

## ABSTRACT

Title of Thesis: THE CHARACTERIZATION AND CRYOPRESERVATION  
OF STRIPED BASS (*Morone saxatilis*) SEMEN

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The increasing popularity of the striped bass as a favorite species for the aquaculture industry worldwide, coupled with the current inability to successfully cryopreserve large amounts of semen efficiently, provide the impetus for this research. This study analyzed the physical and chemical characteristics of striped bass semen as a first step toward the development of a successful cryopreservation protocol. Although no significant differences were found to occur in fish of different age, size, or filial generation near the peak of the spawning season, significant differences were found to occur in striped bass semen at different times during the spawning season. Over the course of three years, eight extenders, three extenders commonly used with other teleosts and five experimental extenders, were evaluated for the short-term storage of striped bass semen. Two experimental extenders, Extenders C4 and C5,

which were modifications of an extender commonly used in brown trout and arctic charr, yielded the highest percentage of motile sperm after 1, 2, and 7d of refrigerated storage. Three cryopreservation trials were performed over three spawning seasons. The best results in terms of post-thaw motility and fertilization capacity were obtained from freezing striped bass semen in Extender D with 5% dimethyl sulfoxide (DMSO) as the cryoprotectant at a rate of  $-40^{\circ}\text{C}/\text{min}$  and thawing the semen in a  $25^{\circ}\text{C}$  water bath for 12s.



THE CHARACTERIZATION AND CRYOPRESERVATION OF STRIPED

BASS (*Morone Saxatilis*) SEMEN

by

Karen Jenkins

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## DEDICATION

Dedicated

to

my parents

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## LIST OF SPECIES NAMES

<u>Common Name</u>	<u>Scientific Name</u>
Ayu	<i>Plecoglossus altivelis</i>
Bass, largemouth	<i>Micropterus salmoides</i>
Bass, smallmouth	<i>Micropterus dolomieu</i>
Bass, striped	<i>Morone saxatilis</i>
Bass, white	<i>Morone chrysops</i>
Bass, yellow	<i>Morone mississippiensis</i>
Bream	<i>Abramis brama</i>
Bream, yellowfin	<i>Acanthopagrus australis</i>
Carp, common	<i>Cyprinus carpio</i>
Carp, Crucian	<i>Carassius carassius</i>
Catfish, channel	<i>Ictalurus punctatus</i>
Chain pickerel	<i>Esox niger</i>
Char, arctic	<i>Salvelinus alpinus</i>
Dace	<i>Phoxinus spp.</i>
Goldfish	<i>Carassius auratus</i>
Grey mullet	<i>Mugil capito</i>
Muskellunge	<i>Esox masquinongy</i>
Ocean pout	<i>Macrozoarces americanus</i>
Perch, white	<i>Morone americana</i>
Pike, northern	<i>Esox lucius</i>

Salmon, Atlantic	<i>Salmo salar</i>
Salmon, Chinook	<i>Oncorhynchus tshawytscha</i>
Salmon, Chum	<i>Oncorhynchus keta</i>
Salmon, Masou	<i>Oncorhynchus masou</i>
Seabream, gilthead	<i>Sparus aurata</i>
Taran roach	<i>Rutilus rutilus</i>
Trout, brook	<i>Salvelinus fontinalis</i>
Trout, brown	<i>Salmo trutta</i>
Trout, rainbow	<i>Oncorhynchus mykiss</i>
Turbot	<i>Scophthalmus maximus</i>

## INTRODUCTION

Aquaculture as defined by Stickney (1994) is the production of an aquatic species under controlled or semi-controlled conditions. The earliest records of aquaculture come from China where the artificial hatching of fish was practiced around 2000 B.C. (Avault 1996). Although aquaculture has ancient origins, it is relatively new to the United States, beginning only about a century ago (Avault 1996). During the past 20 years, aquaculture has evolved at a fast pace where one out of every five fish consumed in the U.S. is farm raised (Lockwood 1999). Currently, the striped bass industry is one of the fastest growing segments of finfish aquaculture in the United States (JSA 1983). This growth is limited however, by the industry's reliance on wild broodstock for seedstock (Harrell et al. 1990). Research is currently being conducted to establish domesticated broodstocks (Woods 1999) and to overcome reproductive problems often encountered in captive and domestic finfish populations (Zohar 1989).

### Historical Background of Striped Bass Culture

The striped bass, *Morone saxatilis*, is one of the most important finfish species in the state of Maryland, the Chesapeake Bay, and the eastern seaboard of the U.S. Although the striped bass is an anadromous teleost, it can successfully complete its entire life cycle in freshwater. The original range of the striped bass was from the St. Lawrence River in Canada to Northern Florida, and along the Gulf coast from western Florida to Louisiana (Whitehurst and Stevens 1990).

In the early 1900's, the basic techniques for the successful culture of striped bass were developed by the U.S. Fish and Fisheries Commission for the purpose of enhancing the commercial fishery (Stickney 1996). The government abandoned the program and these techniques were not put into practice until the 1950's. At this time, striped bass were being stocked into reservoirs to control gizzard shad populations. Since the striped bass were unable to naturally reproduce in these reservoirs, a number of hatcheries were established in the Southeastern U.S. to produce striped bass fingerlings (Stickney 1996). Soon, the striped bass grew in popularity not only as a commercial fish, but also as a sport fish (Fowler 1995).

Around 1973, the striped bass population began to decline along the Atlantic coast (Smith 1989). Catches plummeted from 14.7 million pounds in 1973 to 3.7 million pounds in 1979 (Baker 1994). Minimum size limits and seasons were imposed by marine fishery agencies with little success (Fowler 1995). In 1984, Congress passed the Atlantic Striped Bass Conservation Act, which provided funding for both striped bass research and management programs (Fowler 1995). In 1985, a moratorium on striped bass fishing was imposed in Maryland and severe fishing restrictions were implemented in other states (Baker 1994). Hatchery programs were initiated in an effort to increase the striped bass population back to self-sustainable levels (Fowler 1995). During the next decade, approximately 8 million hatchery-raised fingerlings were released into the wild. In the late 1980's, the striped bass population began to increase and by 1990, the striped bass moratorium in Maryland was lifted (Baker 1994).



Increased demand for striped bass coupled with the inability of natural stocks to satisfy this demand, made the striped bass an ideal candidate for commercial aquaculture production. Commercial striped bass farms were first developed in 1973 (Stickney 1996). These early attempts to commercially produce striped bass failed due primarily to the lack of broodstock (Stickney 1996). Government regulations prohibited private growers from using wild broodfish (Smith 1989, Stickney 1996).

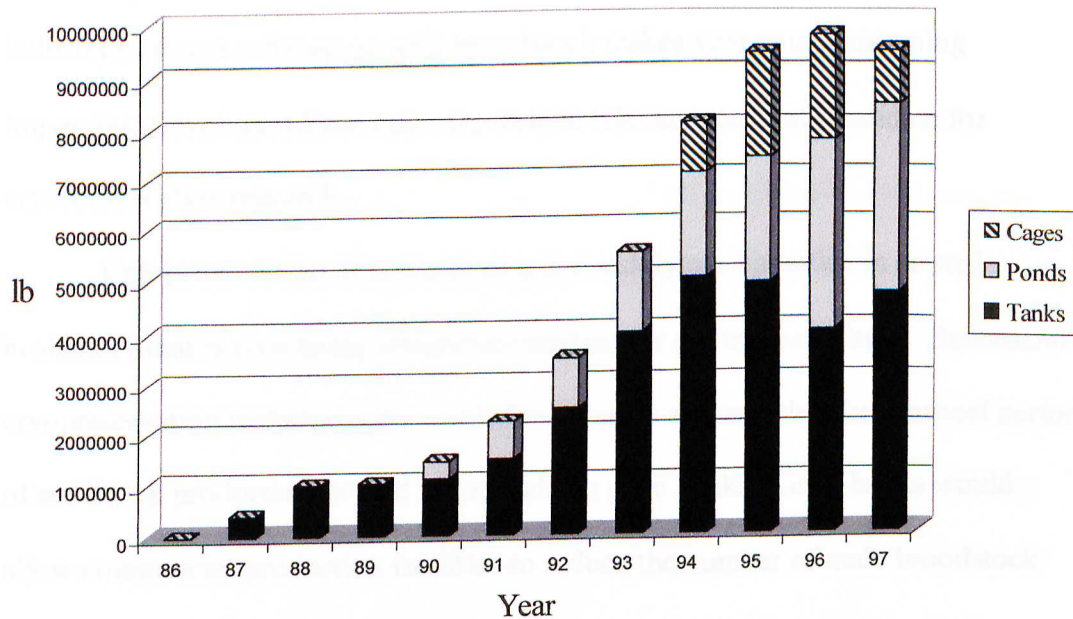
#### Current Striped Bass Aquaculture Industry

Today, the striped bass industry is one of the fastest growing segments of finfish aquaculture in the United States. Hybrid striped bass production increased almost 10-fold from 1986 to 1995 (Figure 0.1). The major limitation to this growth is the industry's reliance on wild broodstock for seedstock. Dependence on the wild population poses significant risks to the industry because the catch is unpredictable and highly seasonal (Woods and Sullivan 1993), the genetic performance of the fish being spawned is unknown, and the number of fish a grower is permitted to collect for spawning is often limited (Leffler 1999, Woods and Sullivan 1993). To overcome these problems, domesticated broodstocks and methods for their controlled reproduction must be developed (Smith 1989, Harrell et al. 1990).

