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Refrigerated storage and cryopreservation of sperm for the production of red snapper and snapper hybrids

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REFRIGERATED STORAGE AND CRYOPRESERVATION OF SPERM FOR THE PRODUCTION OF RED SNAPPER AND SNAPPER HYBRIDS

A Thesis

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in

The School of Renewable Natural Resources

by
Kenneth Lee Pickrell Riley
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To my mom and dad who instilled my love for fishing

"SO IF TO RAISE SOME

HONEST KIDS IS

YOUR FONDEST WISH,

JUST LEAD 'EM TO

THE WATER AND

TEACH 'EM HOW

TO FISH"

Ed Stone, 1973

Acknowledgements

I wish to express my sincere gratitude to Dr. Terrence Tiersch for serving as my major professor and providing guidance throughout my graduate program. I am grateful for the many opportunities Dr. Tiersch allowed me to conduct research and teach. I thank Dr. Edward Chesney for serving on my graduate committee and teaching me the finer details of spawning and rearing marine fish. I also thank Dr. John Chandler for serving on my graduate committee and challenging me to use the commercial-scale cryopreservation standards in the dairy industry to improve current practices for cryopreservation in aquatic species.

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Table of Contents

List of Tables

List of Figures

Abstract

The red snapper *Lutjanus campechanus* is an economically valuable sport and commercial fishery, and because of its high market value and limited commercial harvest, red snapper have received considerable attention as a candidate for marine aquaculture and stock enhancement programs. The goal of this thesis was to improve hatchery techniques for artificial spawning of red snapper *Lutjanus campechanus*. The objectives were to: 1) refine protocols for collecting, handling, transport, and holding of mature red snapper broodstock for induced spawning; 2) establish methods for strip-spawning of red snapper to optimize egg quality; 3) develop procedures for the collection, storage, and use of refrigerated and cryopreserved sperm of red snapper and gray snapper, and 4) evaluate the overall effects of gamete and larval quality on rearing success. The techniques developed were practical methods that hatchery managers can use to collect high-quality broodstock, to preserve sperm, and to collect and incubate eggs.

Red snapper broodstock (N = 101; 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 sampling. The reproductive condition of females was evaluated through samples of oocytes collected by intraovarian biopsy. Females with oocytes ranging in size from 350 to 550 μm were considered good candidates for hormone induced spawning. Female snapper were induced to ovulate with injections of human chorionic gonadotropin. Females were monitored for oocyte maturation and were stripped after ovulation. In a series of 2 x 2 trials, refrigerated and cryopreserved sperm with motilities above 80% were compared to evaluate fertilization and hatching success. Eggs were incubated in plastic bags (200 eggs per L) and fertilization was assessed as embryos developed through 8-cell, neurulation, and hatch. Fertilization and hatch rates ranged from 7 to 99% and were highly correlated ($r^2 = 0.92$). Refrigerated sperm yielded fertilization rates of 52 ± 23% (mean ± SD) and cryopreserved sperm yielded fertilization rates of 44 ± 22%. Use of refrigerated and cryopreserved sperm improved efficiency within the hatchery and each were effective for the fertilization of eggs and production of larvae.

Chapter 1

Foreword

Aquaculture, the farming of aquatic organisms, is one of the fastest growing segments of agriculture. Since the earliest records of carp culture in China 4500 years ago, aquaculture has developed as a result of mankind's inability to control seasonal and annual fluctuations in the productive capacity of natural fisheries. During the last century declines in wild fish stocks from perturbations such as overfishing, pollution, and habitat loss have created economic incentives for the development of competitive aquaculture industries. These industries encompass a broad diversity of stakeholders, organisms, geographic locations, and culture technologies.

Despite fluctuations in global markets, fisheries and aquaculture remain important as a source of food, employment, and revenue in many countries. At the end of the twentieth century, global fisheries production rose to a record 125.2 million metric tons (mmt) with 92.3 mmt from wild capture fisheries and 32.9 mmt from aquaculture (FAO 2001). Food fish production from aquaculture has grown by more than 10% during the past decade; and in worldwide markets, nearly one third of all fish for food is produced by aquaculture. Employment in the primary capture fisheries and aquaculture was estimated at 36 million people, with approximately 9 million people working in aquaculture (FAO 2001). While commercial fishing remains the main source of food fish for world markets, global fisheries resources will not supply the needs of a growing world population. This trend is already being realized in many areas of the world where the demand for seafood consistently exceeds the total catch from local fisheries resources (e.g. Caribbean region) (Tucker and Jory 1991).

Many species of fish have been successfully cultured; however, species of carp, tilapia, catfish, and salmon are among the most widely cultured in the world. These species have been

successful because they are relatively easy to produce in hatcheries, require a low level of culture technology, and are readily accepted in the marketplace. In the past, the culture of marine fish has mostly represented juveniles reared from eggs and larvae collected from unselected, natural stocks (e.g. eels, milkfish, grouper). Within recent years, well-established hatchery techniques have been developed for species such as milkfish *Chanos chanos* and barramundi *Lates calcarifer* in southeast Asia, red sea bream *Pagrus major* in Japan, European sea bass *Dicentrarchus labrax* and gilthead sea bream *Sparus aurata* in the Mediterranean, and red drum *Sciaenops ocellatus* in the United States (Tucker 1998). Worldwide efforts are underway to develop the culture techniques for a broader array of marine species (De Silva 1998).

The ideal culture species would be one that is valuable, with established markets, easy to spawn in captivity, easy to rear and feed, amenable to high stocking densities, resistant to diseases, and fast growing. Although few culture species meet all of these criteria, marine fish are especially difficult to culture because they do not readily spawn in captivity and often have strict dietary or environmental requirements. Other factors that have limited the development of marine aquaculture include health management, economics, and social constraints (Avault 1996).

Despite these limitations marine aquaculture has seen recent breakthroughs in culture technology, feed formulation, and disease diagnosis and treatment, which are in turn, stimulating research with new species. To date, the production of a reliable supply of larvae and juveniles for grow-out remains the limiting factor in marine aquaculture (De Silva 1998). The studies in this thesis address reproduction and gamete quality in snappers (Lutjanidae) with the goal of improving seedstock availability for these and other marine species.

Specifically, this study focuses on improving hatchery techniques for artificial spawning and larval rearing of red snapper *Lutjanus campechanus*. Although efforts have been underway to

develop culture methods for red snapper for the past 20 years (Arnold et al. 1978; Minton et al. 1983), the successful production of larvae, fry, and fingerlings has been very limited. The objectives of the research reported within this thesis were to: 1) develop methods for strip-spawning of red snapper to optimize egg quality; 2) develop methods for the collection, storage, and use of refrigerated and cryopreserved sperm of red snapper and gray snapper, and 3) evaluate the overall effects of gamete and larval quality on rearing success.

The results of this project represented a collaborative effort between the Louisiana State University Aquaculture Research Station (ARS) in Baton Rouge and the Louisiana Universities Marine Consortium (LUMCON) in Cocodrie. Because red snapper and gray snapper are offshore marine species, the hatchery facilities at LUMCON were utilized for holding of broodstock, spawning, and larval rearing trials, while evaluation of sperm quality and methods for refrigerated storage and cryopreservation were developed at ARS. Work of this kind presents a number of challenges including collection of broodstock ~50 km off coastal Louisiana; threats posed by tropical storms and hurricanes, and transport of live samples between the two research stations separated by 200 km. In the end, a total of twenty snapper females were successfully spawned in the hatchery and more than 200 sperm samples were obtained for use in experiments. The results of this project have yielded 13 published abstracts and conference proceedings (Table 1.1). All chapters of this thesis have been prepared in the format of the *Journal of the World Aquaculture Society*, and it is anticipated that chapters three, four, five, and six will be submitted for publication in peer-review journals.

Table 1.1. Conference presentations and abstracts of research presented in this thesis.

Date	Title	Conference	Location
2002	Improved artificial spawning techniques for red snapper *Lutjanus campechanus*	Louisiana Academy of Science	Baton Rouge, Louisiana
2002	Improved hatchery techniques for the culture of red snapper	Louisiana Chapter of the American Fisheries Society	Biloxi, Mississippi
2002	Short-term and long-term storage of red snapper and gray snapper sperm	Louisiana Chapter of the American Fisheries Society	Biloxi, Mississippi
2002	Improved methods for the culture of red snapper	Aquaculture America	San Diego, California
2002	Short-term and long-term storage of red snapper and gray snapper sperm	Aquaculture America[1]	San Diego, California
2001	Defining the role of gamete quality in the production of viable fish larvae: Results with red snapper (Lutjanidae)	International Council for Exploration of the Seas	Oslo, Norway
2001	Cryopreservation of sperm for the production of red snapper	Louisiana Chapter of the American Fisheries Society	Baton Rouge, Louisiana
2001	Refrigerated storage and cryopreservation of gray snapper sperm	Louisiana Chapter of the American Fisheries Society	Baton Rouge, Louisiana
2001	Cryopreservation of sperm for the production of red snapper and snapper hybrids	World Aquaculture Society[2]	Orlando, Florida
2001	Refrigerated storage and cryopreservation of gray snapper sperm	World Aquaculture Society	Orlando, Florida
2000	Defining the role of gamete quality in the production of viable fish larvae: Initial results with red snapper (Lutjanidae)	American Fisheries Society Larval Fish Conference	Mobile, Alabama
2000	Use of cryopreserved sperm for the production of red snapper and snapper hybrids	Louisiana Aquatic and Marine Science Syposium	Cocodrie, Louisiana
1999	New perspectives on the culture of red snapper	Gulf Coast Marine Science Symposium	Ocean Springs, Mississippi

[1] Award received for Best Abstract from United States Chapter of the World Aquaculture Society.
[2] Awards received for Best Abstract and Best Student Presentation from the World Aquaculture Society.

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Chapter 2

Introduction

"*Study nature, not books"*

-Jean Louis Rodolphe Agassiz, 1807-1873

The words from Louis Agassiz, father of ichthyology in the Americas, could not be truer. An adventure outside of the classroom can provide a student the opportunity to experience and study nature's brilliance in design and function. While the technology we have today allows us to experience more intimacy with nature, the lessons learned can be used to improve the lives of those around us. Throughout this project, I have had to examine some of nature's intricate details as I tried to answer questions that developed as a result of laboratory experiments and manipulations. In an effort to aid the reader and reach a variety of audiences, this chapter is intended to provide an overview of snappers, fishes with worldwide importance. Specifically, this chapter examines the biology of red snapper *Lutjanus campechanus* with special reference to the history and management of the fishery in the Gulf of Mexico. The fundamental aspects of reproduction in snappers are discussed as well as global efforts to culture several snapper species.

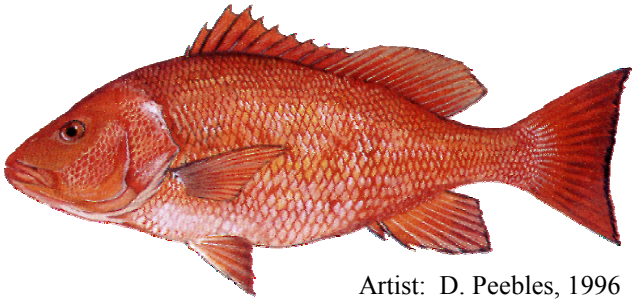
Snappers of the World

Snappers of the family Lutjanidae are represented by 17 genera and approximately 100 species found in the tropical and subtropical oceans around the world (Allen 1987). The genus *Lutjanus* is by far the largest genus with 64 species (Allen and Talbot 1985). While most snapper species are confined to coral reefs and rocky outcrops along continental shelves, several species enter estuaries and even fresh water. Smaller species of snapper are commonly found aggregated along the bottom, while larger individuals are more solitary predators (Anderson 1987). Because snapper are

highly regarded as a food and game fish, the snapper family represents exploited fisheries worldwide (Pauly et al. 1996).

In the warm waters of the Gulf of Mexico, 18 species of snapper are found. Some of the species highly sought after include the red snapper *Lutjanus campechanus*, gray snapper *Lutjanus griseus*, lane snapper *Lutjanus synagris*, mutton snapper *Lutjanus analis*, schoolmaster *Lutjanus apodus*, cubera snapper *Lutjanus cyanopterus*, dog snapper *Lutjanus jocu*, vermillion snapper *Rhomboplites aurorubens*, and yellowtail snapper *Ocyurus chrysurus*. The red snapper is by far the most important sport and commercial snapper fishery throughout the Gulf of Mexico. In 1996, after years of concern for the status of the red snapper stock in the Gulf of Mexico and the possible effects of overexploitation, the Gulf of Mexico Fishery Management Council and the United States Secretary of Commerce determined that the red snapper stock was grossly overfished and needed strict management measures to rebuild and restore the stock size to a sustainable level.

Nomenclature and Taxonomy



Artist: D. Peebles, 1996

Scientific name:	*Lutjanus campechanus*
Class:	Osteichthyes
Order:	Perciformes
Family:	Lutjanidae
Common name:	Red snapper
Other names:	American red snapper, Gulf red snapper, sow snapper, mule snapper, chicken snapper, cherry snapper, rat snapper
Similar species:	Caribbean red snapper, *Lutjanus purpureus*

Range and Distribution

The distribution of red snapper ranges from North Carolina to Florida and throughout the Gulf of Mexico. Red snapper are commonly found in offshore waters at depths of 15-110 m around rocky outcrops and coral reefs (Moran 1988). Habitat utilized by the Gulf stock of red snapper also includes the numerous shipwrecks, oil and gas platforms, and artificial reefs along the continental shelf. Since 1938, the installation of more than 4,500 oil and gas platforms in the Gulf of Mexico has contributed to the production of 5,000 km^2 of artificial reef habitat (Gallaway and Cole 1998). While the Gulf waters abound with ideal natural and artificial habitat for red snapper, it has not been determined if this habitat actually leads to increased populations of snapper or simply redistributes populations throughout the Gulf of Mexico. One hundred years of landings data indicate that the fishery, and possibly the population, has undergone a major shift from natural outcroppings of the West Florida Shelf to oil and gas platforms of the north-central portion of the Gulf of Mexico (Shirripa and Legault 1997).

Biology

Red snapper are a subtropical species capable of tolerating temperatures from 13 to 32 C, salinities from 24 ppt to 35 ppt, and dissolved oxygen as low as 5 mg/L (Moran 1988). Snapper are opportunistic bottom feeders that forage upon a variety of small fish, squid, shrimp, and crabs. During the summer months, spawning aggregations of red snapper move along the shallower waters of the continental shelf in search of food and habitat (Shirripa and Legault 1999). During the winter months, snapper remain relatively sedentary except when searching for food. Adult red snapper may grow to a weight of 25 kg and a total length (TL) of 845 mm at age 13 (Moran 1988). Recent studies have shown that red snapper are actually long-lived with individuals living for more than 50 years (Wilson et al. 1994).

Juveniles typically grow to 177 mm TL in their first year and individuals reach partial sexual maturity at two years and 298 mm TL (Nelson and Manooch 1982). Red snapper are gonochoristic, and following sexual differentiation sex remains fixed throughout life. Stocks within the Gulf of Mexico spawn repeatedly throughout the spring and early fall (May through September). In order to ensure larval survival and dispersal, red snapper are highly fecund broadcast spawners. Estimating the fecundity of red snapper is difficult because as batch spawners they have asynchronous development of oocytes within the ovaries. Batch fecundities can range from 450 oocytes for a fish measuring 349 mm TL to 1.7 million oocytes for a fish measuring 820 mm TL (Collins et al. 1996). Estimates of the annual fecundity for these fish range from 12,000 to 60 million and estimates of annual spawning frequency range from 21 to 35 (Collins et al. 1996).

Spawning usually begins in May and follows the lunar cycle with increases in spawning frequency during the first and last quarters of the moon (Chesney and San Filippo 1994). Research on spawning of red snapper indicates that they spawn in the evening because fish have been collected in the afternoon and early evening with oocytes hydrating and undergoing final oocyte maturation (Collins et al. 1996). Similar spawning strategies (e.g. fecundity, summer season, timing, lunar periodicity) have been documented in at least 38 species of snapper (Grimes 1987).

Subtropical and tropical marine fish such as snapper have distinctly different spawning strategies than those of temperate waters. Intense larval predation appears to exert heavy selection pressure on subtropical and tropical marine fish (Johannes 1978). Additionally, production cycles within these latitudes tends to be dependent upon localized oceanographic and meteorological conditions. Spawning events are highly correlated with peaks in production cycles that provide food for larvae (Cushing 1975) and seasonal environmental conditions affecting water currents, thermoclines, and vertical mixing within the water column (Nzioka 1979). Spawning events correlated with lunar

cycles take advantage of maximum tidal flows to flush embryos and larvae offshore to a more predator-free environment and ultimately return them to recruitment sites along inner shelf areas (McFarland 1982). While adult red snapper are capable of traveling great distances within a year (344 km), it appears that the primary mode of dispersal of individuals may rely on the hydrodynamic transport of eggs and larvae (Patterson 1999).

Detailed observations of snapper spawning are rare. Lane snapper have been observed spawning off southeast Florida (Wiklund 1969). Courtship began in the early evening when fish began to aggregate near the bottom in groups of 5 to 10 fish. The group became active with males chasing females while different males pressed on the swollen bellies of females. The group became condensed as fish swam off the bottom (2 m). The fish swam in all directions depositing sperm and eggs in the water column. A similar account was observed in Japan with the common bluestripe snapper *Lutjanus kasmira* that spawned in a large public aquaria (Suzuki and Hioki 1979). Males congregated near the bottom in the early evening for about 2 h. Pairs of fish then swam in upward spirals with males applying pressure with their snouts on the bellies of females. Groups of fish repeated the behavior for 20 to 30 times until spawning occurred simultaneously among 10 or more fish near the surface. Red snapper have been observed spawning in tanks after photoperiod and temperature manipulation (Arnold et al. 1978). The red coloration of these snapper became noticeably deeper and more vivid as fish entered spawning condition. After 1.5 years in captivity, seven spawns were collected with each consisting of a few thousand eggs. Attempts to repeat this study have not been successful until recently (Maus et al. 2002); however; to date there has been no published observations on red snapper spawning behavior.

Red snapper eggs are typical of marine fish with pelagic eggs. The eggs are small, buoyant, and average 0.82 mm in diameter (Figure 2.1A) (Rabalais et al. 1980). Egg quality is determined by the

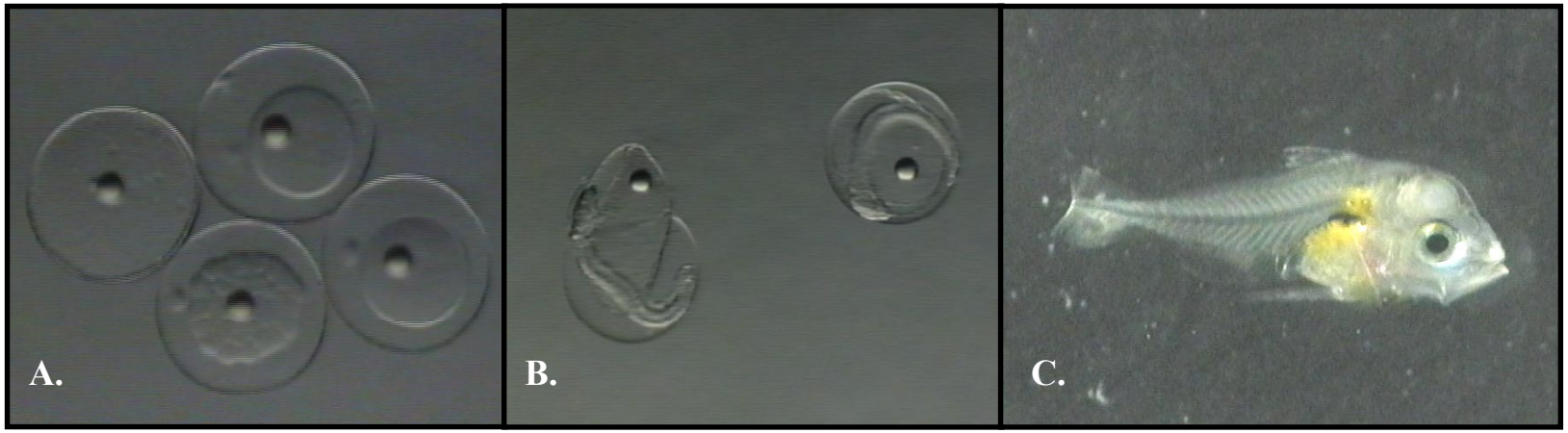


Figure 2.1. A) Development of red snapper embryos 6 h after fertilization. The diameter of the embryos is approximately 0.82 mm. B) Red snapper larvae hatching and emerging from egg. Note the large yolk sac and oil globule. C) Red snapper larva reared in captivity and developed to flexion 10 d after hatching. The total length of the larvae is 4.56 mm.

condition of broodfish and the environment in which they spawn. Red snapper eggs typically have a single oil globule, although eggs from some broodfish have reportedly had several small oil globules (Bourque 2001). The size and number of oil globules within eggs can serve as an indicator of egg quality and correlates with the amount of energy available for developing larvae (Barbaro et al. 1991). The yolk that is deposited during vitellogenesis must provide nutrition for the developing embryo and larvae. Newly hatched red snapper larvae range from 1.8 to 2.2 mm TL and are not well-developed (Figure 2.1B). Depending on developmental rates larvae utilize yolk reserves for 2 to 3 days after hatching.

In red snapper larvae, utilization of yolk reserves coincides with pigmentation of the eyes, mouth formation, and first feeding. Marine fish larvae are visual predators (Hunter and Lasker 1981). Red snapper larvae feed on microplanktonic organisms including ciliates, copepods, and rotifers. Copepods are a major component of marine ecoysystems and adults and their nauplii (early developmental stage) are important prey items for larval red snapper (Piaskoski and Phelps 2000). With proper nutrition, larval growth proceeds through the pre-flexion, flexion, and post-flexion stages of development before they undergo metamorphosis into juveniles 25 to 30 days after hatch (Figure 2.1C). Metamorphosis culminates when fish possess fully developed fin spines and rays and settle onto benthic habitats with structure (Bootes 1998). While larval development in red snapper was recently described (Drass et al. 2000), the early life history is relatively unknown for most snapper species. Descriptions of development from larvae to juvenile have been based upon observations of rearing larvae under captive conditions.

Morphometrics

A variety of techniques have been used in age and growth analysis of red snapper. These methods include estimates of age by microscopic analysis of otoliths, scales, and vertebrae. Early

estimates using all three methods yielded statistically similar population parameters and degrees of reliability for red snapper in the northern Gulf of Mexico (Bortone and Hollingsworth 1980); however, over the past 20 years a number of life history studies have yielded increased estimates of longevity (Table 2.1). Counting annulus formations within sagittal otoliths is the current standard for ageing red snapper (Shirripa and Burns 1997; Szedlmayer and Shipp 1994; Wilson et al. 1994). Recent investigations have found that examining radioisotopes (e.g. radium-226) within otoliths is a rapid and effective procedure for ageing of long-lived fish (Baker 1999).

Collection of data on the length and weight of red snapper have yielded two significantly different growth models for the southwestern Atlantic and Gulf of Mexico stocks (Table 2.1). The Gulf stock of red snapper demonstrates faster growth in length and weight and increased longevity as compared to the southwestern Atlantic stock. Studies have also examined the differences in growth characteristics among populations of red snapper within the Gulf of Mexico. These results have indicated there are slight regional differences in the growth characteristics; however these calculated differences are small and appear to have little biological significance (Nelson and Manooch 1982; Wilson et al. 1998). Genetic analyses of variation within mitochondrial DNA from different populations of red snapper across the Gulf also support the hypothesis that they comprise a single stock (Camper et al. 1993).

History of the Red Snapper Fishery

The red snapper fishery developed during the 1840's around Pensacola, Florida (Collins 1885). Fishermen from New England moved to the Gulf coast in search of this highly prized fish. The first fish house for handling and shipping red snapper was built by S. C. Cobb and A. F. Warren under the name of Pensacola Fish Company. After several years and changes in ownership two separate companies finally emerged and became the dominant red snapper distribution centers. The Warren

Table 2.1. Von Bertalanffy growth parameters of red snapper from the northern Gulf of Mexico and Southwestern Atlantic. (O, otoliths; S, scales; F, female; M, male, L_∞, maximum length; k, growth coefficient; t_o, initial time) (*Adapted from Baker 1999*).

Area	Method	Maximum Age	L_∞	k	t_o	Source
Gulf of Mexico	S	4+	***	***	***	Moseley 1966
Gulf of Mexico	S	9	***	***	***	Wade 1981
Gulf of Mexico	S, O	13	941 TL	0.17	-0.10	Nelson and Manooch 1982
Southwest Atlantic	O	16	975 TL	0.16	0.00	Nelson and Manooch 1982
Gulf of Mexico	O	10	925 TL	0.14	0.00	Nelson et al. 1985
Gulf of Mexico	O	42	1025 TL	0.15	***	Szedlmayer and Shipp 1994
Gulf of Mexico	O	53	772 $FL^{(F)}$ 859 $FL^{(M)}$	0.18 0.09	0.00 2.21	Wilson et al. 1994
Southwest Atlantic	O	25	955 TL	0.15	0.18	Manooch et al. 1998
Gulf of Mexico	O	49	913 $FL^{(F)}$ 842 $FL^{(M)}$	0.16 0.17	0.72 0.58	Wilson et al. 1998

Fish Company and E. E. Saunders Company distributed fresh red snapper across the southeastern United States. In 1880, 1.5 million pounds of red snapper were sold at dockside in Pensacola (Collins 1887). The snapper fishery around Pensacola was becoming rapidly exploited and overfished. By 1883, observations from fishermen and scientists indicated that most of the old fishing grounds were barren, and vessels had to travel longer distances in search of fish (Stearns 1883). With increased demand, fishermen were building bigger boats and moving along the Gulf coast looking for new red snapper fishing grounds. The fishery expanded along the Florida coast to Panama City, Apalachicola, Carrabelle, and Tampa. Fishermen kept the red snapper in live wells on their vessels until ice became available along the Gulf coast in 1895 (Camber 1955). With ice available, the fishery rapidly expanded with many new markets for fresh snapper.

Two types of boats were utilized in the early snapper fishery (Camber 1955). "Smacks" were large sailing schooners (60 – 100 ft) that carried 8 to11 men offshore for up to 32 days. Smacks ranged in size from 50 to 60 tons and were capable of carrying 20 tons of ice. Smaller sailing vessels called "chings" carried 4 to 5 fishermen to near shore snapper fishing grounds. Fishermen used handlines until the 1950's when power and hand-driven wheels were introduced. During the 1960's new technologies in boat designs, diesel engines, depth recorders, and navigation aids (e.g. LORAN) revolutionized the snapper fishing industry (Richard 1996).

During the 1960's and 1970's fishing efforts for red snapper increased with demand. Red snapper became the most valuable of the snappers and were highly sought after as one of the finest marine fish. The white, delicate flesh was ranked among the very best in fresh markets and restaurants (Russell 1967). During this period snapper were caught using a variety of gears including the new hydraulic snapper reel. Other gears included use of longlines and fish traps. The use of these gears increased fishing effort with a growing fleet. During the 1970's, scientists and

fishery managers became concerned about the health of the snapper stock with the increased fishing pressure. Each year fewer red snapper were being landed and sold at dock while fishing pressure and demand continued to increase with market demand and prices (Figure 2.2).

Red Snapper Fishery Management

Snapper fisheries worldwide have experience similar histories of exploitation. The red snapper provides a representative picture. During the 1980's scientists and fishery managers recognized the Gulf stock of red snapper needed management measures to restore its size to a sustainable level. A 12-inch minimum size limit was implemented for commercial and recreational fishers. The Gulf of Mexico Fishery Management Council officially classified the Gulf stock of red snapper as overfished in 1990. The Council established quotas for commercial fishers and prohibited the nearshore longline snapper fishery. Additionally, the recreational minimum size limit was raised to 13 inches and a bag limit was established consisting of seven red snapper per day per person. In 1996, the Gulf of Mexico Fishery Management Council and the Secretary of Commerce determined that the red snapper stock in the Gulf of Mexico was highly exploited and needed strict management measures to rebuild and restore the stock size to a sustainable level. The Gulf of Mexico Fishery Management Council drafted a Fishery Management Plan (FMP) that targeted rebuilding the stock by 2019.

The accepted FMP set the total allowable catch for red snapper in the Gulf of Mexico at 9.12 million lbs. This total allowable catch was divided almost equally between commercial and recreational fishers. Once either group of fishers had reached their annual limit the fishery was closed. At present, commercial fishers are allotted 4.65 million lbs with a minimum size limit for individual snapper set at 15 inches TL. The commercial season is open the first 10 days of each month until the quota is filled. Commercial fishers must also purchase a Federal Permit for Reef

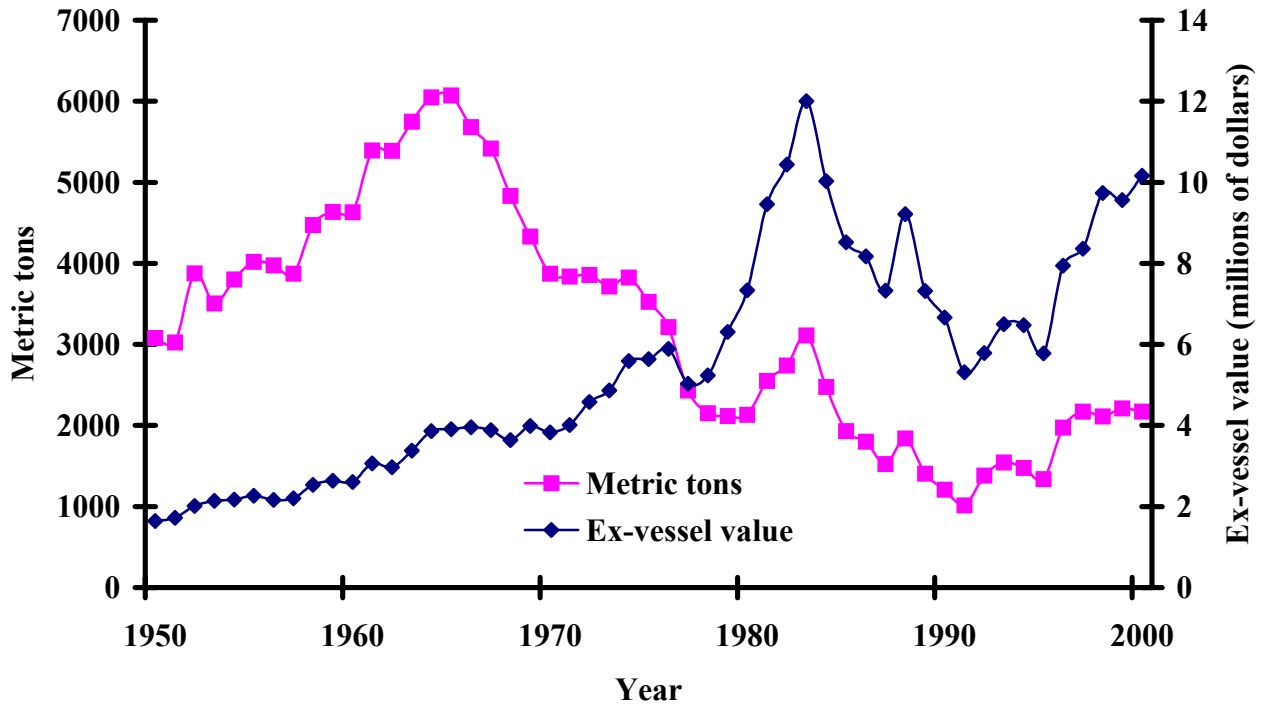


Figure 2.2. Since 1965, the reported commercial landings for red snapper caught in the Gulf of Mexico have significantly declined (NMFS 2002). The dramatic increase during 1950 to 1960 can be attributed to an increase in the number of commercial fishing vessels, implementation of new fishing technologies, and increased fishing effort. Over the years, consumer demand and declining availability has led to higher prices paid dockside and market.

Fish and either a Class I or II Red Snapper License. A Class I Red Snapper License permits the fisher to collect 2000 lb per trip. A Class II Red Snapper License permits the fisher to collect 200 lb per trip. Additional permits are required for the use of traps or longline fishing gears. Recreational fishers are allotted 4.47 million lbs with a minimum size limit for individual snapper set at 16 inches TL. The daily bag limit is four snapper per person and the season extends from April 21 to October 31. Reef fish taken under a recreational bag limit may not be sold. Commercial and recreational fishers must avoid closed areas or those classified as stressed.

Outlook for Red Snapper Fishery

The first step to restoring a depleted fishery includes recognizing that it is overfished and exploited. Over the past 30 years, researchers have watched the red snapper fishery in the Gulf of Mexico slowly decline as a result of overfishing and high juvenile mortality caused by bycatch from the commercial shrimp fishery (Gallaway and Cole 1999). The development of the Gulf of Mexico Red Snapper FMP will work towards rebuilding the stock by 2019. Recent estimates of the stock by year and age class show that the portion of the stock that is age 14 or older is increasing (Gallaway and Cole 1999). Because of recent management strategies, older snapper are contributing to a higher spawning potential. Fishery management plans need to be adaptive and during the next 20 years the red snapper FMP needs to adapt to highly variable year classes and fluctuations in environmental conditions. It is evident that more research is needed on the current status of the red snapper stock and the effects of management measures.

History of Snapper Aquaculture

Interest in the culture of snappers has developed throughout the world because of declines of wild snapper stocks combined with a consistent high demand and market value. With regards to snapper in the United States, domestic landings (4,832 metric tons) are inadequate to meet demand

and imports of snappers into the U. S. have risen dramatically from 1.1 metric tons (valued at $4,289) in 1989 to 11,374 metric tons (valued at $38.7 million) in 1998 (Watanabe et al. 2001; NMFS 2002). Along the coasts of the Gulf of Mexico and the Southwestern Atlantic, increasingly restrictive fishing regulations and the potential utility of stock enhancement to restore natural populations have stimulated interest in the culture of red snapper.

Efforts have been underway to develop and refine the methods to culture red snapper for the past 20 years. Early attempts to spawn red snapper used temperature and photoperiod manipulation to produce a small number of fertilized eggs from tank spawns (Arnold et al. 1978; Rabalais et al. 1980). Larvae produced from these spawns died within a five days of hatching. Although recent efforts have had limited success with hormone-induced tank spawns of red snapper (Laidley and Ostrowski 2001), the use of gonadotropic hormones with mature red snapper has been the most reliable method for induction of ovulation and production of eggs that can be stripped and fertilized (Minton et al. 1983; Bourque 2001; Watanabe 2001). Interests and efforts to culture red snapper along the Gulf coast have seen a resurgence within the past 5 years. For example, during this period a review of 23 published abstracts from conference proceedings of the World Aquaculture Society describes research programs addressing the culture of red snapper in Florida, Alabama, Mississippi, Texas, and Hawaii. The goals of these programs were varied and included: 1) domestication of broodstock; 2) artificial spawning of broodfish collected from the wild; 3) development of techniques for tank spawning; 4) refinement of methods for strip spawning; 5) evaluation of gamete and larval quality; 6) investigation of larval diets and nutrition, and 7) development of culture systems to rear larvae through metamorphosis. While past research has had success with spawning of red snapper in captivity, the successful production of larvae, fry, and fingerlings continues to remain a major impediment for the development of commercial culture.

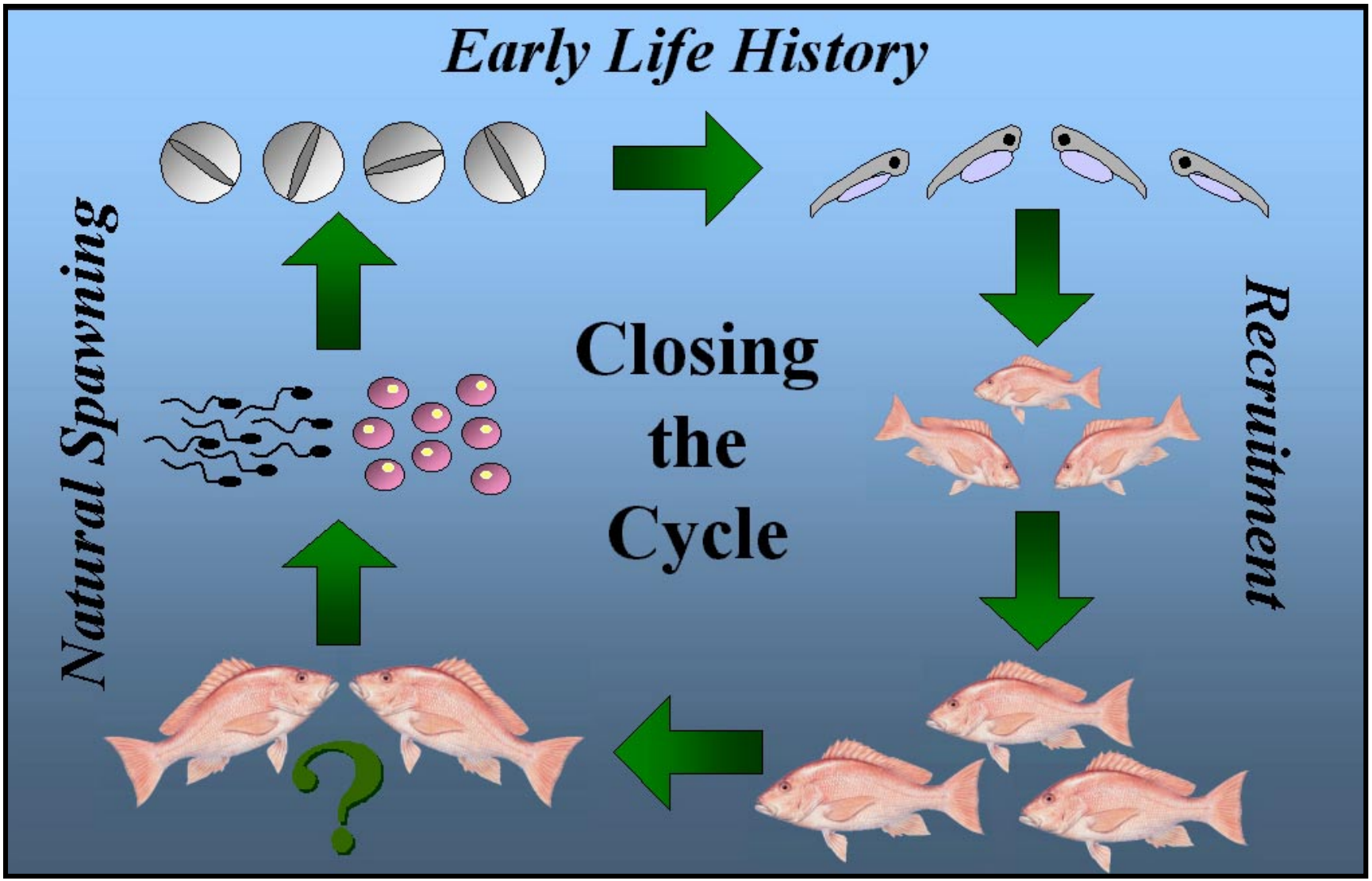


Figure 2.3. Information on the biology and life cycle of red snapper in the wild can be valuable in developing hatchery techniques for artificial spawning and larval rearing.

Successful rearing with other snappers, such as the mangrove red snapper *Lutjanus argentimaculatus* in Asia (Emata et al. 1994; Chou et al. 1995; Liao et al. 1995), the John's snapper *Lutjanus johni* (Lim et al 1985), the lane snapper *Lutjanus synagris* (Clarke et al. 1992; Domier and Clarke 1992), the mangrove snapper *Lutjanus griseus* (Richards and Saksena 1980); the mutton snapper *Lutjanus analis* (Watanabe et al. 1998; 2001), and the yellowtail snapper *Ocyurus chysurus* (Turano et al. 2000) suggests that red snapper culture is feasible. These studies have developed methods for artificial spawning of each species; however, similar to the situation for red snapper, a reliable supply of larvae and juveniles for grow-out remains a limiting factor. In order to achieve success and transfer technology to commercial aquaculture operations, techniques for the culture of red snapper should incorporate applied research, innovations in culture techniques, and a thorough knowledge of their biology and behavior in the wild (Figure 2.3).

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Chapter 3

Techniques for the Hatchery Production of Red Snapper and Snapper Hybrids

The worldwide demand for fish has increased dramatically in the last thirty years, primarily because of the increasing human population (Brown et al. 2000), but also because of per-capita increases in consumption (FAO 2001) and increases in capture fisheries that have been attributed to the sophisticated technologies used in modern fishing fleets (Dayton et al. 1995). The red snapper *Lutjanus campechanus*, an economically valuable recreational and commercial fishery of the southeastern United States, has received considerable attention within recent years because increasing demands and high market values have resulted in a highly exploited fishery (Shirripa and Legault 1999). Red snapper have been classified as overfished by the Gulf of Mexico Fishery Management Council and are under strict management measures to restore the declining fishery to sustainable levels (GOMFMC 2001). Interest in the culture of red snapper has developed as a result of high market values and limited harvests; however, the inability to control the natural spawning of captive broodstocks and the lack of fry and fingerlings have been major impediments to developing culture techniques for this species.

Efforts have been underway to spawn and rear red snapper since the 1970's (Arnold et al. 1978; Rabalais et al. 1980; Minton et al.1983). While these early studies were successful in the production of fertilized eggs and larvae from natural tank spawns and hormone-induced stripped spawns, most larvae died within a few days of hatching. Current research programs throughout the southeastern United States are focused on improving hatchery techniques to bolster larval survival in this species (Watanabe et al. 2001). The culture of red snapper can thus be considered to be underdevelopment and many years from commercial application because significant advancement is required to close the life cycle in captivity and to fully understand the bottlenecks in larval culture.

