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[Article]

Field Collection, Handling, and Refrigerated Storage of Sperm of Red Snapper and Gray Snapper

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Abstract.—Red snapper *Lutjanus campechanus* and gray snapper *L. griseus* support valuable sport and commercial fisheries. Because of their high market value and limited commercial harvests, these species are prime candidates for aquaculture and stock enhancement. Development of culture techniques for snapper species has been attempted over the past 30 years, but use of gonadotropic hormones with mature, wild-caught red snapper remains the most reliable method for inducing ovulation and producing eggs that can be fertilized. In this study, procedures for sperm collection, handling, and refrigerated storage were developed to improve strip-spawning techniques for these snapper species. Use of refrigerated sperm allows efforts to be focused on maintaining female broodstock, monitoring of ovarian development, and increasing efficiency during the strip-spawning process. Sperm were collected from male red snapper ($n = 199$) and gray snapper ($n = 83$) captured in the recreational fishery during the summers of 2000 and 2001. Sperm were diluted 1:4 with calcium-free Hanks' balanced salt solution (HBSS), placed in 4-L plastic bags, and transported to the laboratory on ice. Osmotic pressure (mean ± SE) of seminal plasma was 428 ± 15 milliosmoles (mOsm) per kilogram for red snapper ($n = 19$) and 411 ± 5 mOsm/kg for gray snapper ($n = 13$). Blood plasma osmolality was 440 ± 7 mOsm/kg for red snapper and 421 ± 7 mOsm/kg for gray snapper. Activation studies of red and gray snapper sperm indicated that sperm motility was suppressed by decreasing the osmotic pressure of artificial seawater to a level less than 400 mOsm/kg. Refrigerated storage experiments demonstrated that sperm samples suspended in 200-mOsm/kg HBSS retained motility for 10 d when refrigerated at 4°C. These results show that red snapper and gray snapper sperm can be stored for short-term repeated use in a hatchery.

In tropical and subtropical oceans, almost all species of snapper (Lutjanidae) are exploited because they are highly regarded as food and game fish (Pauly et al. 1996). Increasing demand for snapper species in domestic and foreign seafood markets, high market values, and declining wild stocks have stimulated interest in the culture of several snapper species. In the southwestern Atlantic Ocean and the Gulf of Mexico, red snapper *Lutjanus campechanus* and gray snapper *L. griseus* constitute a significant proportion of the sport and commercial fisheries for snappers. Commercial annual landings of red and gray snappers were estimated at 2,200 and 175 metric tons, respectively (NMFS 2005). Because of their high market value and limited commercial harvest, red and gray snappers have received considerable attention as candidates for marine aquaculture and stock enhancement programs.

Red and gray snappers have ranges extending from the Yucatan Peninsula, Mexico, to Massachusetts (Stark and Schroeder 1971); however, both species are primarily distributed in the subtropical waters from Cape Hatteras, North Carolina, to Florida and throughout the Gulf of Mexico (Bortone and Williams 1986; Moran 1988). Red snapper are moderate-sized fish that grow to 25 kg and 955 mm total length (TL), and individuals can live for more than 50 years (Wilson et al. 1994). In contrast, gray snapper are smaller, can grow to 8 kg and 890 mm TL, and have a life span of over 20 years (Manooch and Matheson 1984; Burton 2001). Adults of red and gray snappers are commonly found together in offshore waters at depths of 15–110 m around rocky outcrops, coral reefs, shipwrecks, and oil and gas platforms (Shipp 1999). While spawning behavior in natural aggregations has not been docu-

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mented for either species, examination of gonads has shown that these species spawn repeatedly offshore along the continental shelf throughout the summer and fall (Rutherford et al. 1983; Grimes 1987).

For the past 30 years, efforts have been made to develop hatchery techniques suitable for mass culture of red and gray snappers. The use of photoperiod and temperature manipulation or injections of gonadotropic hormones has yielded limited success in producing natural spawning in tanks or ponds (Arnold et al. 1978; Rosas et al. 1997). To date, injection of human chorionic gonadotropin (500–1,000 international units/kg of fish body weight) into mature red or gray snapper has been the most reliable method for inducing ovulation and producing eggs that can be stripped and fertilized with sperm (Papanikos et al. 2003).