Development of domesticated striped bass broodstock is a slow process, which is currently in the research phase (Leffler 1999). Although progress has been made (Woods et al. 1999), researchers are still trying to find solutions to many of the reproductive problems associated with striped bass held in captivity. The



Figure 0.1: Production of hybrid striped bass (adapted from Striped Bass growers Association 1998).



main problems faced by striped bass hatcheries are differing spawning times between fish of different sex, species, and geographical location and the inability to spawn fish year round.

Most of the male striped bass for any geographical area are ready to spawn at the beginning of their season, prior to the female reaching final oocyte maturation. Therefore, when most of the females are ready to spawn, there may be an insufficient supply of flowing males. This problem becomes especially acute when trying to produce hybrids. The current industry standard, the sunshine bass, is a white bass female, *Morone chrysops*, crossed with a striped bass male. Because the spawning season of the white bass is earlier than that of the striped bass, producing hybrids can be logistically difficult. Spawning fish from different

regions is equally difficult since the striped bass depends on environmental cues (i.e. photoperiod, temperature) to initiate spawning (Sullivan 1997). Finally, the industry's current reliance on wild broodstock makes year-round spawning impossible. Such problems make the striped bass an excellent candidate for cryopreservation research.

Cryopreservation of spermatozoa is a widely used practice in animal husbandry that is now being intensively studied for use in aquaculture. Successful cryopreservation techniques are needed to enhance and broaden the seasonal period of seedstock production as well as to establish gene banks. Gene banks would allow commercial production facilities to reduce the number of male broodstock maintained on site, expedite shipping of sperm to other facilities, preserve superior genomes long after a fish's death (Kerby 1983), increase the genetic diversity of a population (Piironen 1993), and possibly aid in the conservation of important genetic information and/or the protection of threatened and endangered species (Gwo et al. 1991).

There has been very little research on the cryopreservation of striped bass sperm. The only published works to date are two papers, both by Kerby (1983, 1984). Kerby had limited success in cryopreserving striped bass sperm. The best results he obtained were from his "OH-189" extender with 5% dimethyl sulfoxide (DMSO) which yielded an average of 23.6% fertilization. Most of his samples exhibited no motility and there was a wide range of variability between individual samples in each trial (from 0 to 88% fertilization). However, Kerby did find that between glycerol, methanol, and DMSO, DMSO was the only cryoprotectant that



yielded any motility or fertilization post-thaw. Of the concentrations he tested, 5% yielded the best results (Kerby 1983).

Clearly research is needed to establish successful, repeatable cryopreservation techniques for striped bass sperm. Because there is a large variation in the characteristics and composition of the spermatozoa and seminal fluid of different teleosts (Lahnsteiner et al. 1995), there is a large variation in the cryopreservation techniques needed for the growing number of important aquaculture species. Analyzing the physical and chemical characteristics of striped bass semen is the first step in developing a successful cryopreservation protocol. The initial data gathered in these studies will be pertinent for the development of appropriate extenders for short-term and long-term cryopreservation. Once appropriate extenders are developed, cryopreservation trials can be performed to determine an optimal cryopreservation protocol that will yield viable and motile spermatozoa post-thaw for fertilization.

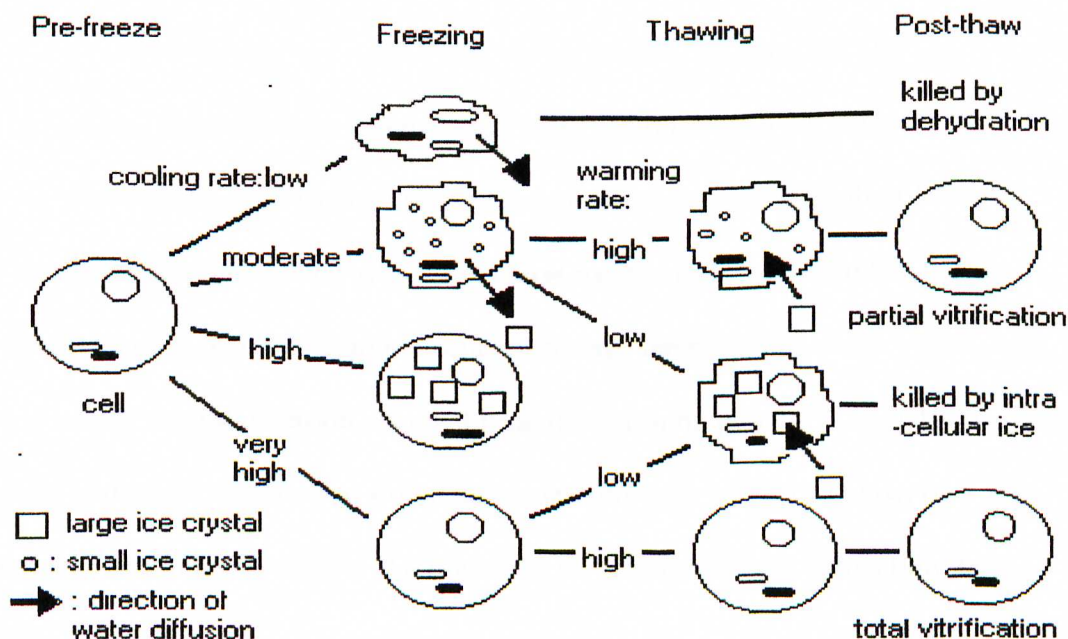
#### Principles of Cryopreservation

Cryopreservation is defined for the purposes of these reported studies, simply as the preservation of the viability of living cells for an indefinite period of time by deep-freezing to temperatures of  $-130^{\circ}\text{C}$  or below. The freezing and thawing of biological material involves complex physical and chemical processes of heat and water transport between cells and their surrounding media (Leung 1991). There are three basic steps in the cryopreservation process: freezing, storage, and

thawing. Different rates of freezing and thawing affect the cell in different ways (Figure 0.2).

The appropriate freezing rate is needed to prevent destruction of the cell. If the freezing rate is too slow, the water in the extracellular solution freezes out and the remaining solution becomes more concentrated. Intracellular water then diffuses out and the cell dies of dehydration. A moderate freezing rate doesn't allow enough time for dehydration and only some of the water leaves the cell. The remaining intracellular water undergoes vitrification or crystallization. Vitrification is freezing without ice crystal formation. In highly concentrated solutions, the lowering of the temperature increases the viscosity of the solution, which prevents crystallization. If crystallization occurs, it is small enough so that it doesn't damage

Figure 0.2: The effects of cryopreservation on the cell (adapted from Leung 1991).





the cell unless recrystallization occurs during the thawing process. A high freezing rate doesn't allow enough time for water to leave the cell and large intracellular ice crystals form that physically damage the cell causing death. Theoretically, an extremely high freezing rate allows total vitrification to occur in which intracellular nuclei may form, but there is insufficient time for crystals to grow. Instead the cell undergoes a glass transformation. These cells only remain viable if they are thawed at a similarly fast rate so recrystallization doesn't occur. Such high freezing and thawing rates are only theoretically possible and to date are not feasible for biological material.

After the freezing process, samples should be stored at  $-130^{\circ}\text{C}$  or below (Mazur 1984, Leung 1991). At these temperatures, biochemical reactions do not occur; therefore, samples can theoretically be stored for an infinite amount of time. In reality, however, reactions can still take place at the atomic level and background radiation can cause DNA damage (Mazur 1984, Leung 1991).

The same processes that occur during the freezing process also occur during the thawing process but in reverse order. The main problem encountered during the thawing process is recrystallization. Recrystallization is the growth of larger crystals from smaller ones, which can cause mechanical damage to the cells. "Spontaneous" recrystallization occurs at storage temperatures above  $-100^{\circ}\text{C}$  and "irruptive" recrystallization occurs during the warming process (Luyet 1966). Since most biological samples are stored at temperatures of  $-130^{\circ}\text{C}$  or below, only the latter situation is of concern. To minimize the degree of recrystallization, high

warming rates are suggested because there is insufficient time for dehydrated cells to reabsorb water (Leung 1991).

The percentage of surviving cryopreserved cells has never been 100%, therefore some type of lethal injury must occur during the process. In order to prevent injury, one must understand the cause. Different types of injuries are associated with different rates of cooling and thawing. The major lethal injuries associated with cryopreservation are related to cold shock, pH effects, solute effects, dehydration, extracellular ice formation, and/or intracellular ice formation. Most cryoinjuries occur in the 0 to -40°C range (Leung 1991).