The challenges in spawning red snapper and rearing larvae in captivity are not unique to this species and have been observed in efforts to culture other subtropical and tropical marine fishes such as other snappers (Lutjanidae), groupers (Serranidae), jacks (Carangidae), and seabream (Sparidae) (Chou et al. 1995). Factors limiting the development of culture techniques for these fishes are often attributed to problems incurred with broodstock management and reproduction, and larval rearing through critical periods such as: first feeding, yolk and oil globule exhaustion, gas bladder inflation, diet transitions, transition to gill gas exchange, and at metamorphosis into juveniles (Tucker 1998).

While in recent years a number of marine species have been conditioned to spawn in captivity through the use of a photoperiod or temperature manipulation, the most reliable method for the production of sperm, eggs, and larvae has been the use of hormone treatments. The most common practice to administer hormones is through injections of hormone solutions; although, hormones can also be administered through time-release implants or oral dosages (Crim et al. 1988). Hormone-induced spawning has been practiced for almost 70 years to spawn fish that do not breed in captivity, to gain control of fry production, and to produce hybrids that are different from the parent species (Zohar and Mylonas 2001).

Fish treated with hormones can naturally spawn within culture systems or can be stripped of their eggs following ovulation. Ovulation occurs when oocytes within the ovary go through final maturation in which the germinal vesicle degenerates, the first meiotic division is completed, the second meiotic division begins and is arrested, and the hydrated oocytes are released into the ovarian lumen (Wallace et al. 1987). Ideally broodstock maintained under optimal conditions (e.g. environmental stimuli, water quality, nutrition) should spawn in captivity; however, broodstock captured from the wild often fail to progress through oocyte maturation and ovulation

due to differences between natural environmental conditions and the hatchery (Zohar and Mylonas 2001).

A variety of hormones have been used to induce spawning in marine fish. The hormones typically used regulate the levels of: 1) releasing hormones produced in the hypothalamus; 2) gonadotropin hormones produced in the pituitary gland, or 3) steroids and prostaglandins produced within the gonads (Lam 1982). Carp pituitary extract, gonadotropin releasing hormone analogues, and human chorionic gonadotropin are the most commonly used hormones that are injected into broodstock to induce spawning (Tucker 1998). Carp pituitary extract has been used to spawn Malabar grouper *Epinephelus malabaricus* (Tucker 1994), Nassau grouper *Epinephelus striatus* (Watanabe et al. 1995), summer flounder *Paralichthys dentatus* (Alves et al. 1999), and spotted snapper *Lutjanus guttatus* (Valverde and Boza 1999). Gonadotropin releasing hormone analogs, such as lutenizing hormone-releasing hormone analog, have been used to induce spawning in grey mullet *Mugil cephalus* (Lee et al. 1988), sea bass *Lates calcarifer* (Garcia 1992) and Atlantic croaker *Micropogonias undulatus* (Gwo et al. 1993). Human chorionic gonadotropin has been the most widely used hormone to induce spawning in marine fish and has been used with red snapper (Minton et al. 1983), blue-spotted grouper *Epinephelus fario* (Kuo et al. 1988), yellowtail snapper *Ocyurus chrysurus* (Soletchnik et al. 1989), mangrove red snapper *Lutjanus argentimaculatus* (Emata et al. 1994), Nassau grouper (Head et al. 1996), yellowfin porgy *Acanthopagrus latus* (Leu and Chou 1996), gray snapper *Lutjanus griseus* (Rosas et al. 1997), and common snook *Centropomus undecimalis* (Neidig et al. 2000). While a few species held under captive conditions for an extended period of time spawn without the use of hormones (e.g. milkfish *Chanos chanos* (Lee 1995), red drum *Sciaenops ocellatus* (Colura et al. 1991), the use of hormones with proper broodstock management remains the most reliable method for the mass production of fry and fingerlings.

Broodstock sources for marine fish hatcheries operated in the United States are primarily based on animals collected from the wild (Lee and Ostrowski 2001). Fishes collected from the wild can be induced to spawn several weeks after capture, although most studies have had success with hormone-induced spawning of fish within a few days of capture (Tucker 1998). This is especially important with species that are susceptible to the effects of stress (e.g. regression of gonads) from capture and transportation to the hatchery. Because the stage of oocyte maturation is related to spawning frequency (Zohar 1989), broodstock that are to be induced to spawn with hormones should be collected from the wild during the natural spawning season. For example, for red snapper within the Gulf of Mexico, spawning begins in May and continues through early October (Collins et al. 1996) following the lunar cycle with increases in spawning frequency during the first and last quarters of the moon (Chesney and San Fillippo 1994). Efforts to collect broodstock and spawn red snapper should be focused around the peak spawning periods in June, July, and August.

After capture and transport to the hatchery, samples of sperm from males and oocytes from females should be collected to assess reproductive condition. A simple intraovarian biopsy can be used to collect oocytes for microscopic analysis and measurement. In general, females with oocyte diameters ranging from 65 to 70% of the spawned oocyte diameter should be considered candidates for hormone induced spawning (Lee 1998). Following this general rule, red snapper females with oocyte diameters ranging from 400 to 600 μ should be considered as candidates for hormone-induced spawning. Other methods for timing of ovulation have included: 1) time after hormone treatment; 2) change in female coloration; 3) swelling of the abdomen and protrusion of the genital papilla, and 4) variation in hormone levels of blood and ovarian fluid (Tucker 1998).

Timing of ovulation is an essential component in stripping fertile eggs from broodstock. Eggs stripped too early are immature, infertile oocytes, while eggs retained in a female after

ovulation begin a process of overripening and degradation (Bromage and Roberts 1995). Overripening continues from the time eggs are stripped from the female to the time sperm is added for fertilization. Immature and overripe eggs stripped from broodstock can have significantly lower fertilization rates and lower larval survival rates (Kjørsvick et al. 1990). Several studies have examined methods for timing of ovulation to eliminate the overripening of eggs. Research on the timing of stripping in the Atlantic halibut *Hippoglossus hippoglossus* shows that egg quality decreases 4 to 6 hours after ovulation (Bromage et al. 1994). Similar results were found in experiments with turbot *Scophthalmus maximus*, which demonstrated that pH measurement may provide a reliable assessment of overripening (Fuavel et al. 1993). With several species of marine fish like the red snapper, there is a small window of opportunity when eggs must be stripped, evaluated for quality, and fertilized with sperm.

The goal of this study was to evaluate techniques to improve spawning, fertilization, and larval rearing success in red snapper. The objectives were to: 1) develop methods for collecting, handling, transport, and holding of mature red snapper for induced spawning; 2) develop criteria for evaluating the reproductive condition of female broodstock; 3) optimize techniques for strip spawning; 4) evaluate fertilization and larval rearing success from eggs fertilized with fresh and cryopreserved sperm, and 5) evaluate fertilization and larval rearing success from red snapper eggs fertilized with red snapper and gray snapper sperm. In this study, the term "oocyte" will refer to gonial cells undergoing meiotic divisions, maturation, and obtained by biopsy. The term "egg" will refer to oocytes that have been hydrated, released into the ovarian lumen, and obtained by stripping.

Methods

Collection, Handling, Transport, and Holding of Broodstock for Induced Spawning

Collection of Broodstock

Red snapper broodstock were collected during the natural spawning season off coastal Louisiana by hook and line sampling. Efforts were focused on collection during the first and last quarters of the moon within the peak spawning season of May through August. Fish were collected from depths ranging from 15 to 30 m and to minimize stress from capture, and fish were brought to the boat as soon as possible using heavy fishing tackle. Fish with inflated swim bladders were deflated through puncture with a 16 G sterile needle and undersized fish were released. Healthy, mature red snapper greater than 40 cm total length (TL) were retained for spawning and fertilization trials at Louisiana Universities Marine Consortium (LUMCON) in Cocodrie, Louisiana. Fish were transported from offshore (~ 50 km) to the LUMCON hatchery in an onboard oxygenated live-well (450 L) filled with seawater (35 ppt) from the site of collection.

Hormone-Induced Spawning

In the hatchery on the day of capture, fish weights were estimated from length measurements and the use of length:weight relationships developed for red snapper from Louisiana (Moran 1988). All fish were injected intramuscularly with a 500 IU/kg priming dose of human chorionic gonadotropin (HCG) (Chorulon, Intervet, Millsboro, DE) and placed in a recirculating culture system. Temperatures (25-27 C) and salinities (32-35 ppt) were maintained at conditions approximating those in the field at the time of capture. The culture systems were checked hourly for eggs that had been ovulated and released into the water and for broodstock mortalities. Approximately 15 h after administering the hormone injection, the fish were anesthetized (100 mg/L, MS-222, tricaine methanesulfonate) and the sex of the fish was determined by visual

inspection. Ripe males were identified by an extended urogenital papilla and the presence of flowing milt upon palpation of the abdomen. Male snapper were used for the collection of fresh sperm for fertilization experiments.

In order to assess the reproductive condition of female broodstock, a sample of oocytes was collected by intraovarian biopsy. A Teflon® spaghetti-tubing catheter (1.2-mm inner diameter; 1.8-mm outer diameter) was inserted 5 cm into the oviduct, and suction was applied using a 10-mL plastic syringe (Hoff 1972). A sample of oocytes were microscopically examined and measured to assess reproductive condition. Females were measured in length, weighed, and injected with a 1000 IU/kg resolving dose of HCG. Additionally, females were tagged with a color-coded anchor tag (Floy Tag Inc., Seattle, Washington) to identify individuals, and fish were returned to the culture system.

Based upon previous experience spawning red snapper in the LUMCON hatchery, females were monitored for oocyte maturation and ovulation 8 to 12 h after administering the resolving dose of hormone. Criteria for final oocyte maturation were when: 1) protein yolks disappeared; 2) oil droplets coalesced to form a single droplet; 3) the nucleus disappeared, and 4) the cytoplasm within the oocytes became clear. Biopsies were not performed as fish approached final oocyte maturation and ovulation; however, females were monitored for swelling of the abdomen, protruding genital papilla, and release of eggs. After ovulation, females were anesthetized, blotted dry, and stripped of their eggs using slight abdominal pressure. Eggs were collected in a dry, 300-mL Pyrex® dish. Once the complete spawn was collected, eggs were divided into experimental treatments and fertilized immediately except in trials evaluating the short-term storage of eggs.

Evaluation of the Reproductive Condition of Female Broodstock

Oocyte Measurement and Analysis

In an effort to establish criteria to predict potential spawning and ovulation in females, oocyte size frequencies were calculated for each fish. Oocytes collected by biopsy after the priming dose of HCG (12 – 16 h) were evaluated and measured using a dissecting microscope equipped with an ocular micrometer. The oocytes were examined immediately after collection. They were washed into a 35-mm petri dish with FSW, and teased apart and connective tissues were removed. A random sample of 100 oocytes were measured and recorded. Observations were also noted on the shape of oocytes and the presence of yolk proteins or oil globules. Samples were catalogued and preserved in a 5% formalin solution.

Measurement of Intraovarian pH

A solid-state pH meter (Model 240, IQ Scientific Instruments Inc., San Diego, California) with a 3.5-mm diameter probe was used to assess the intraovarian pH of ovulating fish. The pH probe was inserted 3 to 5 cm into the oviduct of female broodstock. In order to avoid the effects of stress on egg quality, pH was measured immediately after eggs were stripped.

Collection and Cryopreservation of Sperm

Collection of Sperm

All sperm used in this study were collected using the same methods. Sperm used in experiments as fresh controls or used in experiments manipulating sperm concentrations were obtained from fish caught on broodstock collecting trips. Additional sperm used for cryopreservation experiments were obtained from red snapper and gray snapper caught on recreational fishing boats during the study period. After capture, fish were placed in an insulated cooler with ice. Sacrificed males were measured in length and blotted to avoid contamination of samples with water, mucus, or blood. Sperm were collected by surgical removal of the testes,

which were blotted and sliced to release sperm. Sperm were collected in 50-mL plastic centrifuge tubes and diluted 1:3 (v:v) with calcium-free Hanks' balanced salt solution (HBSS) prepared at 200 mOsmol/kg (Wayman et al. 1996). Except for experiments manipulating sperm concentrations, sperm solutions were not standardized; however sperm concentrations were determined to be $1.5 \times 10^9 \pm 1.0 \times 10^9$ cells/mL though use of phase-contrast hemacytometer (model 1475, Hausser Scientific Company, Horsham, Pennsylvania). The sperm solutions were evaluated for percent motility and refrigerated at 4 C until used in experiments.

Cryopreservation of Sperm

Sperm samples were cryopreserved at Genex Cooperative, Inc. located at the Louisiana State University T. E. Patrick Dairy Improvement Center in Baton Rouge. The techniques used for the cryopreservation of bull semen in 0.5-ml French straws (Chandler 1984) were utilized for bulk cryopreservation of red snapper and gray snapper sperm. In order to protect cells during the freezing process, 10% dimethyl sulfoxide was added to samples and allowed to penetrate cells for 20 min before freezing. Once frozen, samples were stored in liquid nitrogen storage dewars and transported to LUMCON in nitrogen-vapor shipping dewars.

Thawing Sperm Samples

Cryopreservation straws were removed from dewars and individually thawed in a 40 C water bath for 7 sec. After thawing, the straws were wiped dry and cut to release the sperm solutions for analysis of quality or use in fertilization trials.

Fertilization and Larval Rearing of Red Snapper and Snapper Hybrids

Fertilization of Red Snapper Eggs

From each spawn collected, an 1/8 teaspoon measure (approximately 3200 eggs) was used to distribute aliquots of eggs among dry, 300-mL Pyrex® dishes. Aliquots of eggs from each red snapper female were fertilized with 1 mL of fresh or thawed red snapper sperm. Additional

aliquots were fertilized with fresh or thawed gray snapper sperm. Fresh red snapper sperm were used as a control to evaluate egg quality. Approximately 40 mL of filtered (5 µ) ultraviolet-sterilized seawater (35 ppt) (FSW) were added to activate gametes. Sperm and eggs were swirled to facilitate thorough mixing and fertilization. After 5 min, sperm and eggs were transferred to 2-L Pyrex® beakers and filled to 2000 mL with FSW at 28 C.

Incubation of Embryos

Sixteen Ziplock® freezer bags (S. C. Johnson and Son, Inc., Racine, Wisconsin) were used as incubation containers to evaluate fertilization and hatching of eggs from each spawn. Two replicate 4-L bags per treatment were labeled using a permanent ink marker. Before transferring eggs from beakers to bags, the eggs were thoroughly aerated to homogenize samples. A 100-mL plastic beaker was used to transfer 150 mL of water (~200 eggs) to each bag. The bags were filled with an additional 1 L of FSW, sealed, and floated in a recirculating system maintained at 30 C. At the appropriate time intervals, bags were removed from the culture system and samples were collected by cutting a corner off of the bottom of each bag and pouring the contents through a 120-µm filter. Samples were collected at: 2 h to assess development through the 8-cell stage; 14 h to assess development through neurulation, and 20 h to evaluate percent hatch. All samples were preserved in a 5% solution of formalin and buffered seawater and stored in 20-mL glass scintillation vials for later analysis using a dissecting microscope.

Larval Rearing Trials

Larval survival and growth were evaluated by dividing and stocking the remaining 2000 eggs from each beaker into two static culture tanks (100 L). The conical bottom tanks (N = 16) were filled with 80 L of FSW that was maintained at 28 to 30 C using individual aquarium heaters (Visi-Therm, Mentor, Ohio). Temperatures were monitored daily with a digital thermometer (model PS100A, Baxter, Deerfield, Illinois). The tanks were gently aerated and surface lighting

was maintained at 55 µmol (550 lux) (model LI-189 with 4π submersible probe, LI-COR, Lincoln, Nebraska) for 16 h daily. Two days after hatching, the larval culture tanks were inoculated with cultured microalgae, *Isochrysis galbana* (T-Iso strain), to maintain green water culture conditions, and larvae were fed size-sorted wild zooplankton (53-102 µm). Zooplankton consisted of ciliates, rotifers, copepod nauplii, and copepods that were maintained at densities of one to three individuals per mL. Water quality was maintained with 50% daily water changes (40-L). All larvae were reared for 10 d, and were harvested by siphoning tank water through a 4-cm polyethylene hose into a 500-µm filter. Surviving larvae were removed from the filter, counted, and measured. A dissecting microscope equipped with an ocular micrometer was used to measure the standard length and total length of live fish. All samples were preserved in a 5% solution of formalin and buffered seawater, catalogued, and stored for reference at LUMCON.

Refinement of Hatchery Techniques

Optimizing Sperm Concentrations

Sperm from one red snapper collected in June 2001 were used in preliminary experiments to determine the relationship between sperm concentration and fertilization success. Sperm were collected as previously described, diluted 1:3 (v:v) with HBSS, and sperm motility was estimated using darkfield microscopy at 200-x magnification. Five concentrations were produced using a sequential dilution rate of 1:10 with HBSS. In order to determine the concentration of each sperm solution, duplicate samples were diluted 1:300 with HBSS. Ten µL of sperm were discharged from a pipette onto a phase-contrast hemacytometer and sperm cells were allowed to settle for 1 min. Counts were conducted using darkfield microscopy at 200-x magnification. The average of four counts was determined for each of the two dilutions and the average of these

two values was used to calculate the sperm concentration. Sperm concentrations were estimated by multiplying the average of the hemacytometer counts by 50,000 and the dilution rate (i.e. 300).

The original sperm solution and each of the four dilutions were used to fertilize eggs collected from two red snapper. In petri dishes (100 x 15 mm), aliquots of 200 eggs were fertilized with 1 ml of each sperm solution. Filtered seawater was added to activate sperm and initiate fertilization. Sperm and eggs were mixed with 1 L of FSW, which was poured into 4-L Ziplock® freezer bags and incubated for 14 h at 30 C. Samples collected were preserved in 5% formalin and fertilization was assessed at neurulation by use of a dissecting microscope.

Short-term Storage of Eggs

The effect of short-term storage on fertilization of eggs stripped from red snapper was evaluated. Experiments were conducted at 10-min intervals for 60 min. Aliquots of 3200 eggs from two spawns were maintained at room temperature (25 C) until samples of eggs from each treatment were fertilized with fresh sperm. Treatments were: 1) dry; 2) dry with 10 mL of HBSS added 1 min before addition of sperm; 3) suspended in 30 mL of HBSS, and 4) suspended in 30 mL of FSW. At each timed interval, approximately 200 eggs from each treatment were placed into petri dishes (100 x 15 mm) and mixed with 1 mL of fresh red snapper sperm and 10 mL of FSW. Samples were swirled to ensure thorough mixing. After 5 min, two replicates of each sperm and egg were mixed with 1 L of FSW, which was added to 4-L Ziplock® freezer bags and incubated at 30 C. Samples were collected from the bags at 14 h and were preserved in 5% formalin. Fertilization was assessed using a dissecting scope to determine the percent of embryos that developed to neurulation.

Analysis of Preserved Embryo and Larvae

Within a well-ventilated laboratory, preserved eggs, embryos, and larvae were transferred from 20-mL glass scintillation vials by pipette into 35-mm petri dishes with marked grids. The formalin solution was removed and replaced with 35-ppt artificial seawater (Marinemix, Wiegandt GmbH Inc., Krefeld, Germany). The number of embryos and larvae within each sample were counted against a black background with a dissecting microscope at 40-x magnification. Upon completion, the seawater was removed and samples were returned to the original vials with the 5% formalin solution.

Statistical Analysis

All percent motility, fertilization, hatch, and survival values were arcsine-square root transformed prior to statistical analysis. A one-way analysis of variance (ANOVA) (SAS 8.0; SAS Institute, Cary, NC) was tested for all parameters measured by collection date, spawn date, and weight of broodstock. Linear regression analysis (Microsoft Excel 2000, Microsoft Corp., Redmond, Washington) was used to determine the correlation between: 1) the predicted and measured weights of broodstock; 2) fertilization and hatch rates; 3) fertilization and larval survival rates, and 4) hatch rates and larval survival rates. Variables were considered correlated when $r \geq 0.20$. Differences in the fertilization and hatching rates during the 2-year study were analyzed using a one-way ANOVA. Additionally, a one-way ANOVA was used to evaluate variation in fertilization results with sperm of different concentrations, and variation in the short-term storage of eggs. For all tests, Duncan's multiple range test was used to determine if significant differences existed among treatment means. Differences were considered significant at $P \leq 0.05$.

Results

Collection, Handling, Transport, and Holding of Broodstock for Induced Spawning

Collection of Broodstock

During 2000 and 2001, red snapper broodstock (N = 131) were collected during 10 offshore fishing trips (Table 3.1). Fish were collected by hook and line sampling in depths of 15 to 40 m; however, collection efforts were often hampered by poor weather, hooking of undersized fish, and poor fishing conditions. Use of ice blocks and routine water changes (± 50%) permitted collection and transport of as many as 30 broodstock individuals from offshore. Each year 33 females were collected with total lengths ranging from 41 to 61 cm and weights ranging from 902 to 4248 g (Table 3.2). The mean weight of females collected during the 2-year study was 1681 ± 640 g and was not significantly different among years ($P = 0.67$). Female weights were significantly different among collection dates ($P = 0.003$); although, there was no correlation or trend among weight differences and time of year. Weight differences were simply an artifact of the sampling method.

Hormone Induced Spawning

The use of length:weight relationships to calculate hormone dosages on the day of capture minimized handling of broodstock. Analysis of estimates revealed that weight estimates were significantly less than the actual weight of fish (difference 305 ± 109 g; $P < 0.0001$). While all fish were given the priming dose of HCG, ~20% of the females collected ovulated and released eggs into the culture system on the evening of capture. Because of limited personnel, these females were not strip spawned and the eggs were collected within an egg trap on the culture system and subsamples were counted using a dissecting microscope. Quantities of eggs collected ranged from 18,000 to over 3 million; however, these quantities were often released by several fish and there was no evidence of fertilization. Red snapper females in optimal spawning

condition began ovulating 28 h after the initial injection of HCG. The spawning latency (time between initial hormone injection and stripping of eggs) was 29 ± 1 h. Red snapper strip-spawned in the hatchery ranged in total length from 41 to 51 cm and weight from 1108 to 2280 g (Table 3.3). In an effort to distribute eggs immediately among experiments and replicates for fertilization, the total volume of individual spawns was not determined, but all fish that were spawned and used in experiments produced in excess of 18,000 eggs.

Table 3.1. Summary of research efforts to collect and spawn red snapper during 2000 and 2001. Spawning latency (h) refers to the number of h elapsing between initial hormone injection and stripping of eggs.

Date of collection	Lunar phase	Number of red snapper collected	Number of females collected	Females strip spawned	Spawning latency (h)
2000					
May 30	4 d after last quarter	12	4	0	***
June 14	5 d after first quarter	9	3	0	***
June 28	3 d after last quarter	10	4	1	29.50
July 10	2 d after first quarter	5	7	2	30.8 ± 0.7
July 25	1 d after last quarter	8	3	2	29.75 ± 0.7
August 9	2 d after first quarter	14	12	4	29.8 ± 0.5
2001					
June 17	3 d after last quarter	14	8	2	29.1 ± 0.5
July 4	6 d after first quarter	6	1	0	***
July 16	3 d after last quarter	2	2	0	***
July 18	5 d after last quarter	11	5	3	28.8 ± 0.6
July 31	4 d after first quarter	10	6	1	28.8
August 15	3 d after last quarter	30	11	2	29.1 ± 0.9

Table 3.2. Summary of lengths (cm) and weights (g) of female red snapper collected.

		Length (cm)			Weight (g)		
Year	**Females injected**	**Average**	**Minimum**	**Maximum**	**Average**	**Minimum**	**Maximum**
2000	33	46 ± 4	41	52	1622 ± 317	1046	2200
2001	33	46 ± 6	41	61	1735 ± 835	902	4248

Table 3.3. Summary of lengths and weight of female red snapper strip-spawned.

		Length (cm)			Weight (g)		
Year	**Number of fish**	**Average**	**Minimum**	**Maximum**	**Average**	**Minimum**	**Maximum**
2000	9	45 ± 3	41	48	1559 ± 244	1175	1874
2001	8	46 ± 4	41	51	1568 ± 486	1108	2280

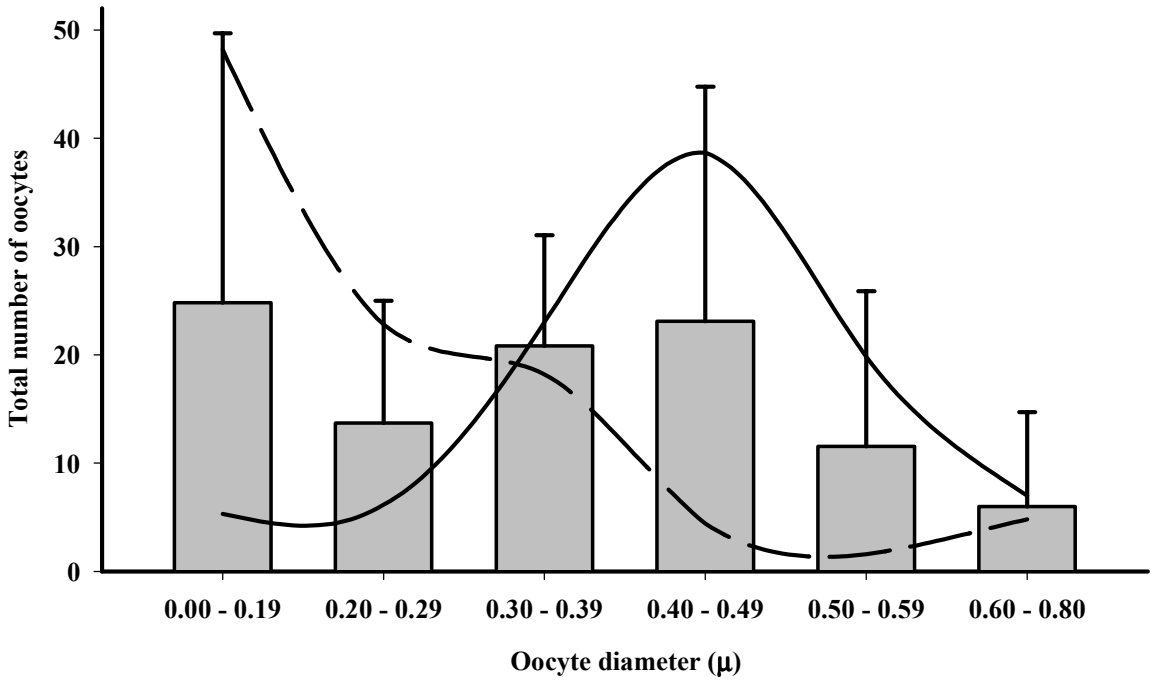

Figure 3.1. Polymodal distribution of oocytes (bars) collected from red snapper by intraovarian biopsy. Size frequency distribution of oocytes from females that ovulated (solid line) (N = 17) and those that did not ovulate (dashed line) (N = 20) as a result of hormone induction. Fish with a majority of oocytes greater than 300 µ were considered good candidates for hormone induced spawning.

Evaluation of the Reproductive Condition of Female Broodstock

Oocyte Measurement and Analysis

Oocytes collected and measured 15 h after the priming dose of HCG had an average diameter of 365 ± 152 µ and ranged in size from 24 to 891 µ. Oocyte appearance and size were useful in predicting females that were likely to proceed through final oocyte maturation and ovulation. Frequency distributions of oocytes exhibited a polymodal distribution pattern (Figure 3.1). The average oocyte diameter for females that ovulated and were stripped spawned (N = 17) was 438 ± 73 µ, and greater than 60% of the oocytes were within the range of 350 to 550 µ. The average oocyte diameter for females that did not ovulate and were not stripped was 348 ± 149 µ with the majority of oocytes less than 300 µ.

Measurement of Intraovarian pH

Intraovarian pH collected from strip-spawned red snapper (N = 17) was 7.44 ± 0.8 and ranged from 6.53 to 9.77. There was no correlation between pH and fertilization, hatch, or total length of fish; however, pH was weakly correlated with fish weight (r = 0.56).

Fertilization and Larval Rearing of Red Snapper and Snapper Hybrids

Fertilization of Red Snapper Eggs

Fresh and cryopreserved sperm of red snapper and gray snapper were used to fertilize eggs collected from 17 red snapper during the two-year study. The use of plastic freezer bags was effective for incubating red snapper and hybrid red snapper embryos through hatch. During the first year, eggs collected from seven females were used in bag studies and in larval rearing trials. Eggs from two additional fish were used in bag studies only. During the second year, eggs collected from two females were used in bag studies and larval rearing trials, and eggs from six females were used in bag studies only.

While fertilization and hatch rates were varied among treatments, variation was low between treatment replicates. The fertilization of eggs treated with fresh red snapper sperm (89 ± 7% motility) as a control treatment ranged from 44 to 95% for development through 8-cell stage, 25 to 83% for development through neurulation, and 8 to 87% for development through hatch. Fertilization and hatch rates were not correlated with the weight or length of broodstock, and were not correlated with the spawn date.

During the first year of the study, fresh red snapper sperm yielded comparable fertilization rates (57 ± 16%) with thawed sperm (50 ± 15%) (Figure 3.2), and similar results were observed with fresh gray snapper sperm which yielded comparable fertilization rates (38 ± 13%) with thawed sperm (42 ± 11%) (Figure 3.3). Although fertilization rates were significantly lower in the second year of the study (P = 0.004), fresh red snapper sperm again yielded comparible

fertilization rates (17 ± 10%) with thawed sperm (12 ± 8%) (Figure 3.4), and fresh gray snapper sperm again yielded comparable higher fertilization rates (9 ± 4%) with thawed sperm (2 ± 1%) (Figure 3.5). Fertilization with fresh and thawed sperm were not compared statistically because sperm concentrations were not standardized and egg quality was highly variable.

Larval Rearing Trials

During this study, larvae were reared from eggs of nine strip-spawned red snapper. Overall survival of red snapper larvae reared through 10 d after hatching was 0.1 ± 0.5% and ranged from 0.0 to 2.0%. Overall survival of hybrid red snapper larvae was 0.4 ± 0.8% and ranged from 0.0 to 2.5%. All larvae were reared at 29.5 ± 0.5 C and growth was variable among treatments (Figure 3.6).

While larval survival for red snapper produced from fresh sperm was 1.1 ± 1.3% for one rearing trial, no larvae survived in the other eight trials and there was no survival in red snapper larvae produced from thawed sperm. Larval survival for hybrid red snapper produced from fresh sperm was 0.7 ± 0.9% for five rearing trials and no larvae survived in the other four trials. Survival of hybrid red snapper larvae produced from thawed sperm was 0.3 ± 0.5% for two rearing trials and no larvae survived in the other seven trials. Survival of larvae was not correlated with fertilization, hatch rates, broodstock size, or spawn date.

Refinement of Hatchery Techniques

Optimizing Sperm Concentrations

The motility of sperm was greater than 95% and there was no effect of dilution on sperm motility. The final concentration of sperm solutions diluted with HBSS were 8.5×10^4, 8.1×10^5, 1.0×10^7, 9.5×10^7, and 8.5×10^8 cells/mL and when used to fertilize eggs resulted in sperm to egg ratios ranging from 500:1 to 4.3×10^6:1. Fertilization of red snapper eggs with fresh sperm as a control (standard dilution of 1:3 with HBSS) was 42 ± 5%. The fertilization rates of

diluted sperm solutions were calculated relative to the control. Fertilization rates were significantly reduced ($P = 0.001$) when sperm concentrations were less than 8.5 x 10^8 cells/mL (Figure 3.7).

Short-term Storage of Eggs

Evaluation of storage treatments revealed that fertilization of red snapper eggs from two spawns was significantly reduced within 10 min of collection ($P = 0.03$). Eggs placed in seawater and fertilized immediately after collection had significantly higher fertilization rates than the other treatments ($P = 0.01$). Fertilization of eggs was optimized when eggs were stripped, stored in seawater, and fertilized within 10 min. There were no significant differences among storage treatments and fertilization rates at 10, 20, 30, and 60 min ($P = 0.66$).

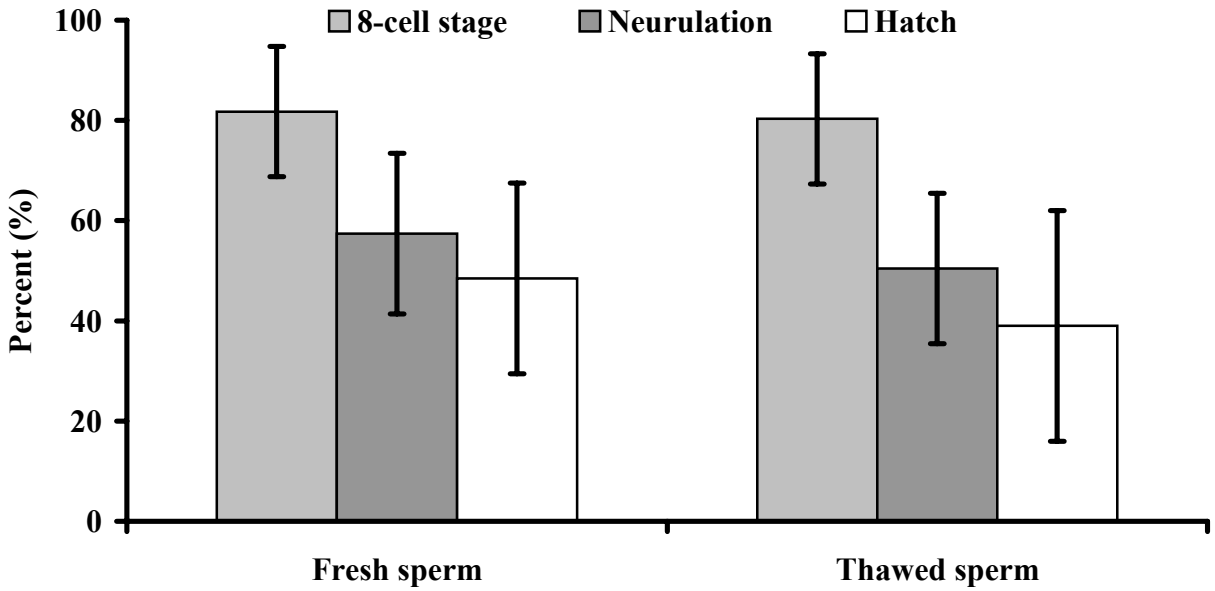

Figure 3.2. Fertilization and hatching results from red snapper eggs strip-spawned during the summer of 2000. Fresh and thawed sperm of red snapper was applied to eggs after stripping. Fertilization and hatch rates for fresh sperm were highly correlated ($r = 0.99$), and fertilization and hatch rate for thawed sperm were highly correlated ($r = 0.98$).

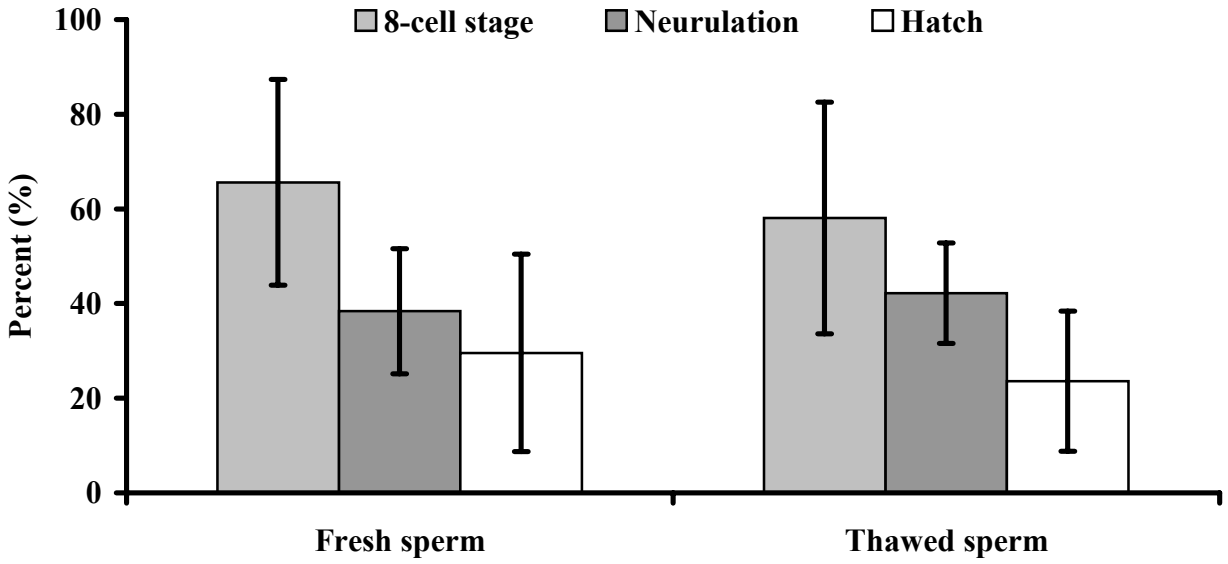

Figure 3.3. Fertilization and hatching results from gray snapper eggs strip-spawned during the summer of 2000. Fresh and thawed sperm of gray snapper was applied to eggs after stripping. Fertilization and hatch rates for fresh sperm were highly correlated ($r = 0.98$), and fertilization and hatch rate for thawed sperm were highly correlated ($r = 0.80$).

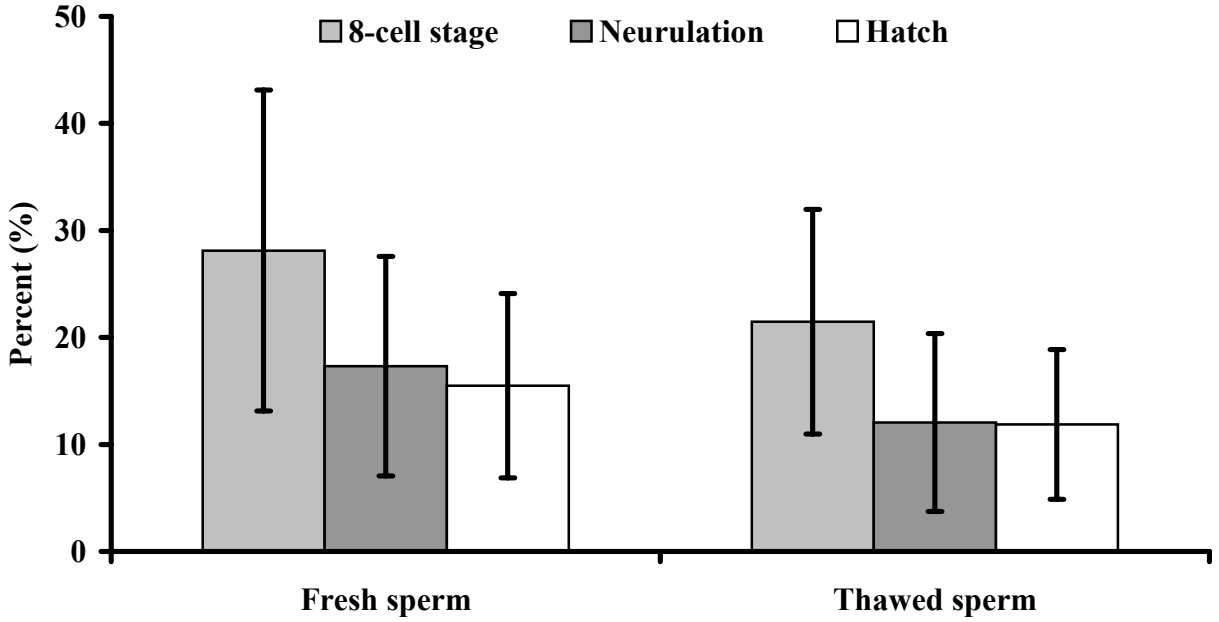

Figure 3.4. Fertilization and hatching results from red snapper eggs strip-spawned during the summer of 2001. Fresh and thawed sperm of red snapper was applied to eggs after stripping. Fertilization and hatch rates for fresh sperm were highly correlated ($r = 0.99$), and fertilization and hatch rate for thawed sperm were highly correlated ($r = 0.99$).

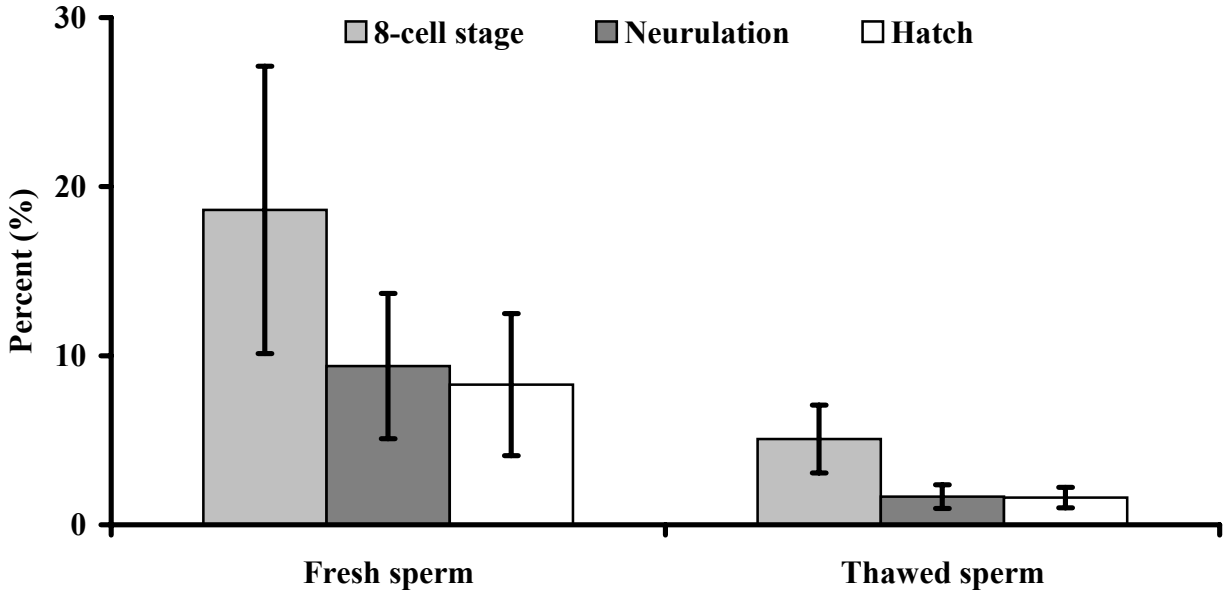

Figure 3.5. Fertilization and hatching results from red snapper eggs strip-spawned during the summer of 2001. Fresh and thawed sperm of gray snapper was applied to eggs after stripping. Fertilization and hatch rates for fresh sperm were highly correlated (r = 0.97), and fertilization and hatch rate for thawed sperm were highly correlated (r = 0.99).