As part of a study to improve artificial spawning techniques for red and gray snappers, procedures were developed for the collection, handling, and refrigerated storage of sperm. Sperm collection and storage are useful for genetic improvement via artificial spawning, cryopreservation of sperm from valuable strains or individuals, and production of hybrids (Leung 1991). The specific objectives of this study were to (1) develop methods for collecting red and gray snapper testes and sperm from fish caught in the recreational fishery; (2) determine the osmotic pressures of blood and seminal fluid from the two snapper species; (3) establish the relationship between sperm motility and osmotic pressure, which would permit development of safe storage methods; (4) evaluate the motility of refrigerated sperm extended in Hanks' balanced salt solution (HBSS) at various concentrations; and (5) evaluate the motility of refrigerated sperm to which an antibiotic–antimycotic cocktail (AAC) was added. The motility of sperm was used as a measure of sperm viability. This is the first published report on the collection and refrigerated storage of sperm from red and gray snappers.

Methods

Collection of fish, testes, and sperm.—During the 2000 and 2001 spawning seasons (May–August), live red and gray snapper males were captured for collection of blood and seminal fluid. Fish were collected off the coast of Louisiana using conventional hook-and-line techniques. After capture, males were placed in an onboard oxygenated live well (450 L) filled with seawater (salinity = 35‰) from the site of collection. Fish were transported from about 60 km offshore to the Louisiana Universities Marine Consortium, Cocodrie. Fish were held in a recirculating aquaculture system until used in experiments.

All sperm used in this study were obtained by the removal of testes from dead snapper collected by recreational anglers on charter boats off coastal Louisiana. Fish were caught by conventional hook-and-line techniques. After capture, fish were placed in insulated coolers with ice. Ice was replenished throughout the day to prevent spoilage. Fish were sampled within 4–8 h of capture before they were cleaned and filleted for customers.

Red and gray snapper males were measured (TL) and blotted to avoid contamination of samples. When possible, a digital thermometer (Baxter, Deerfield, Illinois; Model PS100A) was inserted into the abdomen to assess core body temperature. Testes were surgically removed and placed in 4-L Ziploc freezer bags (S.C. Johnson and Son, Inc., Racine, Wisconsin). To maintain the testes, calcium-free HBSS was added at a concentration of 200 milliosmoles (mOsm) per kilogram (Wayman et al. 1996). The samples were placed on ice and immediately transported to the Louisiana Universities Marine Consortium hatchery (<1 km); the testes were removed from the bags, blotted, weighed, and prepared for sperm collection. Except for experiments evaluating the use of crushed testes for sperm collection, all sperm were obtained by dissecting and slicing each testis into approximately 2-cm^2 cross sections. Each section was gently squeezed or stripped to release the sperm, which were collected in 50-mL plastic centrifuge tubes until use in experiments. Only fish with testes producing at least 1 mL of sperm were selected for use in experiments. The total time from the collection of testes in the field to preparation of sperm in the laboratory was less than 60 min.

Blood and seminal plasma osmolality.—In August 2001, blood samples were collected from 10 red snapper and 5 gray snapper, and sperm samples were collected from 19 red snapper and 13 gray snapper. The osmolality of plasma from blood samples was measured with a vapor pressure osmometer (Wescor, Inc., Logan, Utah; Model 5500). The salinity of the surface water from the collection site was 32–35‰, and osmolality of the surface water was 800–1,000 mOsm/kg. Blood (0.5–2.0 mL) was sampled by syringe from the caudal artery of each fish, placed in a 3.0-mL Vacutainer (Becton, Dickinson and Company, Franklin Lakes, New Jersey), and allowed to clot. Blood plasma (200 μL) was pipetted into labeled, 1.8-mL plastic centrifuge tubes and refrigerated at 4°C until analysis. After blood was collected, the fish were euthanized and testes were surgically removed for collection of sperm. Sperm were collected in 50-mL plastic centrifuge tubes as described above. Undiluted sperm were drawn into 75-μL microhematocrit tubes, and seminal plasma was obtained by centrifugation (7,000 revolutions/min for 10 min). After centrifuga-

TABLE 1.—Concentrations of ingredients (g/L) in calcium-free Hanks' balanced salt solution (HBSS) prepared at various osmolalities (milliosmoles [mOsm]/kg) using distilled water. Final osmolalities were adjusted by measurement with a vapor pressure osmometer (Wescor, Inc., Logan, Utah; Model 5500) and diluted as necessary.