Cold shock occurs at temperatures above 0°C and is caused by the change of the lipid membrane from liquid to solid phase. Studies so far have shown that most biological materials are not susceptible to cold shock with the exception of pig embryos and most mammalian spermatozoa (Leung 1991). Watson (1981) theorized that the susceptibility of spermatozoa to cold shock is species dependent and varies based on the lipid characteristics of the membrane. The cholesterol content, phospholipid composition, and fatty acid distribution of the membrane vary with species and the combination that allows maintenance of membrane fluidity has greater resistance to cold shock. Watson also believes that an increase in intracellular calcium may have something to do with membrane damage, but further research is needed.

Solute effects, pH effects, and dehydration are all caused by a slow cooling rate and are collectively termed “solution effects” (Mazur et al. 1972). As water freezes out, the concentration of the remaining intracellular and extracellular fluid



increases. It has been reported that the high concentration of solute can damage the cell membrane in two ways. The lipoproteins may be denatured by the high salt content (Leung 1991) or hypertonic induced cell shrinkage may cause an alteration in the phospholipid bilayer structure or a loss of membrane components (Morris 1981). The loss of membrane components inhibits the cell from returning to its normal isotonic volume upon thawing and cell lysis may occur (Morris 1981). The slow freezing and thawing process is also thought to destroy the buffering capacity of some biological salts (Leung 1991). When the intra/extracellular solution has reached the eutectic temperature (the lowest possible temperature at which a solution can remain liquid), the pH tends to drop, especially in solutions containing NaCl (Meryman 1966). The prolonged exposure to this pH during a slow cooling/thawing rate can cause denaturation of vital proteins (Meryman 1966). Finally, as previously discussed, dehydration occurs when the cooling rate is slow enough so that most of the intracellular water leaves in an attempt to balance the osmotic gradient (Mazur et al. 1972, Meryman 1974).

While “irruptive” recrystallization of extracellular ice may cause detrimental physical stress to the cell during the thawing process, it is not thought to be detrimental to most biological cells. Researchers Fujikawa and Miura reported mechanical membrane damage from extracellular ice during extremely slow cooling rates (Leung 1991). This may be due to the fact that slower cooling rates promote the growth of a few, large ice crystals whereas faster cooling rates promote the growth of many smaller ice crystals (Mazur et al. 1972). Slower cooling rates are not commonly used with most biological materials and the commonly used cooling

rates have shown no evidence of extracellular ice damage (Meryman 1966). Rather, the injury is presumed to be caused by solute effects (Meryman 1966, Leung 1991).

As the cooling rate increases, so does the amount of intracellular ice formed (Leung 1991). As extracellular ice forms, intracellular water begins to leave the cell to balance the osmotic gradient. The rapid cooling rate doesn't allow enough time for all the intracellular water to leave and some freezes within the cell (Mazur and Schmidt 1968). As stated previously, faster cooling rates promote smaller ice crystals. The small intracellular ice crystals that are formed during the freezing process may not be damaging, but the recrystallization that occurs during the thawing process, if significant, can cause lethal mechanical membrane damage (Mazur 1972, Leung 1991).

### Extenders and Cryoprotectants

In addition to finding the appropriate freezing and thawing rate, extenders and cryoprotectants are used to increase cell survival during the cryopreservation storage process. An extender is a solution of salts and/or other organic compounds used to dilute the semen sample and prolong the life of the cells during refrigeration (Scott and Baynes 1980). In the case of extending sperm, a "suitable" extender would protect the viability of the spermatozoa without activating them. Because of the physical and chemical variation between different cell types, research must be conducted to determine an effective extender for any given species. Often, extenders are used by themselves to prolong the survival of cells under short-term



refrigerated storage. However, they must be used in conjunction with a cryoprotectant if the cells are to undergo the freezing process.

A cryoprotectant is a chemical added to the cell solution in an attempt to prevent or minimize cryoinjuries by suppressing or reducing ice formation (Leung 1991). Cryoprotectants are classified as permeating or non-permeating. Permeating cryoprotectants, such as glycerol, methanol, and DMSO, act to 1) reduce the salt concentration 2) minimize the change in cell volume, 3) decrease the rate of ice formation, 4) decrease the rate of water diffusion from the cell to the ice crystal, and 5) increase the vitrification temperature (Leung 1991). Nonpermeating cryoprotectants such as sugars (e.g. sucrose and glucose), polymers (e.g. polyvinylpyrrolidone and dextran), and proteins (e.g. egg yolk and milk), are usually used in conjunction with permeating cryoprotectants and act to reduce the freezing point and increase the vitrification temperature of the extracellular medium (Leung 1991, Rowe 1966).

Not all cryoprotectants will be effective on all cell types (Rowe 1966). Many cryoprotectants are toxic to cells in high concentrations. Unfortunately, these higher concentrations are usually required for effectiveness (Leung 1991). When using permeating cryoprotectants, proper equilibration time must be determined. The cells must be exposed to the cryoprotectant long enough to allow it to cross the cell membrane, but not so long as to allow the toxic effects to significantly reduce cell viability. Because of these factors, experiments must be performed on each biological type of cell (i.e. sperm cell, red blood cell, etc.) for each species to

determine which cryoprotectant, cryoprotectant concentration, and equilibration time are most effective.

#### Project Relevance

This study analyzes the physical and chemical characteristics of striped bass semen and uses the information obtained as the first step to develop a successful cryopreservation protocol. The increasing popularity of the striped bass as a favorite species for the aquaculture industry worldwide, coupled with the current inability to successfully cryopreserve large amounts of semen efficiently, provide the impetus for this research.

In Experiment 1, conducted in 1997, the physical and chemical characteristics of striped bass semen were analyzed as close to the peak of the spawning season as possible. Semen was collected from three different filial generations of captive and domesticated broodstock and tested for generation, age, and/or size-related differences. The performance of three different extenders for the short-term storage of striped bass semen was also evaluated.

In Experiment 2, conducted in 1998, the physical and chemical characteristics of semen collected from randomly selected captive and domestic striped bass were analyzed weekly for six weeks during the spawning season. The performance of the best extender from Experiment 1 was then compared to the performance of three additional extenders.

In Experiment 3, conducted in 1999, the physical and chemical characteristics of striped bass semen were analyzed across the entire spawning

season (11 weeks). Semen was collected each week from the same twelve individual striped bass and from six striped bass drawn randomly from the general population. To test the effects of a mammalian analog of gonadotropin-releasing hormone (GnRHa) on the seminal characteristics of striped bass, six of the twelve individual fish were given GnRHa implants on a biweekly basis. The other six individuals and the fish drawn randomly from the population were not given implants. The performance of the top two extenders from Experiment 1 and Experiment 2 were then tested against two new extenders.

In Experiment 4, the final experiment which spanned all three years, cryopreservation trials were performed. Four different extenders, three different cryoprotectant concentrations, three different freezing rates, and two different thawing rates were examined for their effectiveness in generating the highest rates of post-thaw sperm motility and fertilization.

## CHAPTER 1

The characterization of striped bass semen near the peak of the spawning season from fish of different filial generations and the analysis of three extenders for the short term storage of striped bass semen.



## ABSTRACT

Semen was collected from captive and domesticated striped bass of three different filial generations and analyzed for differences in physical and chemical characteristics. No significant differences were found in the concentration of sperm ( $97.2 \pm 4.2 \times 10^9$  spermatozoa/mL), the percentage of live sperm ( $68 \pm 2.9\%$ ), the percentage of motile sperm ( $93 \pm 2.6\%$ ), the duration of sperm motility ( $40 \pm 0.7$ s), the osmolality of the semen ( $341 \pm 2.5$  mmol/kg) and seminal plasma ( $353 \pm 3.4$  mmol/kg), the pH of the semen ( $8.0 \pm 0.03$ ) and seminal plasma ( $7.7 \pm 0.06$ ), and the  $\text{Na}^+$  ( $2646 \pm 159.9$  mg/L),  $\text{K}^+$  ( $2189 \pm 217.6$  mg/L), and  $\text{Ca}^{2+}$  ( $20 \pm 9.5$  mg/L) concentrations of seminal plasma between fish of different filial generations.

The performance of three different extenders was evaluated for the short-term storage of striped bass semen. Freshly collected semen samples were extended in a 2:1 ratio of extender:semen and stored at  $4^\circ\text{C}$ . The percentage of motile sperm and the duration of sperm motility were measured from samples of the control (neat) and extended semen after 1, 2, and 7d of storage. Semen stored in Extender A yielded the highest percentage of motile sperm after 1, 2, and 7d of refrigerated storage.