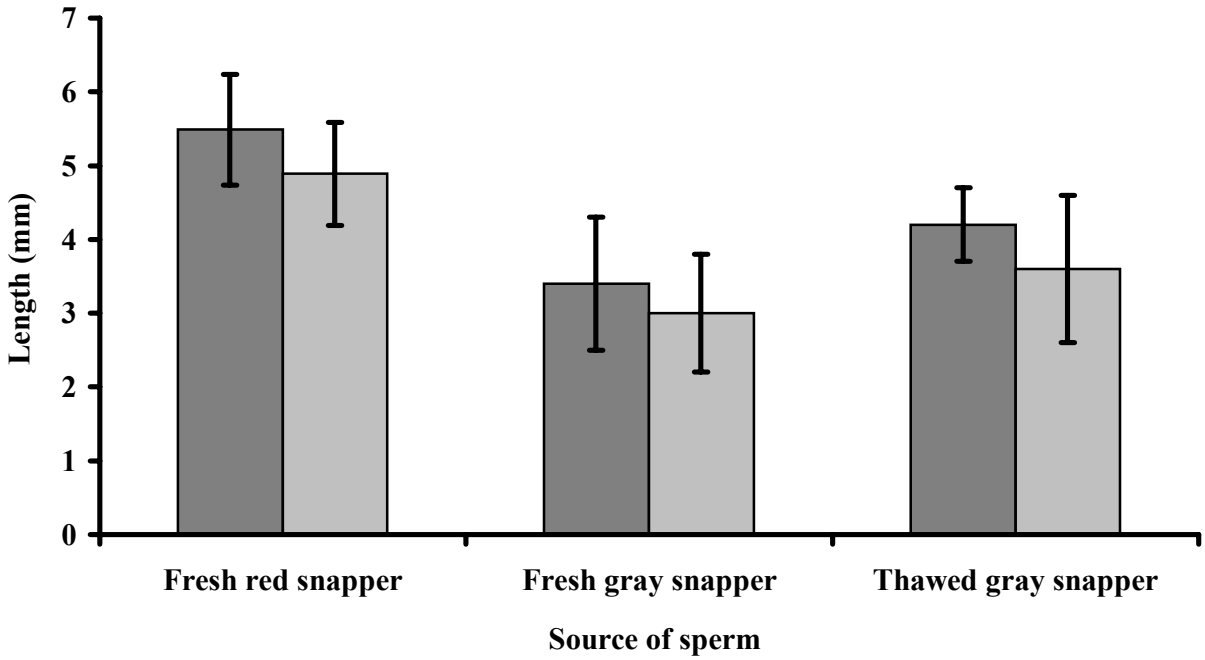

Figure 3.6. Length at 10-d after hatch of red snapper and hybrid snapper larvae produced from fresh red snapper sperm (N = 24), fresh gray snapper sperm (N = 79), and thawed gray snapper sperm (N = 18). Larvae were stocked 12 individuals per L and reared in 80-L conical tanks with filtered seawater (35 ppt) maintained at 29.5 ± 0.5 C.

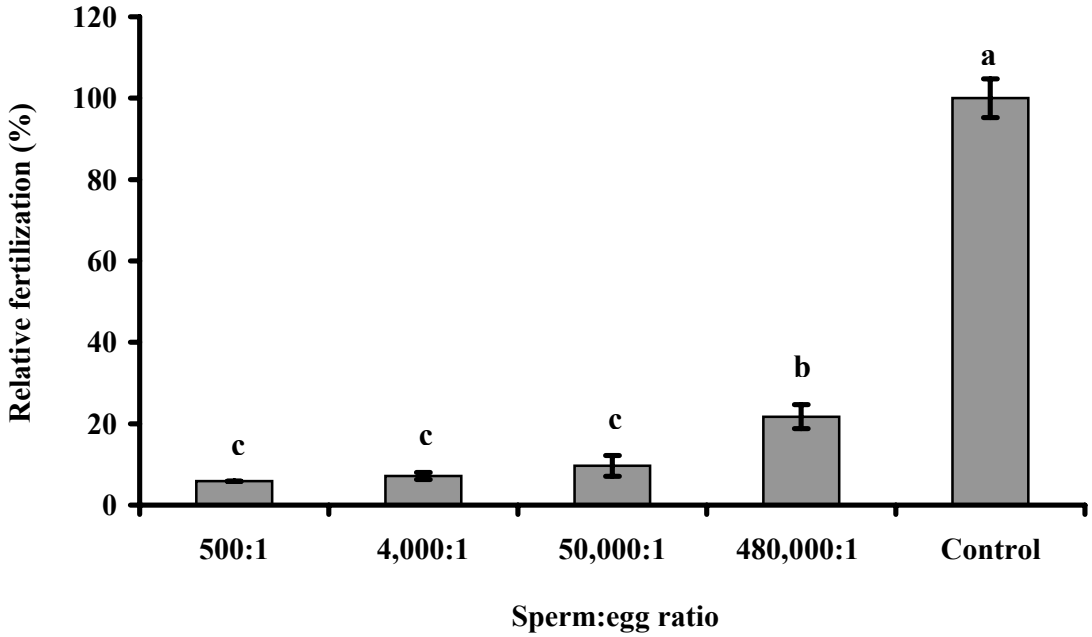

Figure 3.7. Fertilization rates (relative to control) for red snapper eggs from two females fertilized at different ratios of sperm and eggs. For the control, sperm were obtained from sliced testes, diluted 1:3 (v:v), and were used at a ratio of 4.2 x 10^6 sperm per egg.

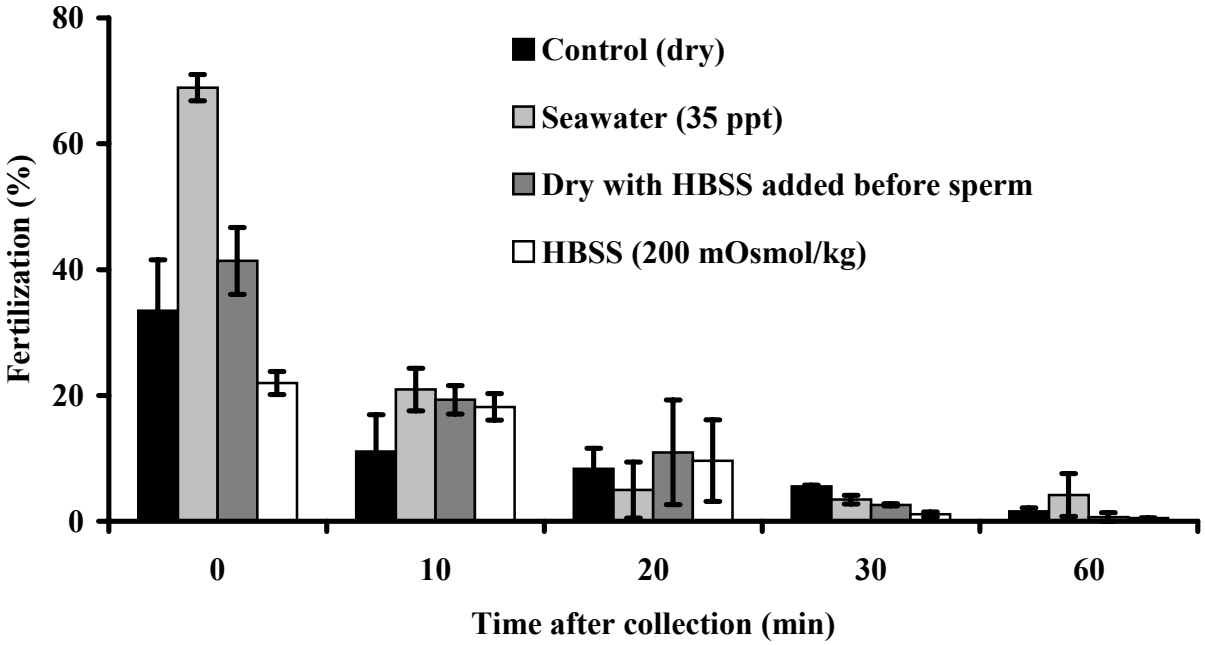

Figure 3.8. Red snapper eggs were fertilized with fresh sperm at 10-min intervals after stripping. Fertilization rate was assessed at neurulation (12 h), and was significantly reduced within 10 min of collection ($P = 0.03$).

Discussion

The growth of marine aquaculture in the future relies on the development of genetic improvement programs. Before genetic improvement can occur in cultured fish, methods must be developed for the controlled reproduction of captive broodstocks and mass production of high quality fry and fingerlings. In the present study, techniques for the collection, handling, and hormone-induced spawning of red snapper were developed to improve fertilization and larval rearing success in hatchery operations. Additionally, techniques were developed for the production of snapper hybrids (red snapper female x gray snapper male), which were used as a measure to evaluate quality of fresh and thawed gray snapper sperm, fertilization of red snapper eggs, and overall survival of larvae.

Because most marine fish hatcheries in the United States rely on wild broodstocks (Lee and Ostrowski 2001), it is essential that techniques be developed to minimize additional stress to broodstock during collection and transport to the hatchery. The techniques employed in this study included use of a 450-L oxygenated live-well capable of safely transporting 30 broodstock (1.0 – 4.0 kg) from the offshore environment to the coastal hatchery. Special attention to deflation of inflated swim bladders allowed fish to orient themselves and maintain equilibrium within the live-well.

In the hatchery, handling was minimized by calculating hormone dosages on the day of capture through use of length:weight relationships. Priming dosages of hormones were underestimated because measured weights were significantly larger than estimates. Caution should be used to ensure that correct dosages of hormone are administered when using length:weight relationships because they may not accurately represent local populations of fish or the seasonal condition of fish. Regression analysis of lengths and weights of broodstock

during this study will permit more accurate estimates of weights future artificial spawning research at the LUMCON hatchery (Figure 3.9).

The collection of oocyte biopsies from females was simple and useful for staging oocyte maturation and predicting ovulation. Fish with oocyte diameters larger than 300 μ and peaks within the range of 400 to 500 μ were deemed as good candidates for strip-spawning. In this study, the critical oocyte diameter values calculated for red snapper were similar to the critical values observed for other cultured species (Table 3.4). The distribution of oocytes collected from each biopsy provided evidence that red snapper have several populations (i.e. size ranges) of oocytes maturing synchronously within the ovary.

This characteristic is common among teleosts with group-synchronous ovarian development, which may be described graphically by a polymodal distribution of oocytes within the ovary (Wallace and Selman 1981). Because red snapper are serial spawners and spawn repeatedly for several days, recruitment and maturation of oocytes within the ovary is a continuous process during the spawning season. The distribution of oocytes at various stages of development can significantly affect how ovaries and developing oocytes will respond to hormone induction. In species that have group-synchronous development of oocytes, injection of HCG causes all oocytes regardless of their stage of development to undergo final oocyte maturation and ovulation, which results in eggs of variable quality and low fertility (Wallace and Selman 1981).

To date, the use of HCG has been the most reliable hormone for stimulating oocyte maturation and ovulation in red snapper (Minton et al. 1983; Lee and Ostrowski 2001; Watanabe et al. 2001). The low fertilization and hatch rates reported in this and previous studies are probably a result of variable egg quality strongly influenced by the use of hormones to induce maturation and ovulation of oocytes. In order to optimize spawning induction in red snapper, hatchery techniques should correlate artificial spawning efforts with the natural spawning cycle.

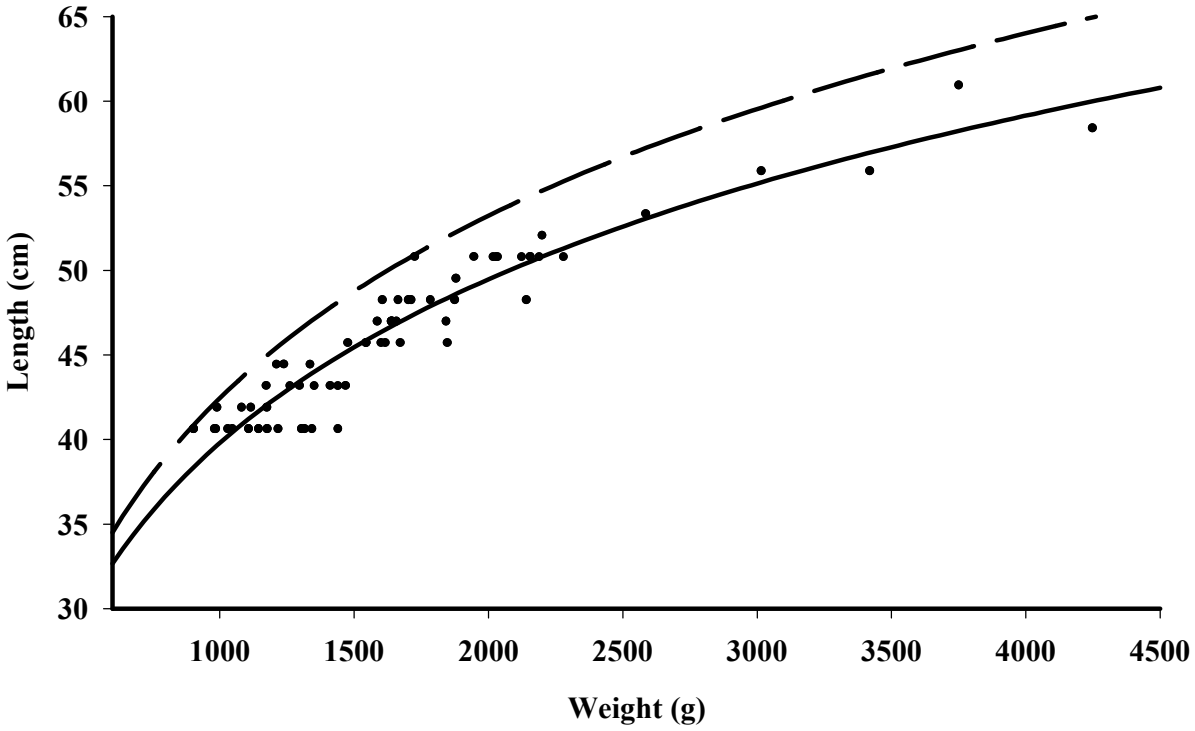

Figure 3.9. Regression analysis of length:weight relationship for red snapper broodstock collected during this study (N = 131). Solid line indicates measured values and dashed line indicates predicted values.

Table 3.4. Egg diameters and critical oocyte diameters in several cultured marine species (adapted from Lee 1998).

Common name	Species	Egg diameter (u)	Critical oocyte diameter (u)	Percent of egg diameter (%)
Red snapper	*Lutjanus campechanus*	750 - 850	300	40
Mutton snapper	*Lutjanus analis*	725 - 875	300	40
Greater amberjack	*Seriola dumerilii*	820 - 880	450	50
Grouper	*Epinephelus striatus*	820 - 920	450	50
Red seabream	*Pagrus major*	800 - 1000	500	60
Mullet	*Mugil cephalus*	850 - 950	650	70
Milkfish	*Chanos chanos*	1100 - 1200	700	60

Although inclement weather often prevented collection of broodstock during peak spawning periods, every effort was made to collect red snapper on the first and last quarter of the lunar cycle during the peak spawning months of June, July, August. Timing of the collection of broodstock from the wild is essential to make use of the natural spawning rhythms of red snapper. It is possible that if collection of female broodstock was synchronized with natural spawning, fish could be strip-spawned in the field or hatchery without the use of hormones or with the use of minimal dosages. Although red snapper have never been observed spawning in the wild, research on their natural spawning behavior could play an important role in developing techniques for artificial spawning red snapper in the hatchery.

The use of plastic bags facilitated the incubation of embryos and larvae from 2 to 20 h. The plastic bags were especially useful for isolating the many treatments and replicates produced during this study. Evaluation of development through 8-cell stage, neurulation, and hatch served as useful measures of fertilization; however, a large number of samples (50 per spawn) were generated and counting of samples was time consuming. Evaluation of the results showed that development of red snapper and hybrid snapper embryos through neurulation was an accurate measure of fertilization and hatching success.

Fresh and cryopreserved gray snapper sperm was useful for the production of hybrid snapper. This study is the first to report hybridization between red snapper females by gray snapper males, although laboratory-produced and natural hybrids have been reported for several snapper species (e.g. lane snapper *Lutjanus synagris* female x yellowtail snapper *Ocyurus chrysurus* male) (Domeier and Clarke 1992, Luftus 1992). Examples of natural hybridization are not uncommon among fishes, especially those that are closely related and share habitats (Hubbs 1955). In aquaculture, hybridization has long been considered an option in genetic improvement programs whose goals are to improve fish size, disease resistance, nutritional efficiency, or survival within

a short period of time (Hallerman 1994). In this study, the production of hybrid snapper embryos and larvae was useful in evaluating fertilization, hatching, and larval rearing success. Hybrid snapper larvae had greater survival than red snapper larvae; however, the few red snapper larvae that survived were significantly larger at 10 d. Survival rates were low for both red snapper and hybrid snapper larvae (less than 2.5%), but growth rates for fish reared in this study were higher than growth rates for larvae of similar cultured species of snapper (Lutjanidae) (Table 3.5).

The use of cryopreserved sperm in artificial spawning of red snapper improved efficiency in the hatchery. With frozen sperm, it was possible to focus efforts on monitoring oocyte maturation and timing ovulation in female broodstock. In fertilization of stripped eggs, several straws could be thawed quickly (7 s) and applied to batches of eggs. Also, because cryopreserved sperm could be stockpiled for use in the hatchery, efforts could be focused on the collection of all-female broodstock.

Optimizing sperm concentrations in fertilization trials would permit the repeated use of valuable or high quality males in the hatchery. In this study, the dilution of sperm concentrations from the control samples resulted in significantly lower rates of fertilization. Future studies should optimize fertilization of red snapper eggs with sperm concentrations ranging from 9.5 x 10^7 to 8.5 x 10^8 cells/mL.

The lack of fertilization of red snapper eggs stored for 60 min revealed the necessity to refine methods to predict ovulation and the need to improve efficiency throughout the artificial spawning process. Although data were collected only for spawns of two fish, the results show timing is essential and sperm treatments must be applied immediately after eggs are collected from a female. The use of traditional "dry" methods for fertilization of red snapper eggs should be evaluated in future studies considering the significantly higher results that were obtained when eggs were placed in seawater after stripping. Although the dry method is the most

Table 3.5. Summary of larval survival and growth of cultured snapper species (genus *Lutjanus*) to 10 d after hatch.

Common name	Species	Mean total length (mm)	Growth rate ($mm \cdot d^{-1}$)	Rearing Temperature (C)	Survival (%)	Reference
Red snapper	*L. campechanus*	5.5	0.33	29.5	0.0 - 2.0	Present study
Hybrid snapper	*L. campechanus x L. griseus*	4.2	0.20	29.5	0.0 - 2.5	Present study
Mutton snapper	*L. analis*	4.1	0.18	28.7	62.0	Watanabe et al. 1998
Mangrove red snapper	*L. argentimaculatus*	3.5	0.11	28.0	***	Emata et al. 1994
Mangrove red snapper	*L. argentimaculatus*	2.6	0.04	30.0	0.0 - 10.0	Singhagraiwain and Doi 1993

commonly practiced technique today, this method could be stressful on eggs such as those from red snapper, which are small (800 μ), delicate and planktonic. In the field these eggs are suspended in water out of contact with other objects. Stripping eggs into seawater or an embryo medium would cushion the eggs and protect them from temperature changes and desiccation, although more experiments are needed to confirm these observations.

The techniques developed in this study were designed to improve methods for artificial spawning of red snapper. The techniques included practical methods that hatchery managers can use to collect high-quality broodstock, to preserve sperm, and to collect and incubate eggs. The results of this research demonstrate that the culture of red snapper is feasible; however, sophisticated hatcheries with high quality seawater, a reliable supply of larval foods, and a dedicated staff are essential for rearing of red snapper larvae.

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Chapter 4

Field Collection, Handling, and Refrigerated Storage of Sperm of Red Snapper *Lutjanus campechanus* and Gray Snapper *Lutjanus griseus*

Of the 17 genera in the family Lutjanidae, only one, *Lutjanus*, has distributions of species in the Atlantic, Pacific, and Indian oceans. In the tropical and subtropical regions of these oceans, almost all species of snapper are exploited because they are highly regarded as food and game fish (Pauly et al. 1996). Increasing demand for snapper in domestic and foreign seafood markets, high market values, and limited harvests from wild stocks have stimulated interest in the culture of several snapper species. In the southwestern Atlantic and Gulf of Mexico, the red snapper *Lutjanus campechanus* and gray snapper *Lutjanus griseus* compose a major portion of the sport and commercial snapper fishery. Because of their high market value and limited commercial harvest (Figure 4.1), red snapper and gray snapper have received considerable attention as potential candidates for marine aquaculture and stock enhancement programs.

Red snapper and gray snapper have ranges extending from the Yucatan peninsula to Massachusetts (Stark and Schroeder 1971); however, both species are primarily distributed in the subtropical waters south of Cape Hatteras, North Carolina to Florida and throughout the Gulf of Mexico (Moran 1988; Bortone and Williams 1986). Red snapper are a moderate-sized snapper that grow to 25 kg and 955 mm total length with individuals living for more than 50 years (Wilson et al. 1994). In contrast, gray snapper are smaller and can grow to 8 kg and 890 mm total length with individuals living for more than 20 years (Manooch and Matheson 1981; Burton 2001). Adult red snapper and gray snapper are commonly found together in offshore waters at depths of 15 to 110 m around rocky outcrops, coral reefs, shipwrecks, and oil and gas platforms (Shipp 1986). While spawning behavior in natural aggregations has not been documented for either species, both spawn repeatedly offshore along the continental shelf during the summer and fall months (May to October). The eggs are typical of Lutjanids and are small (0.80 - 0.90 mm),

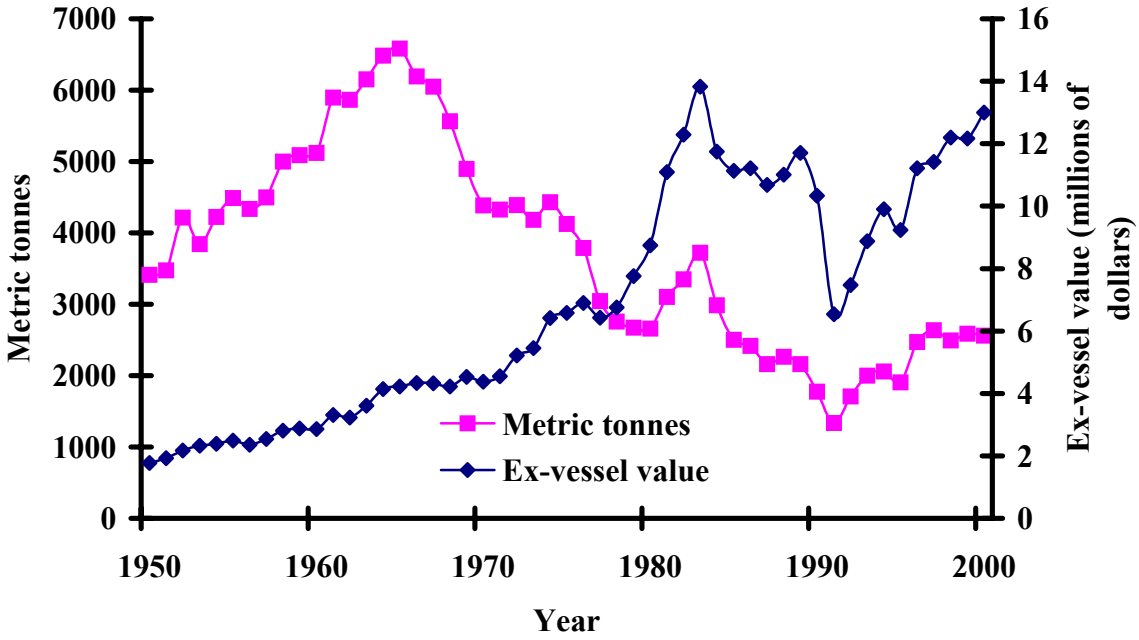

Figure 4.1. Commercial landings for red snapper and gray snapper caught in United States have significantly declined through the last 30 years. Also during this time consumer demand and declining availability have led to higher prices paid dockside and in seafood markets. Data were extracted from the commercial fishery data summary program for the Fisheries Statistics & Economics Division of the National Marine Fisheries Service (NMFS 2002).

spherical, and buoyant (Grimes 1987). Larvae are undeveloped at hatch and feed on yolk reserves until first feeding 2 to 3 days after hatching. While red snapper juveniles recruit to benthic, structured habitat along inner continental shelf areas, gray snapper juveniles recruit to a variety of inshore estuarine habitats including tidal creeks, seagrass beds, and mangroves (Miller and Richards 1980). Adult gray snapper move offshore in search of more abundant, larger prey and to prepare for spawning (Rutherford et al. 1983).

For the past 25 years, efforts have been underway to develop hatchery techniques to culture red snapper and gray snapper. The use of photoperiod and temperature manipulation or injections of gonadotropic hormones has yielded limited success for either species in producing natural spawning in tanks or ponds spawns (Arnold et al. 1978; Rosas et al. 1997). To date, injections of human chorionic gonaodtropin (500-1000 I U per kg body weight) with mature red

snapper or gray snapper has been the most reliable method for induction of ovulation and production of eggs that can be stripped and fertilized with sperm (Watanabe 2001).

As part of a larger study to improve artificial spawning techniques for red snapper and gray snapper, procedures were developed for the collection, handling, and refrigerated storage of sperm. Collection and storage of sperm is useful for genetic improvement through 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 collection of testis and sperm from fish caught in the recreational snapper fishery; 2) determine the osmotic pressures of blood and seminal fluid; 3) establish the relationship between sperm motility and osmotic pressure to allow for safe storage; 4) evaluate the refrigerated storage of sperm extended in Hanks' balanced salt solution at various concentrations, and 5) evaluate the refrigerated storage of sperm with the addition of an antibiotic/antimycotic cocktail. The motility of sperm in samples was used as a measure of sperm viability. This is the first published report on the collection and refrigerated storage of red snapper and gray snapper sperm.

Methods

Collection of Fish, Testes, and Sperm

During the 2000 and 2001 spawning seasons (May to August), live male red snapper and gray snapper were used for collection of blood and seminal fluid. Fish were collected by hook and line sampling off coastal Louisiana (~50 km). All sperm used in this study were obtained by the removal of testes from dead red snapper and gray snapper collected by recreational fishers on charter boats off coastal Louisiana. Fish were collected by conventional hook and line techniques, and after capture, fish were placed in insulated coolers with ice and transported to coastal marinas for cleaning. Fish were sampled within 6 h of capture and before they were cleaned and filleted for customers.

Red snapper and gray snapper males were measured in length, blotted to avoid contamination of samples, and when possible, a digital thermometer (model PS100A, Baxter, Deerfield, Illinois) was inserted into the abdomen of the donor fish to assess core body temperature. Sperm were collected by surgical removal of the testes. The testes were placed in 4-L Ziplock® freezer bags (S. C. Johnson and Son, Inc., Racine, Wisconsin) and Hanks' balanced salt solution (HBSS) was 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 immediately transported to the LUMCON hatchery (1 km) where the testes were removed from the bags, blotted, weighed, and prepared for sperm collection. The total time from the collection of testes in the field to preparation of sperm in the laboratory was less than 60 min.

Table 4.1. Concentration of ingredients (g/L) for calcium-free Hanks' balanced salt solution prepared at various osmolalities using distilled water. Prior to use, osmolalities of the solutions prepared were verified using a vapor pressure osmometer (model 5500, Wescor Inc., Logan, Utah) and diluted if necessary.

Ingredient	**200 mOsmol/kg**	**300 mOsmol/kg**	**400 mOsmol/kg**
NaCl	5.26	8.00	10.53
KCl	0.26	0.40	0.53
$MgSO_4 \bullet 7H_2O$	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

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 the samples was measured with a vapor pressure osmometer (model 5500, Wescor Corp., Logan, Utah). The salinity of the surface water from the collection site was 32 to 35 ppt with an osmolality ranging from 800 to 900 mOsmol/kg. Blood (0.5-2.0 mL) was sampled from the caudal vessel of fish by syringe, placed in 3-mL Vacutainers® (Becton, Dickinson and Company, Franklin Lakes, New Jersey), and allowed to clot. Blood plasma (200 µl) was pipeted into labeled 1.8-mL plastic centrifuge tubes and refrigerated at 4 C until analyzed. After blood was collected, the fish were killed and testes surgically removed for collection of sperm. Testes were sliced to release sperm and fluids, which were collected in 50-ml plastic centrifuge tubes. Undiluted sperm were drawn into 75-µL microhematocrit tubes and seminal plasma was obtained by centrifugation (7000 revolutions/min for 10 min). After centrifugation, the osmolality of 10 µL of seminal plasma was measured.

Preparation of Hanks' Balanced Salt Solution

Hanks' balanced salt solution is a physiological solution commonly used to dilute sperm for refrigerated storage (Wayman et al. 1996). Extender solutions were prepared at an osmolality that would not activate sperm cells. Hanks' balanced salt solution was prepared without calcium at 200, 300, and 400 mOsmol/kg (Table 4.1). Typically extender solutions were prepared in large volumes (8 L), filtered through a 0.22-µm filter (Gelman Sciences, Ann Arbor, Michigan, USA), and frozen in sterile 1-L Nalgene® bottles (Nalge Nunc Inc., Rochester, New York) until used in experiments.

Estimation of sperm motility

The percent motility of each sperm sample was estimated using darkfield microscopy at 200-x magnification. Motility was determined by the percentage of sperm actively moving forward. Activation of sperm was initiated by placing 2 µl of sperm onto a microscope slide and diluting it with 20 µl of activating solution. Except for studies evaluating the effects on sperm activation, the activating solution used was artificial seawater (ASW) (Marinemix®, Wiegandt GmbH Inc., Krefeld, Germany) prepared at 870 mOsmol/kg.

Motility Characterization

Marine fish sperm are not typically motile before addition of an activating solution or natural seawater (35 ppt). Sperm motility from 5 red snapper and 6 gray snapper was characterized by activating with 870 mOsmol/kg ASW. All sperm samples used exhibited greater than 95% motility after activation. In order to characterize each sample, motility was separated into three periods: time to reach maximum motility, duration of motility, and the time until all motility ceased.

Osmotic Analysis of Sperm Activation

In order to assess the relationship between osmotic pressure and sperm activation, undiluted sperm were collected from 13 red snapper and 10 gray snapper in June through August, 2000. Activating solutions were prepared at osmotic pressures ranging from 16 to 870 mOsmol/kg by mixing distilled water with Marinemix ® artificial sea salt. Activation of sperm was initiated by placing 2 µl of sperm onto a microscope slide and diluting it 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 by 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 Experiments

Experiment 1: Refrigerated Storage of Red Snapper and Gray Snapper Sperm

During May, June, July, and August, 2000, testes from red snapper and gray snapper were used for the collection of sperm for refrigerated storage experiments. After weighing the testes, sperm were collected by either slicing the testes to release sperm cells or by crushing the testes in a 4-L Ziplock® freezer bag. Sperm obtained from sliced testes were stripped into 50-ml plastic centrifuge tubes and diluted 1:3 (v:v) with calcium-free HBSS prepared at 200, 300, or 400 mOsmol/kg. Testes used for collection of sperm by crushing were placed in a Ziplock® bag with 1 mL of 200, 300, or 400 mOsmol/kg HBSS added per g of testes. After the testes were crushed, the 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 and samples in excess of 25 mL were discarded. Sperm were stored in a refrigerator at 4 C in loosely capped 50-mL tubes. The osmotic effects of refrigerated storage were evaluated by daily estimates of sperm motility that continued until sperm no longer exhibited motility.

Experiment 2: Refrigerated Storage of Red Snapper Sperm with Antibiotics and Antimycotic

During the summer of 2001, testes from 16 red snapper were used for the collection of sperm used in experiments evaluating refrigerated storage with the addition of antibiotics and an antimycotic. The antibiotic/antimycotic cocktail (A/AC) selected for use was a commercially prepared solution that contained two antibiotics and an antimycotic (Product No. A-7292; Sigma Chemical Co., St. Louis, Missouri). The A/AC was formulated to contain 10,000 units of penicillin, 10 mg of streptomycin, and 25 µg of amphotericin per mL in 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 with 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-mOsmol/kg HBSS. Sperm suspensions from each fish were divided into three aliquots of 10 mL and placed into labeled 50-ml centrifuge tubes without A/AC, with 0.1% A/AC, or with 1% A/AC. Sperm were stored in a refrigerator at 4 C in loosely capped 50-mL tubes. Sperm motilities were checked daily until all sperm exhibited no motility.

Statistical Analysis

Percentage motility values were arcsine-square root transformed before statistical analysis. Blood and seminal plasma osmotic pressures were compared using a Student's t-test assuming equal variances (Microsoft Excel 2000, 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 (Microsoft Excel 2000). In the first refrigerated storage experiment (summer 2000), sperm motility was evaluated over time using a repeated measures analysis of variance (ANOVA) (SAS 8.0; SAS Institute, Cary, NC) to determine the effects of osmolality on refrigerated storage time. In the second refrigerated storage experiment (summer 2001), sperm motility was evaluated over time using a repeated measures ANOVA (SAS 8.0) to determine if A/AC affected refrigerated storage time. For all tests, Duncan's multiple range test was used to determine if significant differences existed among treatment means. Differences were considered significant at $P \leq 0.05$.

Results

Collection of Fish, Testes, and Sperm

Sampling of sperm from fish collected in the recreational fishery was an effective method for obtaining viable red snapper and gray snapper sperm. During the 2-year study, testes were

collected from 199 red snapper and 83 gray snapper. The red snapper ranged in size from 39 to 84 cm in total length with testes ranging from 1 to 398 g. The gray snapper ranged in size from 22 to 71 cm in total length with testes ranging from 16 to 374 g. For both species, testes size was variable among similar–sized fish throughout the sampling period (Figure 4.2). Sperm were not present in approximately 10% of the testes collected. Although there were slight differences in the vascularization and organization of testes from red snapper and gray snapper, testes from both species could be classified in four distinct categories: 1) testes clear, less than 15 mm wide, ⅓ to ½ of the length of body cavity, sperm fluids not visible; 2) testes pinkish to white, opaque, 15 to 30 mm wide, ½ to ¾ length of body cavity, fatty tissues present adjacent to testes; 3) testes white, 30 to 50 mm wide, ¾ length of body, large fatty tissues adjacent to testes, sperm present but not flowing, or 4) testes white, greater than 50 mm wide, ¾ length of body, sperm flows easily.

The quality of the fish collected at the marinas sampled was high because of the standard icing practices used by charter captains to ensure a quality product for their clients. After capture, fish were placed under ice within an insulated cooler, and ice was replenished throughout the day to prevent spoilage. The core body temperature of 40 red snapper sampled in July and August 2001 was 11 ± 5 C with a range of 2 to 25 C. Air temperatures during the collection period averaged 32 C. No statistics were used to correlate sperm motility (i.e. quality) with core body temperature because of the relatively high level of motility (82 ± 27%) in most sperm samples collected.

Blood and Seminal Plasma Osmolality

Blood plasma osmolality was 440 ± 7 mOsmol/kg (mean ± SD) for red snapper and 421 ± 7 mOsmol/kg for gray snapper. Seminal plasma osmolality was 428 ± 15 mOsmol/kg for red snapper and 411 ± 5 mOsmol/kg for gray snapper. The osmolality of red snapper blood plasma

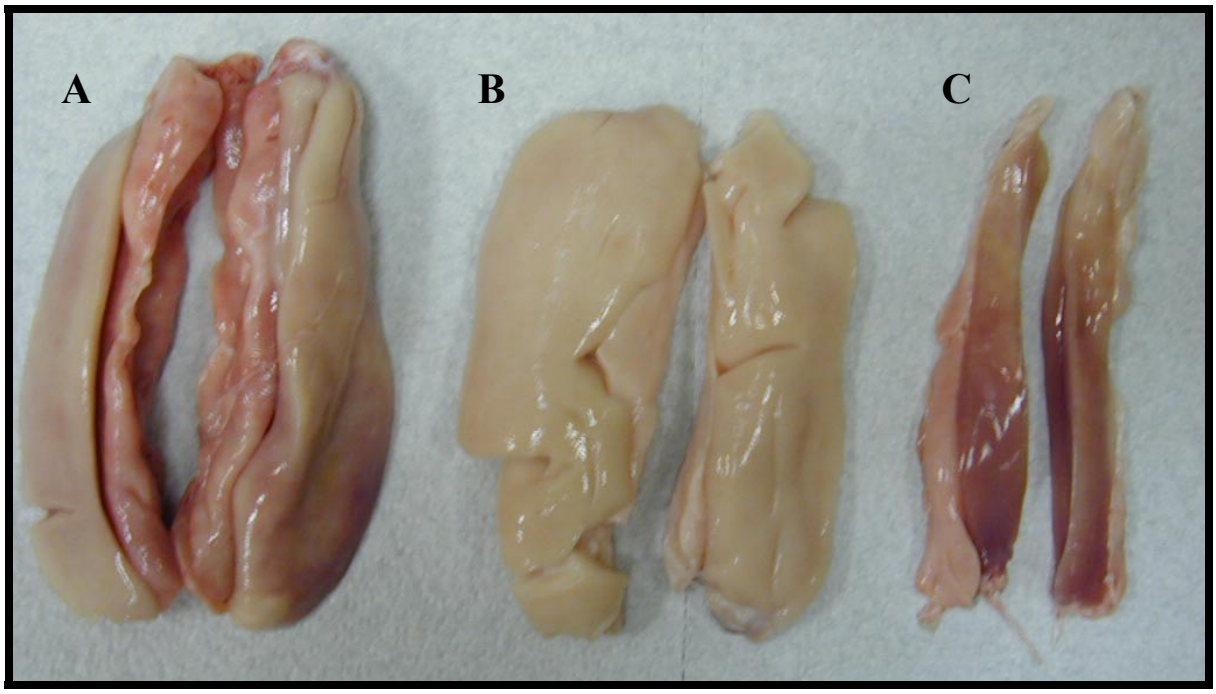

Figure 4.2. During the peak-spawning season (July-August), similar-sized red snapper exhibited testes in various conditions. These testes were collected from red snapper (46 cm total length) in August 2001. While sperm were easily collected from samples A and B, no sperm were obtained from sample C.

Table 4.2. Important osmolalities (mOsmol/kg) relative to red snapper and gray snapper. Experiments determining blood and seminal plasma osmolalities and studies on sperm activation did not represent the same fish.

Species	Blood plasma	Seminal plasma	Threshold activation	Complete activation
Red Snapper	440 ± 7	428 ± 15	439 ± 44	742 ± 58
Gray Snapper	421 ± 7	411 ± 5	486 ± 30	861 ± 39

and seminal plasma was not significantly different ($P = 0.47$). The osmolality of gray snapper blood plasma and seminal plasma was not significantly different ($P = 0.56$). Significant differences were found between the blood and seminal plasma of the two species ($P < 0.001$) (Table 4.2).

Motility Characterization

Sperm began swimming vigorously when activated with 870 mOsmol/kg ASW and motility was characterized in 3 periods (Figure 4.3). After activation, sperm reached maximum motility immediately and sustained maximum motility for 60 ± 9 sec. There was no significant difference in the time to reach maximum motility or duration of maximum motility in sperm of red snapper or gray snapper ($P = 0.32$).