	HBSS osmolality (mOsm/kg)		
Ingredient	200	300	400
NaCl	5.26	8.00	10.53
KCl	0.26	0.40	0.53
$MgSO_4 \cdot 7H_2O$	0.13	0.20	0.26
Na_2HPO_4	0.04	0.06	0.07
KH_2PO_4	0.04	0.06	0.07
$NaHCO_3$	0.23	0.35	0.46
$C_6H_{12}O_6$	0.66	1.00	1.32

tion, the osmolality of 10 μL of seminal plasma was measured.

Preparation of extender solutions.—Hanks' balanced salt solution is a physiological saline that can suppress sperm motility and can be used to dilute sperm for refrigerated storage (Wayman et al. 1996). In this study, HBSS was prepared without calcium at 200, 300, and 400 mOsm/kg (Table 1). Typically, extender solutions were prepared in large volumes (8 L), filtered through a 0.22-μm filter (Gelman Sciences, Ann Arbor, Michigan), and frozen in sterile, 1-L Nalgene bottles (Nalge Nunc International, Rochester, New York) until use in experiments.

Estimation of sperm motility.—The percent motility in each sperm sample was estimated by experienced personnel using dark-field microscopy at 200× magnification. Motility was determined as the percentage of sperm that were actively moving forward. Sperm were activated by placing 2 μL of sperm onto a microscope slide and diluting this quantity with 20 μL of activating solution. Except in studies evaluating effects on sperm activation, the activating solution used was artificial seawater (ASW; Wiegandt GmbH, Inc., Krefeld, Germany; Marinemix) prepared at 870 mOsm/kg. To maintain a high level of quality control, sperm motility was routinely estimated by a second reader for consistency and consensus.

Motility characterization.—Sperm of marine fishes are not typically motile before addition of an activating solution or natural seawater (35‰). Motility of samples from 5 red snapper and 6 gray snapper was characterized by activating the sperm with 870-mOsm/kg ASW. All sperm samples exhibited over 95% motility after activation. To characterize each sample, motility was separated into three periods: time (s) to reach maximum motility, duration of motility, and the time until all motility ceased.

Osmotic analysis of sperm activation.—To assess the relationship between osmotic pressure and sperm activation, undiluted sperm were collected from 13 red snapper and 10 gray snapper during June–August 2000. Thirteen activating solutions at osmotic pressures of 16–870 mOsm/kg were prepared by mixing distilled water with Marinemix artificial sea salt. Sperm were activated by placing 2 μL of sperm onto a microscope slide and diluting the sample with 20 μL of activating solution. After the motility of the sample was estimated, the osmolality of the activated sperm mixture was determined by removing 10 μL of diluted sample directly from the microscope slide for analysis with the vapor pressure osmometer. Threshold activation was defined as the osmotic pressure that elicited 10% motility (Wayman et al. 1996). The complete activation point was the lowest osmotic pressure that elicited the highest percentage of motile sperm.

Refrigerated storage experiment 1: red and gray snapper sperm.—During May–August 2000, red and gray snapper testes were used to obtain sperm for refrigerated storage experiments. After testes were weighed, sperm were collected by either slicing the testes to release sperm or by crushing the testes in a 4-L Ziploc freezer bag. Sperm obtained from sliced testes were stripped into 50-mL plastic centrifuge tubes and diluted 1:3 (volume per volume [v/v]) with HBSS prepared at 200, 300, or 400 mOsm/kg. For sperm collection by crushing, testes were placed in a Ziploc bag with 1 mL of HBSS (200, 300, or 400 mOsm/kg) added per gram of testes. On the laboratory benchtop, testes were manually crushed and homogenized within a plastic bag. Sperm solutions were strained through a 102-μm screen into a 50-mL plastic centrifuge tube. Sperm samples used in refrigerated storage experiments were limited to 25 mL of total volume. Sperm were stored in a refrigerator at 4°C in loosely capped 50-mL tubes. The osmotic effects of refrigerated storage were evaluated by estimating sperm motility each day until sperm no longer exhibited motility.