## INTRODUCTION

The striped bass, *Morone saxatilis*, is of great importance in the U.S. as both a game and a food fish (JSA 1983). The increased popularity of the striped bass coupled with the decline of natural populations, has led to the establishment and rapid growth of the striped bass and hybrid striped bass aquaculture industry. The industry's current reliance on wild fish for seedstock has been a major limiting factor to this growth (Leffler 1999). To alleviate this problem, extensive research efforts to develop domesticated striped bass broodstocks have recently been undertaken (Woods and Sullivan 1993, Woods et al. 1992). With domesticated striped bass, commercial facilities could conceivably spawn the same fish for 20 years or more (Vuthiphandchai and Zohar 1999). The long-term use of these fish raises the question of whether or not age could affect reproduction, especially gamete quality. Sperm quality has already been shown to change with age in a few teleost species such as rainbow trout (Büyükhatipoglu and Holtz 1984) and Atlantic salmon (Kazakov 1981).

Knowledge of age-related differences in striped bass sperm are important for many reasons. Hatchery managers can use this information to determine 1) the maximum age broodstock can reach before their performance deteriorates, 2) the earliest age at which young broodstock should be spawned, and 3) whether a sperm sample should be used for short-term storage and/or cryopreservation.

Recently, some of the physical characteristics of striped bass semen were shown to be affected by age. Vuthiphandchai and Zohar (1999) compared semen samples from captive striped bass aged 1, 3, and 12 years. No significant



differences were found in the percentage of motile sperm, the percentage of live sperm, or the duration of sperm motility from freshly collected samples of semen. The relative volume of semen expressed did not change with age either. However, the sperm density, spermatocrit, and total number of spermatozoa were found to be significantly greater in the 3-yr-old striped bass as compared to the 1 and 12-yr-old fish. Also, extended sperm from the 3-yr-old fish remained viable for a significantly longer period of time than extended sperm from the older and younger age groups.

Knowledge of sperm biochemistry and physiology are essential to improve methods of artificial fertilization (Wang and Crim 1997). To date, there has been no published work on the chemical characteristics of striped bass semen. If chemical factors, such as osmolality and pH, change with age, then the effectiveness of an extender and/or cryopreservation technique may be altered with the age of the broodstock. These changes may, in part, account for the sustained viability Vuthiphandchai and Zohar observed in extended semen from 3-yr-old striped bass as opposed to the extended semen from 1 and 12-yr-old striped bass.

The purpose of this study was to analyze both the physical and chemical characteristics of semen collected from three different filial generations of captive and domesticated striped bass. Semen samples from these fish were also used to evaluate the performance of three different extenders for the short-term refrigerated storage of striped bass semen.

## MATERIALS AND METHODS

### Animals

Eighteen captive ( $F_1$ ) and domestic ( $F_2$  and  $F_3$ ) striped bass produced and maintained at Crane Aquaculture Facility were used. The  $F_3$ 's were 5 years old, the  $F_2$ 's were 6 years old, and the  $F_1$ 's ranged from 8 to 14 years old. Six fish from each of the three different filial generations were moved from a 6m diameter circular tank with flow through water from the Chesapeake Bay to a 4m diameter tank with a recirculating water system. The fish were allowed to acclimate to the system for two weeks. Each fish was given a mammalian analog of gonadotropin-releasing hormone (GnRHa) implant to offset the effects of stress due to handling and density associated with the smaller tank (Woods and Sullivan 1993, Mylonas et al. 1998). Samples were collected on May 8, 1997, near the peak of the spawning.

### Sample Collection

The striped bass broodstock were anesthetized using a 70mg/L quinaldine bath (Woods et al. 1992). The length and weight of each fish was measured and recorded before sample collection. Urine was expressed from the bladder with repeated palpitations and the urogenital vent was then blotted clean and dry with absorbent towelettes to prevent the semen from becoming contaminated with water, urine, or feces. Semen was expressed directly into sterile 50mL conical tubes by applying gentle pressure to both lobes of the testes simultaneously beginning anterior and moving posterior toward the vent. Blood samples were drawn from the caudal vein using heparinized needles (21 gauge) and syringes (3cc). All samples



were kept on ice or under refrigeration until chemical and physical analyses were performed.

#### Physical Characterization

The color, viscosity, and total volume of semen collected was observed and recorded immediately after collection. The sperm was activated in replicate samples of three, using Fisher's deionized ultra-filtered water (DIUF) with an osmolality of 0 to 5 mmol/kg. Sperm motility was recorded on videotape using a Hitachi Model KP-140 video camera attached to a Zeiss Model D-7082 compound microscope at a magnification of 400x. The percentage of motile sperm and the amount of time the sperm remained motile were determined later from the videotapes. The percentage of motile sperm was estimated for all samples by the same observer. Duration of motility was timed beginning the instant the DIUF was mixed with the semen and ending when the majority (approximately 90%) of the sperm in the field of view had stopped moving. Spermatozoa that simply vibrated or did not show progressive forward movement were not considered motile. Duration of motility for each sample replicate was measured three times with a digital stopwatch and recorded to the nearest 1/100s. The percentage of live sperm was determined by an eosin-nigrosin differential staining technique described by Blom (1950). Semen samples were diluted by a factor of 200 and sperm density was measured using a Makler Counting Chamber.

## Chemical Characterization

Semen and blood samples were centrifuged using an Eppendorf Model 5415C centrifuge. Semen samples were centrifuged at 14000 RPM for 30min and blood samples were centrifuged at 10000 RPM for 5 min. Blood and seminal plasma samples were removed and placed in 1.5mL snap-cap vials and stored on ice or under refrigeration until further analyses were performed.

Neat semen, seminal plasma, and blood plasma were measured for osmolality (mmol/kg) and pH using a Wescor Model 5400 vapor pressure osmometer and an Orion Model 9810 micro-combination pH electrode, respectively. The remaining blood and seminal plasma samples were frozen in an -80°C freezer for later sodium, potassium, and calcium measurements using a Perkin-Elmer Model 5100 PC atomic absorption spectrophotometer.

## Extender Evaluation

The formulas of three experimental extenders tested in this experiment are listed in Table 1.1. Extender A, Stein's Extender, has been used successfully in the cryopreservation of semen from both rainbow trout and brown trout (Stein and Bayrle 1978) as well as Arctic charr (Piironen 1993). Extender B, Hank's Balanced Salt solution, is commonly used for the cryopreservation of channel catfish sperm (Tiersch et al. 1994). Extender C, Crane Extender, was developed to mimic the osmolality, pH, and sodium and potassium values from a pooled semen sample of six striped bass collected early in the spawning season of the initial year of this study.

Immediately after collection, 0.3mL of semen from each fish was gently mixed with 0.6mL of each extender and then placed into a 1.5mL snap-cap vial. As a control, 0.9mL of neat semen from each fish was also placed into a snap-cap vial without any extender. The samples were stored upright and refrigerated at 3-4°C. After 1, 2, and 7 days, each extended and neat sample was activated with DIUF in replicates of three. Sperm motility was recorded on videotape using a Hitachi Model KP-140 video camera attached to a Zeiss Model D-7082 compound microscope at a magnification of 400x. The percentage of motile sperm and the amount of time the sperm remained motile were determined later from the videotapes by the same methods described under Physical Characterization.

Table 1.1: Extender formulas for Experiment 1.<sup>1</sup>

Extender A <sup>2</sup>	Extender B <sup>3</sup>	Extender C
100mg glucose 750mg NaCl 200mg NaHCO <sub>3</sub> 40mg KCl 100mL water <sup>4</sup> 20mL egg yolk Osmolality: 290 mmol/kg pH: 7.6	2000 mg NaCl 100mg KCl 40mg CaCl <sub>2</sub> ·2H <sub>2</sub> O 50mg MgSO <sub>4</sub> ·7H <sub>2</sub> O 100mL water <sup>4</sup>  Osmolality: 287 mmol/kg pH: 7.6	10g glucose 1000 mg NaCl 130mg KHCO <sub>3</sub> 200mL water <sup>4</sup>  Osmolality: 435 mmol/kg pH: 7.9

<sup>1</sup> Extenders pH adjusted with HCl or NaOH.

<sup>2</sup> Formula taken from Stein and Bayle (1978).

<sup>3</sup> Formula taken from Tiersch et al. (1994)

<sup>4</sup> Water used was Fisher's deionized ultra-filtered water (DIUF).



## Statistical Analysis

One-way analysis of variance was used to detect differences in the physical and chemical characteristics of striped bass semen from fish of different generations. Statistical analyses were performed using the general linear model procedures in SAS version 6.12 statistical software (SAS 1996). Pairwise contrasts between treatment means were made to detect significant differences at the 5% level. Results, unless otherwise noted, are reported as the mean  $\pm$  S.E.M.