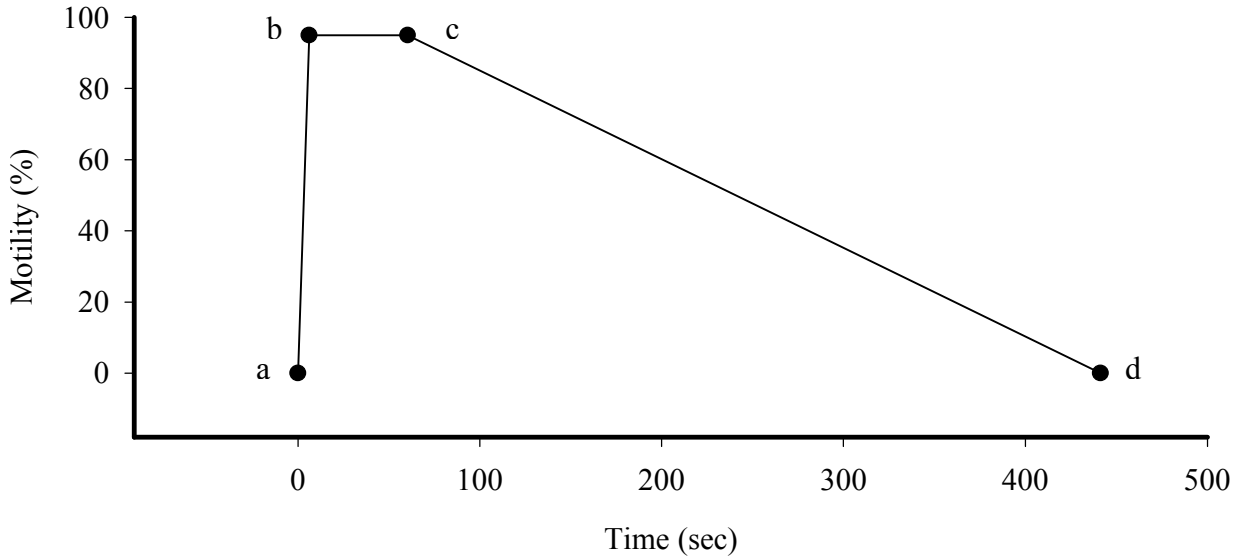

Figure 4.3. Motility characterization of red snapper and gray snapper sperm activated with 870 mOsmol/kg artificial seawater. Motility was characterized in three intervals: 1) time to reach maximum motility (segment a-b); 2) duration of maximum motility(b-c), and 3) time until all motility ceased (c-d). Each point represents the mean of samples from 5 red snapper and 6 gray snapper. There was no significant difference in the criteria used to characterize red snapper and gray snapper sperm.

Osmotic Analysis of Sperm Activation

Initial motility of all sperm used in experiments was 95%. The osmolality that initiated activation was 407 ± 48 mOsmol/kg in red snapper and 437 ± 15 in gray snapper. Threshold activation for red snapper sperm occurred at 439 ± 44 mOsmol/kg with complete activation at 742 ± 58 mOsmol/kg. Similar values were seen in gray snapper sperm with threshold activation at 486 ± 30 mOsmol/kg and complete activation at 861 ± 39 mOsmol/kg. Sperm activation for red snapper and gray snapper were highly correlated ($r = 0.99$). For both species, sperm motility increased as the osmolality of ASW increased (Figure 4.4) and the osmolality values at complete activation were significantly higher than the threshold activation values ($P < 0.0001$).

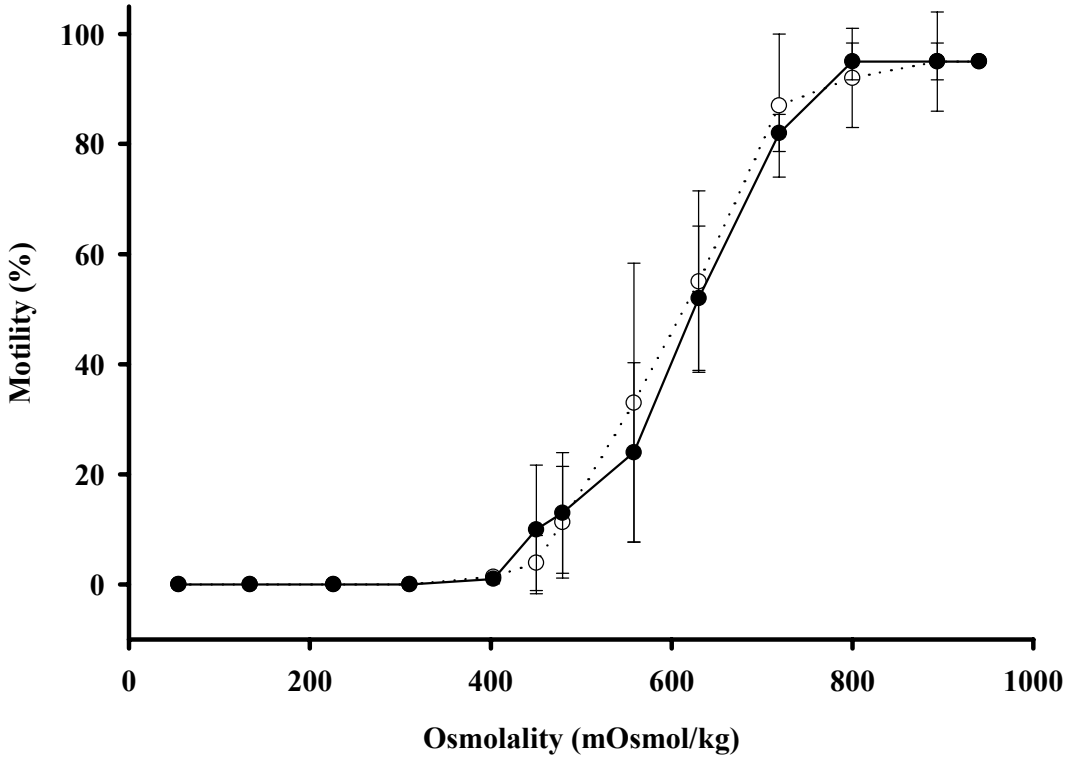

Figure 4.4. Percent motility of red snapper (filled circles) and gray snapper (open circles) sperm at various osmolalities of artificial seawater. Sperm activation for red snapper and gray snapper were highly correlated ($r = 0.99$). Each point represents the mean of samples from 13 red snapper and 10 gray snapper.

Refrigerated Storage Experiments

Experiment 1: Refrigerated Storage of Red Snapper and Gray Snapper Sperm

Red snapper sperm from sliced testes of 14 fish and crushed testes of 11 fish were used for refrigerated storage experiments. Storage times for sperm collected from sliced testes and crushed testes were significantly different ($P < 0.0001$), with sperm from sliced testes retaining motility for 9 d and crushed sperm retaining motility for 7 d. There were no significant differences in the motilities of red snapper sperm from crushed or sliced testes on the day of collection ($P = 0.88$). However, all sperm exhibited significant declines in motility daily ($P < 0.0001$). Sperm from sliced testes retained motility for 9 d when stored in 200 mOsmol/kg HBSS; 5 d when stored in 300 mOsmol/kg HBSS, and 4 d in 400 mOsmol/kg HBSS (Figure 4.5). Sperm from crushed testes retained motility for 7 d when stored in 200 mOsmol/kg HBSS; 6 d when stored in 300 mOsmol/kg HBSS, and 4 d in 400 mOsmol/kg HBSS (Figure 4.6).

Gray snapper sperm from sliced testes of 16 fish and crushed testes of 13 fish were used for refrigerated storage experiments. The refrigerated storage of sperm collected from sliced testes and crushed testes were not significantly different ($P = 0.21$). In all samples, sperm exhibited significant losses in motility each day ($P < 0.0001$). Sperm from sliced testes retained motility for 6 d when stored in 200 mOsmol/kg HBSS; 5 d when stored in 300 mOsmol/kg HBSS, and 4 d in 400 mOsmol/kg HBSS (Figure 4.7). Sperm from crushed testes retained motility for 6 d when stored in 200 mOsmol/kg HBSS; 5 d when stored in 300 mOsmol/kg HBSS, and 3 d in 400 mOsmol/kg HBSS (Figure 4.8).

Red snapper sperm was stored for a significantly longer period of time than gray snapper sperm ($P < 0.0001$); although sperm from both species exhibited motilities less than 50% after 4 d. The collection of sperm by slicing testes resulted in sperm that could be stored for the longest

period of time, and storage times were optimal when sperm were diluted with 200 mOsmol/kg HBSS.

Experiment 2: Refrigerated Storage of Red Snapper Sperm with Antibiotics and Antimycotic

The addition of the A/AC did not improve storage of red snapper sperm. Red snapper sperm diluted with 200 mOsmol/kg HBSS with and without the addition of A/AC retained motility for 9 d (Figure 4.9). There was no significant difference in storage duration or motility of sperm in samples with or without A/AC ($P = 0.93$).

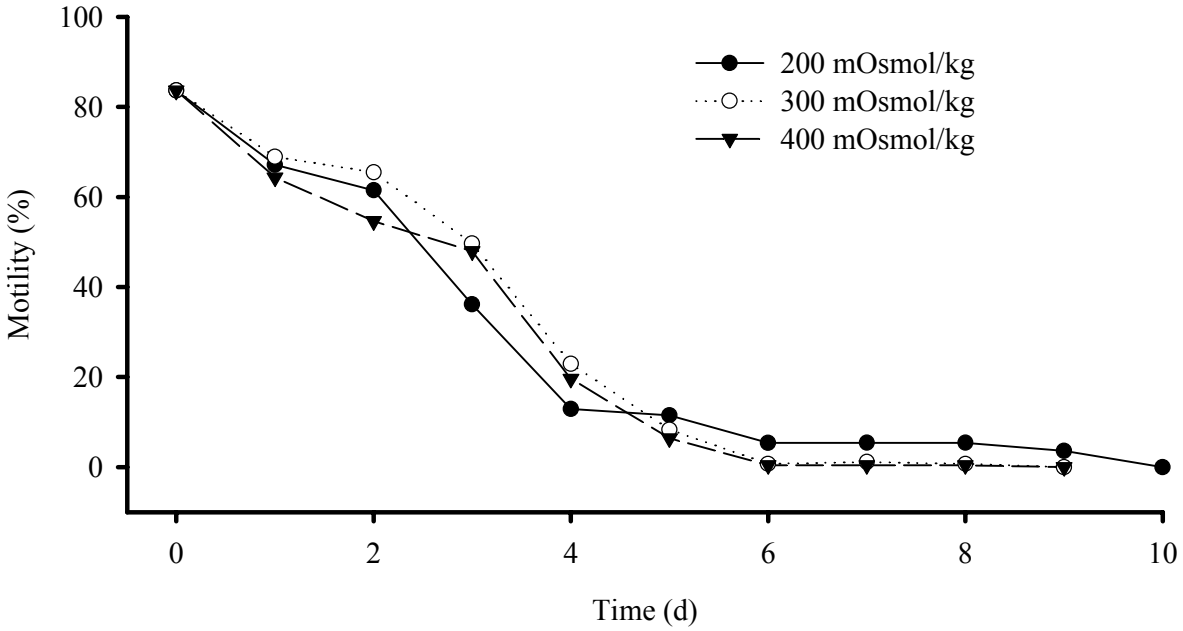

Figure 4.5. Refrigerated storage time of sperm stripped from the testes of 14 red snapper. Sperm were diluted 1:3 (v:v) with various concentrations of Hanks' balanced salt solution. There was no significant difference in daily motility values among the extender solutions; although, sperm stored in 200 mOsmol/kg Hanks' balanced salt solution retained motility for 9 d.

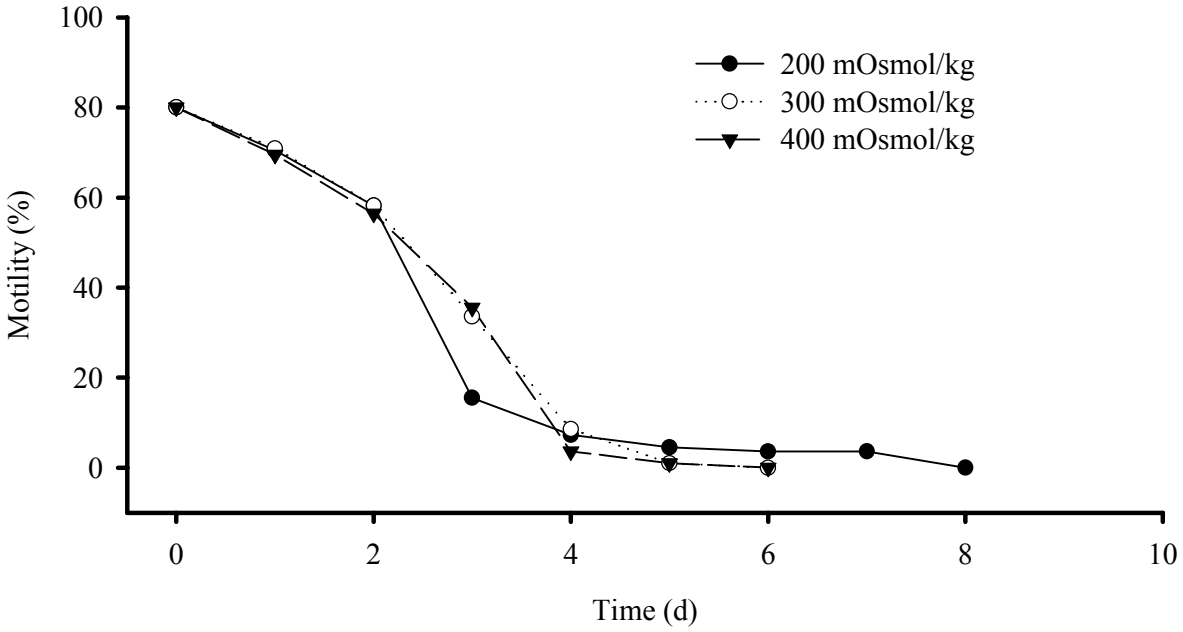

Figure 4.6. Refrigerated storage time of sperm from 11 red snapper. Sperm were obtained by crushing testes suspended in Hanks' balanced salt solution prepared at 200, 300, or 400 mOsmol/kg. While no extender solution resulted in significantly higher sperm motilities, sperm stored in 200 mOsmol/kg Hanks' balanced salt solution retained motility for 7 d.

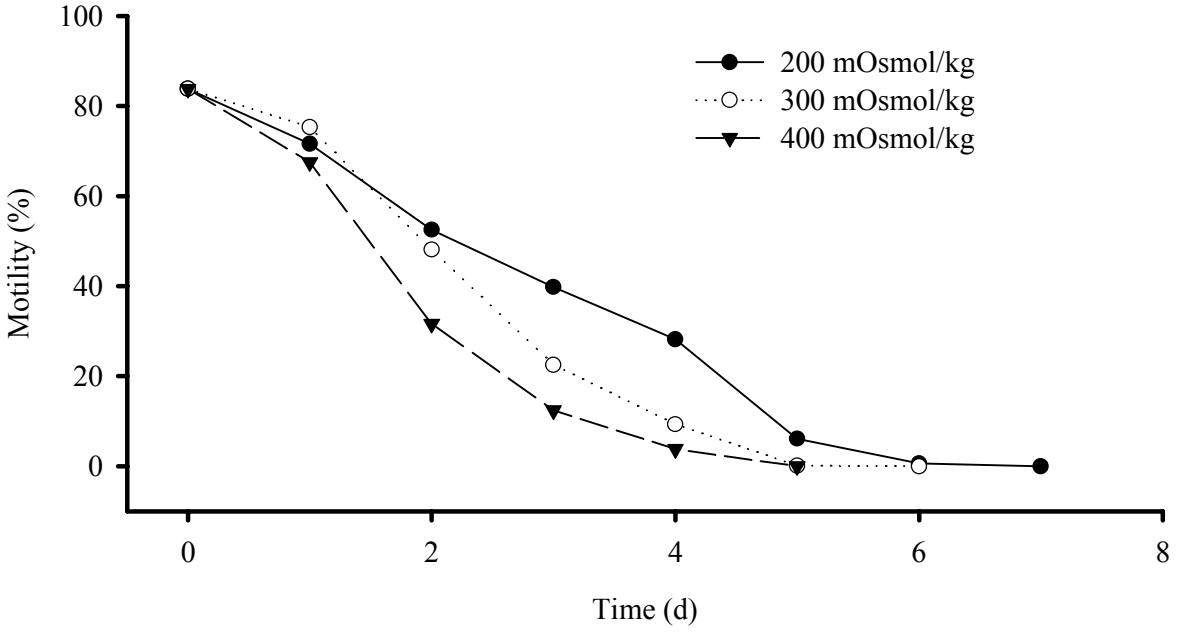

Figure 4.7. Refrigerated storage time of sperm stripped from the testes of 16 gray snapper. Sperm were diluted with various concentrations of Hanks' balanced salt solution. In each extender solution, sperm exhibited significant losses in motility daily ($P < 0.0001$). Sperm diluted with 200 mOsmol/kg Hanks' balanced salt solution retained motility for 7 d.

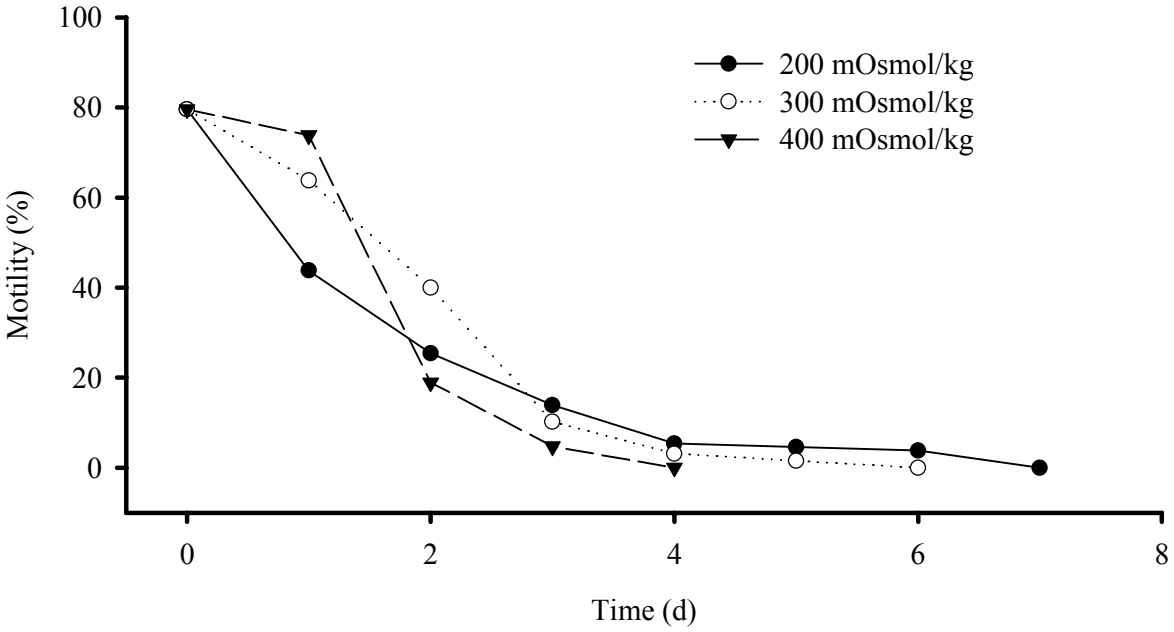

Figure 4.8. Refrigerated storage time of sperm from 13 gray snapper. Sperm were obtained by crushing testes suspended in various concentrations of Hanks' balanced salt solution. Sperm stored in 300 and 400 mOsmol/kg Hanks' balanced salt solution retained significantly higher motilities for the first 2 d ($P = 0.0001$), however, sperm stored in 200 mOsmol/kg retained motility longer than the other extender solutions.

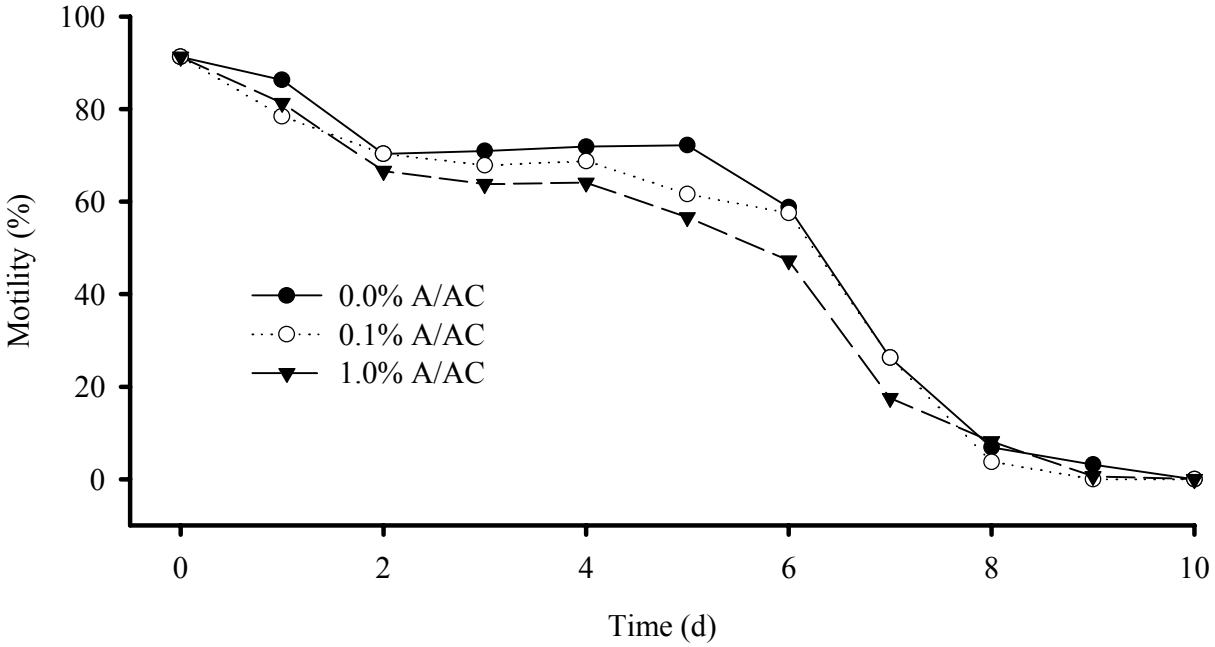

Figure 4.9. Motility of red snapper sperm diluted with 200 mOsmol/kg Hanks' balanced salt solution with and without the addition of antibiotic/antimycotic cocktail (A/AC) during refrigerated storage (4 C). There was no significant difference in storage duration or motility of sperm in samples with or without A/AC ($P = 0.93$). Each point represents the mean of 16 fish.

Discussion

Use of refrigerated sperm during the strip spawning process allows efforts to be focused on maintaining female broodstock, monitoring ovarian development, and increasing efficiency. In this study, techniques were developed for the short-term storage of sperm, which allows repeated use of high-quality males for production of larvae. The results suggest that red snapper and gray snapper sperm can be stored in a refrigerator at 4 C for six to ten days. Furthermore, the techniques developed would facilitate shipping of sperm samples, hybridization and genetic studies, and conservation of sperm of endangered species.

Development of collection, handling and storage techniques requires adequate knowledge and understanding of the characteristics of marine fish sperm. Sperm are typically not motile in the testes or seminal fluid of fish, and during reproduction motility is induced after sperm are released into the water (Grier 1981). Sperm motility is stimulated by the ionic composition, pH, or the osmolality of water (Stoss 1983). Sperm of freshwater fish are activated by suspension in hypotonic solution (Morisawa and Suzuki 1980, Christensen and Tiersch 1996), while sperm of marine fish are activated by suspension in hypertonic solution greater than 400 mOsmol/kg (Morisawa and Suzuki 1980; Gwo et al. 1991; Wayman 1998). Because fish sperm are quiescent while in the seminal plasma within the testes, preparation and use of extender solutions that are similar in chemical concentration and osmolality are essential to 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 (mOsmol/kg) of blood and seminal plasma. Collection of data on the osmolality of fish blood and seminal plasma 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 of both species were highly correlated ($r = 0.73$). The values reported for red snapper (blood plasma 440 mOsmol/kg; seminal plasma 421 mOsmol/kg) and those for gray snapper (blood plasma 428 mOsmol/kg; seminal plasma 411 mOsmol/kg) are similar to the osmolalities reported for blood plasma of other marine species (Table 4.3). Increases in osmotic pressures above that of blood and seminal plasma were associated with the activation of red snapper and gray snapper sperm. Complete activation of snapper sperm diluted with ASW occurred at osmolalities above 700 mOsmol/kg. This value corresponds to the osmolality of the natural waters in which these species spawn (800 – 900 mOsmol/kg).

Most research on the refrigerated storage of sperm has addressed the more commonly cultured freshwater species. In order to evaluate the refrigerated storage of red snapper and gray snapper sperm, 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. Since red snapper and gray snapper sperm become motile at osmolalities above 400 mOsmol/kg, extender solutions should be prepared at or below 400 mOsmol/kg.

Techniques for observing sperm can be highly objective and identification of sperm actively swimming can be confused with cells moving due to dilution on the microscope slide, Brownian movement, or contamination of samples with bacteria. Although care was taken to minimize contamination of samples at the time of collection, bacteria were observed swimming in most samples. Bacteria were identified as any microorganism without tails that were 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, antibiotics and an antimycotic were added to sperm suspended in 200 mOsmol/kg HBSS. At the concentrations tested (0.1% and 1.0%), the addition of the antibiotics and antimycotic did not significantly

Table 4.3. Osmolality of blood plasma from some teleostean fishes in three aquatic environments of various salinity ranges.*

Environment	Salinity (ppt)	Common name	Species	Osmolality (mOsmol/kg)	Reference
Marine	32 - 35			800 - 1100	
		Great barracuda	*Sphyraena barracuda*	476	Becker et al. 1958
		Yellowfin grouper	*Mycteroperca venenosa*	467	Becker et al. 1958
		Black grouper	*Mycteroperca bonasi*	461	Becker et al. 1958
		Anglerfish	*Lophius piscatorius*	452	Evans 1979
		Bluefin tuna	*Thunnus thynnus*	437	Becker et al. 1958
		Goliath grouper	*Promicrops itaiara*	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

*These data were extracted from Hoar and Randall (1969), Evans (1979), and Evans (1993).

improve the storage capacity of red snapper sperm. Future research should examine the effects of different concentrations of antibiotics and antimycotics on sperm motility and growth of bacteria.

The fish caught by recreational fishers on charter boats proved to be a valuable and easily accessible source of sperm for artificial spawning. While motility is a good estimator of sperm quality, 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 sec), care should be taken to ensure prompt and thorough mixing of gametes during artificial spawning of red snapper 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.

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Chapter 5

Cryopreservation of Red Snapper Sperm

The red snapper *Lutjanus campechanus* provides economically valuable sport and commercial fisheries throughout the Gulf of Mexico and Southwestern Atlantic. Within recent years, fishery managers have become concerned with the status of red snapper stocks and the possible effects of overexploitation (Goodyear 1995). These stocks are not only experiencing pressures from fishing, but also problems caused by environmental factors such as hypoxia, pollution, and changing weather patterns (Rabalais et al. 1996; Chesney et al. 2000). The decline of the fisheries combined with a high global demand and high market value have stimulated interest in the development of red snapper for marine aquaculture.

The hatchery production of red snapper could be optimized through the use of cryopreserved sperm during artificial spawning. The use of cryopreserved sperm improves efficiency in artificial spawning and allows for long-term, repeated use in the hatchery. Additional applications 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). To date, cryopreservation protocols have been developed and published for more than 32 species of marine fish (Tiersch 2000). Attempts to cryopreserve marine fish sperm have been more successful than those for freshwater fish (Gwo 2000). Research on cryopreservation of sperm from marine fish has addressed several temperate and subtropical species (Table 5.1).

Table 5.1. Since the successful cryopreservation of Atlantic herring sperm fifty years ago, global efforts have been underway to cryopreserve sperm from a variety of temperate and subtropical species.

Common name	Species	Reference
Atlantic herring	*Clupea harengus*	Blaxter 1953
Atlantic cod	*Gadus morhua*	Mounib et al. 1968
American plaice	*Pleuronectes platessoides*	Pullin 1972
Grey mullet	*Mugil cephalus*	Chao 1982
Bluefin tuna	*Thunnus thynnus*	Doi et al. 1982
Milkfish	*Chanos chanos*	Hara et al. 1982
Black grouper	*Mycteroperca bonaci*	Whithler and Lim 1982
Black porgy	*Acanthopagrus schlegeli*	Chao et al. 1986
Atlantic halibut	*Hippoglossus hippoglossus*	Bolla et al. 1987
Barramundi	*Lates calcarifer*	Leung 1987
Gilthead seabream	*Sparus aurata*	Chambeyron and Zohar 1990
Atlantic croaker	*Micropogonias undulatus*	Gwo et al. 1991
Summer whiting	*Sillago ciliata*	Young et al. 1992
Yellowfin seabream	*Acanthopagrus latus*	Gwo 1994
Cobia	*Rachycentron canadum*	Caylor et al. 1994
Ocean pout	*Macrozoarces americanus*	Yao et al. 1995
Spotted seatrout	*Cynosion nebulosus*	Wayman et al. 1996
Black drum	*Pogonias cromis*	Wayman et al. 1997
Turbot	*Scophthalmus maximus*	Dreanno et al. 1997
Red drum	*Sciaenops ocellatus*	Wayman et al. 1998
Yellowtail flounder	*Pleuronectes ferrugineus*	Richardson et al. 1999

Since the first attempts to cryopreserve sperm for hybridizing spring and autumn herring 50 years ago (Blaxter 1953), several methods have been developed for cryopreservation of fish sperm. These methods address protocols for freezing, long-term storage, and thawing of samples. 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).

Similar to the dairy industry, fish sperm that has been collected must be diluted to an optimal concentration with extender solutions, which prolong cell viability by suppressing motility and preventing desiccation and death of sperm cells. Extender solutions selected for use are typically 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) formulated at 200 to 300 mOsmol/Kg has been used successfully in the refrigerated storage and cryopreservation of several marine species (Wayman and Tiersch 2000).

Prior to freezing, permeating and nonpermeating cryoprotectants are 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. Examples of nonpermeating cryoprotectants include sugars, milk and egg proteins, and polymers such as dextran. 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 after exposure at concentrations as low as 5, 10 or 15%. 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-ml 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. In this study, the cryopreservation techniques developed and in practice in the dairy industry were evaluated for use with sperm from red snapper.

The goal of this study was to developed 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 cryopreservation of red snapper sperm with dairy procedures; 3) evaluate fertilization of red snapper eggs with cryopreserved sperm, and 4) evaluate the effects of cryoprotectant concentration on the fertilization of red snapper eggs. To our knowledge this is the first published report on the successful production of red snapper with cryopreserved sperm.

Methods

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 sampling. After capture,

inflated 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 and 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 used in this study were obtained from fish (N = 21) caught on recreational fishing boats during the study period and during preliminary investigations in the summer of 1998.

Collection of Sperm

Males were measured in length and blotted dry to avoid contamination of samples. Sperm were collected by surgical removal of the testes. The testes were placed in 4-L Ziplock® freezer bags (S. C. Johnson and Son, Inc., Racine, Wisconsin) and HBSS was 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, where the testes were removed from the bags, blotted dry, 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 evaluated for percent motility and refrigerated at 4 C until use in cryopreservation experiments.

Estimation of sperm motility

The percent motility of each sperm sample was estimated using darkfield microscopy at 200-x magnification immediately after addition of 870 mOsmol/kg artificial seawater (Marinemix®, Wiegandt GmbH Inc., Krefeld, Germany) used as an activating solution. Motility was determined as the percentage of sperm actively moving forward.

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 Chemical Corp., St. Louis, Missouri) 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 mOmol/kg) 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.

Cryopreservation of Sperm

In order to further examine the effectiveness of DMA, DMSO, and MeOH as cryoprotectants, sperm were cryopreserved with final concentrations of 5% or 10% of each chemical. All sperm samples were cryopreserved at Genex Cooperative, Inc. located at the Louisiana State University T. E. Patrick Dairy Improvement Center in Baton Rouge. In a walk-in cooler held at 5 C, sperm solutions were mixed with 10% DMSO and allowed 20 min to equilibrate before beginning the freezing process. Using an automated straw filler (model MRS 1, IMV Int. Corp., Minneapolis, Minnesota), 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). The samples were placed in the freezing chamber held at –140 C. During the first 3 min of the freezing process, the chamber was allowed to warm from –140 C 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 returning the chamber to –140 C (Figure 5.1) (Chandler et al. 1984). Once frozen, the samples were removed and placed in a liquid nitrogen storage container for sorting and preparation for storage.

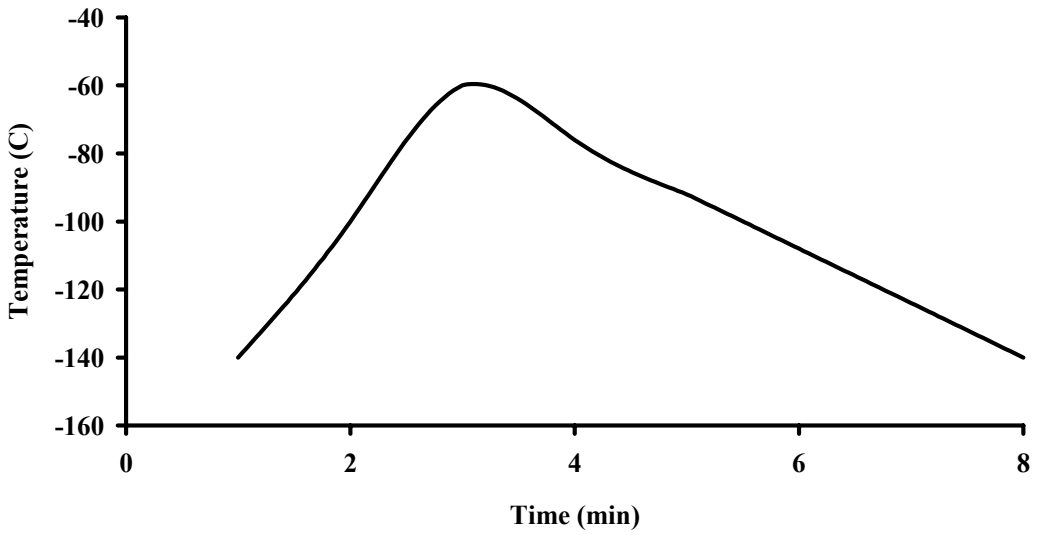

Figure 5.1. Cooling profile of the dairy method of cryopreserving sperm used at Genex Inc. at the T. E. Patrick Dairy Improvement Center of the Louisiana State University Agricultural Center. After the freezing chamber warmed from the heat load of the samples, the chamber was cooled at a rate of 16 C/min. Samples were plunged into liquid nitrogen after 8 min.

Thawing 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 sec. The rest of the cryopreserved sperm samples were held in nitrogen-vapor shipping dewars for use in the LUMCON hatchery and bulk samples were placed into long-term cryogenic storage at Genex Inc.

Hormone Injection, Spawning, and Fertilization

Females were injected with a 500 IU/kg priming dose of human chorionic gonadotripin (HCG) (Chorulon, Intervet, Millsboro, Delaware), tagged with a colored anchor tag (Floy Tag Inc., Seattle, Washington), and placed in a recirculating culture system. Female red snapper were given a 1000 IU/kg resolving dose of HCG 16 h later. The females were monitored for oocyte maturation and were stripped after ovulation. In a series of 2 x 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 roughly 1.0 x 10^9 cells/mL . 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, which was added to 4-L Ziplock® freezer bags and incubated at 30 C. Fertilization was assessed at neurulation (14 h) by use of a dissecting microscope. All samples were preserved in a solution of 5% formalin in buffered seawater.

Long-term Storage of Sperm

In order to evaluate the long-term storage and fertilizing capacity of red snapper sperm, sperm samples cryopreserved in 1998 and 2000 were used for fertilization trials in 2001. The motility of all samples used in fertilization trials were greater than 90%. Samples of 200 eggs were fertilized with 1 ml of fresh or cryopreserved sperm. Ultraviolet sterilized seawater was added to activate sperm and initiate fertilization. Sperm and eggs were then mixed with 1 L of FSW, which was added to 4-L Ziplock® freezer bags and incubated at 30 C. Fertilization was assessed at neurulation (14 h).

Cryoprotectant Effects on Fertilization

Eggs stripped from two female red snapper were evaluated in fertilization 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. 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, which was added to 4-L Ziplock® freezer bags and incubated at 30 C. Fertilization was assessed at neurulation.

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. For all tests, Duncan's multiple range test was used to determine if significant differences existed among treatment means. Differences were considered significant at $P \leq 0.05$.

Results

Cryoprotectant Toxicity Study

Sperm samples from five males collected during the 2000 spawning season were used in the acute toxicity study. The average motility of the samples prior to exposure to cryoprotectants was 95% and during the 60 min experimental period (Figure 5.2). Within 30 min, the motility of samples diluted with 20% and 25% concentrations of all cryoprotectants were reduced below 50% ($P < 0.0001$). This loss of motility was likely due to acute toxic effects of the chemicals on sperm. 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 samples. These chemicals and respective concentrations were thus selected for use in cryopreservation trials.

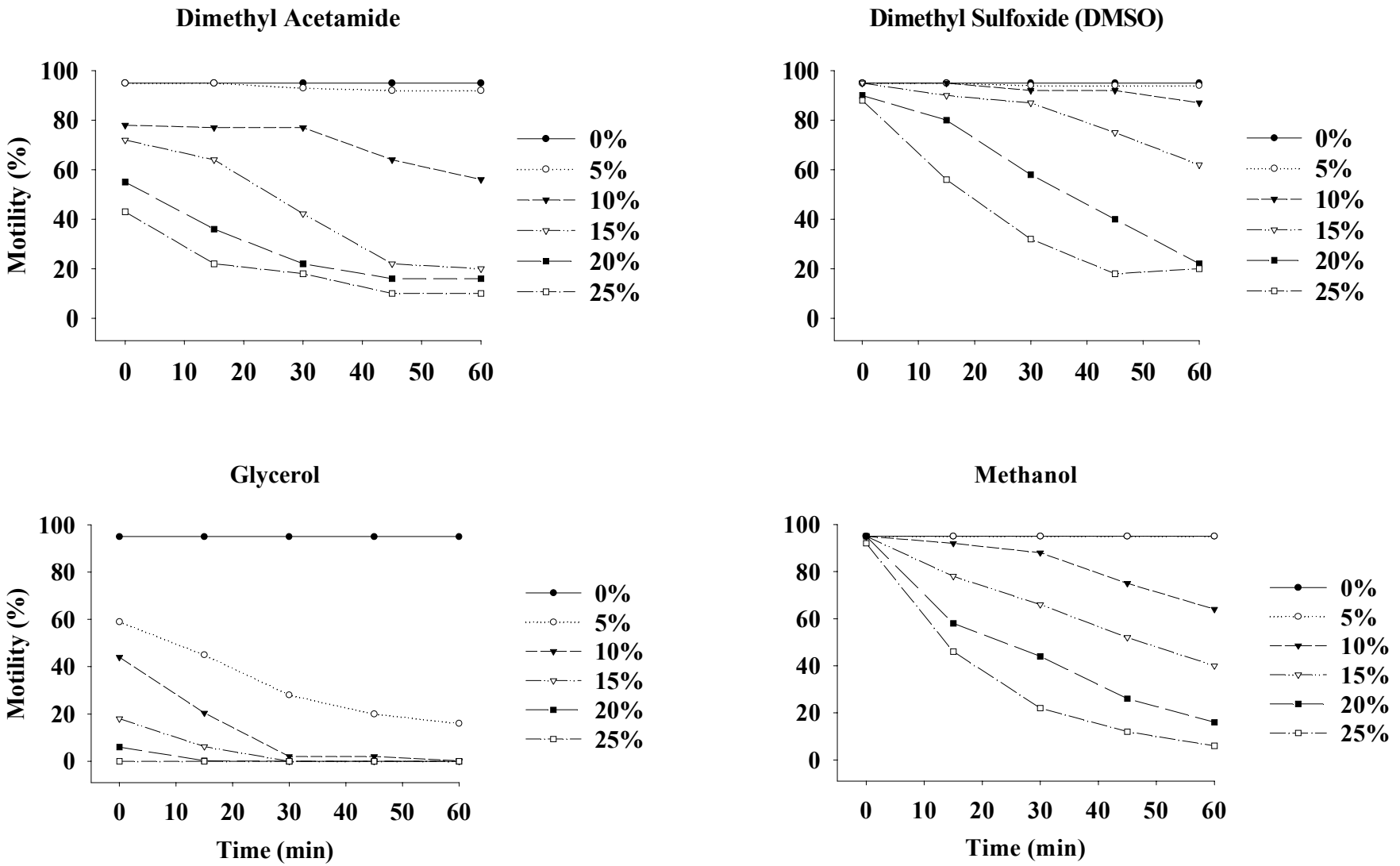

Figure 5.2. Acute toxicity of various concentrations of four cryoprotectants to sperm of red snapper *Lutjanus campechanus*.