Refrigerated storage experiment 2: red snapper sperm mixed with antibiotics and an antimycotic.—During the summer of 2001, sperm were collected from the testes of 16 red snapper for experiments evaluating the motility of refrigerated sperm stored in AAC. The AAC selected for use was a commercially prepared solution that contained two antibiotics and one antimycotic (Sigma Chemical Co., St. Louis, Missouri; Product A-7292). The AAC was formulated to contain penicillin at 10,000 units/mL, streptomycin at 10 mg/mL, and amphotericin at 25 μg/mL in a 0.9% sodium chloride solution when reconstituted with sterile water. Concentrations selected for use were based upon the manufacturer's recommendation for cell culture and

previous refrigerated storage studies of fish sperm (Christensen and Tiersch 1996). In the laboratory, testes were sliced to release sperm cells, which were collected in 50-mL plastic centrifuge tubes and diluted 1:3 (v/v) with 200-mOsm/kg HBSS. Sperm suspensions from each fish were divided into three aliquots of 10 mL and placed into labeled 50-mL centrifuge tubes without AAC, with 0.1% AAC, or with 1.0% AAC. Sperm were stored in a refrigerator at 4°C in loosely capped 50-mL tubes. Sperm motility was checked daily until all sperm were nonmotile.

Statistical analysis.—Percentage motility values were normalized by arcsine–square-root transformation before statistical analysis. Blood and seminal plasma osmotic pressures were compared using a Student's *t*-test that assumed equal variances (Excel 2003; Microsoft Corp., Redmond, Washington). In sperm activation with ASW of different osmolalities, the threshold activation point was compared to the complete activation point using a paired Student's *t*-test (Excel 2000). In refrigerated storage experiment 1 (summer 2000), the effect of osmolality on refrigerated sperm motility over time was evaluated using a repeated-measures analysis of variance (ANOVA) in the Statistical Analysis System (SAS) version 8.0 (SAS Institute, Cary, North Carolina). In refrigerated storage experiment 2 (summer 2001), the effect of AAC on sperm motility over time was evaluated using a repeated-measures ANOVA (SAS). Duncan's multiple-range test was used to determine whether significant differences existed among treatment means. Differences were considered significant at *P*-values of 0.05 or less.

Results

Collection of Fish, Testes, and Sperm

Sampling of red or gray snapper collected in the recreational fishery was an effective method for obtaining viable sperm. During the 2-year study, testes were collected from 199 red snapper (mean TL ± SE = 57.8 ± 11.4 cm) and 83 gray snapper (57.7 ± 7.9 cm TL). For both species, testis size was highly variable among similar-sized fish throughout the sampling period (Figure 1). Red snapper testes ranged from 1 to 398 g, and the volume of sperm collected per testis was 15.2 ± 6.1 mL. Gray snapper testes ranged from 16 to 374 g, and the volume of sperm collected per testis was 8.7 ± 3.4 mL. Approximately 10% of the sampled testes contained no sperm.

Although there were slight visible differences in the vascularization and organization of testes between the two species, testes could be classified into four distinct developmental categories: (1) clear, less than 15 mm wide, one-third to half of the body cavity length, and sperm not visible; (2) pinkish to white, opaque, 15–30 mm wide, between half and three-fourths of the body cavity length, and fatty tissues present adjacent to testes; (3) white, 30–50 mm wide, three-fourths of the body cavity length, large fatty tissues adjacent to testes, and sperm present but not flowing; and (4) white, over 50 mm wide, three-fourths of the body cavity length, and sperm flowing easily. Because of the relatively high abundance of fish available for sampling, collections of testes and sperm for experiments and hatchery trials were limited to males that represented testis developmental categories 3 and 4. No significant differences were observed in sperm motility estimates among fish with these stages of development ($P = 0.35$).

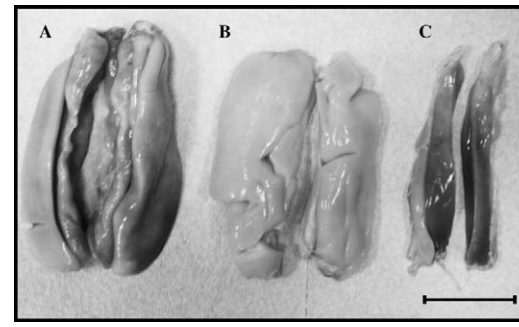

FIGURE 1.—Photograph of testes from three red snapper of similar size (46 cm total length) but exhibiting various stages of testis development (defined in text): **(A)** category 4, **(B)** category 3, and **(C)** category 2 (scale bar = 5 cm). Sperm were easily collected from A and B, but no sperm were obtained from C. Fish were collected off the coast of Louisiana in August 2001.

The mean (±SE) core body temperature of 40 red snapper sampled in July and August 2001 was 11 ± 5°C (range = 2–25°C). Air temperature during the collection period was 32 ± 4°C (range = 26–37°C). There was no correlation ($r = 0.27$) between sperm motility and core body temperature, because a relatively high level of sperm motility (82 ± 27%) was observed in all samples.