## RESULTS AND DISCUSSION

### Physical Characterization

Most of the semen samples were white in color. A few had a hint of pink color due to a small amount of blood contamination. The viscosity of all the samples were low as compared to pre-experimental samples collected early in the spawning season, which were highly viscous. The mean total volume of milt collected varied with each fish. However, no significant difference was found in the relative semen volume, that is, the amount of semen collected per kg of body weight (Figure 1.1). Also, there was no significant difference in the percentage of live spermatozoa, the percentage of motile sperm, or the duration of sperm motility in semen from striped bass of different filial generations (Table 1.2). Vuthiphandchai and Zohar (1999) obtained similar results when comparing the semen from striped bass aged 1, 3, and 12 years (Table 1.3). In contrast to their results, no significant difference was found in the sperm density (number of spermatozoa/mL) between the three different filial generations. These conflicting

Figure 1.1: The volume of semen obtained from striped bass of three different filial generations. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other at  $P < 0.05$ .

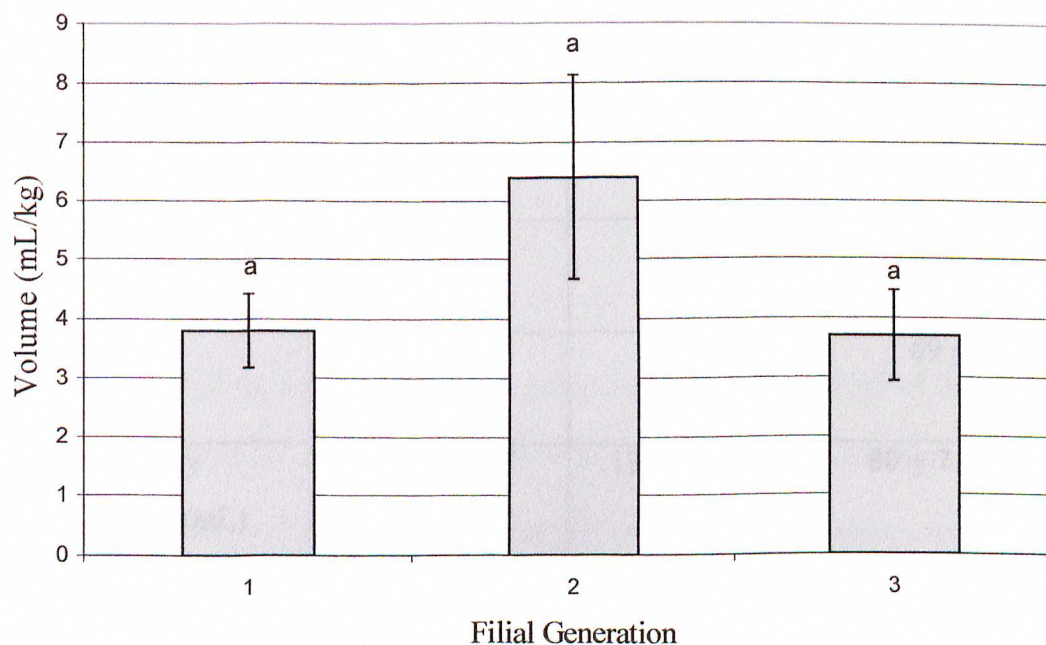


Table 1.2: The percentage of live sperm, percentage of motile sperm, duration of sperm motility, and sperm density in three generations of captive and domestic striped bass.

Characteristic	Generation (Age in years)		
	F <sub>1</sub> (8-10)	F <sub>2</sub> (6)	F <sub>3</sub> (5)
Live Sperm (%)	68 $\pm$ 3.8	67 $\pm$ 6.1	68 $\pm$ 5.4
Motile Sperm (%)	99 $\pm$ 3.7	98 $\pm$ 0.5	100 $\pm$ 4.7
Duration of Motility (s)	37 $\pm$ 0.5	34 $\pm$ 1.1	36 $\pm$ 0.7
Sperm Density (spermatozoa/mL)	106 $\pm$ 16.6 $\times 10^9$	89 $\pm$ 16.9 $\times 10^9$	96 $\pm$ 2.1 $\times 10^9$

Table 1.3: The percentage of live and motile sperm, duration of motility, and sperm density from three age groups of captive striped bass (taken from Vuthiphandchai and Zohar 1999).

Characteristic	Age (year)		
	1	3	12
Live Sperm (%)	$93 \pm 2$	$91 \pm 2$	$91 \pm 2$
Motile Sperm (%)	$58 \pm 3$	$55 \pm 6$	$60 \pm 2$
Duration of Motility (s)	$72 \pm 5$	$72 \pm 4$	$69 \pm 3$
Sperm Density (spermatozoa/mL)	$46 \pm 6 \times 10^9$	$120 \pm 8 \times 10^9$	$80 \pm 7 \times 10^9$

results may be explained by the use of different age groups in each study. Vuthiphandchai and Zohar's study compared striped bass aged 1, 3, and 12 years whereas our study compared striped bass aged 5, 6, and 8-14 years. Also, the average values obtained in this experiment were higher for the percentage of motile sperm and lower for the percentage of live sperm and the duration of sperm motility than those obtained by Vuthiphandchai and Zohar (Tables 1.2 and 1.3). These differences may be the result of the subjective method of measurement.

Although no generation or age-related differences were observed in the viability of freshly collected semen samples, semen samples stored in Extender A exhibited significantly higher viability (in terms of percent motile sperm and duration of motility) in the  $F_3$  generation than in the  $F_1$  and  $F_2$  generations. After 48h of refrigerated storage, sperm from the  $F_3$  generation yielded 32% motile sperm for 36s whereas the  $F_1$  and  $F_2$  generations yielded 17 and 11% motile sperm for 31



and 29s, respectively. Extender A was the only extender yielding results adequate to allow a comparison between filial generations. These results together with those obtained by Vuthiphandchai and Zohar (1999) suggest that the quality of striped bass semen may be highest in fish 3 to 5 years of age. However, further research is needed to verify this theory.

#### Chemical characterization

No significant differences were found in the osmolality and pH of the semen, seminal plasma, and blood plasma of the three different generations of striped bass examined (Table 1.4). The average osmolality of the semen was close to that of the blood plasma. Similar results have been reported for other teleost fish including turbot (Suquet et al.1994), seabream (Chambeyron and Zohar 1990), carp (Morisawa et al.1983a), and rainbow trout, masu salmon and ayu (Morisawa et al.1983b). The osmolality of the seminal plasma was slightly higher but not significantly different than that obtained from neat semen and blood plasma.

The pH of the seminal plasma resembled that of the blood plasma and the pH of the neat semen was only slightly higher in comparison. Similar pH values have been observed in freshwater species such as rainbow trout and carp (Suquet et al. 1994). Solutions having a pH below 7.5 have been shown to totally inhibit the motility of rainbow trout sperm (Billard and Cosson 1992). However, most researchers have reported the pH of seminal plasma in rainbow trout as higher (Billard and Cosson 1992, Baynes et al. 1981). Therefore, pH is not thought to be a major factor in keeping intratesticular sperm immotile, but the inhibiting effect of

Table 1.4: The osmolality (mmol/kg) and pH of striped bass semen, seminal plasma, and blood plasma from three different filial generations of striped bass near the peak of the spawning season.

Characteristic	Generation (Age in years)		
	F <sub>1</sub> (8-14)	F <sub>2</sub> (6)	F <sub>3</sub> (5)
Semen Osmolality	343 $\pm$ 4.2	340 $\pm$ 4.2	340 $\pm$ 5.9
Seminal Plasma Osmolality	348 $\pm$ 4.4	363 $\pm$ 4.9	349 $\pm$ 6.3
Blood Plasma Osmolality	344 $\pm$ 1.4	342 $\pm$ 1.4	345 $\pm$ 2.0
Semen pH	8.0 $\pm$ 0.05	8.0 $\pm$ 0.05	8.0 $\pm$ 0.05
Seminal Plasma pH	7.7 $\pm$ 0.04	7.5 $\pm$ 0.12	7.8 $\pm$ 0.16
Blood Plasma pH	7.6 $\pm$ 0.05	7.6 $\pm$ 0.05	7.5 $\pm$ 0.07

hydrogen ions should be kept in mind when developing an extender.

The sodium, potassium, and calcium concentrations of the blood plasma and seminal plasma samples showed no significant difference between filial generations (Table 1.5). Sodium levels were lower in the seminal plasma than in the blood plasma of the striped bass. Similar findings have been observed in the goldfish and carp (Morisawa et al. 1983a). The effects of sodium on the motility of rainbow trout sperm are not thought to be as important as the effects of potassium and calcium (Tanimoto and Morisawa 1988). However, sodium has been shown to overcome the inhibitory action of potassium in some teleosts (Billard and Cosson 1992). Also, the sodium content of the seminal plasma in Atlantic salmon has



Table 1.5: The ion concentration (mg/L) of seminal and blood plasma from three different filial generations of striped bass.