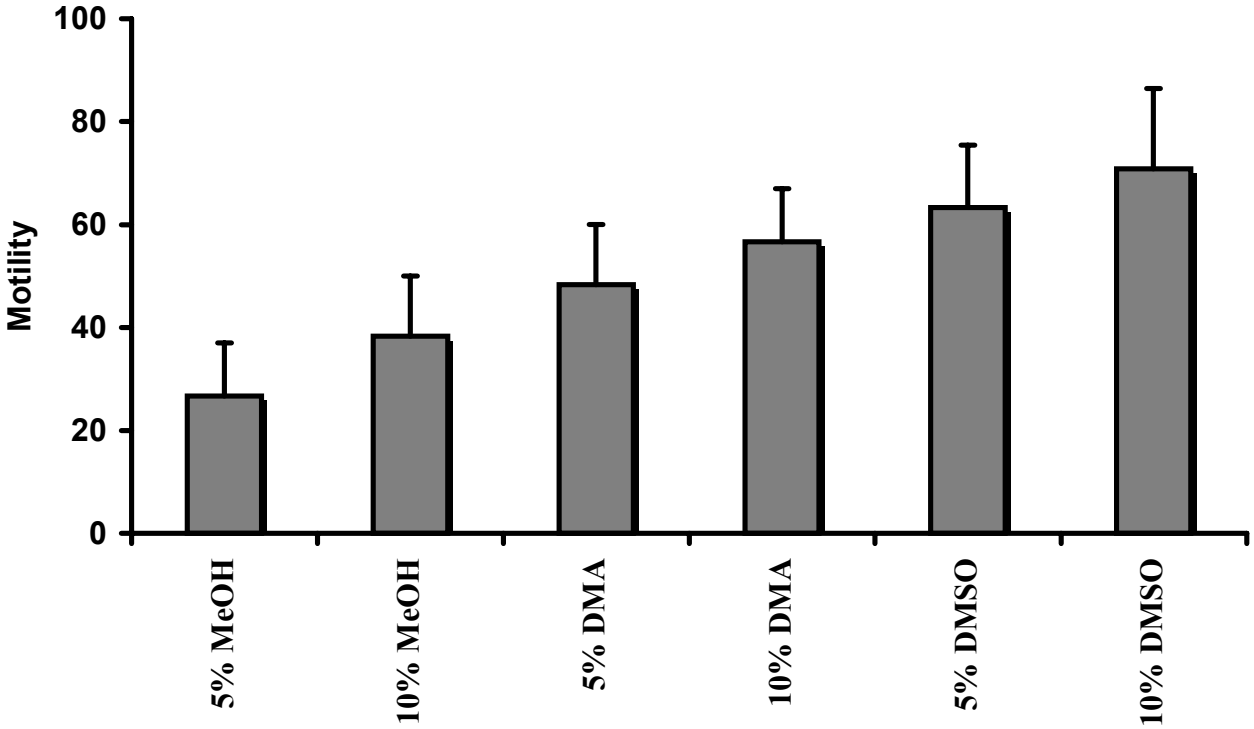

Figure 5.3. Mean post-thaw motility (± SD) of red snapper sperm from the preliminary cryoprotectant evaluation. Sperm samples from six males were frozen in 5% and 10% methanol (MeOH), dimethyl acetamide (DMA), and dimethyl sulfoxide (DMSO). The motility of sperm before addition of chemicals was 93 ± 3%.

Sperm samples from six males were cryopreserved using 5% or 10% DMA, DMSO, or MeOH. The motility of sperm before addition of chemicals was 93 ± 3 %. Ten percent DMSO produced the highest post-thaw sperm motility (71%) of the cryoprotectants studied ($P < 0.0001$) (Figure 5.3). All other chemical treatments except 5% DMSO and 10% DMA yielded post-thaw sperm motilities below 50%.

Cryopreservation of Sperm

During the two-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 average motility of samples before freezing was 90 ± 6% while the average motility of samples after thawing was 80 ± 23%. While few samples experienced a loss of motility below 80% after the freezing and thawing process, comparison with fresh samples revealed a significant difference in motility ($P = 0.048$).

Hormone Injection, Spawning, and Fertilization

Refrigerated and cryopreserved sperm were evaluated in experiments with eggs collected from eight female red snapper in 2000 and three female red snapper in 2001. While fertilization rates were highly variable among females (6% - 89%), results with refrigerated and cryopreserved sperm were highly correlated ($r = 0.85$) (Table 5.2).

Long-term Storage of Sperm

Eggs collected from two females were fertilized with fresh sperm and cryopreserved sperm from males collected in 1998 and 2000. Fertilization rates for female 1 averaged 19% while fertilization rates for female 2 averaged 66% (Figure 5.4). Although no statistical analysis was performed on these data due to the small sample size, cryopreserved sperm yielded fertilization of 60 to 70% comparable to fresh sperm.

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, this difference was not statistically significant (Figure 5.5).

Discussion

In the present study, procedures were developed 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). From our experience, collection of samples at fishing tournaments can provide large volumes of sperm from a diversity of species. During the study period, red snapper males were readily available from marinas and fishing tournaments along coastal Louisiana.

Sperm from red snapper 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).

Table 5.2. Comparison of fertilization of red snapper eggs with fresh and cryopreserved sperm. No attempts were made to standardize the concentrations of refrigerated and cryopreserved sperm samples. While fertilization rates were variable among females, cryopreserved sperm was effective in fertilizing eggs. The low rates of fertilization for some spawns were due to poor egg quality.

Female	Refrigerated	Cryopreserved
Summer 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
Summer 2001		
9	56 ± 8	38 ± 6
10	23 ± 3	17 ± 2
11	20 ± 0	11 ± 6

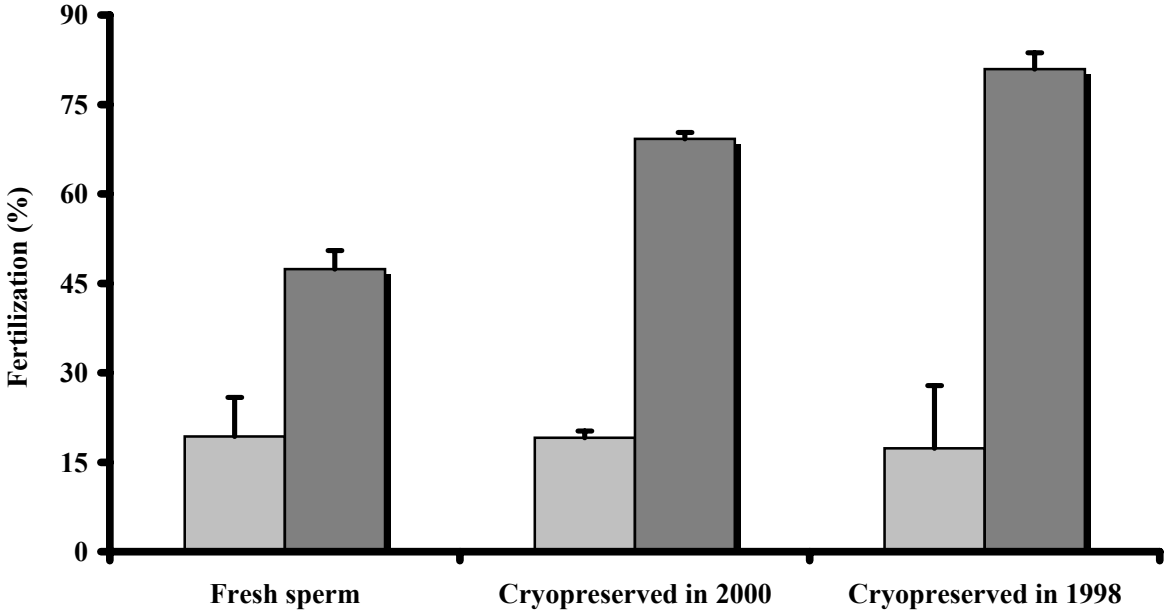

Figure 5.4. Fertilization rates (mean ± SD) of eggs from two females (female 1 = grey bars; female 2 = dark bars) fertilized with fresh and cryopreserved sperm. Sperm samples cryopreserved and stored in liquid nitrogen retained fertilizing ability over 2 years. No statistical comparisons were made due to the small sample size.

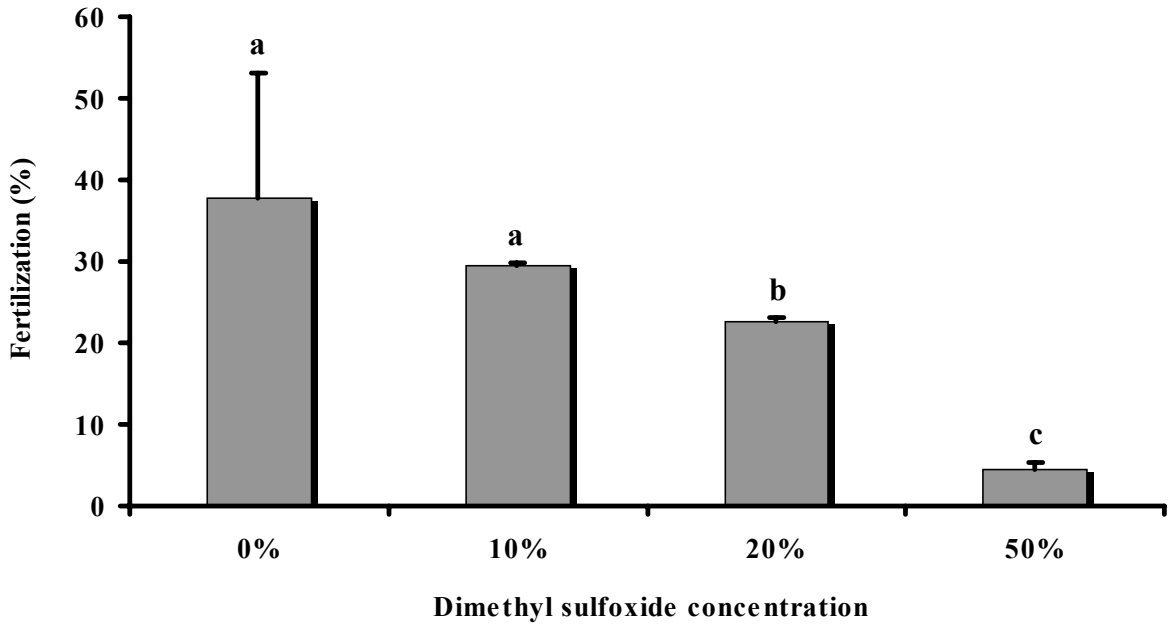

Figure 5.5. Fertilization rates (mean ± SD) of red snapper eggs presented with fresh sperm containing various concentrations of DMSO. Although sperm solutions containing 10% DMSO resulted in lower rates of fertilization than did sperm solutions without DMSO, this difference was not statistically significant. Eggs fertilized with sperm containing 20% and 50% DMSO had significantly lower rates of fertilization ($P < 0.001$).

While there is no universal cryoprotectant and concentration for fish sperm, 10% and 20% DMSO has produced the best post-thaw motility and fertilization results with many 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 prepared 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 was not detrimental in the fertilization of 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 sec) and once thawed, sperm are ready to be applied to eggs. In this study, the use of cryopreserved sperm provided the opportunity to collect a greater number of female broodstock on offshore collecting trips and ensured greater success in spawning of red snapper in the hatchery.

Use of the commercial-scale dairy methods for cryopreserving sperm was efficient for freezing of 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 1500 0.5-ml French cryopreservation straws per hour. If cryopreservation of fish sperm is to be integrated into marine fish hatcheries, the use of commercial dairy cryopreservation centers should be considered as a valuable option.

The use of semen cryopreservation in the dairy industry has resulted from decades of study, refinement and integration. At the heart of any dairy breeding cooperative 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 almost five decades of research on the cryopreservation of fish sperm, the techniques evaluated in this study are among the most practical for the integration and use of the large volumes of sperm needed in marine fish hatcheries. Future studies should evaluate practical considerations such as optimal densities of red snapper sperm needed for freezing, thawing, and use in fertilization of red snapper eggs. Additional studies should also evaluate the long-term survival of larvae and juveniles produced from cryopreserved sperm.

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Chapter 6

Evaluation of Sperm Quality in Red Snapper and Gray Snapper

Securing a consistent supply of good quality eggs and larvae is a major constraint to the development of marine aquaculture. Larvae and fingerlings of many marine species are still harvested from the wild in many parts of the world; however, increasing efforts are focusing on development of methods for spawning of captive broodstocks and rearing of larvae in the marine fish hatchery. Although highly variable egg quality is the largest limiting factor for the fertilization of eggs and mass production of marine fish larvae (Kjørsvick et al. 1990), surprisingly few studies have developed clear, concise criteria for evaluation of sperm quality in fish.

Sperm quality is variable and can depend on factors such as the culture environment (e.g. temperature, salinity, dissolved oxygen), feeding regime and quality of the feed, broodstock condition, individual genetic variability, and the methods for artificial spawning or inducing spermiation. Furthermore, sperm quality can be variable among males or within the same individual (Rana 1995). Methods used for the collection of sperm can significantly affect quality. Stripped sperm can be contaminated with urine, feces, water, or mucus (Dreanno et al. 1998). More rigorous methods for collection of sperm can catheterization of the sperm duct or surgical removal of the testes, often resulting death of the male. Immediately after collection, sperm should be evaluated for quality and application. Current procedures for assessing sperm quality in fish are time-consuming, subjective, variable among species, and lack repeatability.

Estimation of sperm motility is the most commonly used method to assess sperm quality and sperm viability (Stoss and Holtz 1983). Motility estimates are measurements of the percentage of motile cells within a sample (Stoss 1983), the total duration of sperm movement (Baynes et al. 1981), or a combination of these parameters. Sperm cells that are motile can be observed by use

of 100-x or 200-x magnification and dark-field microscopy with standard microscope slides or a hemacytometer (Stoss 1983). While techniques for observing the motility of fish sperm are simple, inexperienced personnel can mistake microscopic organisms (e.g. bacteria) that are motile for sperm (Jenkins and Tiersch 1997). 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 cell are responsible for motility and fertility.

Recently, the use of flow cytometry to evaluate sperm quality has gained considerable attention because it is rapid (500 – 4000 cells per sec), simple to perform with proper protocols, and may be a better measure for evaluating quality (McNiven et al. 1992). Flow cytometry allows the simultaneous measurement of characteristics of cells in a system designed to deliver particles in single file past a point of measurement that records fluorescence and scattered light (Figure 6.1) (Ormerod 1994). Flow cytometric techniques in combination with fluorescent staining have been used to evaluate the quality of sperm in animals such as birds (Donoghue et al. 1995), mammals (Garner and Johnson 1995), fishes (McNiven et al. 1992) and mollusks (Paniagua-Chavez and Tiersch 2001). In these studies, the fluorescent dyes SYBR-14 and propidium iodide (PI) were used to stain nucleic acids within sperm cells to identify the ratio of live and dead cells. SYBR-14 penetrates the cell membrane of the sperm head and stain live cells green. Propidium iodide, a red dye, penetrates cells with damaged nuclear membranes and stain the nucleic acids by intercalating between base pairs (Garner et al. 1994).

Flow cytometric evaluation of stained fish sperm has been reported for species such as turbot *Scophthalmus maximus* (Ogier de Baulny et al. 1996), rainbow trout *Onchorhynchus mykiss* (Ogier de Baulny et al. 1997), Nile tilapia *Oreochromis niloticus* (Segovia et al. 2000), Atlantic salmon *Salmo salar*, steelhead trout *Onchorhynchus mykiss*, and lake trout *Salvelinus namaycush*

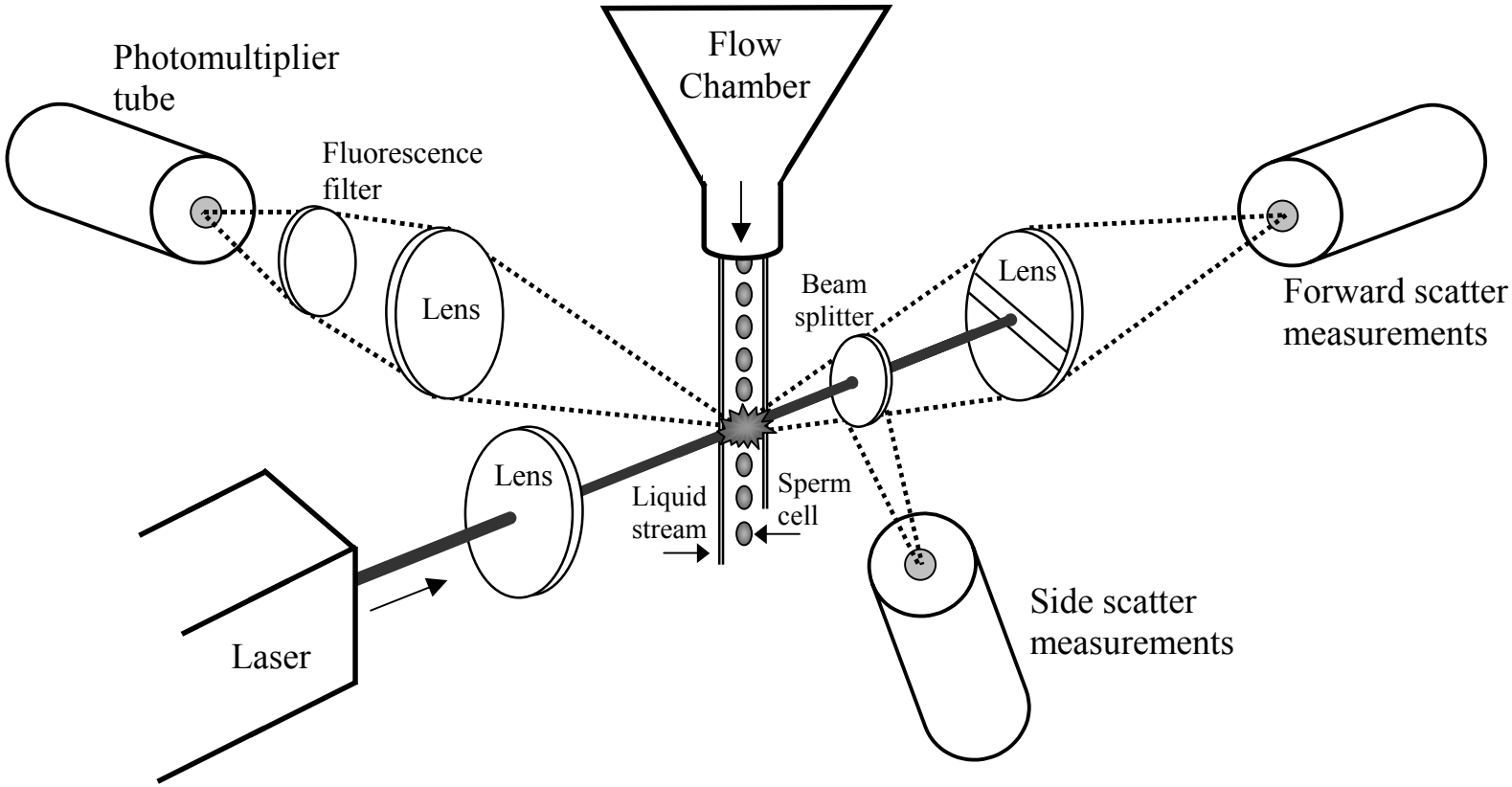

Figure 6.1. Conceptual model of a flow cytometer used to evaluate sperm cells stained with fluorescent dyes.

(Honeyfield and Krise 2000). These studies used flow cytometric techniques to assess membrane damage and viability of sperm that has been stored for a short period of time in a refrigerator or cryopreserved. To date, only one study has correlated fertilization rates with estimates of sperm quality based on flow cytometric analyses (Honeyfield and Krise 2000); however, flow cytometric analysis of bovine semen has repeatedly demonstrated a high correlation with motility and fertility (Garner and Johnson 1995).

The development of protocols for evaluating sperm quality can be an effective management tool for short-term and long-term storage of disease-free sperm for production of desirable strains, hybrids, or germplasm conservation. The objectives of this study were to: 1) develop flow cytometric techniques to assess the viability of fresh and killed sperm from Nile tilapia *Oreochromis niloticus*, 2) apply flow cytometric techniques to assess the viability of thawed sperm from red snapper *Lutjanus campechanus* and gray snapper *Lutjanus griseus*, and 3) evaluate variation in the motility, fertility, and viability of thawed sperm. There is often confusion and controversy in the use of the terms "viable" and "non-viable" to describe sperm quality. In this study, the term viable refers to sperm with intact cellular membranes. This is the first report on sperm quality and fertilization success in red snapper or gray snapper.

Methods

Collection, Handling, and Cryopreservation of Snapper Sperm

Sperm were obtained by the removal of testes from red snapper and gray snapper collected by recreational fishermen off coastal Louisiana. The fish were measured in length and blotted dry to avoid contamination of samples. Testes were surgically removed, sliced to release sperm which were collected in 50-ml plastic centrifuge tubes. Sperm were diluted 1:3 (v:v) with 200 mOsmol/kg Hanks' balanced salt solution (HBSS) (Wayman et al. 1996). Sperm solutions were

transported to the laboratory in an insulated cooler with ice and evaluated for percent motility using dark-field microscopy. Sperm with motilities greater than 95% were refrigerated at 4 C until use in experiments.

Cryopreservation of Sperm

Sperm samples were cryopreserved at Genex Cooperative, Inc. (GCI) located at the Louisiana State University T. E. Patrick Dairy Improvement Center in Baton Rouge. Genex Cooperative, Inc. specializes in the custom collection and cryopreservation of bull semen. The commercial facilities at GCI utilize new technologies in computerized straw labeling, automated straw-fillers, and large freezing and storage chambers. In this study, the same techniques for the cryopreservation of bull semen in 0.5-ml French straws were utilized for cryopreservation of red snapper and gray snapper sperm. In order to protect cells during the freezing process, 10% dimethyl sulfoxide was added to samples and allowed to penetrate cells for 20 min before freezing. Once frozen, samples were stored in liquid nitrogen storage dewars and transported to the coastal hatchery in nitrogen-vapor shipping dewars.

Thawing Sperm Samples

Cryopreservation straws were removed from dewars and individually thawed in a 40 C water bath for 7 sec. After thawing, straws were wiped dry and cut to release the sperm solutions for analysis of quality or use in fertilization trials.

Flow Cytometric Analysis of Sperm

Experiment 1: Standard curve for sperm viability

In order to evaluate the viability of red snapper and gray snapper sperm, a standard curve for known ratios of viable and non-viable sperm was developed using fresh sperm of Nile tilapia. Sperm were collected from one Nile tilapia, diluted with 300 mOsmol/kg HBSS (Tiersch et al.

1994), standardized to 4.0 x 10^6 cells/mL using a phase-contrast hemacytometer (model 1475, Hausser Scientific Company, Horsham, Pennsylvania), and motility of the sperm was estimated.

Non-viable sperm (i.e. killed sperm) was produced by heating 2.5 mL of sperm in 15-mL plastic centrifuge tubes. Tubes were heated in a water bath at 70 C for 5 min. Viable and non-viable sperm were mixed in five ratios (100:0, 75:25, 50:50, 25:75, 0:100) and were stained with the fluorescent dyes (Molecular Probes, Inc., Eugene, Oregon) SYBR-14 and PI. A SYBR-14 stock solution was prepared using 1 mM of dye and 99 µL of 300 mOsmol/kg HBSS.

Viable and non-viable sperm solutions were analyzed using a flow cytometer (FACSCalibur®, Becton Dickinson, San Jose, California) equipped with an air-cooled 480-nm argon (blue) laser. The FACSComp® software provided by the manufacturer was used to calibrate the flow cytometer prior to analysis of samples. To estimate sperm viability and develop a standard curve for known ratios of viable and non-viable sperm, 5 µL of SYBR-14 stock solution was added to 500 µL of sperm and incubated without light for 20 min. After the incubation period, 5 µL of PI was added to samples, which were incubated for another 20 min.

Data were collected based on the green fluorescence of viable sperm (SYBR-14) and red fluorescence of non-viable sperm (PI). Fluorescent-stained sperm populations were quantified using forward-scattered and side-scattered light. A total of 10,000 sperm cells were analyzed per sample.

Experiment 2: Viability of thawed sperm from red snapper and gray snapper

Two 0.5-mL straws were thawed and mixed for each red snapper and gray snapper male. Sperm motilities were estimated using dark-field microscopy. The sperm in each sample were diluted with 200 mOsmol/kg HBSS and standardized to 4.0 x 10^6 cells/mL by use of a phase-contrast hemacytometer. The viability of red snapper and gray snapper sperm were analyzed using a flow cytometer as described previously, however, the SYBR-14 stock solution was

prepared using 1 mM of dye and 99 µL of 200 mOsmol/kg HBSS. Quantitative data on fluorescent-stained sperm populations were collected on three samples from each male. The results of this experiment were used to compare flow cytometric techniques for assessing sperm quality with sperm motility estimates and fertilization results.

Fertilization of Red Snapper and Gray Snapper Eggs

During July and August of 2001, two red snapper and two gray snapper females captured from the wild were induced to spawn in the laboratory following injections of human chorionic gonadotripin (Chorulon, Intervet, Millsboro, Delaware). The females were stripped of eggs and aliquots of 200 eggs were fertilized with 1 ml of fresh (control) or cryopreserved sperm. No attempts were made to standardize the sperm concentrations of fresh or cryopreserved samples, although the concentrations were approximately 1.5 x 10^9 cells/mL. 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, which was added to 4-L Ziplock® freezer bags and incubated at 30 C. Fertilization was assessed at neurulation (14 h) by use of a dissecting microscope. All samples were preserved in a 5% solution of formalin and buffered seawater.

Statistical Analysis

Flow cytometric data were analyzed using Cell Quest® Software (Becton Dickinson). All percentage motility and fertilization values were arcsine-square root transformed prior to statistical analysis. Linear regression analysis (Microsoft Excel 2000, Microsoft Corp., Redmond, Washington) was used to determine the correlation between the predicted and measured ratios of viable and non-viable tilapia sperm. A one-way factorial analysis of variance (SAS 8.0; SAS Institute, Cary, North Carolina) was used to analyze male-to-male variability in samples from red snapper and gray snapper. Means were separated using Duncan's multiple

range test and were considered significant when $P < 0.05$. Linear regression analysis (Microsoft Excel 2000) was used to determine the correlation between motility and sperm viability, and between sperm viability and fertilization results.

Results

Cryopreservation of Sperm

During June of 2001, sperm from five red snapper and six gray snapper were commercially cryopreserved. Two straws from each male were thawed to estimate motility. Sperm motilities in all samples were greater than 95% before freezing and after thawing.

Fluorescent Staining of Sperm

Viable and non-viable sperm of tilapia, red snapper, and gray snapper were successfully stained with SYBR-14 and propidium iodide at concentrations recommended by the manufacturer (Figure 6.2).

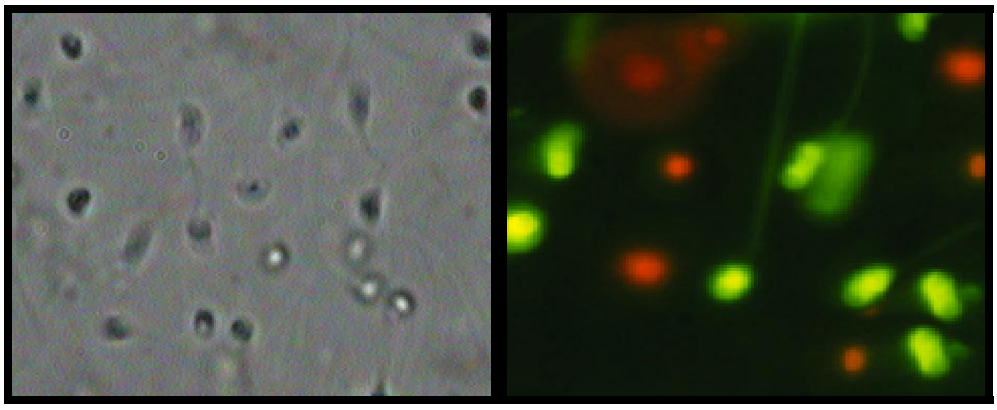

Figure 6.2. Photomicrographs of unstained (left) and fluorescently stained (right) sperm of red snapper. Sperm were stained with SYBR-14 (green) and propidium iodide (red).

Flow Cytometric Analysis of Sperm

Experiment 1: Standard curve for sperm viability

Using Cell Quest® Software to analyze the data, populations of viable and non-viable sperm were separated using gates, which permitted precise calculations of viable or non-viable sperm in each population (Figure 6.3). A standard curve was generated for sperm viability using various ratios of live and killed tilapia sperm (Figure 6.4). Linear regression analysis revealed that the fluorescent molecular stains were effective for identifying populations of live and dead sperm ($r = 0.98$).

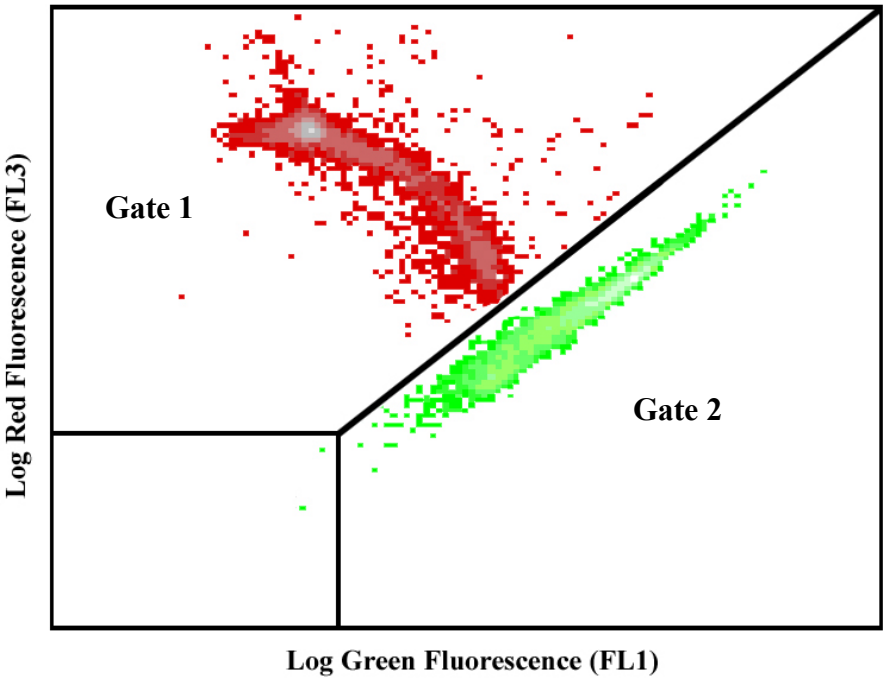

Figure 6.3. A dot-plot of live and killed tilapia sperm (mixed 50:50) stained with SYBR-14 and propidium idodide. Populations of viable (green) and non-viable (red) sperm were separated into gates. Gate one shows the population of dead sperm (56%), while gate two shows the population of live sperm (44%).

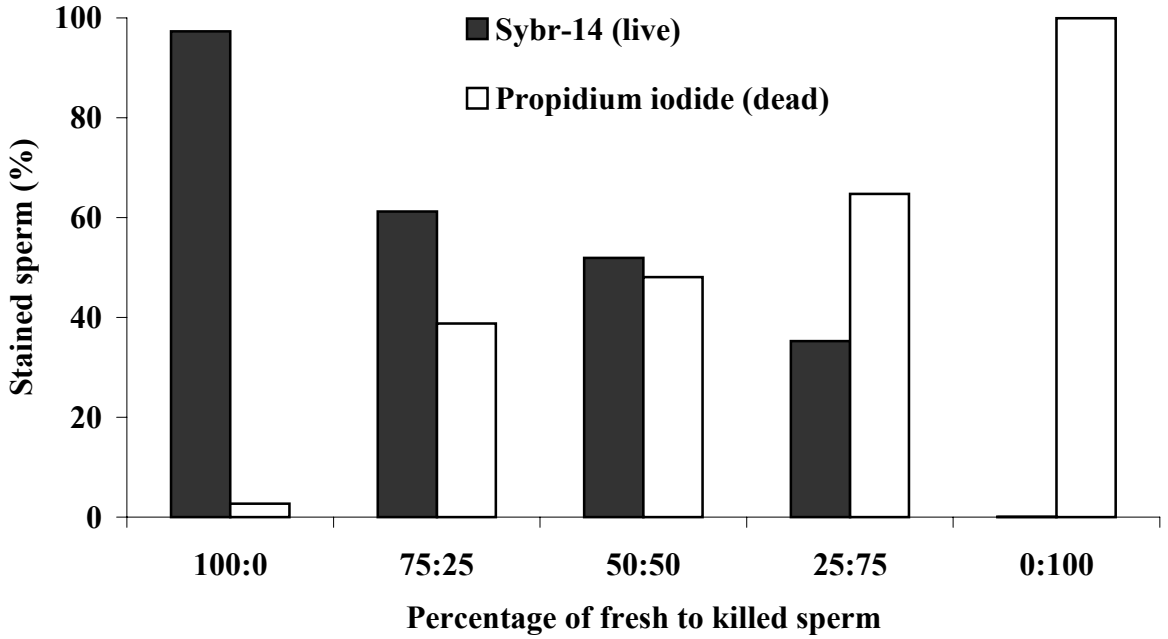

Figure 6.4. Flow cytometric results with use of SYBR-14 and propidium iodide to stain known concentrations of live and dead tilapia sperm.

Experiment 2: Analysis of viability with thawed sperm of red snapper and gray snapper

Flow cytometric methods were used to analyze thawed sperm of five red snapper and six gray snapper. In all thawed samples, sperm motilities were greater than 95%. The viability of red snapper sperm ranged from 47% to 82% and there were significant differences among individuals ($P < 0.0001$) (Figure 6.5). Similar results were observed with sperm of gray snapper in which the viability of sperm ranged from 44% to 75% and was also significantly different among individuals ($P < 0.0001$) (Figure 6.6). A comparison of sperm from both species revealed that the viability of red snapper sperm was significantly higher than that of gray snapper sperm ($P = 0.002$). There was no correlation between sperm motility and sperm viability; however, motility estimates were significantly higher than were the viability of sperm tested ($P < 0.0001$)

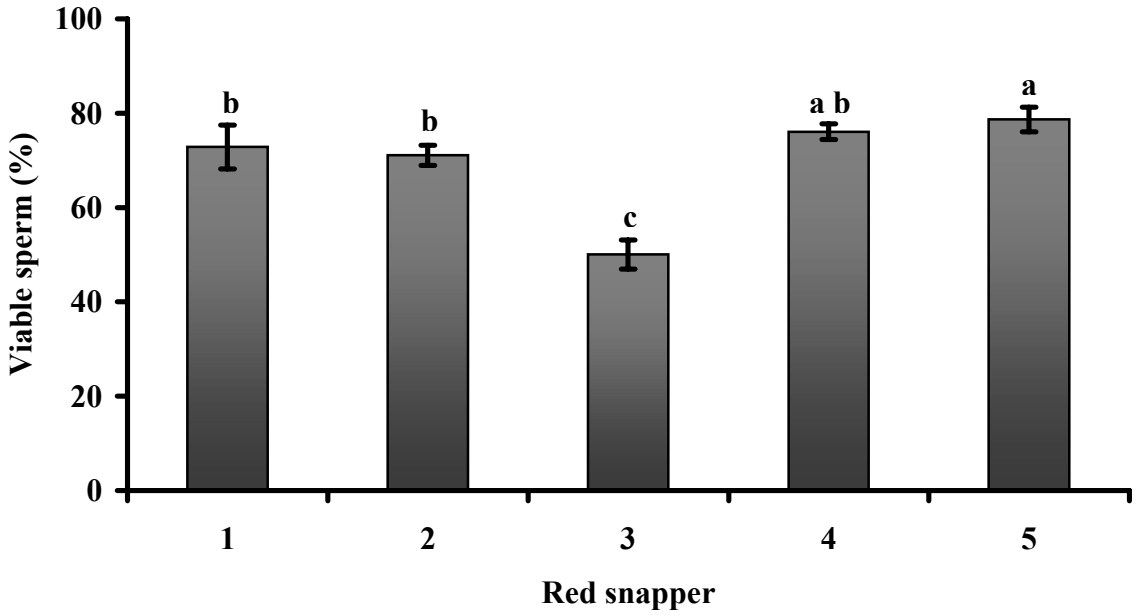

Figure 6.5. Viability (means ± SD) of thawed sperm from five red snapper as determined by flow cytometry.

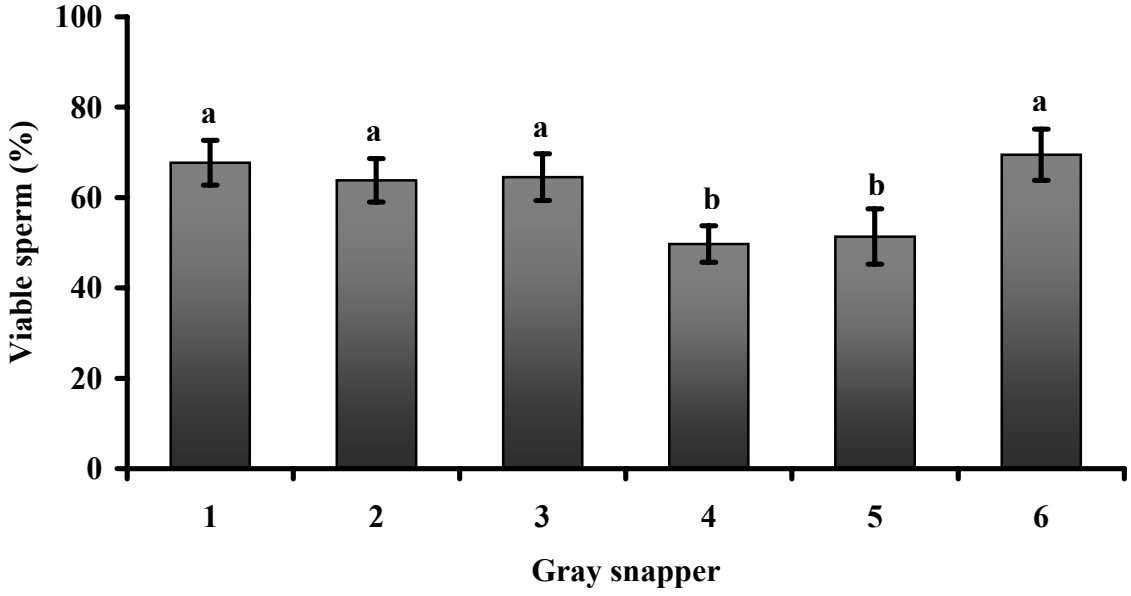

Figure 6.6. Viability (means ± SD) of thawed sperm from six gray snapper as determined by flow cytometry.

Fertilization of Red Snapper and Gray Snapper Eggs

Fertilization of spawns collected from red snapper and gray snapper were varied (Table 6.1). Fertilization rates were less than 50% in almost all treatments. No significant differences were found in the fertilizing capability of sperm from the fresh control and red snapper ($P = 0.28$), while all other cryopreserved samples resulted in significantly lower fertilization rates than the fresh control ($P < 0.0001$). A weak correlation ($r = 0.50$) was found between sperm viability and the fertilizing capability of red snapper sperm (Figure 6.7). Similarly, a weak correlation ($r = 0.31$) was also found between sperm viability and the fertilizing capability of gray snapper sperm. Red snapper and gray snapper sperm were equally effective in the production of red snapper, gray snapper, and hybrid snapper embryos.

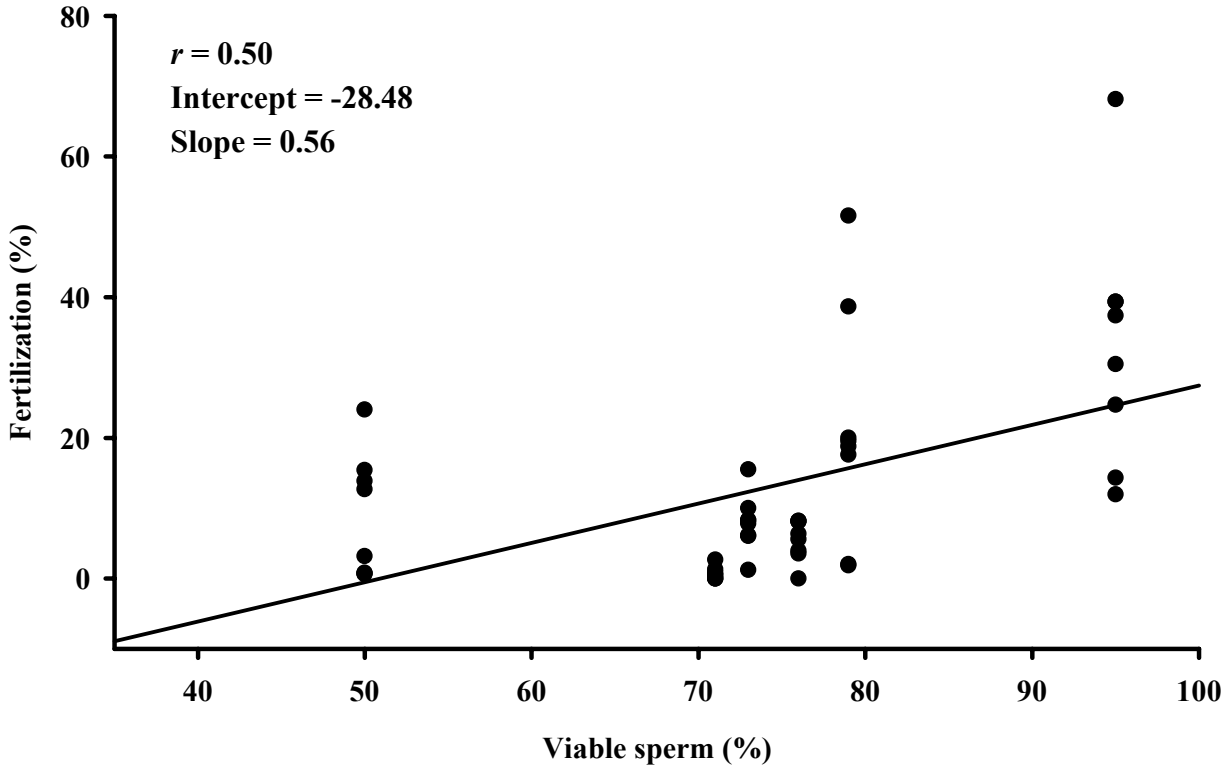

Figure 6.7. Linear regression of red snapper sperm viability and the fertilizing capability of sperm with red snapper and gray snapper eggs.

Table 6.1. Results of red snapper and gray snapper eggs fertilized with fresh and thawed sperm. Values represent the average of replicates.