Blood and Seminal Plasma Osmolality

Mean (±SE) blood plasma osmolality was 440 ± 7 mOsm/kg for red snapper and 421 ± 7 mOsm/kg for gray snapper. Seminal plasma osmolality was 428 ± 15 mOsm/kg for red snapper and 411 ± 5 mOsm/kg for gray snapper. Osmolality was not significantly different between blood plasma and seminal plasma for red snapper ($P = 0.47$) or gray snapper ($P = 0.56$). Significant between-species differences ($P < 0.001$) were found for osmolality of blood plasma and seminal plasma.

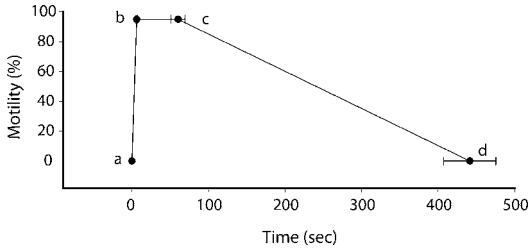

FIGURE 2.—Motility characterization (percent motile after sample activation) of red and gray snapper sperm activated with artificial seawater at an osmolality of 870 milliosmoles/kg: (1) time (s) to reach maximum motility (segment a–b); (2) duration of maximum motility (b–c); and (3) time to cessation of motility in all sperm cells (c–d). Each point represents the mean of samples from 5 red snapper and 6 gray snapper, as motility criteria did not differ between the two species.

Motility Characterization

Sperm began swimming vigorously when activated with 870-mOsm/kg ASW, and motility in each sample was characterized into three periods (Figure 2). After activation, sperm reached maximum motility immediately (i.e., $\leq$2 s) and sustained maximum motility for 60 $\pm$ 9 s (mean $\pm$ SE). Time required to reach maximum motility and duration of maximum motility did not differ between red and gray snappers ($P = 0.32$).

Osmotic Analysis of Sperm Activation

Initial motility of all sperm used in experiments was 95%. The mean ($\pm$SE) osmolality that initiated activation was 407 $\pm$ 48 mOsm/kg in red snapper and 437 $\pm$ 15 mOsm/kg in gray snapper. Threshold activation for red snapper sperm occurred at 439 $\pm$ 44 mOsm/kg, and complete activation was at 742 $\pm$ 58 mOsm/kg. Threshold activation for gray snapper sperm occurred at 486 $\pm$ 30 mOsm/kg, and complete activation was at 861 $\pm$ 39 mOsm/kg. Sperm activation was highly correlated between red and gray snappers ($r = 0.99$). For both species, sperm motility increased as the osmolality of ASW increased (Figure 3), and osmolality values at complete activation were significantly higher than threshold activation values ($P < 0.0001$).

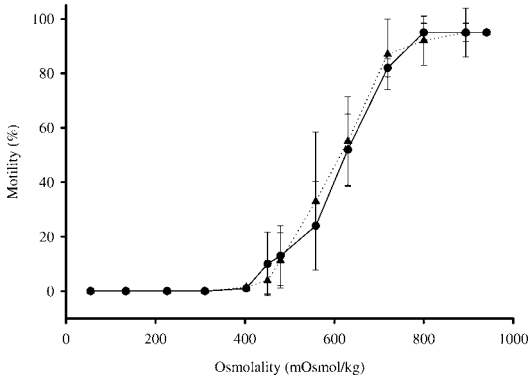

FIGURE 3.—Percent motility (mean $\pm$ SE) in samples of red snapper sperm (circles and solid line; samples from 13 fish) and gray snapper sperm (triangles and dotted line; 10 fish) activated with artificial seawater of various osmolalities (milliosmoles [mOsm]/kg). Sperm activation was highly correlated between the species ($r = 0.99$).