Characteristic	F <sub>1</sub>	F <sub>2</sub>	F <sub>3</sub>
Seminal Plasma Sodium	2767 ± 245.9	2412 ± 301.1	2733 ± 347.7
Seminal Plasma Potassium	2025 ± 249.0	2800 ± 287.5	1600 ± 352.1
Seminal Plasma Calcium	18 ± 8.9	20 ± 12.5	24 ± 12.5
Blood Plasma Sodium	3767 ± 72.1	3633 ± 72.1	3633 ± 33.3
Blood Plasma Potassium	102 ± 4.1	105 ± 4.5	88 ± 5.8
Blood Plasma Calcium	113 ± 1.6	110 ± 1.6	116 ± 2.3

been positively correlated with fertilization capacity of the semen (Aas et al. 1991). If future research proves this correlation is valid in other species, measuring sodium concentrations of the seminal plasma may be used as a method of evaluating semen quality (Aas et al. 1991).

Potassium levels were much higher in the seminal plasma than in the blood plasma of the striped bass. Similar findings have been well documented in other species such as rainbow trout, masu salmon, and ayu (Morisawa et al. 1983b). Schlenk and Kahman (1938) were the first to suggest that potassium ions may have inhibitory effects on sperm motility (Billard and Cosson 1992). Since then, various concentrations of potassium have been shown to inhibit sperm motility in several



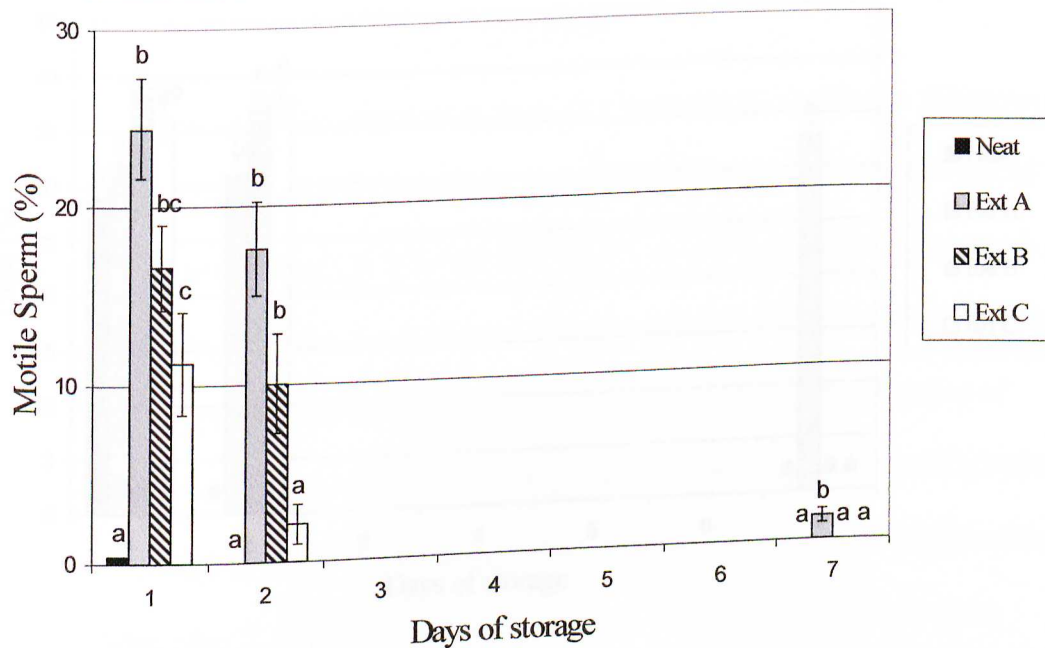
teleosts such as rainbow trout (Morisawa et al. 1983b) and chum salmon (Morisawa and Suzuki 1980). Sperm activation in these species is thought to be more dependent upon dilution of the seminal potassium concentration rather than a change in osmolality (Morisawa et al. 1983b). However, the inhibitory effects of potassium appear to be species dependent since sperm motility in the perch (Lahnsteiner et al. 1995) is unaffected by potassium levels. In fact the presence of potassium has been shown to maintain sperm motility and even increase the speed and/or duration of sperm movement in muskellunge (Lin and Dabrowski 1996) and goldfish, carp, crucian carp, and dace (Morisawa et al. 1983a). Sperm activation in these species is thought to be dependent upon osmotic change.

Calcium levels measured in the striped bass seminal plasma were lower than the blood plasma levels and much lower than seminal plasma potassium levels. The low concentration may be due to the counteractive effect calcium and magnesium ions have on the action of potassium (Tanimoto and Morisawa 1988). Calcium has been shown to counteract both the inhibitory effects of potassium in rainbow trout semen (Baynes et al. 1981) and the increased sperm motility effects in muskellunge sperm (Lin and Dabrowski 1996).

#### Evaluation of Extenders

Extender A yielded a significantly higher percentage of motile sperm than Extender C and the neat control after 1, 2, and 7d of storage (Figure 1.2). Although Extender A and Extender B showed no significant difference in sperm motility after

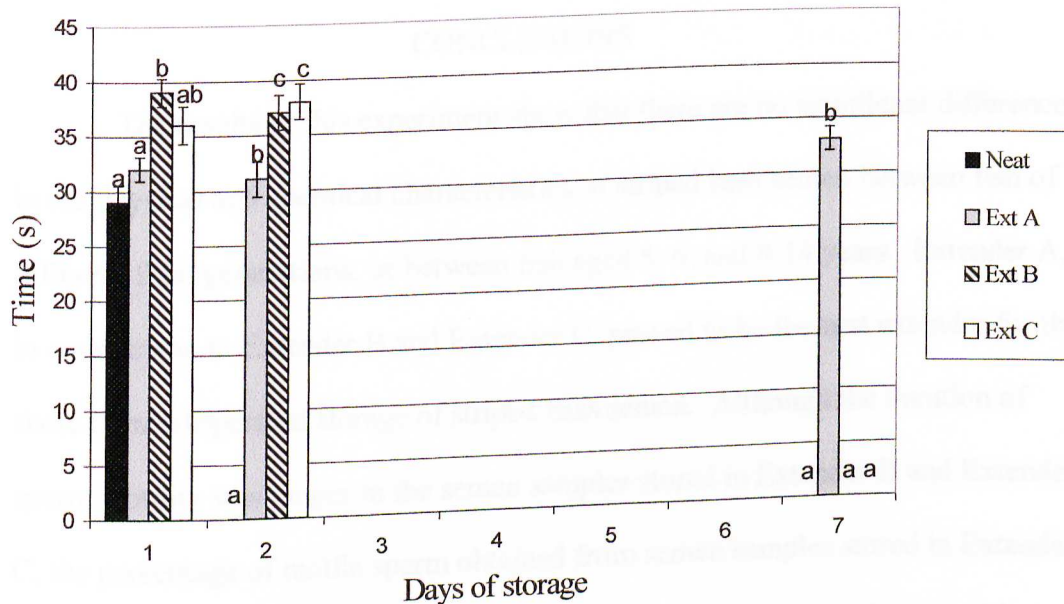
Figure 1.2: The percentage of motile sperm in neat and extended striped bass semen samples after short-term refrigerated storage. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other within each day at  $P < 0.05$ .



the 1<sup>st</sup> and 2<sup>nd</sup> day of storage, Extender A always had a higher percentage of motile sperm and was the only extender yielding any motility after 7d of storage.

Although Extender A maintained a higher percentage of motile sperm, the duration of sperm motility after 1d of storage was shorter in Extender A than in Extender B and C. Also, the duration of motility of the few sperm that were active in the neat semen samples, showed no significant difference from the duration of motility of sperm in Extender A or Extender C. After 2d of storage, the duration of sperm motility was significantly shorter in Extender A than Extender B and C (Figure 1.3). The differences observed in the duration of sperm motility can be attributed in part to the vigorous action of sperm in Extender A and the

Figure 1.3: The duration of sperm motility in neat and extended striped bass semen samples after short-term refrigerated storage. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other within each day at  $P < 0.05$ .



progressively slower movement of sperm in Extender C and B, respectively.

The semen stored in Extender A may have yielded the best results due to the fact that the osmolality of Extender A was closer than the other extenders to the average semen osmolality. Although sperm in Extender B yielded similar results during the first 2d of storage, the lower osmolality of Extender B may have induced motility in more sperm than Extender A upon initial mixing. This could account for the observation that sperm stored in Extender B always had a slightly lower percentage of motility than the sperm stored in Extender A. The poor results obtained from Extender C may be the result of osmotic damage. Although semen samples taken early in the spawning season measured around 400 mmol/kg, the samples taken near the peak of the spawning season measured around 340 mmol/kg.



The high osmolality of Extender C resulted in lower initial activation of sperm, but the cells may not have been able to adjust to so large an osmotic gradient.

## CONCLUSIONS

The results of this experiment show that there are no significant differences in the physical and chemical characteristics of striped bass semen between fish of different filial generations, or between fish aged 5, 6, and 8-14 years. Extender A, in comparison to Extender B and Extender C, proved to be the best extender for the short-term refrigerated storage of striped bass semen. Although the duration of sperm motility was longer in the semen samples stored in Extender B and Extender C, the percentage of motile sperm obtained from semen samples stored in Extender A was higher after 1, 2, and 7d of storage. In fact, the semen samples stored in Extender A were the only samples that exhibited any sperm motility after 7d of storage.