Male	Treatment	Motility (%)	Average viability (%)	Average Fertilization Rate			
				Red snapper female 1	Red snapper female 2	Gray snapper female 1	Gray snapper female 2
Red snapper							
Control	Fresh	95	***	39	13	28	53
1	Cryopreserved	95	73	13	7	7	7
2	Cryopreserved	95	71	2	0	1	1
3	Cryopreserved	95	50	13	2	20	1
4	Cryopreserved	95	76	8	6	6	2
5	Cryopreserved	95	79	18	20	45	2
Gray snapper							
1	Cryopreserved	95	68	3	0	2	1
2	Cryopreserved	95	64	1	0	0	0
3	Cryopreserved	95	64	1	1	7	1
4	Cryopreserved	95	50	0	0	0	2
5	Cryopreserved	95	51	0	0	***	***
6	Cryopreserved	95	69	***	***	0	7

Discussion

Flow cytometric analysis of sperm stained with fluorescent dyes provided an alternative to traditional methods for evaluating sperm viability. While motility remains the easiest method to assess the quality of sperm in routine situations (i.e. in a fish hatchery during spawning), this study suggests that flow cytometric methods for evaluating stained sperm can provide a more accurate measure of sperm quality. The combination of SYBR-14 and PI was effective in differentiating between viable and non-viable sperm of tilapia, red snapper, and gray snapper. The standard curve developed with stained tilapia sperm demonstrated that various concentrations of live and killed sperm were highly correlated when analyzed with a flow cytometer.

In this study, eggs of red snapper and gray snapper were fertilized with red snapper and gray snapper sperm. While the production of hybrid snapper was not the focus of this study, use of red snapper and gray snapper eggs was valuable for evaluating sperm quality in these species. The fertilization results were useful in comparing the fertilizing ability of fresh and thawed sperm of red snapper and gray snapper; however, poor egg quality may have contributed to the low rates of fertilization because similar values were observed for all treatments. Interestingly, thawed sperm from red snapper five had a significantly higher viability and consistently higher fertilization rates as compared to the fresh sperm and other thawed samples. Because cryopreservation of sperm does not improve sperm motility, fertility, or overall quality (Lahnsteiner et al. 1992, 1996), this enhanced performance could be attributed to individual variability from a superior male (e.g. age, condition, or sperm fertility) or from some experimental error (e.g. sperm concentration). Further studies are needed to evaluate the effects of male-to-male variation on fertilization success.

While the most reliable indicator of sperm quality is the fertilization of eggs, the techniques developed in this study are especially useful for evaluating the viability of sperm that has been cryopreserved and thawed. Freezing and thawing of sperm can result in a number of injuries to cells and cellular compartments caused by changing pH and osmotic pressures within cells, formation of intracellular and extracellular ice, and the toxic effects of cryoprotectants (Leung 1991). The simple, rapid method of analyzing sperm stained with Syber-14 and PI using a flow cytometer can be used to improve cryopreservation techniques by identifying cells with damaged membranes. Thus, cryopreservation techniques may be optimized to store sperm of highly valuable species for selective breeding programs or conservation of genetic resources of threatened or endangered populations. The flow cytometric techniques developed in this study can be used to assess the quality of sperm used in fish hatcheries, laboratories, or germplasm repositories. While the high cost (e.g. greater than US $100,000) of a flow cytometer may limit their widespread application, use of molecular probes with flow cytometry offers more objectivity and precision in evaluating sperm quality.

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Chapter 7

Summary and Conclusions

Although aquaculture has been widely practiced for thousands of years, culture has only begun to shift from extensive farming to more intensive practices during the last century. The further growth and industrialization of aquaculture will require the development and use of technologies that are highly advanced, competitive, and environmentally sound. These considerations are especially important in the development of marine aquaculture, which has received much attention in recent years as people search for alternative methods of producing high-quality foods, and as natural resource managers look to aquaculture for stock enhancement programs to replenish depleted natural populations.

A major hurdle to the growth of marine aquaculture is the lack of adequate fry production for commercially important species. While many successful aquaculture programs have been developed using seedstock (i.e. fertilized eggs, larvae, and fry) collected from the wild, these practices are unreliable, unpredictable, and are not sustainable or ecologically sound. The technological bottleneck that currently limits the production of larvae and fry is the need for hatchery techniques for controlled reproduction in broodstock (Zohar and Mylonas 2001). Once reproduction of broodstock can be controlled, a steady supply of fish can be produced by year-round spawning (Bromage and Roberts 1995) and genetic improvement programs can be employed that focus on survival and growth of offspring (Purdom 1993).

The goal of this thesis work was to improve hatchery techniques for artificial spawning of red snapper *Lutjanus campechanus*. Broodstock management techniques were developed to optimize gamete quality for fertilization and larval-rearing success. The techniques developed

herein include practical methods that hatchery managers can use to collect high-quality broodstock, to preserve sperm, and to collect and incubate eggs.

In the first part of this work, (Chapter 3) methods were developed for collection, handling, transport, and holding of mature red snapper for induced spawning. Broodstock were collected during their peak spawning periods within June, July, and August. Fish were transported from the offshore waters of the Gulf of Mexico to the hatchery at Louisiana Universities Marine Consortium (LUMCON). Collection and handling techniques were developed to minimize stress to broodstock. Females that ovulated were strip-spawned 29 ± 1 h after initial injection of human chorionic gonadotropin. Sampling of oocytes by biopsy provided an accurate measure female reproductive condition. Females with oocyte size distributions ranging from 350 µ to 550 µ were good candidates for hormone-induction and artificial spawning.

The use of food-grade plastic bags facilitated the incubation of embryos and larvae from 2 to 20 h. The plastic bags were especially useful for isolating the many treatments and replicates produced during this study. The fertilization of eggs treated with fresh red snapper sperm ranged from 44 to 95% for development through 8-cell stage, 25% to 83% for development through neurulation, and 8 to 87% for development through hatch. Evaluation of the embryonic development of red snapper showed that development of embryos through neurulation served as a good predictor of fertilization and hatching success. This study was the first to document the production of red snapper and snapper hybrids (red snapper x gray snapper *Lutjanus griseus*) using fresh and cryopreserved sperm. Survival of red snapper and hybrid snapper larvae to 10 d post-hatch was low (< 2.5%), but was the highest survival reported to date for efforts to culture this species.

In the second part of this work (Chapters 4 and 5), techniques were developed for the collection, handling, refrigerated storage, and cryopreservation of red snapper sperm. Additional techniques (Chapter 7) were developed for evaluation of sperm quality. Testes and sperm were collected from fish (~400) captured and donated by recreational anglers on commercial charter operations. All samples were collected from fish that had been caught, killed, and placed into insulated coolers with ice. Although samples were collected from fish 4 to 8 hours after death, samples remained viable when fish were covered in ice for the duration of the fishing trip. Sampling from the recreational and commercial snapper fishery has been used for the collection of otoliths, scales, and vertebrae in ageing studies; however, this study is the first to report the collection and use of viable sperm from dead red snapper and gray snapper. Because sperm could not be stripped from fish, techniques had to be developed for the surgical removal of testes to obtain sperm cells.

Sperm were obtained from sliced and crushed testes, and calcium-free Hanks' balanced salt solution (HBSS) was used to dilute samples. Red snapper sperm diluted 1:3 (v:v) with Hanks' balanced salt solution prepared without calcium at 200 mOsmol/kg retained motility for 10 d when maintained at 4 C. Gray snapper sperm collected using the same methods retained motility for 7 d. Based upon preliminary research done in 1998 on the cryopreservation of red snapper sperm (Roppolo 1999), red snapper and gray snapper sperm were cryopreserved using commercial-scale techniques applied in the dairy industry. Sperm samples were cryopreserved at Genex Cooperative, Inc. located at the Louisiana State University T. E. Patrick Dairy Improvement Center in Baton Rouge. The use of cryopreserved sperm in the marine fish hatchery improved efficiency in fertilizing stripped eggs and enables the long-term repeated use of particular males for specific breeding designs. Because sperm were able to be collected from

fish donated by recreational anglers, broodstock collecting trips were specifically focused on the collection and handling of females in spawning condition.

Sperm quality is highly variable and can depend on factors such as the culture environment, feeding regime, broodstock condition, and individual genetic variability. Estimation of motility is commonly used to assess sperm quality, but motility estimates do not necessarily correlate with sperm fertility. Flow cytometric methods were developed to evaluate the quality of thawed sperm of red snapper and gray snapper. The fluorescent dyes Sybr-14 and propidium iodide were used to stain sperm cells to identify the ratio of viable and non-viable cells. Motility estimates were not correlated with fertility or viability; however, flow cytometric results were correlated with fertilization results ($r = 0.58$). Flow cytometric analysis of sperm was simple to perform and provided an objective alternative to traditional methods for estimating sperm quality through estimation of motility.

The hatchery production of red snapper holds promise, provided that further research is undertaken to optimize existing techniques for: 1) hormone-induced spawning, 2) prediction of ovulation; 3) collection and fertilization of eggs; 4) incubation of embryos; and 5) larval rearing. While research conducted in this study was performed within a small-scale marine hatchery, the techniques developed could be adopted in commercial-scale operations that culture other subtropical and tropical marine species (Figure 7.1).

References

Bromage, N. R. and R. J. Roberts. 1995. *Broodstock Management and Egg and Larval Quality*. Blackwell Science, Cambridge, Massachusetts.

Purdom, C. E. 1993. *Genetics and Fish Breeding*. Chapman and Hall Publishing Company, New York, New York.

Zohar, Y., and C. C. Mylonas. 2001. Endocrine manipulations of cultured fish: from hormones to genes. Aquaculture 197:99-136.

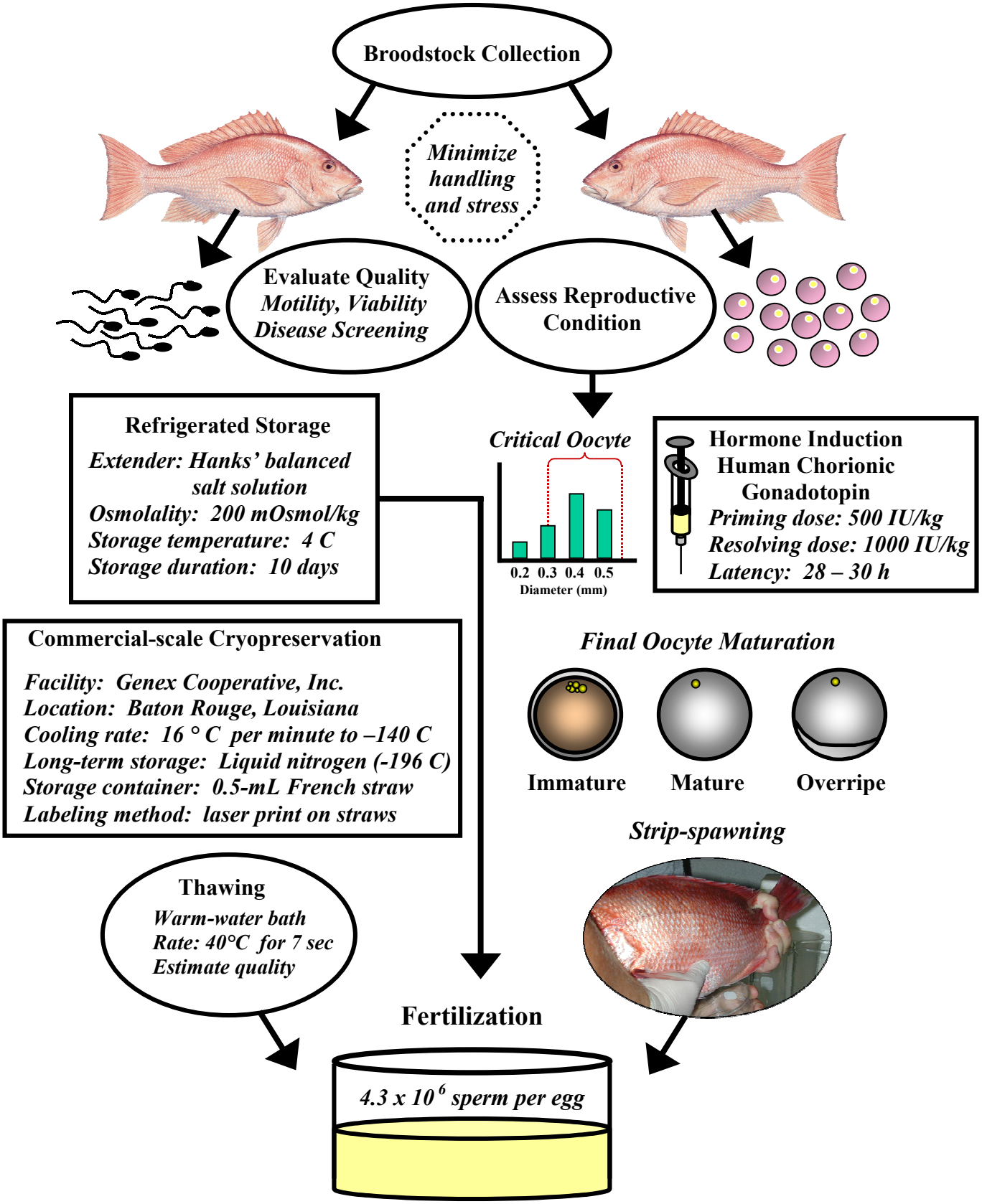


Figure 7.1. Summary of hatchery techniques developed in this thesis for artificial spawning of red snapper Lutjanus campechanus.

Appendix A

Standard Operating Procedures

SOP-1. Identification codes for fish

Each fish collected and used in experiments received a unique identification code to track distribution of samples and offspring.

Examples:

RS00M01	**GS01F01**
RS: red snapper	**GS: gray snapper**
00: year 2000	**01: year 2001**
M: male	**F: female**
01: sample number	**01: sample number**

SOP-2. Collection of testes from fish donated by recreational fishermen on commercial charter operations

Materials needed:

Laboratory notebook
Permanent pen
Paper towels
Latex gloves
Measuring tape
Fillet knife
Tweezers
4-L Ziploc® Freezer Bags
1 L of Hanks' balanced salt solution (HBSS)
Digital thermometer

Procedure:

1. Obtain fish on ice from marina personnel prior to cleaning.
2. Identify males and measure total length.
3. Remove fish from ice and blot with paper towels to remove slime and water.
4. Make a 2.5-cm incision below the pelvic girdle on the fish to be sampled.
5. Gently insert the temperature probe four to five cm into the fish.
6. Record temperature in notebook. Ideal temperatures should range from 2 to 12 C.
7. Extend incision from pelvic girdle to anus being careful not to cut through the stomach, intestines or anus.
8. Remove complete testes and wipe excess blood away.
9. Place testes in Ziploc® bag and add enough HBSS to suspend the testes.
10. Using a permanent marker label bag with fish identification number.
11. Place samples in an insulated cooler with ice.
12. Repeat as necessary rinsing fillet knife and tweezers in HBSS between fish.
13. Transport samples to laboratory immediately after collection.

SOP-3. Collection of sperm from testes

Materials needed:
Laboratory notebook
Paper towels
Latex gloves
Scale
Scissors
50-mL centrifuge tubes
1 L of calcium-free Hanks' balanced salt solution (HBSS)

Procedure:

1. Remove testes from Ziploc® bag and blot with paper towels.
2. Remove non-testicular tissues from testes (e.g. fat, mesentery).
3. Weigh testes and record in notebook.
4. Cut each testis beginning with the anterior end and strip sperm from the testis into centrifuge tubes. Continue to cut the testis moving towards the posterior end until all sperm is collected. Discard testis after collection.
5. Dilute the sperm collected 1:3 (v:v) with HBSS.
6. Store loosely-capped centrifuge tubes in refrigerator at 4 C.

SOP-4. Estimation of motility

Materials needed:
10 µL pipette and tips
20 µL pipette and tips
Glass microscope slide
Microscope with darkfield filter and 200-x magnification
Artificial seawater (35 ppt; 870 mOsmol/kg)

Procedure:

1. Place 2 µL of sperm on microscope slide.
2. Rapidly examine sample for motile sperm.
3. Add 20 µL of artificial seawater and mix gently with the tip of the pipette.
4. Estimate the percent of sperm that are vigorously swimming forward.

SOP-5. Preparation of Hanks' balanced salt solution (HBSS)

Hanks' balanced salt solution is a physiological solution that has been used to dilute marine fish sperm for refrigerated storage and cryopreservation. In this study, HBSS was prepared without calcium, filtered through a 0.22-μm filter (Gelman Sciences, Ann Arbor, Michigan), and frozen in sterile 1-L Nalgene® bottles (Nalge Nunc Inc., Rochester, New York) until used in experiments. Solutions were prepared by mixing the chemicals below in distilled water. Osmolalities were verified using a vapor pressure osmometer (model 5500, Wescor Inc., Logan Utah).

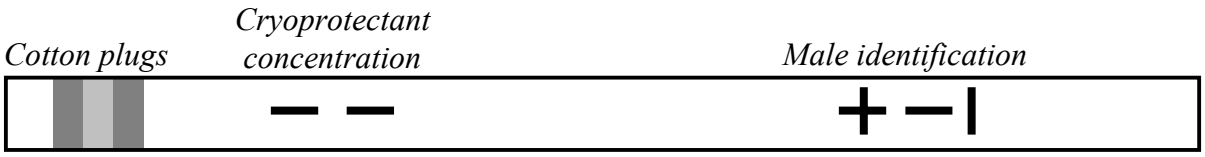
	Concentration (g/L)		
Ingredient	**200 mOsmol/kg**	**300 mOsmol/kg**	**400 mOsmol/kg**
NaCl	5.26	8.00	10.53
KCl	0.26	0.40	0.53
$MgSO_4 \bullet 7H_2O$	0.13	0.20	0.26
Na_2HPO_4	0.04	0.06	0.07
KH_2PO_4	0.04	0.06	0.07
$NaHCO_3$	0.23	0.35	0.46
$C_6H_{12}O_6$	0.66	1.00	1.32

SOP-6. Commercial-scale freezing procedures

1. Select samples for freezing and estimate initial motility
2. Transport samples to T.E. Patrick Dairy Improvement Center
3. Label 0.5-mL cryopreservation straws with species name and sample identification code
4. Label goblets with initials, date, species, fish number, cryoprotectant, and concentration
5. Label canes with initials, date, species
6. Place samples in walk-in cooler (5 C)
7. Add cryoprotectant and start equilibration time
8. Fill straws using automated straw-filler
9. Place straws on racks and add enough liquid-filled straws to make 660 total straws
10. Place straws in freezing chamber
11. Allow dairy personnel to freeze samples
12. At the end of the freezing process, plunge straws into liquid nitrogen
13. Transfer straws into goblets, and place goblets on canes
14. Place canes into long-term storage dewars at the T.E. Patrick Dairy Improvement Center or transfer to a shipping dewar for transport to LSU Aquaculture Research Station or LUMCON

SOP-7. Methods used to label cryopreservation straws

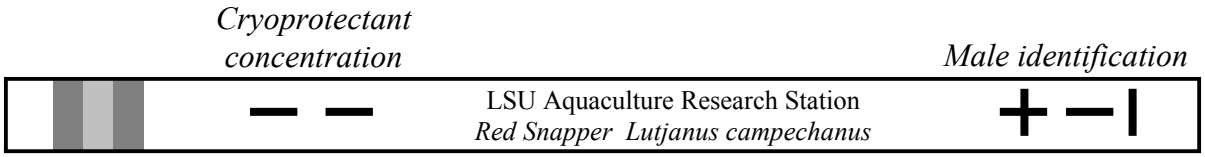
Year 2000



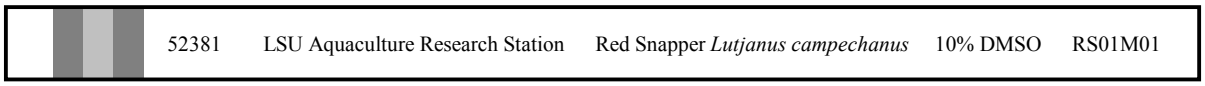
Colors:
Red straws: red snapper
Gray straws: gray snapper

Symbols:
| male 1; || male 2; ||| male 3; — male 5; —| male 6; + male 10; +— male 15; +—| male 16

Year 2001a



Year 2001b



SOP-8. Broodstock collecting supplies

The following supplies were used in collection of snapper broodstock

Holding tank
Oxygen cylinder
Oxygen regulators (2)
Ceramic air stone and tubing
96-qt cooler
Ice blocks (15-20)
Ice bags (2)
Offshore fishing rods
Live bait
Frozen bait (menhaden, squid)
Dip nets (2)
Buckets (3)

Toolbox containing the following items:
Airline tubing (10 ft)
Rope (20 ft)
Fillet knife
Duct tape
Electrical tape
Teflon tape
Hose menders (3)
Airline tubing connectors (3)
100-mL Amquel®
16G – 18G syringe needles (30)
1 1/8" Crescent wrench

SOP-9. Collection and preparation of larval feeds

Wild size-sorted zooplankton were collected to feed larval snapper. Plankton were collected from Terrebonne Bay, Terrebonne Parish, Louisiana (29° 10' N, 90° 35' W). A plankton net with 53 µ mesh was towed by boat for two minute intervals. Plankton were sieved through 500 µ, 333 µ, 183 µ, and 102 µ screens. Plankton were concentrated by capturing organisms on a 41 µ screen and rinsing into an aerated tank. Species of plankton were grossly identified and concentrations were determined through use of a dissecting microscope and 1-mL Hensen-Stempel pipette.

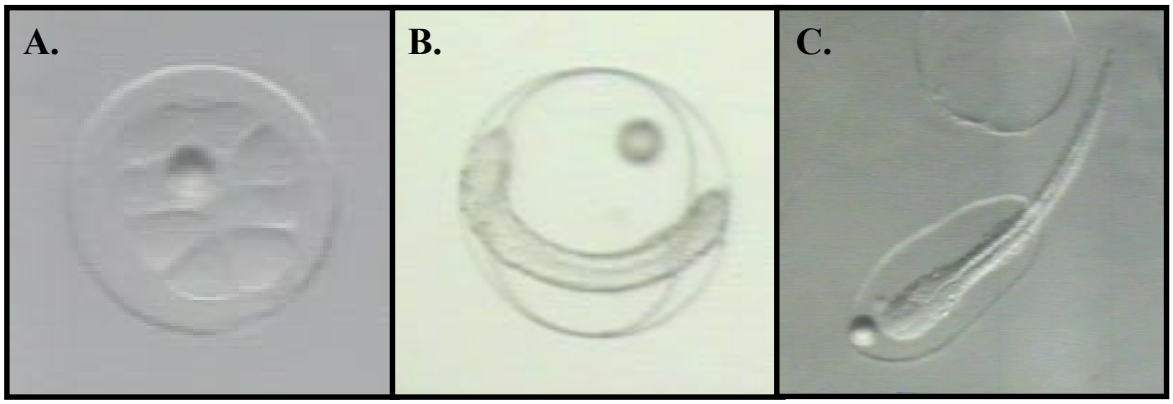
SOP-10. Egg, embryo, and larval fixative

5% Buffered Formalin

Ingredient
100 mL of Fomalin (37.5% Formaldehyde)
650 mL of seawater (35 ppt)
3 g Sodium borate (Borax®; $Ba_4Na_2O_7 \bullet 10H_2O$)

SOP-11. Assessment of fertilization and hatching rates

Within a well-ventilated laboratory, preserved eggs, embryos, and larvae were transferred from 20-mL glass scintillation vials by pipette into 35-mm petri dishes with marked grids. The formalin solution was removed and replaced with 35-ppt artificial seawater (Marinemix, Wiegandt GmbH Inc., Krefeld, Germany). The number of embryos and larvae within each sample were counted against a black background with a dissecting microscope at 40-x magnification. Fertilization was assessed as embryos developed through 8-cell stage (A), neurulation (B), and hatch (C). Upon completion, the seawater was removed and samples were returned to the original vials with the 5% formalin solution.



Appendix B

Thesis Unanalyzed Data

Chapter 2

Table B.1. Fifty years of red snapper landings from the Gulf of Mexico.

YEAR	METRIC TONS (mmt)	POUNDS (lbs)	EX-VESSEL VALUE (millions of dollars)
1950	3,079	6,788,300	1.64
1951	3,026	6,670,400	1.72
1952	3,877	8,546,700	2.02
1953	3,505	7,727,300	2.14
1954	3,804	8,385,500	2.17
1955	4,020	8,862,500	2.27
1956	3,978	8,769,300	2.16
1957	3,874	8,540,600	2.20
1958	4,472	9,858,900	2.53
1959	4,635	10,218,700	2.64
1960	4,633	10,214,200	2.61
1961	5,392	11,887,900	3.06
1962	5,389	11,880,500	2.97
1963	5,749	12,675,100	3.38
1964	6,048	13,334,000	3.86
1965	6,072	13,386,800	3.91
1966	5,682	12,527,100	3.96
1967	5,417	11,942,500	3.88
1968	4,832	10,651,800	3.64
1969	4,331	9,547,200	3.99
1970	3,873	8,537,600	3.83
1971	3,837	8,459,700	4.01
1972	3,858	8,504,700	4.58
1973	3,714	8,188,500	4.87
1974	3,825	8,431,500	5.59
1975	3,526	7,773,000	5.64
1976	3,215	7,086,800	5.89
1977	2,431	5,358,600	5.03
1978	2,151	4,741,070	5.23
1979	2,118	4,668,781	6.31
1980	2,134	4,703,824	7.34
1981	2,550	5,620,716	9.46
1982	2,742	6,043,937	10.44
1983	3,113	6,861,811	12.00
1984	2,475	5,455,841	10.03
1985	1,931	4,256,994	8.52
1986	1,799	3,965,149	8.17

YEAR	METRIC TONS (mmt)	POUNDS (lbs)	EX-VESSEL VALUE (millions of dollars)
1987	1,522	3,356,284	7.33
1988	1,841	4,058,979	9.21
1989	1,405	3,098,454	7.32
1990	1,207	2,660,845	6.66
1991	1,017	2,240,893	5.31
1992	1,380	3,042,877	5.79
1993	1,544	3,404,705	6.50
1994	1,475	3,252,041	6.47
1995	1,339	2,950,950	5.78
1996	1,973	4,348,859	7.95
1997	2,171	4,786,431	8.36
1998	2,114	4,660,971	9.73
1999	2,212	4,876,635	9.56
2000	2,172	4,788,081	10.16

Chapter 3

Table B.2. Lengths (cm) and weights (g) of female red snapper collected.

Date	Fish Identification	Length (in)	Length (cm)	Weight (g)
05/30/00	RS00F1	19.0	48.26	1713
05/30/00	RS00F2	17.5	44.45	1238
05/30/00	RS00F3	18.0	45.72	1476
06/14/00	RS00F4	17.0	43.18	1352
06/14/00	RS00F5	20.0	50.80	2155
06/28/00	RS00F6	18.5	46.99	1639
06/28/00	RS00F7	19.5	49.53	1879
06/28/00	RS00F8	17.5	44.45	1336
06/28/00	RS00F9	16.0	40.64	1304
07/10/00	RS00F10	20.5	52.07	2200
07/10/00	RS00F11	18.5	46.99	1642
07/10/00	RS00F12	18.0	45.72	1846
07/10/00	RS00F13	17.0	43.18	1468
07/10/00	RS00F14	16.0	40.64	1216
07/10/00	RS00F15	16.0	40.64	1315
07/10/00	RS00F16	18.0	45.72	1544
07/25/00	RS00F20	18.5	46.99	1638
07/25/00	RS00F21	18.0	45.72	1616
08/09/00	RS00F22	20.0	50.80	2017
08/09/00	RS00F23	20.0	50.80	2124
08/09/00	RS00F24	16.5	41.91	1175

Date	Fish Identification	Length (in)	Length (cm)	Weight (g)
08/09/00	RS00F25	16.0	40.64	1046
08/09/00	RS00F26	20.0	50.80	2034
08/09/00	RS00F27	18.0	45.72	1600
08/09/00	RS00F28	16.0	40.64	1440
08/09/00	RS00F29	16.0	40.64	1343
08/09/00	RS00F30	20.0	50.80	1725
08/09/00	RS00F31	20.0	50.80	2028
08/09/00	RS00F32	19.0	48.26	1664
08/09/00	RS00F33	19.0	48.26	1874
06/17/01	RS01F1	17.0	43.18	1296
06/17/01	RS01F2	18.5	46.99	1842
06/17/01	RS01F3	17.0	43.18	1440
06/17/01	RS01F4	19.0	48.26	1784
06/17/01	RS01F5	19.0	48.26	1702
06/17/01	RS01F6	19.0	48.26	2141
06/17/01	RS01F7	20.0	50.80	2280
06/17/01	RS01F8	24.0	60.96	3751
07/04/01	RS01F9	20.0	50.80	2189
07/16/01	RS01F10	18.0	45.72	1671
07/16/01	RS01F11	16.0	40.64	902
07/18/01	RS01F12	16.5	41.91	1116
07/18/01	RS01F13	20.0	50.80	1945
07/18/01	RS01F14	22.0	55.88	3419
07/18/01	RS01F15	17.0	43.18	1172
07/18/01	RS01F16	16.0	40.64	1108
07/31/01	RS01F17	17.0	43.18	1261
07/31/01	RS01F18	18.0	45.72	1544
07/31/01	RS01F19	18.5	46.99	1586
07/31/01	RS01F20	17.0	43.18	1411
07/31/01	RS01F21	16.0	40.64	1176
07/31/01	RS01F22	22.0	55.88	3015
08/15/01	RS01F23	23.0	58.42	4248
08/15/01	RS01F24	21.0	53.34	2585
08/15/01	RS01F25	18.5	46.99	1658
08/15/01	RS01F26	16.0	40.64	1144
08/15/01	RS01F27	16.0	40.64	1030
08/15/01	RS01F28	16.0	40.64	980
08/15/01	RS01F29	16.0	40.64	984
08/15/01	RS01F30	16.5	41.91	989
08/15/01	RS01F31	19.0	48.26	1605
08/15/01	RS01F32	17.5	44.45	1211
08/15/01	RS01F33	16.5	41.91	1081

Table B.3. Lengths, weights, and pH of female red snapper strip-spawned.

Date	Fish Identification	Length (in)	Length (cm)	Weight (g)	pH
06/28/00	RS00F9	16.0	40.64	1304	7.83
07/10/00	RS00F12	18.0	45.72	1846	6.58
07/10/00	RS00F15	16.0	40.64	1315	7.33
07/25/00	RS00F20	18.5	46.99	1638	7.10
07/25/00	RS00F21	18.0	45.72	1616	7.16
08/09/00	RS00F24	16.5	41.91	1175	7.35
08/09/00	RS00F27	18.0	45.72	1600	7.23
08/09/00	RS00F32	19.0	48.26	1664	7.40
08/09/00	RS00F33	19.0	48.26	1874	7.35
06/17/01	RS01F6	19.0	48.26	2141	9.77
06/17/01	RS01F7	20.0	50.8	2280	8.43
07/18/01	RS01F13	20.0	50.8	1945	7.74
07/18/01	RS01F15	17.0	43.18	1172	7.46
07/18/01	RS01F16	16.0	40.64	1108	7.46
07/31/01	RS01F18	18.0	45.72	1544	7.20
08/15/01	RS01F26	16.0	40.64	1144	6.62
08/15/01	RS01F32	17.5	44.45	1211	6.53

Table B.4. Diameters of oocytes (μ) collected from red snapper. During the summer of 2000, 100 oocytes were randomly sampled from each fish. Because measurement of oocytes was time consuming, only 50 oocytes were sampled from each fish during the summer of 2001. Fish indicated by bold print were strip-spawned.

Year 2000

RS00F1	RS00F2	RS00F3	RS00F4	RS00F5	RS00F6	RS00F7	RS00F8	**RS00F9**
24	48	48	48	48	48	165	132	99
24	48	48	72	48	48	165	132	99
48	48	48	72	48	48	182	149	99
48	48	48	72	48	72	198	165	99
48	48	48	72	48	72	198	165	132
48	48	48	72	48	72	198	165	132
48	48	48	72	48	72	198	165	132
48	48	48	96	48	72	198	198	165
48	48	48	120	48	72	198	198	165
48	48	48	144	48	72	198	198	165
48	48	48	144	48	72	198	198	165
48	48	48	144	48	72	215	198	165
48	48	48	144	48	72	231	198	165
48	48	48	144	48	96	231	215	198
48	48	48	168	48	96	231	215	198
48	48	48	168	48	120	231	231	198
48	48	48	168	48	120	231	231	198
48	48	48	168	48	120	231	231	198
48	48	48	168	48	120	231	231	198
48	48	48	192	48	120	231	231	198
48	48	48	192	72	120	231	231	198
48	48	48	192	72	120	248	231	198
48	48	48	240	72	120	264	231	198
48	48	48	240	72	120	264	231	198
48	48	48	240	72	168	264	231	198
48	48	48	240	72	168	264	248	198
48	48	48	240	72	192	264	264	198
72	48	48	240	72	192	264	264	215
72	48	48	264	72	192	264	264	215
72	48	48	288	96	240	264	264	231
72	72	48	288	120	240	264	264	231
72	72	48	288	120	240	264	264	231
72	72	48	312	120	240	281	264	231
72	96	48	312	120	240	297	264	231
72	96	48	312	120	264	297	264	231
72	96	48	312	120	264	297	264	231
72	96	48	336	120	288	297	281	248
72	120	48	336	120	288	297	297	264
72	120	48	336	144	288	297	297	264
96	144	48	336	144	288	297	297	264

RS00F1	RS00F2	RS00F3	RS00F4	RS00F5	RS00F6	RS00F7	RS00F8	**RS00F9**
96	144	48	336	144	288	297	297	264
96	144	48	336	168	288	297	297	264
96	144	48	336	192	288	297	297	264
96	144	48	336	192	288	297	297	264
96	144	48	360	216	312	297	297	264
96	144	48	360	240	312	297	297	264
96	144	48	360	264	312	297	330	264
96	168	48	360	264	312	297	330	297
120	168	48	360	288	312	297	330	297
120	168	72	360	288	336	297	330	297
144	216	72	360	288	336	297	330	330
168	240	72	360	288	336	314	330	330
168	240	72	360	288	336	330	330	330
168	240	72	360	288	336	330	330	330
168	240	72	360	288	336	330	330	330
168	264	72	384	288	336	330	330	330
168	264	96	384	312	336	330	347	330
192	264	96	384	312	336	330	347	363
192	264	96	384	312	336	330	363	363
216	264	144	384	336	360	347	363	380
216	288	144	384	336	360	347	363	396
240	288	144	384	336	360	347	363	396
240	288	144	384	336	360	363	363	396
240	288	144	384	336	360	363	363	396
240	288	144	384	336	360	363	363	413
264	288	168	384	336	360	363	363	429
264	312	216	384	360	360	363	363	429
264	336	216	384	360	360	363	380	429
288	336	216	408	360	360	363	380	429
288	336	240	408	360	384	396	396	429
288	336	240	408	360	384	396	396	429
288	336	264	408	384	384	396	396	429
288	336	264	408	384	384	396	396	429
288	336	264	408	384	384	396	396	429
288	336	288	408	384	384	396	396	462
312	336	288	432	384	384	396	396	462
312	360	288	432	384	384	396	396	462
336	360	288	432	384	384	396	396	462
336	360	288	432	384	384	396	396	462
336	360	288	480	384	384	413	413	462
336	360	288	480	408	384	413	429	462
336	360	288	504	408	384	413	429	462
336	360	288	600	408	408	429	429	462
360	360	288	672	408	408	429	429	462
360	360	312	696	408	408	429	429	479
360	360	336	696	408	408	429	429	495

RS00F1	RS00F2	RS00F3	RS00F4	RS00F5	RS00F6	RS00F7	RS00F8	**RS00F9**
384	384	336	744	408	432	429	462	495
384	384	336	768	408	504	429	462	495
384	384	336	768	408	552	429	462	528
384	384	360	768	408	696	446	462	545
408	384	360	768	408	696	446	462	561
408	408	360	792	432	744	462	479	561
408	408	360	792	504	744	462	495	594
480	408	384	792	552	768	462	495	594
480	408	408	840	744	768	462	512	594
504	456	432	840	744	792	495	627	611
504	480	816	912	768	792	594	660	611

RS00F10	RS00F11	**RS00F12**	RS00F13	RS00F14	**RS00F15**	RS00F16	RS00F17
96	165	99	48	132	165	189	165
96	165	99	48	132	165	189	165
96	165	99	48	132	165	189	165
120	165	99	48	165	165	189	165
120	165	99	48	165	165	189	165
120	198	99	48	165	198	189	165
120	198	99	48	198	198	189	165
120	231	99	48	198	231	189	165
120	231	99	48	198	231	189	165
120	231	99	48	198	231	189	165
144	231	132	48	198	231	252	165
144	231	132	48	198	231	252	165
168	231	132	48	198	231	252	198
168	231	132	48	231	231	252	198
168	231	132	48	231	231	252	198
192	264	132	48	231	264	252	198
192	264	132	48	231	264	252	198
192	264	132	48	231	264	252	198
192	264	132	48	231	264	252	198
192	264	132	48	231	264	252	198
240	264	132	48	231	264	252	198
240	264	132	48	231	264	252	198
264	297	165	48	231	297	315	198
264	297	165	48	231	297	315	198
264	297	165	48	231	297	315	198
288	297	165	48	264	297	315	198
288	297	165	48	264	297	315	198
288	330	165	48	264	330	315	198
288	330	165	48	264	330	315	198
288	330	165	48	297	330	315	198
288	330	165	48	297	330	315	198
288	330	165	48	297	330	315	198

RS00F10	RS00F11	**RS00F12**	RS00F13	RS00F14	**RS00F15**	RS00F16	RS00F17
288	330	165	48	330	330	315	198
288	330	165	48	330	330	315	198
312	330	165	48	330	330	315	198
312	330	165	48	330	330	315	198
312	330	165	48	330	330	315	198
312	330	165	72	330	330	315	231
336	363	165	72	330	363	315	231
336	363	165	72	330	363	315	231
336	363	165	72	330	363	315	231
336	363	165	72	330	363	315	231
336	363	165	72	330	363	315	231
336	363	165	72	330	363	315	264
336	363	165	72	330	363	315	264
336	363	165	72	330	363	315	264
336	363	165	72	330	363	315	264
336	363	165	72	330	363	315	264
336	396	165	72	330	396	315	264
360	396	165	72	330	396	315	264
360	396	165	72	330	396	315	264
360	396	165	72	330	396	315	264
360	396	165	72	330	396	315	264
360	396	198	72	330	396	378	264
360	396	198	72	330	396	378	264
360	396	198	72	330	396	378	264
360	396	198	96	330	396	378	264
384	396	198	96	363	396	378	264
384	396	198	96	363	396	378	264
384	396	198	144	363	396	378	264
384	429	198	144	363	429	378	264
384	429	198	144	363	429	378	264
384	429	198	144	363	429	378	264
384	462	231	144	363	462	441	297
384	462	231	144	363	462	441	297
384	462	231	168	363	462	441	297
384	462	231	216	363	462	441	297
384	462	231	216	363	462	441	297
384	462	231	216	363	462	441	297
384	462	231	240	363	462	441	297
384	495	231	240	363	495	441	297
384	528	231	264	363	528	441	330
384	528	231	264	363	528	441	330
408	594	231	264	363	594	441	330
408	594	231	288	363	594	441	330
408	627	231	288	363	627	441	330
408	627	231	288	363	627	441	330
408	627	231	288	396	627	441	330

RS00F10	RS00F11	**RS00F12**	RS00F13	RS00F14	**RS00F15**	RS00F16	RS00F17
408	660	231	288	396	660	441	330
408	693	231	288	396	693	441	330
432	693	231	288	396	693	441	330
432	693	231	288	396	693	441	330
432	726	231	312	396	726	441	330
504	726	231	336	396	726	441	330
504	726	231	336	396	726	441	330
552	726	231	336	396	726	441	330
600	726	231	336	429	726	441	330
696	726	264	336	429	726	504	363
696	726	264	336	429	726	504	363
744	726	264	336	429	726	504	363
744	726	264	336	429	726	504	363
768	759	264	336	429	759	504	363
792	759	264	336	462	759	504	363
792	759	264	336	495	759	504	363
792	759	264	336	528	759	504	363
792	825	264	432	561	825	504	363
792	891	297	456	726	891	567	363

RS00F18	RS00F19	**RS00F20**	**RS00F21**	RS00F22	RS00F23	**RS00F24**	RS00F25
132	165	165	132	165	99	132	132
132	165	165	165	165	99	132	132
165	165	165	198	165	99	132	198
165	165	165	198	165	99	132	198
198	165	198	198	198	132	132	198
198	165	198	198	198	132	132	198
198	165	198	198	198	132	165	231
198	165	198	231	198	132	165	231
231	165	198	231	198	198	165	231
231	165	198	264	198	198	165	231
231	165	198	264	198	198	165	264
231	165	198	264	198	198	165	264
231	198	198	264	198	231	198	264
231	198	198	264	198	231	198	264
231	198	198	264	198	231	198	264
231	198	198	264	198	231	198	264
231	231	198	297	198	231	198	297
231	231	198	297	198	231	198	297
264	231	198	297	198	231	198	297
264	231	198	297	198	231	198	297
264	231	198	297	198	264	231	297
264	231	198	330	198	264	231	297
264	231	198	330	198	264	231	297
264	231	198	330	198	264	231	297