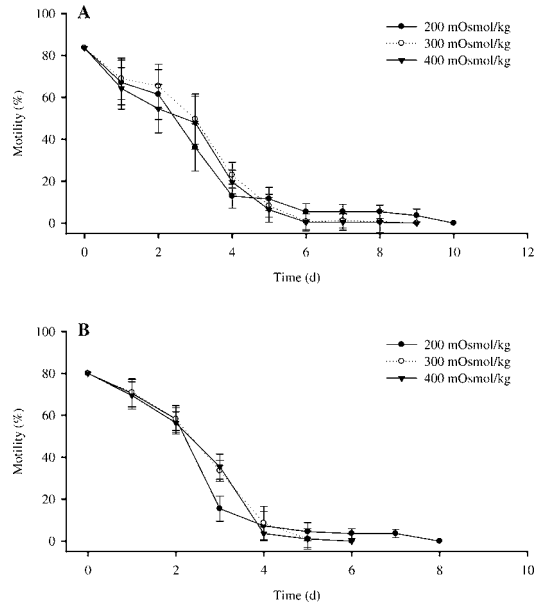

FIGURE 4.—Red snapper sperm motility (percent of sample; mean $\pm$ SE) in relation to refrigerated storage time (d) for samples collected by **(A)** stripping from dissected testes or **(B)** crushing the testes. Samples were suspended in Hanks' balanced salt solution (HBSS) at 200, 300, or 400 milliosmoles (mOsm) per kilogram. The storage time for which motility was retained (stripping: 9 d; crushing: 7 d) was significantly different between collection methods ($P < 0.0001$). Sample motility did not differ significantly among HBSS concentrations, but sperm stored in 200-mOsm/kg HBSS retained motility for the longest period.

Refrigerated Storage Experiment 1

Red snapper sperm from stripped testes ($n = 14$ fish) and crushed testes ($n = 11$ fish) were compared in refrigerated storage experiments (Figure 4). Storage times were significantly different between sperm collected from stripped testes (motility retained for 9 d) and those collected from crushed testes (motility retained for 7 d; $P < 0.0001$). There was no significant difference in motility of red snapper sperm from

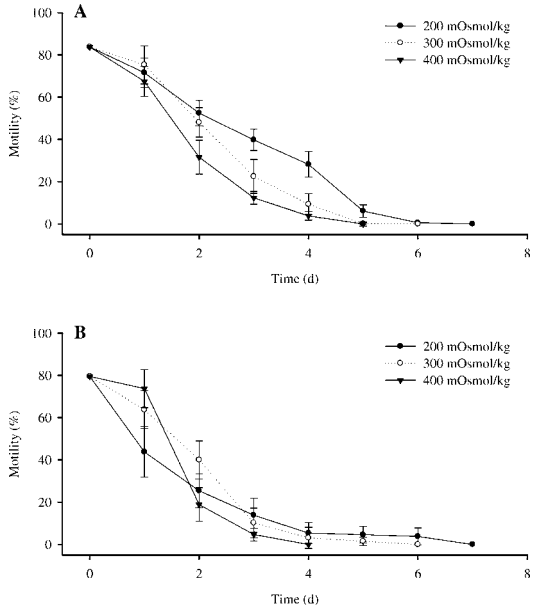

FIGURE 5.—Gray snapper sperm motility (percent of sample; mean ± SE) in relation to refrigerated storage time (d) for samples collected by **(A)** stripping from dissected testes or **(B)** crushing the testes. Samples were suspended in Hanks' balanced salt solution (HBSS) at 200, 300, or 400 milliosmoles (mOsm) per kilogram. Samples exhibited significant losses in motility each day ($P < 0.0001$), but the storage time for which motility was retained did not differ significantly between collection methods ($P = 0.21$). Sperm stored in 200-mOsm/kg HBSS retained motility for the longest period (6 d).

stripped or crushed testes on the day of collection ($P = 0.88$). However, all sperm exhibited significant declines in motility each day ($P < 0.0001$). The HBSS concentration did not significantly affect sperm motility, but sperm stored in 200-mOsm/kg HBSS retained motility for the longest duration.

Gray snapper sperm from stripped testes ($N = 16$ fish) and crushed testes ($N = 13$ fish) were also compared in experiment 1 (Figure 5). The refrigerated storage time was not significantly different between sperm from stripped testes and those from crushed testes ($P = 0.21$). In all samples, sperm exhibited significant losses in motility each day ($P < 0.0001$), and sperm retained motility for the longest duration when stored in 200-mOsm/kg HBSS.

Red snapper sperm retained motility for a significantly longer storage period than did gray snapper sperm ($P < 0.0001$), but sperm motility declined to less than 50% after 4 d in both species. Sperm from stripped testes and sperm extended in 200-mOsm/kg HBSS exhibited the longest storage periods.