## CHAPTER 2

The characterization of striped bass semen during the spawning season and the analysis of four extenders for the short-term storage of striped bass semen.

## ABSTRACT

The physical and chemical characteristics of striped bass semen were measured once a week for six weeks during the spawning season. Semen samples were collected from six fish drawn randomly each week from a population of captive and domesticated striped bass broodstock. The viscosity of the semen, percentage of live sperm, percentage of motile sperm, duration of sperm motility, osmolality of the semen and seminal plasma, pH of the semen and seminal plasma, and the concentration of sodium and potassium in the seminal plasma all changed significantly during the spawning season. The sperm density ( $71.5 \pm 3.29 \times 10^9$  spermatozoa/mL) and the concentration of calcium in the seminal plasma ( $61.4 \pm 1.36$  mg/L) did not change significantly during the six week period.

Four experimental extenders were evaluated for use in the short-term refrigerated storage of striped bass semen. Performance was based on the amount of sperm motility obtained after 1, 2, and 7d of storage. The best extender from the previous experiment, Extender A, was compared to three new extenders, Extender C3, Extender C4, and Extender D. On average, semen stored in Extender A and/or Extender C3 contained a higher percentage of motile sperm than semen stored in Extender C2 or Extender D after 1 and 2d of refrigerated storage. The only semen samples exhibiting any motility after 7d of refrigerated storage were those extended with Extender A.



## INTRODUCTION

In the previous experiment, Experiment 1, the physical and chemical characteristics of striped bass semen were measured near the peak of the spawning season. No differences were observed between fish of different age, size, and filial generation. However, when developing Extender C, semen samples were collected early in the spawning season and measured for osmolality, pH, and sodium and potassium concentrations. Differences between these values and those collected from the fish near the peak of the spawning season a few weeks later, suggest that the quality and composition of striped bass semen may vary over the course of any given spawning season.

Variation in the physical and chemical composition of semen during the spawning season has been reported in several teleost species such as turbot (Suquet et al. 1998), ocean pout (Wang and Crim 1997), and carp (Christ et al. 1996). Knowledge of the natural changes that occur in semen composition during the spawning season is necessary for 1) more accurate measurement of semen quality, 2) more reliable short-term storage of semen under refrigeration, and 3) higher quality semen for cryopreservation.

This experiment, Experiment 2, was designed to compare the physical and chemical characteristics of striped bass semen during the spawning season. In addition, three new extenders were compared to Extender A, the extender yielding the best results in Experiment 1.

## MATERIALS AND METHODS

### Animals

Thirty-six captive and domestic striped bass from the F<sub>1</sub> and F<sub>2</sub> generations were used. Results obtained from the first experiment showed no generation or age-related differences in the characteristics of striped bass semen. Therefore, the older fish were used because their larger size would yield a greater volume of semen. A few striped bass were checked mid-March for the onset of spermiation and the majority of the fish were producing copious amounts of semen. Therefore, semen samples were collected once a week for 6 weeks during the spawning season beginning March 30, 1998. The experimental fish were allowed to remain in their normal 6m diameter circular tank receiving flow-through ambient water from the Chesapeake Bay. No hormone implants were necessary.

### Sample Collection

The striped bass broodfish were anesthetized with quinaldine at a concentration of 70mg/L (Woods et al. 1992). The urine in the bladder was expressed and the urogenital vent was wiped clean and dry to prevent contamination by water, urine, and feces. Semen was expressed directly into sterile 50-mL conical tubes by applying gentle abdominal pressure. Blood samples were taken in the last 4 weeks of the study. Blood samples were drawn from the caudal vein using heparinized needles (21 gauge) and syringes (3cc). All samples were kept on ice or under refrigeration until further analysis could be performed.

## Physical Characterization

The color and viscosity of semen collected was observed and recorded immediately after collection. The sperm was activated in replicate samples of three, using Fisher's deionized ultra-filtered water (DIUF) with an osmolality of 0 to 5 mmol/kg. Sperm motility was recorded on videotape using a Hitachi Model KP-140 video camera attached to a Zeiss Model D-7082 compound microscope at a magnification of 400x. The percentage of motile sperm and the amount of time the sperm remained motile were determined later from the videotapes. The percentage of motile sperm was estimated for all samples by the same observer. Duration of motility was timed beginning the instant the DIUF was mixed with the semen and ending when the majority (approximately 90%) of the sperm in the field of view had stopped moving. Spermatozoa that simply vibrated or did not show progressive forward movement were not considered motile. Duration of motility for each sample replicate was measured three times with a digital stopwatch and recorded to the nearest 1/100s. The percentage of live sperm was determined by an eosin-nigrosin differential staining technique described by Blom (1950). Semen samples were diluted by a factor of 200 and sperm density was measured using a Makler Counting Chamber.

## Chemical Characterization

Semen samples were centrifuged at 14000 RPM for 30min and blood samples were centrifuged at 10000 RPM for 5 min. To keep the samples at 4°C while spinning, a Heraeus Model 400R refrigerated centrifuge was used. Blood and



seminal plasma samples were removed and placed in 1.5mL snap-cap vials and stored on ice or under refrigeration until further analysis could be performed.

Neat semen, seminal plasma, and blood plasma were measured for osmolality (mmol/kg) and pH using a Wescor Model 5400 vapor pressure osmometer and an Orion Model 9810 micro-combination pH electrode, respectively. The remaining blood and seminal plasma samples were frozen in a -80°C freezer for later sodium, potassium, and calcium measurements using a Perkin-Elmer Model 5100 PC atomic absorption spectrophotometer.

#### Extender Evaluation

The formulas for the four extenders tested in this experiment are listed in Table 2.1. Extender A, Stein's extender, was carried over from the first experiment to test its performance in relation to three other extenders. Extender C2, is a modification of the original Extender C from the first experiment. The  $\text{Na}^+:\text{K}^+$  ratio remained the same, but the osmolality was lowered to 330mmol/L and egg yolk was added. Egg yolk is a common ingredient in many extenders and has been shown to have cryoprotective effects in some teleost species such as summer whiting (Young et al. 1992). Extender C3 was developed a week into the experiment as a further modification of the original Extender C. The osmolality was lowered to 315mmol/kg and the  $\text{Na}^+:\text{K}^+$  ratio was raised in case the higher levels of potassium in Extender C may have prevented semen activation. Extender D has been used successfully for the short-term storage of paddlefish semen (Brown and Mims 1995). Although the performance of Extender D on the short-term storage of

striped bass semen has not been published, it is currently used by many others conducting striped bass research (G. Brown, Iowa State University, personal communication).

Immediately after collection, 0.3mL of semen from each fish was gently mixed with 0.6mL of each extender and pipetted into a 1.5mL snap-cap vial. As a control, 0.9mL of neat semen from each fish was pipetted into a snap-cap vial without any extender. The samples were stored upright and refrigerated at 3-4°C. After 1, 2, and 7d, each extended and neat sample was activated with DIUF in replicates of three. Sperm motility was recorded on videotape using a Hitachi Model KP-140 video camera attached to a Zeiss Model D-7082 compound microscope at a magnification of 400x. The percentage of motile sperm and the

Table 2.1: Extender Formulas for Experiment 2.<sup>1</sup>

Extender A <sup>2</sup>	Extender C2	Extender C3	Extender D <sup>3</sup>
100mg glucose	9.5g glucose	3.75g glucose	860mg NaCl
750mg NaCl	500mg NaCl	360mg NaCl	100mL water <sup>4</sup>
200mg NaHCO <sub>3</sub>	63mg KCl	4.2mg KCl	
40mg KCl	200mL water <sup>4</sup>	100mL water <sup>4</sup>	
100mL water <sup>4</sup>			
20mL egg yolk			
Osmolality:	Osmolality:	Osmolality:	Osmolality:
290 mmol/kg	330 mmol/kg	315 mmol/kg	270 mmol/kg
pH: 7.6	pH: 7.6	pH: 7.8	pH: 7.6

<sup>1</sup> All extenders were mixed and then pH adjusted with HCl or NaOH.

<sup>2</sup> Formula taken from Stein and Bayrle (1978).

<sup>3</sup> Formula taken from Brown and Mims (1995).

<sup>4</sup> Water used was Fisher's deionized ultra-filtered water (DIUF).



amount of time the sperm remained motile were determined later from the videotapes by the same procedures described under Physical Characterization.

### Statistical Analysis

Each individual semen characteristic was subjected to analysis of variance in order to determine if any changes occurred during the spawning season. The mixed model procedures from SAS version 6.12 statistical software were used (SAS 1996). Pairwise contrasts between weeks were made to detect significant differences at the 5% level. Results unless otherwise noted, are reported as means  $\pm$  S.E.M.