RS00F18	RS00F19	**RS00F20**	**RS00F21**	RS00F22	RS00F23	**RS00F24**	RS00F25
264	231	198	330	198	264	231	297
264	231	198	330	198	264	264	297
264	231	198	330	198	264	264	297
264	231	231	330	231	264	297	297
264	231	231	330	231	264	297	297
264	231	231	330	231	264	297	297
264	231	231	330	231	264	297	297
264	231	231	330	231	264	297	297
264	231	231	330	231	264	297	297
264	231	231	330	231	297	297	297
264	231	231	330	231	297	297	297
297	231	231	363	231	297	297	330
297	231	231	363	231	330	297	330
297	264	264	363	264	330	330	330
297	264	264	363	264	330	330	330
297	264	264	363	264	330	330	330
297	264	264	363	264	330	330	330
297	264	264	363	264	330	330	330
297	264	264	363	264	330	330	330
297	264	264	363	264	330	330	330
297	264	264	396	264	330	330	330
330	297	264	396	264	330	330	330
330	297	264	396	264	330	330	330
330	297	264	396	264	330	330	330
330	297	264	396	264	330	330	330
330	330	297	396	297	330	330	330
330	330	297	396	297	330	330	330
330	330	330	396	330	330	330	330
330	330	330	396	330	330	330	330
330	330	330	429	330	330	330	330
330	330	330	429	330	363	330	330
330	330	330	429	330	363	330	330
330	330	330	429	330	363	330	330
330	330	330	429	330	363	363	330
330	330	330	429	330	363	363	330
330	330	330	429	330	363	363	330
330	330	330	429	330	363	363	330
330	330	330	429	330	363	363	330
330	330	330	429	330	363	363	330
330	330	330	429	330	363	363	330
330	330	330	429	330	363	363	363
330	363	330	462	330	363	363	363
330	363	330	462	330	363	363	363
330	363	330	462	330	363	363	363
330	363	330	462	330	363	363	363
330	363	330	462	330	363	363	363

RS00F18	RS00F19	**RS00F20**	**RS00F21**	RS00F22	RS00F23	**RS00F24**	RS00F25
330	363	330	462	330	363	363	363
330	363	330	462	330	363	363	363
330	363	330	462	330	363	363	363
330	363	330	462	330	363	363	363
363	363	330	462	330	363	396	396
363	363	330	462	330	363	396	396
363	363	363	462	363	396	396	396
363	363	363	462	363	396	396	396
363	363	363	462	363	396	396	396
363	363	363	495	363	396	396	396
363	363	363	495	363	396	396	396
363	363	363	495	363	396	396	396
363	396	396	528	396	396	396	396
363	396	396	528	396	396	396	462
363	396	396	528	396	396	396	462
363	396	396	528	396	396	396	462
363	396	396	528	396	429	396	462
363	396	396	561	396	429	396	462
363	396	396	561	396	429	396	462
363	396	396	561	396	429	396	462
396	396	396	561	396	429	396	495
396	396	396	561	396	462	396	495
396	396	396	594	396	462	396	693
396	396	396	594	396	462	396	693
396	396	396	594	396	495	396	726
396	396	396	594	396	528	396	726
396	396	396	627	396	528	396	759
396	396	396	660	396	693	396	759

RS00F26	**RS00F27**	RS00F28	RS00F29	RS00F30	RS00F31	**RS00F32**	**RS00F33**
165	165	132	264	132	165	165	132
165	165	132	264	132	165	165	132
198	165	132	297	198	165	165	165
198	165	132	297	198	165	165	165
198	165	198	297	198	165	231	198
198	198	198	297	198	165	231	198
231	198	198	297	198	198	330	198
231	198	198	297	198	198	330	198
231	198	198	330	198	198	330	198
231	198	198	330	198	198	330	198
264	198	198	330	231	198	330	198
264	198	198	330	231	198	330	198
264	198	231	330	231	198	330	198
264	198	231	330	231	198	330	198
264	198	231	330	231	198	330	231

RS00F26	**RS00F27**	RS00F28	RS00F29	RS00F30	RS00F31	**RS00F32**	**RS00F33**
297	198	231	330	231	198	363	231
297	231	231	330	231	198	363	231
297	231	264	330	231	231	363	264
297	231	264	330	231	231	363	264
297	231	264	330	231	231	363	264
330	231	264	330	231	231	363	264
330	231	264	330	264	231	363	264
330	264	264	330	264	231	363	264
330	264	264	330	264	231	363	264
330	264	264	330	264	231	363	264
330	264	297	330	264	231	363	264
330	264	297	330	264	231	363	264
330	264	297	330	264	231	363	264
330	297	297	330	264	231	363	264
330	330	330	330	297	231	363	264
330	330	330	330	297	231	363	264
330	330	330	330	297	231	363	297
330	330	330	330	297	264	363	297
330	330	330	330	297	264	396	297
330	330	330	330	297	264	396	297
330	330	330	330	297	264	396	297
330	330	330	330	297	264	396	297
330	330	330	363	297	264	396	297
330	330	330	363	297	264	396	297
330	330	330	363	330	264	429	297
330	330	330	363	330	264	429	297
330	330	330	363	330	264	429	330
330	330	330	363	330	264	429	330
330	330	330	363	330	264	429	330
363	330	330	363	330	264	429	330
363	330	330	396	330	264	429	330
363	363	330	396	330	264	429	330
363	363	330	396	330	264	429	330
363	363	330	396	330	264	429	330
363	363	330	396	330	264	462	330
363	363	330	396	330	264	462	330
363	363	330	396	363	264	462	330
363	363	330	396	363	297	462	330
363	363	363	396	363	297	462	330
363	363	363	396	363	297	462	330
363	363	363	396	363	297	462	330
363	396	363	396	363	297	462	330
363	396	363	396	363	297	462	330
363	396	363	396	363	297	462	330
363	396	363	396	363	297	462	330
363	396	363	396	363	330	462	330

RS00F26	**RS00F27**	RS00F28	RS00F29	RS00F30	RS00F31	**RS00F32**	**RS00F33**
363	429	363	396	396	330	462	330
396	429	363	396	396	330	462	330
396	429	363	396	396	330	462	330
396	429	363	396	396	330	462	330
396	462	363	396	396	330	495	330
396	462	363	396	396	330	495	330
396	462	363	396	396	330	495	330
396	462	363	396	396	330	495	330
396	462	396	396	429	330	495	363
396	462	396	396	429	330	495	363
396	462	396	462	495	330	495	363
396	462	396	462	495	330	495	363
396	462	396	462	495	330	495	363
396	462	396	462	495	330	495	363
396	462	396	462	495	330	495	363
396	462	396	462	495	330	495	363
396	495	396	462	495	396	495	363
396	495	396	462	495	396	495	363
396	495	462	462	495	396	495	363
396	495	462	462	495	396	495	363
396	495	462	462	528	396	528	363
396	495	462	462	528	396	528	363
462	495	462	462	528	396	561	363
462	495	462	462	528	396	561	363
462	495	462	462	528	396	561	363
462	495	462	462	528	462	561	363
462	495	462	495	528	462	561	396
462	528	462	495	528	462	561	396
462	561	462	495	528	462	561	396
462	561	462	495	528	462	561	396
462	561	462	495	561	462	561	396
495	561	462	495	561	462	561	396
495	561	495	660	561	462	561	396
495	561	495	660	561	495	561	396
660	561	495	726	561	495	594	396
726	594	495	726	561	495	594	396

Year 2001

RS01F1	RS01F2	RS01F3	RS01F4	RS01F5	**RS01F6**	**RS01F7**	RS01F8	RS01F9
33	189	165	132	165	165	165	48	165
66	189	165	165	165	231	231	48	165
66	189	165	198	165	297	297	48	165
99	189	165	198	165	297	297	48	165
99	189	165	231	165	297	297	48	165
99	189	165	231	165	330	330	48	165
99	189	198	231	198	330	330	48	198

RS01F1	RS01F2	RS01F3	RS01F4	RS01F5	**RS01F6**	**RS01F7**	RS01F8	RS01F9
99	189	198	231	198	330	330	165	198
132	189	198	231	198	330	330	165	198
132	198	198	231	198	330	330	165	198
132	198	198	231	198	330	330	165	198
132	198	198	231	231	330	330	165	198
132	198	198	231	231	330	330	198	198
132	198	198	231	231	330	330	198	198
132	198	198	231	231	330	330	198	198
132	198	198	231	231	330	330	198	198
132	198	198	231	231	330	330	198	198
149	198	264	264	231	330	330	198	198
165	231	264	264	231	363	363	198	231
165	231	264	264	231	363	363	198	231
165	231	264	264	231	363	363	198	231
165	252	264	264	231	363	363	198	264
182	252	264	264	231	363	363	198	264
198	252	264	264	231	363	363	198	264
198	252	264	264	231	363	363	198	264
198	252	297	264	231	363	363	231	264
198	252	297	264	264	396	363	231	264
198	252	297	264	264	396	363	231	264
198	252	297	264	264	396	429	264	264
198	264	297	264	264	396	429	264	264
198	264	330	297	264	396	429	264	264
198	264	330	297	264	396	462	264	297
198	264	330	330	297	396	462	396	297
198	264	330	330	330	429	462	396	297
215	264	330	330	330	429	462	165	297
215	264	330	330	330	462	495	165	330
215	264	330	330	330	462	495	165	330
231	264	330	330	330	462	495	198	330
231	264	330	330	330	462	495	198	330
231	297	330	330	330	462	528	198	330
231	297	330	363	330	495	528	198	330
231	528	380	363	330	495	561	198	330
231	561	396	363	330	495	561	198	330
248	561	396	363	347	495	561	198	330
248	660	396	363	363	561	561	198	363
264	693	462	363	363	561	561	198	363
264	726	462	363	363	561	561	198	363
264	726	495	380	363	561	594	330	363
264	759	528	462	363	594	594	330	363

RS01F10	RS01F11	RS01F12	**RS01F13**	RS01F14	**RS01F15**	**RS01F16**	RS01F17
132	165	165	132	165	165	132	132
165	165	165	132	165	165	165	132
198	165	198	132	165	231	198	165
198	165	198	165	198	330	198	165
231	165	198	165	231	330	198	198
231	165	198	165	231	330	198	198
231	198	198	198	231	330	198	198
231	198	198	198	231	330	231	198
231	231	198	198	264	363	231	198
264	231	198	198	264	363	264	198
264	231	198	231	264	363	264	198
264	231	198	231	264	363	264	198
264	231	198	231	264	363	264	231
264	231	198	264	264	363	264	231
264	231	231	297	264	363	264	231
264	231	231	297	264	363	264	264
264	231	231	297	297	363	297	264
264	231	231	297	297	396	297	264
297	231	231	297	297	396	297	264
297	264	264	330	297	396	297	264
297	264	264	330	297	429	297	264
297	264	264	330	297	429	330	264
297	264	264	330	330	429	330	330
330	297	264	330	330	429	330	330
330	297	264	330	330	429	330	330
330	330	297	330	330	462	330	330
330	330	330	330	330	462	330	330
330	330	330	330	330	462	330	363
330	330	330	330	330	462	330	363
330	330	330	363	330	462	330	363
330	330	330	363	330	462	330	363
330	330	330	363	330	462	330	363
330	330	330	363	330	462	330	363
330	363	330	363	330	462	330	363
330	363	330	363	363	495	330	396
330	363	330	363	363	495	330	396
330	363	330	363	363	495	363	396
330	363	330	363	363	495	363	396
363	363	330	396	363	495	363	396
363	363	363	396	363	495	363	396
363	363	363	396	363	495	363	396
363	363	363	396	363	495	363	396
363	396	396	396	363	528	363	396
363	396	396	396	363	561	363	594
363	396	396	396	363	561	363	660

RS01F10	RS01F11	RS01F12	**RS01F13**	RS01F14	**RS01F15**	**RS01F16**	RS01F17
396	396	396	396	363	561	396	693
396	396	396	396	363	561	396	693
396	396	396	396	363	561	396	726
396	396	396	396	363	594	396	726

RS01F18	RS01F19	RS01F20	RS01F21	RS01F22	RS01F23	RS01F24	RS01F25
264	99	264	231	165	132	132	363
264	132	297	264	165	198	132	363
264	198	297	264	198	198	198	429
264	231	297	297	198	198	198	429
264	231	330	297	198	198	198	429
264	264	330	297	198	231	198	429
264	264	330	297	231	231	231	462
264	264	330	297	231	231	231	462
264	264	330	297	231	231	231	462
264	297	330	297	231	231	264	462
264	330	330	297	264	231	264	462
264	330	330	297	264	264	264	462
297	330	330	297	264	264	264	462
330	330	330	297	264	264	297	462
330	330	330	297	264	264	297	462
363	330	330	297	297	297	330	462
396	363	330	297	297	297	330	462
396	363	330	297	330	297	330	462
396	363	330	297	330	297	330	462
396	363	363	297	330	297	330	462
396	363	363	297	330	330	330	462
396	363	363	330	330	330	330	462
396	363	363	330	330	330	330	462
396	363	396	330	330	330	330	495
462	363	396	330	330	330	330	495
462	363	396	330	330	330	330	495
495	363	396	330	363	363	330	495
495	363	396	330	363	363	363	495
495	396	396	330	363	363	363	495
528	396	396	330	363	363	363	495
528	396	396	330	363	363	363	495
561	396	396	330	363	396	363	495
561	396	396	330	363	396	363	528
594	396	396	330	363	396	363	528
594	396	396	330	363	396	363	528
594	396	396	330	363	396	363	528
594	396	396	330	363	429	396	528
627	396	462	330	363	495	396	528
792	429	462	363	363	495	396	528
792	429	462	363	363	495	396	528

RS01F18	RS01F19	RS01F20	RS01F21	RS01F22	RS01F23	RS01F24	RS01F25
792	429	462	396	363	495	462	528
792	429	462	396	396	528	462	528
792	462	462	396	396	528	462	528
792	462	462	693	396	528	462	528
792	462	495	693	396	528	462	528
792	495	495	726	396	528	462	528
792	528	495	726	396	561	462	528
825	528	660	759	396	561	495	528
825	693	726	759	462	561	495	528

RS01F26	RS01F27	RS01F28	RS01F29	RS01F30	RS01F31	**RS01F32**	RS01F33
165	363	132	99	264	198	132	264
231	363	198	132	264	363	132	264
429	363	198	165	264	363	132	264
429	363	198	165	264	363	132	264
462	363	198	165	264	396	132	264
462	363	198	198	264	396	363	264
462	363	231	198	297	396	363	264
462	363	231	198	297	396	363	264
462	363	330	198	297	396	363	297
462	363	330	231	297	429	363	330
462	363	363	231	297	429	363	330
462	363	363	231	330	429	363	396
462	363	396	264	363	429	363	396
462	363	396	264	363	429	363	396
462	363	396	264	363	429	363	396
462	363	396	330	363	462	363	396
495	363	396	330	363	462	363	429
495	363	396	330	363	462	462	429
495	363	429	363	363	462	462	429
495	363	429	363	363	462	462	429
495	363	429	363	363	528	462	429
495	363	462	363	363	528	462	429
495	363	462	363	363	528	462	429
495	363	462	396	363	528	462	462
495	363	462	396	363	528	462	462
528	363	462	396	363	528	462	462
528	363	462	396	363	528	462	462
528	363	462	396	363	528	462	462
528	363	462	429	363	561	462	462
528	363	462	462	363	561	462	462
528	462	462	462	363	561	462	462
528	462	462	462	363	561	462	462
528	462	462	462	363	561	462	462
528	462	462	462	363	561	462	462

RS01F26	RS01F27	RS01F28	RS01F29	RS01F30	RS01F31	**RS01F32**	RS01F33
528	462	462	462	363	561	462	462
561	462	495	462	363	594	462	462
561	462	495	462	363	594	462	462
561	462	495	495	363	594	462	462
561	462	495	495	363	594	462	462
561	462	495	495	363	627	462	462
561	495	495	495	363	627	462	462
561	495	495	495	363	660	462	495
561	495	495	495	363	660	462	528
561	528	495	495	363	660	462	528
561	528	495	495	363	660	462	528
594	528	495	495	363	693	462	528
594	528	495	528	363	693	462	528
594	528	528	528	363	693	462	528
627	528	528	594	396	726	462	561

Table B.5. Fertilization and hatching results from 2000 and 2001.

Date	Fish ID	Description	Sperm Motility	Percent 8-Cell (R1)	Percent 8-Cell (R2)	Percent 8-Cell (R3)	Percent Neurulation (R1)	Percent Neurulation (R2)	Percent Neurulation (R3)
06/29/2000	**RS00F09 X RS00M52**	Fresh Red	0/90	70.13	51.55	43.70	44.00	48.79	52.08
06/29/2000	**RS00F09 X GS00M35**	Fresh Grey	0/95	47.25	26.32	61.96	42.50	35.58	38.86
06/29/2000	**RS00F09 X RS00M49**	Cryo Red	0/80	52.38	31.62	30.67	9.95	19.37	12.57
06/29/2000	**RS00F09 X GS00M34**	Cryo Grey	0/80	29.14	41.55	36.67	2.90	2.55	1.83

Date	Fish ID	Description	Sperm Motility	Percent 8-Cell (R1)	Percent 8-Cell (R2)	Percent Neurulation (R1)	Percent Neurulation (R2)	Percent Hatch (R1)	Percent Hatch (R2)
07/11/2000	**RS00F15 X RS00M58**	Fresh Red	0/80	80.59	65.27	78.64	57.65	47.86	57.48
07/11/2000	**RS00F15 X GS00M42**	Fresh Grey	0/90	81.08	76.04	48.60	52.13	47.97	44.38
07/11/2000	**RS00F15 X RS00M49**	Cryo Red	0/80	66.48	72.93	39.53	53.25	41.61	36.40
07/11/2000	**RS00F15 X GS00M34**	Cryo Grey	0/80	22.87	17.78	37.04	22.47	29.89	21.52
07/11/2000	**RS00F12 X RS00M58**	Fresh Red	0/80	77.52	74.16	80.77	80.49	71.48	71.11
07/11/2000	**RS00F12 X GS00M42**	Fresh Grey	0/90	88.74	83.11	63.91	85.29	93.53	88.89
07/11/2000	**RS00F12 X RS00M50**	Cryo Red	0/95	86.99	90.30	89.07	80.50	59.36	80.09
07/11/2000	**RS00F12 X GS00M23**	Cryo Grey	0/80	54.92	56.22	42.90	38.34	33.05	38.84
07/26/2000	**RS00F20 X RS00M59**	Fresh Red	0/90	44.26	46.94	33.33	24.83	23.15	38.18
07/26/2000	**RS00F20 X GS00M44**	Fresh Grey	0/95	18.37	14.95	16.67	1.98	5.00	6.82
07/26/2000	**RS00F20 X RS00M50**	Cryo Red	0/95	19.40	35.85	17.46	20.00	10.42	17.78
07/26/2000	**RS00F20 X GS00M24**	Cryo Grey	0/80	58.25	54.43	14.58	14.49	12.94	6.78
07/26/2000	**RS00F20 X LS00M01**	Fresh Lane	0/10	25.00	25.00	14.81	30.36	13.46	13.73

Date	Fish ID	Description	Sperm Motility	Percent 8-Cell (R1)	Percent 8-Cell (R2)	Percent Neurulation (R1)	Percent Neurulation (R2)	Percent Hatch (R1)	Percent Hatch (R2)
07/26/2000	**RS00F21 X RS00M59**	Fresh Red	0/90	93.51	87.50	37.25	40.96	23.26	27.42
07/26/2000	**RS00F21 X GS00M44**	Fresh Grey	0/95	79.17	83.33	10.37	6.00	4.35	5.31
07/26/2000	**RS00F21 X RS00M50**	Cryo Red	0/95	92.08	94.12	37.11	37.50	45.28	68.00
07/26/2000	**RS00F21 X GS00M24**	Cryo Grey	0/80	3.15	3.47	26.89	32.14	14.29	16.07
07/26/2000	**GS00F04 X GS00M44**	Fresh Grey	0/95	5.13	3.23	41.13	51.49	2.26	0.00
07/26/2000	**GS00F04 X RS00M59**	Fresh Red	0/90	85.71	92.47	82.61	78.32	62.09	63.14
07/26/2000	**GS00F04 X GS00M24**	Cryo Grey	0/80	86.49	83.93	1.41	1.86	50.49	55.17
07/26/2000	**GS00F04 X RS00M50**	Cryo Red	0/95	89.24	92.98	80.05	82.03	78.45	75.98
07/26/2000	**GS00F04 X LS00M01**	Fresh Lane	0/10	12.69	11.21	6.55	5.65	1.02	2.67
08/10/2000	**RS00F24 X RS00M44**	Cryo Red	0/90	72.05	63.19	18.29	19.88	23.08	17.93
08/10/2000	**RS00F24 X Red Snap1**	Cryo Red - 1998	0/90	78.81	72.42	14.66	23.94	20.00	16.29
08/10/2000	**RS00F24 X GS00M24**	Cryo Grey	0/80	85.71	61.60	17.20	18.12	25.93	27.19
08/10/2000	**RS00F33 X RS00M71**	Fresh Red	0/95	98.41	97.46	82.87	78.99	87.84	80.33
08/10/2000	**RS00F33 X RS00M44**	Cryo Red	0/90	95.27	92.31	68.47	***	60.45	73.72
08/10/2000	**RS00F33 X Red Snap1**	Cryo Red - 1998	0/90	85.71	85.09	49.59	45.18	79.78	68.32
08/10/2000	**RS00F33 X GS00M29**	Cryo Grey	0/50	94.13	85.61	52.69	31.71	53.76	72.43
08/10/2000	**RS00F32 X RS00M71**	Fresh Red	0/95	92.52	96.88	39.81	26.46	12.50	8.16
08/10/2000	**RS00F32 X RS00M44**	Cryo Red	0/90	98.92	94.32	35.73	45.57	14.71	6.83
08/10/2000	**RS00F32 X GS00M24**	Cryo Grey	0/80	96.87	91.21	2.94	3.83	0.28	0.00
08/10/2000	**RS00F27 X RS00M70**	Fresh Red	0/95	90.52	86.54	46.15	53.16	47.22	60.29
08/10/2000	**RS00F27 X RS00M71**	Fresh Red	0/95	94.57	95.16	74.47	67.16	64.29	65.52
08/10/2000	**RS00F27 X RS00M43**	Cryo Red	0/90	92.74	92.86	58.11	48.98	40.48	53.49
08/10/2000	**RS00F27 X GS00M29**	Cryo Grey	0/50	90.79	83.17	67.33	16.00	23.44	6.78

Date	Fish ID	Description	Sperm Motility	Percent 8-Cell (R1)	Percent 8-Cell (R2)	Percent Neurulation (R1)	Percent Neurulation (R2)	Percent Hatch (R1)	Percent Hatch (R2)
07/19/2001	**RS01F13 X RS01M33**	Cryo	0/95	65.76	61.78	42.37	33.33	47.32	31.38
07/19/2001	**RS01F13 X RS01M56**	Fresh	0/95	81.03	89.34	50.61	62.07	49.73	48.43
07/19/2001	**RS01F13 X GS01M3**	Cryo	0/95	12.12	13.22	2.82	2.78	2.06	3.49
07/19/2001	**RS01F13 X GS01M20**	Fresh	0/95	44.16	47.65	20.87	23.98	19.37	22.91
07/19/2001	**RS01F15 X RS01M33**	Cryo	0/95	29.26	26.82	14.87	18.13	12.50	14.67
07/19/2001	**RS01F15 X RS01M56**	Fresh	0/95	36.91	40.83	20.58	24.46	19.46	19.43
07/19/2001	**RS01F15 X GS01M3**	Cryo	0/95	8.38	6.96	5.00	2.48	4.65	1.83
07/19/2001	**RS01F15X GS01M20**	Fresh	0/95	7.35	6.70	3.98	4.62	2.66	3.63
07/19/2001	**RS01F16 X RS01M33**	Cryo	0/95	16.23	18.98	14.98	6.28	11.06	8.68
07/19/2001	**RS01F16 X RS01M56**	Fresh	0/95	33.06	33.46	19.63	19.53	19.92	18.26
07/19/2001	**RS01F16 X GS01M3**	Cryo	0/95	2.49	2.33	0.00	1.11	0.77	0.79
07/19/2001	**RS01F16 X GS01M20**	Fresh	0/95	5.96	4.98	0.84	2.14	1.33	0.93
08/01/2001	**RS01F18 X RS01M33**	Cryo	0/95	6.25	4.49	1.94	1.77	2.30	1.92
08/01/2001	**RS01F18 X RS01M72**	Fresh	0/95	7.48	6.32	3.26	5.11	2.88	4.21
08/01/2001	**RS01F18 X GS01M3**	Cryo	0/95	2.04	1.75	0.70	0.00	1.19	0.63
08/01/2001	**RS01F18 X GS01M21**	Fresh	0/95	16.75	15.53	8.85	9.85	7.11	8.41
08/15/2001	**RS01F32 X RS01M88**	Fresh	0/95	0.27	0.50	0.00	0.53	0.00	0.00
08/15/2001	**RS01F32 X RS01M89**	Fresh	0/95	0.00	0.00	0.00	0.00	0.00	0.00
08/15/2001	**RS01F32 X RS01M33**	Cryo	0/95	0.00	0.00	0.00	0.00	0.00	0.00
08/15/2001	**RS01F32 X GS01M3**	Cryo	0/95	1.06	2.75	2.47	0.00	0.72	1.15

Date	Fish ID	Description	Sperm Motility	Percent 8-Cell (R1)	Percent 8-Cell (R2)	Percent Neurulation (R1)	Percent Neurulation (R2)	Percent Hatch (R1)	Percent Hatch (R2)
08/15/2001	**RS01F26 X RS01M88**	Fresh	0/95	28.29	31.28	17.11	17.37	15.35	17.34
08/15/2001	**RS01F26 X RS01M89**	Fresh	0/95	5.29	3.73	1.82	0.79	1.69	1.92
08/15/2001	**RS01F26 X RS01M33**	Cryo	0/95	15.79	12.33	6.98	3.98	9.04	3.43
08/15/2001	**RS01F26 X GS01M3**	Cryo	0/95	4.97	2.72	1.91	0.75	0.69	1.32
07/11/2001	**GS01F6 X GS01M16**	Fresh	0/95	59.74	60.82	24.29	39.62	21.88	33.93
07/11/2001	**GS01F6 X GS01M3**	Cryo	0/95	26.72	22.54	10.32	6.78	12.59	8.09
07/11/2001	**GS01F6 X RS01M33**	Cryo	0/95	58.71	63.43	26.71	38.83	32.89	36.17
07/11/2001	**GS01F6 X RS01M36**	Cryo	0/95	63.27	57.40	38.46	31.82	32.10	41.22
07/05/2001	**GS01F3 X RS01M41**	Fresh	0/95	49.43	45.65	22.52	34.98	31.23	30.20
07/05/2001	**GS01F3 X RS01M33**	Cryo	0/95	43.08	46.30	25.15	27.84	24.47	25.16
07/05/2001	**GS01F3 X GS01M3**	Cryo	0/95	20.04	22.61	11.27	8.33	10.11	9.30
07/05/2001	**GS01F3 X GS01M13**	Cryo	0/95	8.13	6.94	1.25	1.82	1.95	1.49

Table B.6. Average larval survival and growth at 10 d after hatching.

Spawn	Total Larvae (N)	Average Temperature (C)	Method	Average Live Length (mm)	Average Preserved Length (mm)
RS00F09xGS00M35	4	26.8	Standard length	4.18	3.22
			Total length	4.52	3.56
RS00F12xGS00M23	2	29.8	Standard length	3.27	2.91
			Total length	3.72	3.72
RS00F12xGS00M23	1	29.3	Standard length	3.37	3.13
			Total length	3.63	3.51
RS00F12xGS00M42	30	29.26	Standard length	2.61	2.46
			Total length	2.93	2.86
RS00F12xGS00M42	14	29.72	Standard length	3.77	3.39
			Total length	4.02	3.88
RS00F15xGS00M42	26	29.06	Standard length	2.56	2.40
			Total length	2.88	2.70
RS00F21xGS00M44	2	29.46	Standard length	4.05	4.02
			Total length	4.72	4.56
RS00F20xGS00M44	1	30.56	Standard length	3.99	3.66
			Total length	4.75	4.64
RS00F21xGS00M44	2	30.23	Standard length	4.91	4.55
			Total length	5.42	5.01
RS00F33xGS00M29	1	29.81	Standard length	3.75	3.25
			Total length	4.30	3.87
RS00F33xGS00M29	15	29.56	Standard length	3.61	3.41
			Total length	4.30	3.82
RS00F33xRS00M71	2	29.64	Standard length	5.67	4.78
			Total length	6.06	5.54
RS00F33xRS00M71	25	29.65	Standard length	4.83	4.24
			Total length	5.45	4.72
RS01F13xGS01M20	1	29.92	Standard length	4.73	4.18
			Total length	5.48	4.80

Table B.7. Live and preserved larval lengths 10 d after hatching.

Spawn	Method	Live length (mm)	Preserved length (mm)
RS00F09xGS00M35	Standard length	4.49	2.32
	Standard length	4.19	3.35
	Standard length	3.87	3.99
	Standard length	4.19	Lost Sample
	Total length	4.84	2.74
	Total length	4.64	3.75
	Total length	4.10	4.19
	Total length	4.52	Lost Sample
RS00F12xGS00M23	Standard length	2.95	2.44
	Standard length	3.59	3.38
	Total length	3.42	2.96
	Total length	4.02	3.76
RS00F12xGS00M42	Standard length	1.90	1.90
	Standard length	1.53	1.98
	Standard length	1.60	2.31
	Standard length	2.33	2.69
	Standard length	2.16	2.51
	Standard length	2.36	2.71
	Standard length	2.29	2.46
	Standard length	2.65	2.84
	Standard length	2.31	2.59
	Standard length	2.23	2.66
	Standard length	2.92	2.54
	Standard length	2.98	2.54
	Standard length	3.12	2.13
	Standard length	3.36	2.42
	Standard length	2.60	2.45
	Standard length	1.40	2.34
	Standard length	2.78	2.15
	Standard length	2.61	2.88
	Standard length	2.77	2.28
	Standard length	2.20	2.20
	Standard length	2.65	2.14
	Standard length	3.11	3.17
	Standard length	3.16	2.45
	Standard length	2.84	3.22
	Standard length	2.35	2.40
	Standard length	2.53	2.15
	Standard length	3.08	2.27
	Standard length	3.27	2.85
	Standard length	3.42	2.41
	Standard length	3.75	2.22
	Total length	2.42	2.39

Spawn	Method	Live length (mm)	Preserved length (mm)
RS00F12xGS00M42	Total length	1.79	2.95
	Total length	2.58	3.20
	Total length	2.44	2.75
	Total length	2.66	3.38
	Total length	2.51	2.89
	Total length	3.15	3.60
	Total length	2.62	3.05
	Total length	2.56	2.88
	Total length	3.23	3.10
	Total length	3.31	2.87
	Total length	3.55	2.37
	Total length	3.71	2.79
	Total length	2.91	2.78
	Total length	1.64	2.70
	Total length	3.31	2.40
	Total length	2.99	3.20
	Total length	3.09	2.79
	Total length	2.47	2.39
	Total length	3.04	2.57
	Total length	3.41	3.61
	Total length	3.40	2.76
	Total length	3.05	3.75
	Total length	2.70	2.60
	Total length	2.98	2.48
	Total length	3.26	2.59
	Total length	3.45	3.27
	Total length	3.80	2.79
	Total length	4.20	2.67
RS00F12xGS00M23	Standard length	3.37	3.13
	Total length	3.63	3.51
RS00F12xGS00M42	Standard length	4.42	2.96
	Standard length	4.37	4.21
	Standard length	3.39	2.86
	Standard length	4.09	2.43
	Standard length	3.14	3.77
	Standard length	4.31	2.84
	Standard length	4.71	2.02
	Standard length	3.22	4.25
	Standard length	4.69	4.61
	Standard length	3.38	4.41
	Standard length	3.21	4.26
	Standard length	2.87	3.15
	Standard length	2.40	2.82
	Standard length	4.63	2.86
	Total length	4.71	3.54

Spawn	Method	Live length (mm)	Preserved length (mm)
RS00F12xGS00M42	Total length	4.75	3.15
	Total length	3.73	2.99
	Total length	4.82	4.47
	Total length	4.89	3.20
	Total length	3.53	2.45
	Total length	5.10	4.91
	Total length	3.74	5.22
	Total length	3.58	4.65
	Total length	3.40	4.47
	Total length	2.89	3.77
	Total length	4.05	3.55
	Total length	3.23	3.36
RS00F15xGS00M42	Standard length	2.62	1.98
	Standard length	2.52	2.29
	Standard length	2.56	2.00
	Standard length	2.22	2.30
	Standard length	2.66	2.39
	Standard length	2.61	2.49
	Standard length	2.40	2.13
	Standard length	2.46	1.84
	Standard length	2.62	2.11
	Standard length	2.85	2.96
	Standard length	2.81	2.35
	Standard length	2.34	2.21
	Standard length	2.34	2.25
	Standard length	2.92	2.07
	Standard length	2.61	1.82
	Standard length	2.62	2.53
	Standard length	2.40	3.64
	Standard length	2.82	3.17
	Standard length	2.43	2.56
	Standard length	2.57	2.42
	Standard length	3.16	2.46
	Standard length	2.33	2.89
	Standard length	2.53	2.15
	Standard length	2.32	2.53
	Standard length	2.30	2.83
	Standard length	2.45	2.06
	Total length	3.03	2.41
	Total length	3.06	2.48
	Total length	2.92	2.26
	Total length	2.67	2.46
	Total length	2.90	2.66
	Total length	3.07	2.90
	Total length	2.58	2.42
	Total length	2.80	2.09

Spawn	Method	Live length (mm)	Preserved length (mm)
RS00F15xGS00M42	Total length	3.13	3.25
	Total length	2.95	2.51
	Total length	2.48	2.64
	Total length	2.72	2.57
	Total length	3.09	2.39
	Total length	3.01	2.15
	Total length	3.01	3.10
	Total length	2.60	4.32
	Total length	3.15	3.46
	Total length	2.71	2.72
	Total length	3.07	2.85
	Total length	3.26	2.78
	Total length	2.62	2.60
	Total length	2.93	2.59
	Total length	2.57	2.79
	Total length	2.61	2.99
	Total length	2.97	2.47
RS00F21xGS00M44	Standard length	4.17	4.01
	Standard length	3.93	4.02
	Total length	4.97	4.67
	Total length	4.46	4.45
RS00F20xGS00M44	Standard length	3.99	3.66
	Total length	4.75	4.64
RS00F21xGS00M44	Standard length	5.16	4.59
	Standard length	4.65	4.51
	Total length	5.61	5.26
	Total length	5.23	4.75
RS00F33xRS00M71	Standard length	5.43	4.86
	Standard length	5.91	4.70
	Total length	5.86	5.71
	Total length	6.27	5.37
RS00F33xGS00M29	Standard length	3.75	3.25
	Total length	4.30	3.87
RS00F33xGS00M29	Standard length	3.22	3.13
	Standard length	0.05	3.58
	Standard length	4.80	3.53
	Standard length	3.32	2.76
	Standard length	4.45	3.46
	Standard length	3.51	3.61
	Standard length	3.53	3.14
	Standard length	3.58	2.73
	Standard length	3.70	3.58

Spawn	Method	Live length (mm)	Preserved length (mm)
RS00F33xGS00M29	Standard length	4.09	3.71
	Standard length	3.92	2.73
	Standard length	3.96	3.08
	Standard length	3.54	3.44
	Standard length	3.82	4.38
	Total length	3.96	3.39
	Total length	4.70	4.08
	Total length	4.10	3.88
	Total length	3.96	3.23
	Total length	2.89	3.80
	Total length	4.23	4.02
	Total length	4.11	3.46
	Total length	4.11	3.19
	Total length	4.52	3.86
	Total length	5.37	4.87
	Total length	4.78	4.24
	Total length	4.38	3.15
	Total length	4.80	3.48
	Total length	4.19	3.85
	Total length	4.43	4.78
RS00F33xRS00M71	Standard length	4.63	4.32
	Standard length	5.12	4.53
	Standard length	4.91	4.85
	Standard length	4.92	4.38
	Standard length	3.71	3.60
	Standard length	4.72	4.20
	Standard length	3.72	3.46
	Standard length	5.42	3.64
	Standard length	4.65	4.81
	Standard length	4.46	4.48
	Standard length	4.79	4.67
	Standard length	3.84	3.68
	Standard length	4.94	5.10
	Standard length	4.80	4.12
	Standard length	4.90	3.50
	Standard length	5.85	3.76
	Standard length	6.04	4.46
	Standard length	6.20	4.13
	Standard length	5.05	4.47
	Standard length	5.30	3.01
	Standard length	5.06	4.56
	Standard length	4.26	4.61
	Standard length	3.47	3.90
	Standard length	5.19	5.14
	Standard length	4.75	4.73
	Total length	5.07	4.91
	Total length	5.74	4.98

Spawn	Method	Live length (mm)	Preserved length (mm)
RS00F33xRS00M71	Total length	5.30	4.99
	Total length	4.26	4.04
	Total length	5.48	4.77
	Total length	4.22	3.93
	Total length	5.77	3.81
	Total length	5.39	5.17
	Total length	4.88	5.16
	Total length	5.18	5.37
	Total length	4.25	4.01
	Total length	5.58	5.64
	Total length	5.51	4.46
	Total length	5.54	3.76
	Total length	6.74	4.07
	Total length	6.76	4.55
	Total length	6.71	4.59
	Total length	5.81	4.93
	Total length	6.39	3.46
	Total length	5.76	5.26
	Total length	4.91	5.20
	Total length	4.07	4.45
	Total length	5.97	6.01
	Total length	5.08	5.06
RS01F13xGS01M20	Standard length	4.73	4.18
	Total length	5.48	4.80

Table B.8. Percent fertilization for red snapper eggs fertilized at different ratios of sperm and eggs.

Sperm:egg ratio	Sperm density (cells/mL)	Motility	Measured		Relative to control		Measured		Relative to control	
			RS01F6 (R1)	RS01F6 (R2)	RS01F6 (R1)	RS01F6 (R2)	RS01F7 (R1)	RS01F7 (R2)	RS01F7 (R1)	RS01F7 (R2)
Control	8.48 x 108	0/95	13.3	9.7	100	100	45.3	38.5	100	100
480,000:1	9.50 x 107	0/95	1.6	2.4	16.1	24.4	8.2	10.0	21.4	10.0
50,000:1	1.00 x 107	0/95	0.6	0.7	5.8	7.7	2.3	1.9	5.8	1.9
4,000:1	8.13 x 105	0/95	0.7	0.3	7.6	2.6	0.9	0.5	2.3	0.5
500:1	1.00 x 105	0/95	0.3	0.2	2.7	2.5	0.5	0.4	1.2	0.4

Table B.9. Red snapper eggs were fertilized with fresh sperm at 10-min intervals after stripping.

Female 1

Time	Treatment	Replicate A			Replicate B		
		Neural embryos	Total eggs	Percent fertilization	Neural embryos	Total eggs	Percent fertilization
0	Dry	53	241	22	376	837	45
	Seawater	429	636	67	290	412	70
	Dry-HBSS	270	598	45	111	295	38
	HBSS	108	522	21	81	348	23
10	Dry	103	853	12	33	327	10
	Seawater	61	531	11	10	650	2
	Dry-HBSS	41	196	21	86	486	18
	HBSS	88	402	22	37	256	14
20	Dry	44	487	9	28	552	5
	Seawater	53	662	8	18	612	3
	Dry-HBSS	34	284	12	7	234	3
	HBSS	44	367	12	4	387	1
30	Dry	19	334	6	54	994	5
	Seawater	21	535	4	4	136	3
	Dry-HBSS	26	1051	2	8	293	3
	HBSS	3	359	1	8	589	1
60	Dry	10	507	2	3	245	1
	Seawater	7	395	2	25	379	7
	Dry-HBSS	7	607	1	1	472	1
	HBSS	4	722	1	4	930	1

Female 2

Time	Treatment	Replicate A			Replicate B		
		Neural embryos	Total eggs	Percent fertilization	Neural embryos	Total eggs	Percent fertilization
0	Dry	153	1148	13	158	1134	14
10	Dry	29	426	7	13	212	6
	Seawater	125	482	26	57	355	16
	Dry-HBSS	3	97	3	9	107	8
	HBSS	6	85	7	49	517	9
20	Dry	7	200	4	24	588	4
	Seawater	12	471	3	52	743	7
	Dry-HBSS	25	321	8	27	613	4
	HBSS	0	136	0	4	790	1
30	Dry	7	368	2	20	512	4
	Seawater	8	251	3	15	381	4
	Dry-HBSS	14	347	4	7	237	3
	HBSS	0	486	0	0	348	0
60	Dry	2	141	1	9	285	3
	Seawater	17	624	3	6	883	1
	Dry-HBSS	2	334	1	3	222	1
	HBSS	0	432	0	0	535	0

Table B.10. Estimated and measured weights of female red snapper collected.

Fish ID	Length (in)	Measured weight (g)	Estimated weight (g)
RS01F11	16	902	873
RS01F16	16	1108	873
RS01F21	16	1176	873
RS01F26	16	1144	873
RS01F27	16	1030	873
RS01F28	16	980	873
RS01F29	16	984	873
RS00F9	16	1304	873
RS00F14	16	1216	873
RS00F15	16	1315	873
RS00F25	16	1046	873
RS00F28	16	1440	873
RS00F29	16	1343	873
RS01F12	16.5	1116	964
RS01F30	16.5	989	964
RS01F33	16.5	1081	964
RS00F24	16.5	1175	964
RS01F1	17	1296	1054
RS01F3	17	1440	1054
RS01F15	17	1172	1054
RS01F17	17	1261	1054
RS01F20	17	1411	1054
RS00F4	17	1352	1054
RS00F13	17	1468	1054
RS01F32	17.5	1211	1152
RS00F2	17.5	1238	1152
RS00F8	17.5	1336	1152
RS01F10	18	1671	1250
RS01F18	18	1544	1250
RS00F3	18	1476	1250
RS00F12	18	1846	1250
RS00F16	18	1544	1250
RS00F21	18	1616	1250
RS00F27	18	1600	1250
RS01F2	18.5	1842	1365
RS01F19	18.5	1586	1365
RS01F25	18.5	1658	1365
RS00F6	18.5	1639	1365
RS00F11	18.5	1642	1365
RS00F20	18.5	1638	1365
RS01F4	19	1784	1479
RS01F5	19	1702	1479
RS01F6	19	2141	1479
RS01F31	19	1605	1479
RS00F1	19	1713	1479
RS00F32	19	1664	1479
RS00F33	19	1874	1479
RS00F7	19.5	1879	1602
RS01F7	20	2280	1725
RS01F9	20	2189	1725
RS01F13	20	1945	1725
RS00F5	20	2155	1725
RS00F22	20	2017	1725
RS00F23	20	2124	1725
RS00F26	20	2034	1725
RS00F30	20	1725	1725
RS00F31	20	2028	1725
RS00F10	20.5	2200	1861
RS01F24	21	2585	1996
RS01F14	22	3419	2306
RS01F22	22	3015	2306
RS01F23	23	4248	2634
RS01F8	24	3751	3006

Table B.11. Commercial landings for red snapper and gray snapper caught in the United States.