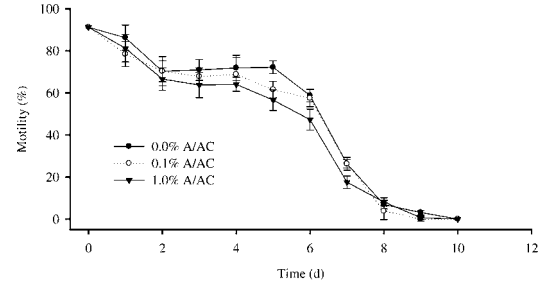

FIGURE 6.—Red snapper sperm motility (percent of sample; mean ± SE) in relation to refrigerated (4°C) storage time (d) for samples ($n = 16$ fish) diluted with Hanks' balanced salt solution (200 milliosmoles [mOsm]/kg) and stored in an antibiotic–antimycotic cocktail (AAC; at 0.0, 0.1, or 1.0%). Sperm motility and the storage duration for which motility was retained did not differ among AAC concentrations ($P = 0.93$).

Refrigerated Storage Experiment 2

The addition of AAC did not improve storage of red snapper sperm. Red snapper sperm diluted with 200-mOsm/kg HBSS retained motility for 9 d whether or not AAC was added (Figure 6). There was no significant difference in storage duration or sperm motility between samples with AAC and those without AAC ($P = 0.93$).

Discussion

The use of refrigerated sperm during the strip-spawning process can be advantageous in hatcheries because it allows efforts to be focused on maintaining female broodstock, monitoring ovarian development, and increasing hatchery production efficiency. In this study, techniques were developed for short-term storage that allows for repeated use of sperm from high-quality males to produce larvae. The results suggest that sperm of red and gray snappers can be stored for 6–9 d at 4°C.

We compared refrigerated storage of sperm from samples collected by two different methods. Although both methods were effective for sperm collection, samples from crushed testes yielded a mixture of cell types (i.e., sperm, testicular tissue, blood), whereas sperm samples collected from sliced, stripped testes were relatively pure. We obtained mixed results from refrigerated storage experiments. The results from red snapper experiments suggest that for optimal storage time, sperm should be collected from stripped testes, while results from gray snapper suggest that storage time is not affected by sperm collection method. Based on our experience, we recommend collection by slicing and stripping the testes when possible, because this method yields pure sperm and poses a lower risk of

TABLE 2.—Osmolality (milliosmoles [mOsm]/kg) of blood plasma from selected teleostean fishes in three aquatic environments of various salinity ranges and osmolalities. Data were summarized by Hoar and Randall (1969), Evans (1979), and Karnaky (1993).

Environment	Salinity range (‰)	Species	Osmolality (mOsm/kg)	Reference
Marine	32–35		800–1000	
		Great barracuda *Sphyraena barracuda*	476	Becker et al. 1958
		Yellowfin grouper *Mycteroperca venenosa*	467	Becker et al. 1958
		Black grouper *M. bonaci*	461	Becker et al. 1958
		Anglerfish *Lophius piscatorius*	452	Evans 1979
		Bluefin tuna *Thunnus thynnus*	437	Becker et al. 1958
		Goliath grouper *Epinephelus itajara*	384	Becker et al. 1958
Estuarine	1–32		100–800	
		Red drum *Sciaenops ocellatus*	375	Wayman et al. 1998
		Spotted seatrout *Cynoscion nebulosus*	356	Wayman et al. 1996
		Black drum *Pogonias cromis*	342	Wayman et al. 1997
		European flounder *Platichthys flesus*	297	Evans 1979
Freshwater	0–1		1–100	
		Lake trout *Salvelinus namaycush*	298	Hoffert and Fromm 1966
		Common carp *Cyprinus carpio*	274	Evans 1979
		Northern pike *Esox lucius*	274	Keys and Hill 1934
		Channel catfish *Ictalurus punctatus*	272	Norton and Davis 1976

contamination. The crushing method should be reserved for testes of lower developmental stages (1–2), from which sperm are difficult to obtain.

