, and MeOH were least toxic to sperm at concentrations of 5% or 10%, these chemicals were evaluated in cryopreservation trials. The cryoprotectant yielding the highest post-thaw motility was 10% DMSO. This finding agreed with studies on the cryopreservation of sperm from species such as rainbow trout *Oncorhynchus mykiss* (Stoss and Holtz, 1983), gilthead seabream *Sparus aurata* (Chambeyron and Zohar, 1990), Atlantic croaker *Micropogonias undulatus* (Gwo et al., 1991), cobia *Rachycentron canadum* (Caylor et al., 1994), spotted seatrout *Cynosion nebulosus* (Wayman et al., 1996), black drum *Pogonias cromis* (Wayman et al., 1997), and red drum *Sciaenops ocellatus* (Wayman et al., 1998). While there is no universal cryoprotectant or concentration for freezing of fish sperm, 10% and 20% DMSO have produced the highest post-thaw motility and fertilization results with several species of marine fish (Gwo, 2000). In the fertilization trials of the present study, sperm solutions with concentrations of DMSO above 10% produced significantly lower rates of fertilization. Dimethyl sulfoxide at 10% concentration was selected for use as the cryoprotectant in bulk freezing of red snapper sperm because it was least toxic to sperm samples, produced the highest post-thaw motility, and consistently produced greater than 60% fertilization in trials with red snapper eggs.

The use of cryopreserved sperm in artificial spawning of red snapper can improve efficiency in the hatchery. Efforts can be focused on monitoring of oocyte maturation and timing of ovulation in female broodstock. Following ovulation, females can be stripped of eggs and sperm can be applied from straws instead of males. Thawing cryopreserved samples is quick (7 s) and once thawed, sperm are ready to be applied to eggs. The use of cryopreserved sperm can provide the opportunity to collect a greater number of female broodstock during offshore collecting trips or reduce handling stress by transporting of fewer fish. This could increase success in spawning of red snapper in the hatchery.

The use of commercial-scale dairy methods for cryopreservation was efficient for freezing the large volumes of sperm needed in artificial spawning of red snapper. The facilities used in this study were capable of filling and freezing approximately 1300 straws per hour with a high level of standardization and quality assurance. If cryopreservation of fish sperm is to be integrated into hatchery operations, the use of specialized cryopreservation centers such as dairy facilities should be considered as a time-saving and cost-effective option.

Moreover, the use of semen cryopreservation in the dairy industry is the result of decades of study, refinement and integration. At the heart of any dairy breeding facility is an organized database that tracks samples to ensure quality across collection, disease screening, cryopreservation, storage, distribution, and use. Proper labeling of straws and organization of storage dewars enable long-term repeated use of superior males. After five decades of research on the cryopreservation of fish sperm, the techniques evaluated in this study are among the most practical yet identified for the integration and use of the large volumes of sperm needed in commercial marine fish hatcheries. Future studies should evaluate practical considerations such as the optimal densities of sperm for freezing, thawing, and use in fertilization of red snapper eggs. Additional studies should evaluate the viability, survival, and development of larvae produced from cryopreserved sperm.

Acknowledgements

This research was supported by the Louisiana Sea Grant College Program, a part of the National Sea Grant College Program. We thank Ron Becker of Louisiana Sea Grant for administrative support. We thank Johnny Glover and the staff of the Coco Marina for donating fish for the collection of samples, and John Chandler and the staff of T. E. Patrick Dairy Improvement Center for assistance in the bulk cryopreservation of samples. We are grateful to Yanli Lee, Jennifer Verburg, Paul Lang, Mark Stead, Eric Herbst, and Josh Porter for their technical assistance throughout this project. This manuscript was approved by the Director of the Louisiana Agricultural Experiment Station as manuscript number 04-11-0043.

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