YEAR	Metric Tons (mmt)	Pounds (lbs)	EX-VESSEL VALUE (millions of dollars)
1950	3,411	7,503,100	1.77
1951	3,477	7,650,060	1.93
1952	4,214	9,271,460	2.17
1953	3,844	8,457,240	2.33
1954	4,225	9,295,440	2.39
1955	4,487	9,870,520	2.49
1956	4,335	9,537,440	2.36
1957	4,498	9,895,820	2.55
1958	5,001	11,002,860	2.81
1959	5,090	11,197,340	2.88
1960	5,122	11,269,060	2.86
1961	5,898	12,974,940	3.32
1962	5,867	12,907,620	3.23
1963	6,151	13,531,760	3.60
1964	6,484	14,264,360	4.14
1965	6,581	14,477,980	4.22
1966	6,193	13,625,480	4.34
1967	6,047	13,304,280	4.33
1968	5,566	12,244,980	4.22
1969	4,898	10,776,040	4.53
1970	4,388	9,653,160	4.37
1971	4,327	9,519,620	4.55
1972	4,390	9,658,440	5.22
1973	4,185	9,206,780	5.45
1974	4,429	9,742,920	6.41
1975	4,125	9,075,440	6.58
1976	3,790	8,338,880	6.89
1977	3,044	6,696,580	6.43
1978	2,757	6,064,520	6.76
1979	2,671	5,875,100	7.76
1980	2,660	5,851,780	8.74
1981	3,105	6,830,560	11.09
1982	3,351	7,372,420	12.29
1983	3,723	8,189,940	13.83
1984	2,985	6,565,900	11.75
1985	2,504	5,508,800	11.13
1986	2,418	5,319,820	11.22
1987	2,163	4,758,380	10.68

YEAR	Metric Tons (mmt)	Pounds (lbs)	EX-VESSEL VALUE (millions of dollars)
1988	2,266	4,984,320	11.01
1989	2,162	4,755,960	11.71
1990	1,776	3,906,320	10.33
1991	1,335	2,936,340	6.54
1992	1,706	3,754,080	7.47
1993	2,000	4,399,560	8.87
1994	2,058	4,526,940	9.91
1995	1,907	4,194,520	9.23
1996	2,471	5,435,760	11.22
1997	2,637	5,802,280	11.42
1998	2,495	5,487,900	12.19
1999	2,587	5,690,960	12.17
2000	2,559	5,629,800	12.99

Table B.12. Osmolality (mOsmol/kg) of red snapper blood and seminal plasma.

Fish	Seminal plasma	Blood plasma
RS00M1	398.00	***
RS00M2	400.00	***
RS00M3	446.00	***
RS00M4	414.00	***
RS00M5	407.00	***
RS00M6	429.00	***
RS00M7	428.00	***
RS00M8	417.00	***
RS00M9	419.00	***
RS00M10	422.00	437.00
RS00M11	443.00	440.00
RS00M12	436.00	432.00
RS00M13	438.00	435.00
RS00M14	446.00	443.00
RS00M15	440.00	451.00
RS00M16	431.00	429.00
RS00M17	432.00	438.00
RS00M18	440.00	450.00
RS00M19	448.00	446.00

Table B.13. Osmolality (mOsmol/kg) of gray snapper blood and seminal plasma.

Fish	Seminal plasma	Blood plasma
GS00M1	410.00	***
GS00M2	419.00	***
GS00M3	403.00	***
GS00M4	416.00	***
GS00M5	408.00	***
GS00M6	411.00	***
GS00M7	415.00	***
GS00M8	403.00	***
GS00M9	414.00	428.00
GS00M10	409.00	420.00
GS00M11	414.00	426.00
GS00M12	413.00	420.00
GS00M13	407.00	411.00

Table B.14. Osmolality (mOsmol/kg) of surface seawater from broodstock collection sites.

Sample	Salinity (ppt)	Osmolality (mOsmol/kg)
1	35	900
2	33	800
3	35	850
4	35	880
5	35	890

Table B.15. Sperm motility, air temperature, and internal body temperature of red snapper collected at coastal marinas.

Fish	Date	Body temperature (C)	Air temperature (C)	Sperm motility (%)
RS01M41	7/4/2001	4.6	35	95
RS01M45	7/11/2001	14.5	35.2	1
RS01M46	7/11/2001	6.4	35.2	70
RS01M47	7/17/2001	18.6	32.2	90
RS01M48	7/18/2001	7.4	32.2	90
RS01M49	7/19/2001	10.2	32.2	80
RS01M50	7/20/2001	10	32.2	95
RS01M52	7/22/2001	12.8	32.2	90
RS01M54	7/24/2001	9.9	32.2	90
RS01M55	7/25/2001	7.1	32.2	95
RS01M56	7/26/2001	6.4	30	95
RS01M57	7/27/2001	9.6	30	95
RS01M58	7/28/2001	10.1	30	95
RS01M59	7/29/2001	9.2	30	90
RS01M60	7/30/2001	11	30	95
RS01M61	7/31/2001	11.3	30	95
RS01M62	7/19/2001	20	33	70
RS01M63	7/19/2001	13.5	33	90
RS01M64	7/19/2001	11.4	33	90
RS01M65	7/24/2001	25.7	30.9	10
RS01M66	7/24/2001	13.5	30.9	90
RS01M67	7/24/2001	11	30.9	80
RS01M68	7/24/2001	16.6	30.9	95
RS01M69	7/24/2001	9.5	30.9	95
RS01M70	7/24/2001	25.6	30.9	10
RS01M74	8/9/2001	4.3	31	95
RS01M75	8/9/2001	7.4	31	95
RS01M76	8/9/2001	8.9	31	95
RS01M77	8/9/2001	8.5	31	95
RS01M78	8/9/2001	7.6	31	95
RS01M79	8/10/2001	14.1	31.6	95
RS01M80	8/10/2001	10.6	31.6	0
RS01M81	8/10/2001	10.3	31.6	80
RS01M82	8/10/2001	14.3	31.6	95
RS01M83	8/10/2001	11.3	31.6	95
RS01M85	8/10/2001	15.2	31.6	95
RS01M86	8/10/2001	15.5	31.6	80
RS01M98	8/14/2001	4	30	95
RS01M99	8/14/2001	3	30	95
RS01M100	8/14/2001	4	30	95

Table B.16. Motility characterization of red snapper and gray snapper sperm activated with 870 mOsmol/kg artificial seawater. Time was measured in seconds.

Fish	Motility	Sample	Time to maximum motility	Duration of maximum motility	Time to cessation
RS01M33	0/95	a	5	63	420
	0/95	b	6	71	400
RS01M40	0/95	a	8	52	490
	0/95	b	8	58	410
RS01M36	0/95	a	7	46	450
	0/95	b	8	41	430
RS01M9	0/95	a	5	61	470
	0/95	b	6	55	460
RS01M11	0/95	a	5	52	400
	0/95	b	7	71	410
GS01M3	0/95	a	5	64	510
	0/95	b	5	68	480
GS01M4	0/95	a	5	72	430
	0/95	b	6	66	450
GS01M9	0/95	a	7	55	390
	0/95	b	6	62	430
GS01M10	0/95	a	8	68	410
	0/95	b	7	76	410
GS01M13	0/95	a	5	48	490
	0/95	b	6	62	460
GS01M34	0/95	a	7	53	470
	0/95	b	5	61	440

Table B.17. Percent motility of red snapper sperm at various osmolalities of artificial seawater.

Artificial seawater (ppt)	Osmolality (mOsmol/kg)	RS00M18	RS00M17	RS00M16	RS00M14	RS00M12	RS00M96	RS00M97	RS00M98	RS00M99	RS00M44	RS00M43
0	46	0	0	0	0	0	0	0	0	0	0	0
4	129	0	0	0	0	0	0	0	0	0	0	0
7	225	0	0	0	0	0	0	0	0	0	0	0
11	314	0	0	0	0	0	0	0	0	0	0	0
14	407	0	0	0	0	0	5	1	0	5	1	0
16	439	0	5	1	0	1	10	1	5	10	5	0
18	479	1	10	5	0	1	80	5	10	50	10	0
20	528	50	50	60	10	10	80	10	10	60	40	10
22	563	80	80	90	10	50	80	80	50	70	60	20
25	636	95	95	95	95	70	90	95	60	80	95	95
28	742	95	95	95	95	95	95	95	95	90	95	95
32	822	95	95	95	95	95	95	95	95	95	95	95
35	923	95	95	95	95	95	95	95	95	95	95	95

Time B.18. Percent motility of red snapper and gray snapper sperm at various osmolalities of artificial seawater.

Artificial seawater (ppt)	Osmolality (mOsmol/kg)	RS00M91	RS00M42	GS00M45	GS00M54	GS00M52	GS00M50	GS00M51	GS00M55	GS00M56	GS00M57	GS00M59	GS00M58
0	46	0	0	0	0	0	0	0	0	0	0	0	0
4	129	0	0	0	0	0	0	0	0	0	0	0	0
7	225	0	0	0	0	0	0	0	0	0	0	0	0
11	314	0	0	0	0	0	0	0	0	0	0	0	0
14	407	0	0	1	0	0	1	1	0	1	5	0	5
16	439	0	0	1	1	5	5	5	1	5	5	1	10
18	479	0	0	30	1	10	10	10	1	10	10	1	30
20	528	1	10	50	10	80	40	40	10	20	30	10	40
22	563	1	10	90	20	90	80	60	50	50	40	20	50
25	636	5	95	95	95	95	90	80	80	90	70	95	80
28	742	95	95	95	95	95	80	95	95	95	95	95	80
32	822	95	95	95	95	95	95	95	95	95	95	95	95
35	923	95	95	95	95	95	95	95	95	95	95	95	95

Table B.19. Refrigerated storage of red snapper sperm. Sperm motilities were estimated daily until samples no longer exhibited motility.

Fish ID	Method	HBSS	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
RS00M45	crushed	200 -Ca	80	50	0	0	0	0	0	0	0
RS00M46	crushed	200 -Ca	95	95	90	70	0	0	0	0	0
RS00M48	crushed	200 -Ca	95	95	95	90	80	50	40	40	0
RS00M49	crushed	200 -Ca	50	50	50	0	0	0	0	0	0
RS00M50	crushed	200 -Ca	70	70	10	0	0	0	0	0	0
RS00M79	crushed	200 -Ca	50	30	10	0	0	0	0	0	0
RS00M80	crushed	200 -Ca	90	60	60	0	0	0	0	0	0
RS00M81	crushed	200 -Ca	80	80	80	0	0	0	0	0	0
RS00M82	crushed	200 -Ca	80	60	60	0	0	0	0	0	0
RS00M83	crushed	200 -Ca	95	90	90	10	0	0	0	0	0
RS00M84	crushed	200 -Ca	95	95	95	0	0	0	0	0	0

Fish ID	Method	HBSS	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
RS00M45	crushed	300-Ca	80	80	50	5	0	0	0
RS00M46	crushed	300-Ca	95	90	70	60	40	1	0
RS00M48	crushed	300-Ca	95	95	95	95	50	10	0
RS00M49	crushed	300-Ca	50	40	40	0	0	0	0
RS00M50	crushed	300-Ca	70	60	0	0	0	0	0
RS00M79	crushed	300-Ca	50	30	20	0	0	0	0
RS00M80	crushed	300-Ca	90	50	50	0	0	0	0
RS00M81	crushed	300-Ca	80	70	50	50	0	0	0
RS00M82	crushed	300-Ca	80	80	80	0	0	0	0
RS00M83	crushed	300-Ca	95	95	95	80	5	0	0
RS00M84	crushed	300-Ca	95	90	90	80	0	0	0

Fish ID	Method	HBSS	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
RS00M45	crushed	400-Ca	80	50	0	0	0	0	0
RS00M46	crushed	400-Ca	95	60	40	60	10	1	0
RS00M48	crushed	400-Ca	95	95	80	95	10	10	0
RS00M49	crushed	400-Ca	50	40	60	10	0	0	0
RS00M50	crushed	400-Ca	70	50	10	0	0	0	0
RS00M79	crushed	400-Ca	50	50	10	0	0	0	0
RS00M80	crushed	400-Ca	90	90	90	5	0	0	0
RS00M81	crushed	400-Ca	80	80	80	50	0	0	0
RS00M82	crushed	400-Ca	80	60	60	10	0	0	0
RS00M83	crushed	400-Ca	95	95	95	80	20	0	0
RS00M84	crushed	400-Ca	95	95	95	80	0	0	0

Fish ID	Method	HBSS	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
RS00M45	stripped	200 -Ca	50	45	40	5	0	0	0	0	0	0	0
RS00M46	stripped	200 -Ca	90	90	50	20	0	0	0	0	0	0	0
RS00M47	stripped	200 -Ca	95	95	90	80	0	0	0	0	0	0	0
RS00M48	stripped	200 -Ca	95	95	95	90	5	1	0	0	0	0	0
RS00M49	stripped	200 -Ca	95	95	95	1	0	0	0	0	0	0	0
RS00M50	stripped	200 -Ca	90	90	95	5	1	0	0	0	0	0	0
RS00M51	stripped	200 -Ca	90	30	40	0	0	0	0	0	0	0	0
RS00M79	stripped	200 -Ca	80	40	5	0	0	0	0	0	0	0	0
RS00M80	stripped	200 -Ca	50	10	10	5	0	0	0	0	0	0	0
RS00M81	stripped	200 -Ca	80	80	80	70	10	10	5	5	5	0	0
RS00M82	stripped	200 -Ca	80	40	30	30	5	0	0	0	0	0	0
RS00M83	stripped	200 -Ca	90	95	95	80	80	70	0	0	0	0	0
RS00M84	stripped	200 -Ca	90	40	40	40	0	0	0	0	0	0	0
RS00M87	stripped	200 -Ca	95	95	95	80	80	80	70	70	70	50	0

Fish ID	Method	HBSS	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9
RS00M45	stripped	300-Ca	50	50	50	5	0	0	0	0	0	0
RS00M46	stripped	300-Ca	90	80	50	50	0	0	0	0	0	0
RS00M47	stripped	300-Ca	95	95	90	95	90	10	0	0	0	0
RS00M48	stripped	300-Ca	95	95	95	95	80	50	0	0	0	0
RS00M49	stripped	300-Ca	95	65	90	10	0	0	0	0	0	0
RS00M50	stripped	300-Ca	90	50	95	60	60	0	0	0	0	0
RS00M51	stripped	300-Ca	90	50	95	20	20	0	0	0	0	0
RS00M79	stripped	300-Ca	80	80	80	60	5	1	0	0	0	0
RS00M80	stripped	300-Ca	50	50	20	10	10	5	5	5	5	0
RS00M81	stripped	300-Ca	80	10	10	10	0	0	0	0	0	0
RS00M82	stripped	300-Ca	80	80	80	70	50	50	5	10	5	0
RS00M83	stripped	300-Ca	90	90	90	50	0	0	0	0	0	0
RS00M84	stripped	300-Ca	90	40	40	80	0	0	0	0	0	0
RS00M87	stripped	300-Ca	95	80	80	80	5	0	0	0	0	0

Fish ID	Method	HBSS	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9
RS00M45	stripped	400-Ca	50	40	20	20	20	20	0	0	0	0
RS00M46	stripped	400-Ca	90	80	50	50	50	20	0	0	0	0
RS00M47	stripped	400-Ca	95	95	80	50	50	20	0	0	0	0
RS00M48	stripped	400-Ca	95	95	70	60	10	0	0	0	0	0
RS00M49	stripped	400-Ca	95	50	80	50	0	0	0	0	0	0
RS00M50	stripped	400-Ca	90	80	95	80	50	0	0	0	0	0
RS00M51	stripped	400-Ca	90	90	10	0	0	0	0	0	0	0
RS00M79	stripped	400-Ca	80	30	20	10	0	0	0	0	0	0
RS00M80	stripped	400-Ca	50	50	50	20	5	10	5	5	5	0
RS00M81	stripped	400-Ca	80	30	30	30	0	0	0	0	0	0
RS00M82	stripped	400-Ca	80	60	60	50	0	0	0	0	0	0
RS00M83	stripped	400-Ca	90	90	90	90	90	20	0	0	0	0
RS00M84	stripped	400-Ca	90	60	60	80	0	0	0	0	0	0
RS00M87	stripped	400-Ca	95	50	50	80	0	0	0	0	0	0

Table B.20. Refrigerated storage of gray snapper sperm. Sperm motilities were estimated daily until samples no longer exhibited motility.

Fish ID	Method	HBSS	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
GS00M09	crushed	200 -Ca	90	50	50	50	10	10	0	0
GS00M10	crushed	200 -Ca	95	10	5	0	0	0	0	0
GS00M11	crushed	200 -Ca	95	40	5	0	0	0	0	0
GS00M12	crushed	200 -Ca	95	20	10	0	0	0	0	0
GS00M13	crushed	200 -Ca	95	10	5	0	0	0	0	0
GS00M15	crushed	200 -Ca	95	40	15	0	0	0	0	0
GS00M19	crushed	200 -Ca	60	50	20	1	0	0	0	0
GS00M21	crushed	200 -Ca	80	60	50	50	0	0	0	0
GS00M23	crushed	200 -Ca	90	90	80	80	60	50	50	0
GS00M24	crushed	200 -Ca	80	80	40	0	0	0	0	0
GS00M34	crushed	200 -Ca	80	90	30	0	0	0	0	0
GS00M48	crushed	200 -Ca	30	10	10	0	0	0	0	0
GS00M49	crushed	200 -Ca	50	20	10	0	0	0	0	0

Fish ID	Method	HBSS	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
GS00M09	crushed	300 -Ca	90	50	50	50	0	0	0	0
GS00M10	crushed	300 -Ca	95	95	20	0	0	0	0	0
GS00M11	crushed	300 -Ca	95	30	10	0	0	0	0	0
GS00M12	crushed	300 -Ca	95	95	95	0	0	0	0	0
GS00M13	crushed	300 -Ca	95	95	20	0	0	0	0	0
GS00M15	crushed	300 -Ca	95	95	50	0	0	0	0	0
GS00M19	crushed	300 -Ca	60	60	5	0	0	0	0	0
GS00M21	crushed	300 -Ca	80	80	70	1	0	0	0	0
GS00M23	crushed	300 -Ca	90	80	80	80	40	20	0	0
GS00M24	crushed	300 -Ca	80	50	60	1	0	0	0	0
GS00M34	crushed	300 -Ca	80	40	30	0	0	0	0	0
GS00M48	crushed	300 -Ca	30	30	20	0	0	0	0	0
GS00M49	crushed	300 -Ca	50	30	10	0	0	0	0	0

Fish ID	Method	HBSS	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
GS00M09	crushed	400 -Ca	90	90	50	50	0	0	0	0
GS00M10	crushed	400 -Ca	95	95	5	0	0	0	0	0
GS00M11	crushed	400 -Ca	95	95	10	0	0	0	0	0
GS00M12	crushed	400 -Ca	95	95	95	0	0	0	0	0
GS00M13	crushed	400 -Ca	95	50	0	0	0	0	0	0
GS00M15	crushed	400 -Ca	95	95	10	0	0	0	0	0
GS00M19	crushed	400 -Ca	60	60	10	0	0	0	0	0
GS00M21	crushed	400 -Ca	80	70	5	0	0	0	0	0
GS00M23	crushed	400 -Ca	90	80	10	10	0	0	0	0
GS00M24	crushed	400 -Ca	80	80	0	0	0	0	0	0
GS00M34	crushed	400 -Ca	80	80	40	1	0	0	0	0
GS00M48	crushed	400 -Ca	30	20	10	0	0	0	0	0
GS00M49	crushed	400 -Ca	50	50	1	0	0	0	0	0

Fish ID	Method	HBSS	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
GS00M19	stripped	200 -Ca	60	60	50	30	0	0	0	0
GS00M21	stripped	200 -Ca	95	90	95	80	50	20	0	0
GS00M23	stripped	200 -Ca	90	70	70	70	50	20	10	0
GS00M24	stripped	200 -Ca	95	90	95	30	20	0	0	0
GS00M26	stripped	200 -Ca	20	10	10	5	1	1	0	0
GS00M27	stripped	200 -Ca	80	40	20	20	10	10	0	0
GS00M28	stripped	200 -Ca	80	80	50	50	20	0	0	0
GS00M29	stripped	200 -Ca	90	90	40	20	20	0	0	0
GS00M30	stripped	200 -Ca	95	40	20	0	0	0	0	0
GS00M31	stripped	200 -Ca	90	90	60	50	40	10	0	0
GS00M32	stripped	200 -Ca	90	90	50	40	40	1	0	0
GS00M33	stripped	200 -Ca	90	90	50	50	40	10	0	0
GS00M34	stripped	200 -Ca	95	95	90	90	80	0	0	0
GS00M49	stripped	200 -Ca	80	20	10	1	0	0	0	0
GS00M50	stripped	200 -Ca	95	95	80	50	30	5	0	0
GS00M51	stripped	200 -Ca	95	95	50	50	50	20	0	0

Fish ID	Method	HBSS	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
GS00M19	stripped	300 -Ca	60	30	30	20	0	0	0
GS00M21	stripped	300 -Ca	95	95	60	10	10	0	0
GS00M23	stripped	300 -Ca	90	90	80	60	0	0	0
GS00M24	stripped	300 -Ca	95	90	70	60	50	0	0
GS00M26	stripped	300 -Ca	20	20	10	5	1	0	0
GS00M27	stripped	300 -Ca	80	80	50	10	10	0	0
GS00M28	stripped	300 -Ca	80	60	5	5	0	0	0
GS00M29	stripped	300 -Ca	90	80	5	5	0	0	0
GS00M30	stripped	300 -Ca	95	80	70	50	40	0	0
GS00M31	stripped	300 -Ca	90	80	20	10	5	0	0
GS00M32	stripped	300 -Ca	90	60	50	5	1	0	0
GS00M33	stripped	300 -Ca	90	90	40	10	1	0	0
GS00M34	stripped	300 -Ca	95	80	80	10	10	0	0
GS00M49	stripped	300 -Ca	80	80	50	0	0	0	0
GS00M50	stripped	300 -Ca	95	95	80	50	20	1	0
GS00M51	stripped	300 -Ca	95	95	70	50	0	0	0

Fish ID	Method	HBSS	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
GS00M19	stripped	400 -Ca	60	40	5	0	0	0
GS00M21	stripped	400 -Ca	95	40	10	0	0	0
GS00M23	stripped	400 -Ca	90	75	50	5	0	0
GS00M24	stripped	400 -Ca	95	90	50	50	20	0
GS00M26	stripped	400 -Ca	20	20	20	5	0	0
GS00M27	stripped	400 -Ca	80	80	20	10	10	0
GS00M28	stripped	400 -Ca	80	50	1	5	1	0
GS00M29	stripped	400 -Ca	90	80	20	1	0	0
GS00M30	stripped	400 -Ca	95	95	50	40	30	0
GS00M31	stripped	400 -Ca	90	40	10	5	0	0
GS00M32	stripped	400 -Ca	90	70	30	1	0	0
GS00M33	stripped	400 -Ca	90	70	20	10	0	0
GS00M34	stripped	400 -Ca	95	60	60	10	0	0
GS00M49	stripped	400 -Ca	80	80	60	1	0	0
GS00M50	stripped	400 -Ca	95	95	80	50	0	0
GS00M51	stripped	400 -Ca	95	95	20	5	0	0

Table B.21. Refrigerated storage of red snapper sperm with and without addition of antibiotic/antimycotic cocktail (A/AC).

0% Antibiotic/Antimycotic cocktail

Fish	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
RS01M47	90	80	80	80	80	80	80	20	10	0	0
RS01M48	90	70	0	0	0	0	0	0	0	0	0
RS01M49	80	80	0	0	0	0	0	0	0	0	0
RS01M50	90	80	0	0	0	0	0	0	0	0	0
RS01M52	90	95	70	70	80	80	80	0	0	0	0
RS01M54	95	80	90	90	90	90	90	90	20	10	0
RS01M55	95	95	95	95	95	95	95	50	0	0	0
RS01M56	95	90	90	90	90	90	90	0	0	0	0
RS01M57	95	95	90	90	95	95	95	0	0	0	0
RS01M58	90	80	95	95	95	95	95	20	0	0	0
RS01M59	95	80	70	70	70	70	70	0	0	0	0
RS01M60	95	95	95	95	95	95	95	90	50	40	0
RS01M61	90	90	80	80	80	90	0	0	0	0	0
RS01M66	80	80	80	90	90	90	70	70	0	0	0
RS01M67	95	95	95	95	95	90	70	70	30	0	0
RS01M68	95	95	95	95	95	95	10	10	0	0	0
RS01M69	90	90	70	70	70	70	60	30	10	5	0

0.1% Antibiotic/Antimycotic cocktail

Fish	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
RS01M47	90	90	80	80	80	80	80	50	10	0	0
RS01M48	90	50	0	0	0	0	0	0	0	0	0
RS01M49	80	95	0	0	0	0	0	0	0	0	0
RS01M52	90	10	5	5	5	5	5	0	0	0	0
RS01M54	90	80	90	90	95	95	80	0	0	0	0
RS01M55	95	90	90	90	90	90	90	90	10	0	0
RS01M56	95	80	95	95	95	95	95	10	0	0	0
RS01M57	95	80	90	90	90	80	80	0	0	0	0
RS01M58	95	95	95	95	95	95	95	0	0	0	0
RS01M59	90	95	95	95	95	95	95	10	0	0	0
RS01M60	95	80	20	20	20	10	10	0	0	0	0
RS01M61	95	50	95	95	95	95	90	70	10	0	0
RS01M66	90	90	90	50	60	50	10	0	0	0	0
RS01M67	80	80	90	90	90	50	70	70	30	0	0
RS01M68	95	95	95	95	95	50	70	70	0	0	0
RS01M69	95	95	95	95	95	95	50	50	0	0	0

1.0% Antibiotic/Antimycotic cocktail

Fish	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
RS01M47	90	70	80	80	70	60	60	20	10	10	0
RS01M48	90	50	0	0	0	0	0	0	0	0	0
RS01M49	80	70	0	0	0	0	0	0	0	0	0
RS01M52	90	90	0	0	0	0	0	0	0	0	0
RS01M54	90	80	95	95	95	95	80	0	0	0	0
RS01M55	95	70	70	70	80	70	70	70	10	0	0
RS01M56	95	90	95	95	95	95	95	20	0	0	0
RS01M57	95	80	70	70	70	50	70	0	0	0	0
RS01M58	95	90	90	90	90	80	80	0	0	0	0
RS01M59	90	75	80	80	80	70	90	30	0	0	0
RS01M60	95	80	20	20	20	10	10	0	0	0	0
RS01M61	95	95	95	95	95	90	90	50	50	0	0
RS01M66	90	90	90	50	50	70	30	0	0	0	0
RS01M67	80	80	90	90	90	70	70	70	30	0	0
RS01M68	95	95	95	90	95	50	10	20	30	0	0
RS01M69	95	95	95	95	95	95	1	0	0	0	0

Table B.22. Cooling profile of the dairy method of cryopreserving sperm used at Genex Inc. at the T. E. Patrick Dairy Improvement Center.

Time	Temperature (C)
1	-140
2	-100
3	-60
4	-76
5	-92
6	-108
7	-124
8	-140

Table B.23. Acute toxicity of various concentrations of four cryoprotectants to sperm of red snapper.

Time 0	Control	5% DMSO	10% DMSO	15% DMSO	20% DMSO	25% DMSO
RS00M41	95	95	95	95	95	95
RS00M96	95	95	95	95	80	70
RS00M97	95	95	95	95	90	90
RS00M98	95	95	95	95	95	95
RS00M99	95	95	95	95	90	90

Time 15	Control	5% DMSO	10% DMSO	15% DMSO	20% DMSO	25% DMSO
RS00M41	95	95	95	95	80	50
RS00M96	95	95	95	95	80	50
RS00M97	95	95	95	95	80	50
RS00M98	95	95	95	70	70	50
RS00M99	95	95	95	95	90	80

Time 30	Control	5% DMSO	10% DMSO	15% DMSO	20% DMSO	25% DMSO
RS00M41	95	90	80	90	50	0
RS00M96	95	95	95	95	70	50
RS00M97	95	95	95	90	60	50
RS00M98	95	95	95	70	50	10
RS00M99	95	95	95	90	60	50

Time 45	Control	5% DMSO	10% DMSO	15% DMSO	20% DMSO	25% DMSO
RS00M41	95	95	80	50	10	0
RS00M96	95	95	95	95	70	50
RS00M97	95	95	95	80	70	40
RS00M98	95	95	95	70	10	0
RS00M99	95	95	95	80	40	0

Time 60	Control	5% DMSO	10% DMSO	15% DMSO	20% DMSO	25% DMSO
RS00M41	95	95	60	5	0	0
RS00M96	95	95	95	95	60	50
RS00M97	95	95	95	60	50	50
RS00M98	95	95	95	70	0	0
RS00M99	95	95	90	80	0	0

Time 0	Control	5% MeOH	10% MeOH	15% MeOH	20% MeOH	25% MeOH
RS00M41	95	95	95	95	95	80
RS00M96	95	95	95	95	95	95
RS00M97	95	95	95	95	95	95
RS00M98	95	95	95	95	95	95
RS00M99	95	95	95	95	95	95

Time 15	Control	5% MeOH	10% MeOH	15% MeOH	20% MeOH	25% MeOH
RS00M41	95	95	95	50	50	40
RS00M96	95	95	95	95	80	60
RS00M97	95	95	90	95	70	60
RS00M98	95	95	90	70	20	10
RS00M99	95	95	90	80	70	60

Time 30	Control	5% MeOH	10% MeOH	15% MeOH	20% MeOH	25% MeOH
RS00M41	95	95	95	40	40	0
RS00M96	95	95	95	95	70	60
RS00M97	95	95	80	95	50	30
RS00M98	95	95	90	50	20	0
RS00M99	95	95	80	50	40	20

Time 45	Control	5% MeOH	10% MeOH	15% MeOH	20% MeOH	25% MeOH
RS00M41	95	95	95	40	10	0
RS00M96	95	95	80	80	70	40
RS00M97	95	95	60	50	50	20
RS00M98	95	95	80	50	0	0
RS00M99	95	95	60	40	0	0

Time 60	Control	5% MeOH	10% MeOH	15% MeOH	20% MeOH	25% MeOH
RS00M41	95	95	40	0	0	0
RS00M96	95	95	80	50	50	20
RS00M97	95	95	60	60	30	10
RS00M98	95	95	80	50	0	0
RS00M99	95	95	60	40	0	0

Time 0	Control	5% DMA	10% DMA	15% DMA	20% DMA	25% DMA
RS00M41	95	95	10	10	0	0
RS00M96	95	95	95	95	80	80
RS00M97	95	95	95	95	95	95
RS00M98	95	95	95	80	50	0
RS00M99	95	95	95	80	50	40

Time 15	Control	5% DMA	10% DMA	15% DMA	20% DMA	25% DMA
RS00M41	95	95	40	10	0	0
RS00M96	95	95	80	95	70	50
RS00M97	95	95	80	95	80	60
RS00M98	95	95	90	60	20	0
RS00M99	95	95	95	60	10	0

Time 30	Control	5% DMA	10% DMA	15% DMA	20% DMA	25% DMA
RS00M41	95	95	50	1	0	0
RS00M96	95	95	80	80	50	40
RS00M97	95	95	80	70	50	50
RS00M98	95	90	80	20	10	0
RS00M99	95	90	95	40	0	0

Time 45	Control	5% DMA	10% DMA	15% DMA	20% DMA	25% DMA
RS00M41	95	90	40	0	0	0
RS00M96	95	95	60	60	40	20
RS00M97	95	95	60	50	40	30
RS00M98	95	90	80	0	0	0
RS00M99	95	90	80	0	0	0

Time 60	Control	5% DMA	10% DMA	15% DMA	20% DMA	25% DMA
RS00M41	95	90	40	0	0	0
RS00M96	95	95	50	50	40	20
RS00M97	95	95	50	50	40	30
RS00M98	95	90	60	0	0	0
RS00M99	95	90	80	0	0	0

Time 0	Control	5% Glycerol	10% Glycerol	15% Glycerol	20% Glycerol	25% Glycerol
RS00M41	95	80	50	10	0	0
RS00M96	95	50	50	10	0	0
RS00M97	95	95	80	50	20	0
RS00M98	95	20	20	10	10	0
RS00M99	95	50	20	10	0	0

Time 15	Control	5% Glycerol	10% Glycerol	15% Glycerol	20% Glycerol	25% Glycerol
RS00M41	95	95	40	1	0	0
RS00M96	95	50	10	0	0	0
RS00M97	95	50	50	30	1	0
RS00M98	95	10	1	0	0	0
RS00M99	95	20	1	0	0	0

Time 30	Control	5% Glycerol	10% Glycerol	15% Glycerol	20% Glycerol	25% Glycerol
RS00M41	95	95	10	0	0	0
RS00M96	95	5	0	0	0	0
RS00M97	95	30	0	0	0	0
RS00M98	95	0	0	0	0	0
RS00M99	95	10	0	0	0	0

Time 45	Control	5% Glycerol	10% Glycerol	15% Glycerol	20% Glycerol	25% Glycerol
RS00M41	95	90	10	0	0	0
RS00M96	95	0	0	0	0	0
RS00M97	95	10	0	0	0	0
RS00M98	95	0	0	0	0	0
RS00M99	95	0	0	0	0	0

Time 60	Control	5% Glycerol	10% Glycerol	15% Glycerol	20% Glycerol	25% Glycerol
RS00M41	95	80	1	0	0	0
RS00M96	95	0	0	0	0	0
RS00M97	95	0	0	0	0	0
RS00M98	95	0	0	0	0	0
RS00M99	95	0	0	0	0	0

Table B.24. Post-thaw motility of red snapper sperm from the preliminary cryoprotectant evaluation.

Male	**Intial Motility**	**5% DMSO**	**10% DMSO**	**5% MeOH**	**10% MeOH**	**5% DMA**	**10% DMA**
RS00M33	90	50	60	30	30	40	50
RS00M34	90	50	50	10	20	30	40
RS00M35	95	70	70	20	40	50	60
RS00M48	95	60	70	30	40	50	60
RS00M49	95	70	80	40	50	60	60
RS00M50	95	80	95	30	50	60	70

Table B.25. Motilities of red snapper sperm before freezing and after thawing.

Fish	Initial motility	Post-thaw motility
RS00M26	80	0
RS00M33	90	60
RS00M34	90	50
RS00M35	95	70
RS00M42	95	90
RS00M43	95	90
RS00M44	95	90
RS00M48	95	70
RS00M49	95	80
RS00M50	95	95
RS01M9	95	95
RS01M11	95	95
RS01M33	80	95
RS01M36	90	95
RS01M38	90	70
RS01M40	90	95
RS01M22	80	80
RS01M23	80	90
RS01M16	90	90
RS01M20	90	90

Table B.26. Fertilization rates of red snapper eggs fertilized with fresh and cryopreserved sperm.

Date	Spawn	Description	Percent Neurulation (R1)	Percent Neurulation (R2)
07/11/2000	**RS00F12 X RS00M50**	Cryo Red	89.1	80.5
07/11/2000	**RS00F15 X RS00M49**	Cryo Red	39.5	53.3
07/11/2000	**RS00F12 X RS00M58**	Fresh Red	80.8	80.5
07/11/2000	**RS00F15 X RS00M58**	Fresh Red	78.6	57.6
07/26/2000	**RS00F20 X RS00M50**	Cryo Red	17.5	20.0
07/26/2000	**RS00F21 X RS00M50**	Cryo Red	37.1	37.5
07/26/2000	**RS00F20 X RS00M59**	Fresh Red	33.3	24.8
07/26/2000	**RS00F21 X RS00M59**	Fresh Red	37.3	41.0
08/10/2000	**RS00F24 X RS00M44**	Cryo Red	18.3	19.9
08/10/2000	**RS00F27 X RS00M43**	Cryo Red	58.1	49.0
08/10/2000	**RS00F32 X RS00M44**	Cryo Red	35.7	45.6
08/10/2000	**RS00F33 X RS00M44**	Cryo Red	68.5	70.0
08/10/2000	**RS00F24 X Red Snap1**	Cryo Red - 1998	14.7	23.9
08/10/2000	**RS00F33 X Red Snap1**	Cryo Red - 1998	49.6	45.2
08/10/2000	**RS00F24 X RS00M70**	Fresh Red	24.8	9.9
08/10/2000	**RS00F27 X RS00M70**	Fresh Red	46.2	53.2
08/10/2000	**RS00F27 X RS00M71**	Fresh Red	74.5	67.2
08/10/2000	**RS00F32 X RS00M71**	Fresh Red	39.8	26.5
08/10/2000	**RS00F33 X RS00M71**	Fresh Red	82.9	79.0
07/19/2001	**RS01F13 X RS01M33**	Cryo	42.4	33.3
07/19/2001	**RS01F15 X RS01M33**	Cryo	14.9	18.1
07/19/2001	**RS01F16 X RS01M33**	Cryo	15.0	6.3
07/19/2001	**RS01F13 X RS01M56**	Fresh	50.6	62.1
07/19/2001	**RS01F15 X RS01M56**	Fresh	20.6	24.5
07/19/2001	**RS01F16 X RS01M56**	Fresh	19.6	19.5

Table B.27. Fertilization rates of red snapper eggs presented with fresh sperm containing various concentrations of dimethyl sulfoxide.

Spawn	DMSO (%)	Percent Neurulation (R1)	Percent Neurulation (R2)
RS01F6 x RS01M16	0	23	31
	10	32	27
	20	28	18
	50	3	6
RS01F7 x RS01M16	0	46	51
	10	34	24
	20	26	19
	50	5	5

Chapter 6

Table B.28. Data collected in developing a standard curve for known amounts of live and dead tilapia sperm.

Live:Dead Sperm Concentration	Flow Cytometric Results	
	Live	Dead
100:0	97.3	2.7
75:25	61.2	38.8
50:50	51.9	48.1
25:75	35.2	64.8
0:100	0.1	99.9

Table B.29. Viability of red snapper and gray snapper sperm as determined by flow cytometry.

	Replicate 1		Replicate 2		Replicate 3	
Fish	Dead cells	Live Cells	Dead cells	Live Cells	Dead cells	Live Cells
RS01M9	23.19	76.17	25.69	73.6	31.38	65.79
RS01M11	26.03	72.31	29.61	67.73	29.19	68.51
RS01M33	49.53	48.85	46.46	53.09	51.59	46.12
RS01M36	24.81	71.26	22.56	76.64	23.07	76.4
RS01M40	21.95	73.91	18.22	81.37	22.54	76.3
GS01M3	27.31	71.64	31.17	66.97	36.32	60.47
GS01M4	30.83	68.48	36.18	61.79	39.62	57.97
GS01M9	30.35	68.51	34.41	64.41	39.4	56.74
GS01M10	45.22	53.33	50.28	47.87	53.15	45.61
GS01M13	44.41	54.58	44.65	53.94	54.71	43.48
GS01M34	24.43	74.55	30.59	68.35	35.37	62.94

Vita

Kenneth Lee Pickrell Riley was born on October 11, 1972, in Durham, North Carolina. He attended the University of North Carolina at Wilmington where he received in 1996 his Bachelor of Science degree in marine biology and secondary science education. While an undergraduate, Ken worked for four years as a research assistant in UNC-Wilmington's Benthic Ecology Laboratory. Upon completion of his undergraduate program, Ken presented his research on *Pit Disturbance By Common Estuarine Predators: Stingrays, Flatfish, and Crabs* at the 1996 Benthic Ecology Meeting in Columbia, South Carolina, where he was awarded Best Student Paper Presentation. After graduation, he moved to Cocodrie, Louisiana, to work as a marine educator for Louisiana Universities Marine Consortium. For three years, he educated thousands of K-12 and college students, teachers, and the public about the functions and values of Louisiana's coastal wetlands and marine resources. In the fall of 1999, Ken and his wife, Kelly, moved to Baton Rouge to enroll in Graduate School at Louisiana State University. While a graduate student, Ken took an active role serving on the executive committees of the LSU Aquaculture and Fisheries Club and the Louisiana Chapter of the American Fisheries Society. In the spring of 2001, Ken was awarded Best Abstract and Best Paper Presentation at the Triennial Conference of the World Aquaculture Society in Orlando, Florida. While his graduate research focused on spawning, larval rearing, and propagation of marine fishes for aquaculture, his research interests extend to marine ecology, fisheries management, and coastal resource management. Ken is currently a candidate for the degree of Master of Science in fisheries (aquaculture), which will be awarded through the School of Renewable Natural Resources on August 8, 2002.