Development of collection, handling, and storage techniques requires adequate knowledge and understanding of the sperm characteristics of marine fish. The sperm are typically nonmotile in the testes or seminal fluid; during reproduction, motility is induced after sperm are released into the water (Grier 1981). Sperm motility is stimulated by the ionic composition, pH, or osmolality of water (Stoss 1983). Sperm of freshwater fish are activated by suspension in hypotonic solution or reduction in concentration of specific ions (Morisawa and Suzuki 1980; Christensen and Tiersch 1996), whereas sperm of marine fish are activated by suspension in hypertonic solutions with osmolalities greater than 400 mOsm/kg (Morisawa and Suzuki 1980; Gwo et al. 1991; Wayman et al. 1998). Because fish sperm are quiescent while in the seminal plasma within the testes, preparation and use of extender solutions with a chemical concentration and osmolality similar to those of seminal plasma are essential for optimizing storage time (Baynes et al. 1981). Physiology and osmoregulation have been well studied in marine fish, but few reports are available on the osmolality of blood and seminal plasma. Collection of such data is an integral part of developing extender solutions that prevent the initiation of sperm motility and ultimately prolong cell viability during storage.

Blood and seminal plasma chemistry were highly correlated in both species ($r = 0.73$). The values reported for red snapper (blood plasma: 440 mOsm/kg; seminal plasma: 421 mOsm/kg) and gray snapper (blood plasma: 428 mOsm/kg; seminal plasma: 411 mOsm/kg) were similar to blood plasma osmolalities reported for other marine species (Table 2). Increases in osmotic pressure above that of blood and seminal plasma were associated with sperm activation for both species. Complete activation of sperm diluted with ASW occurred at osmolalities above 700 mOsm/kg for both species. This value falls within the osmolality range of the natural waters in which these two species spawn (800–1,000 mOsm/kg).

Most research on the refrigerated storage of sperm has addressed the more commonly cultured freshwater species. In the present evaluation, HBSS was prepared at osmotic pressures below that of the blood and seminal plasma to ensure that sperm remained inactive when suspended in the extender for storage. Because sperm of red and gray snappers become motile at osmolalities above 400 mOsm/kg, extender solutions should be prepared at or below this level. However, it should be noted that hypo-osmotic shock can cause structural damage to sperm of marine organisms (e.g., Pacific oysters *Crassostrea gigas*; Dong et al. 2006) and could cause similar damage to sperm of marine fishes.

Techniques for observing sperm can be highly objective, and the identification of sperm motility can be confused with cell movement due to dilution on the microscope slide, Brownian motion, or contamination of samples with bacteria (Jenkins and Tiersch 1997). Although care was taken to minimize contamination of samples at the time of collection, swimming bacteria were observed in most samples. Bacteria were identified as any microorganism without tails that was observed moving actively in samples. No attempts were made to taxonomically classify or quantify the

bacteria in samples; however, their presence raised concerns that storage duration and sperm quality could be compromised by degradation of samples. In an effort to limit bacterial growth, the AAC was added to snapper sperm suspended in 200-mOsm/kg HBSS. Addition of AAC at the concentrations tested (0.1% and 1.0%) did not significantly improve storage duration for red snapper sperm. Future research should examine the effects of different concentrations of antibiotics and antimycotics on sperm motility and bacterial growth.

In general, motile sperm are necessary to achieve fertilization (Jamieson 1991); however, motility estimates are not always correlated with fertility and are not the best indicator of sperm quality, because different parts of the sperm cell are responsible for motility and fertility. While motility is a good estimator of sperm viability, the ultimate test for sperm quality is the ability to fertilize eggs (Bromage and Roberts 1995). Given the short time that snapper sperm were highly motile after activation (60 s), care should be taken to ensure prompt and thorough mixing of gametes during artificial spawning of red or gray snapper. The ratio of sperm to eggs, contact time between gametes, and fertilization method should be refined to optimize fertilization and hatching success.

The snapper caught by recreational fishers on charter boats proved to be a valuable and easily accessible source of sperm for artificial spawning. The quality of the fish collected at the sampled marinas was high because of the standardized icing practices used by experienced charter captains to ensure a quality product for their clients. Although samples were collected 4–8 h after the fish had died, samples remained viable when fish were covered in ice for the duration of the fishing trip. Historical sampling from the recreational and commercial snapper fisheries has been used to collect otoliths, scales, and vertebrae for age estimation studies and to collect data for fisheries modeling and management; however, this study is the first to report the collection and use of viable sperm from dead red or gray snapper. As a result of this study, these procedures have become standard in the cryopreservation of sperm, fertilization of eggs, and production of snappers and other species in our laboratory; the procedures are readily available for application in marine fish hatcheries and commercial, research, stock enhancement, and conservation activities (Riley 2002; Riley et al. 2004; Tiersch et al. 2004).

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