## RESULTS AND DISCUSSION

### Physical Characterization

The viscosity of the semen remained relatively low throughout the entire spawning season. Only at the beginning and end of the season did some samples exhibit high viscosity. The color of the semen was white with the exception of a few blood-contaminated samples having a pink hue.

The percentage of live sperm changed significantly over the spawning season (Figure 2.1). Weeks 2 and 6 had significantly lower numbers of live cells than weeks 3 and 5. Semen quality in teleost fish is usually quantified by the percentage of motile sperm, duration of sperm motility, seminal plasma composition, sperm density, and/or spermatocrit (Rana 1995). Only a few studies have used the percentage of live sperm as a measurement of semen quality. The



percentage of live sperm documented in *Oreochromis mossambicus* and carp were not found to change significantly during the spawning season (Kruger et al. 1984). The change in the percentage of live sperm observed in striped bass of this study may not be accurate due to problems associated with the eosin-nigrosin staining technique. The eosin-nigrosin staining technique differentiates between cells with or without an intact membrane. The color change observed was not dramatic enough to allow an accurate differentiation between these cells. For any given sample, the average percentage of live sperm measured was lower than the percentage of motile sperm obtained (Figures 2.1 and 2.2). Since motility cannot occur without an intact membrane, these results were not considered valid and the eosin-nigrosin staining procedure is not recommended for use with striped bass semen.

The percentage of motile sperm showed a significant change during the spawning season (Figure 2.2). The percentage of motile sperm seemed to increase later in the season. Semen collected in the 6<sup>th</sup> week showed a significantly higher percentage of motile sperm than semen collected in the first 3 weeks. Also, motile sperm from the 4<sup>th</sup> week was significantly greater than the 3<sup>rd</sup> week. These results contrast those obtained for Atlantic salmon where motility decreased by 22% at the end of the season (Kazakov 1981). The typical pattern observed in most species with changing sperm motilities, is for the percentage of motile sperm to be low in the beginning of the season, highest at the peak of the season, and then low again at the end of the season. Such patterns have been observed in ocean pout (Wang and Crim 1997) and rainbow trout (Büyükhatoğlu and Holtz 1984).

Figure 2.1: The percentage of live sperm obtained from neat semen during the spawning season. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other at  $P < 0.05$ .

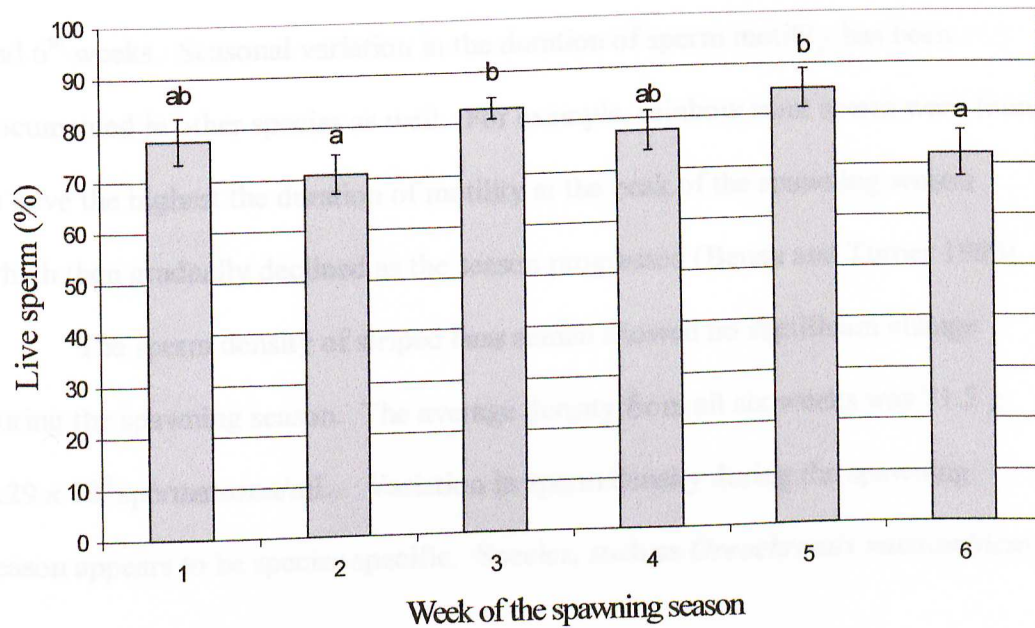
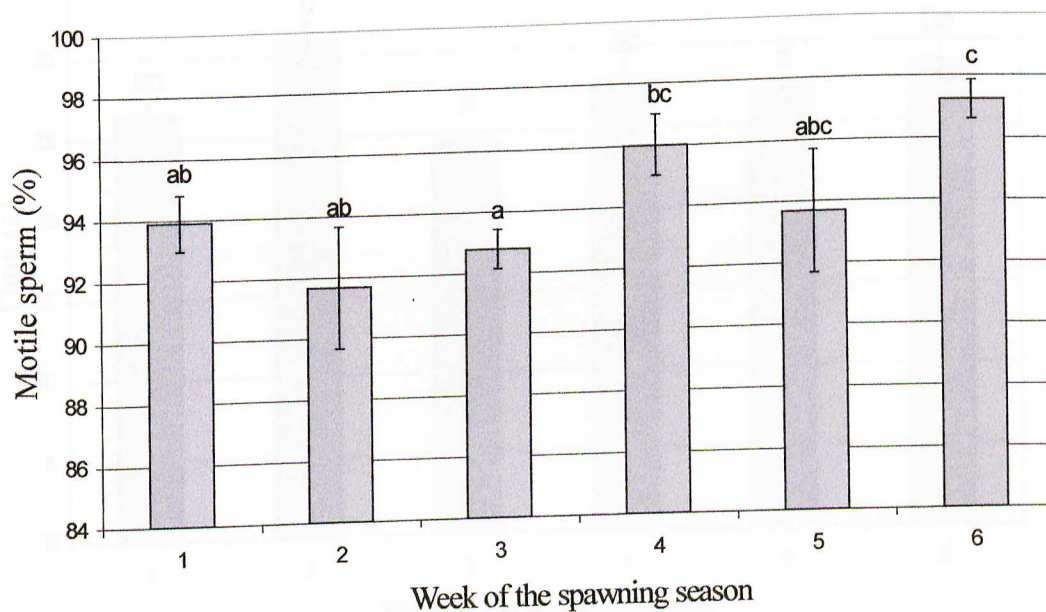


Figure 2.2: The percentage of motile sperm obtained from neat semen during the spawning season. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other at  $P < 0.05$ .

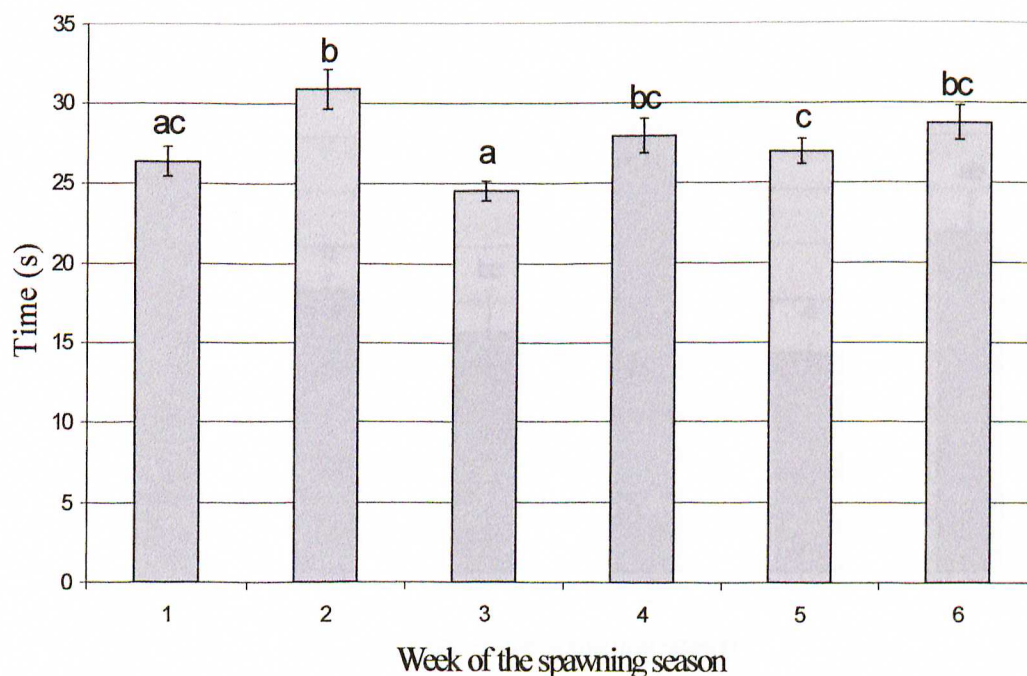




The duration of sperm motility in the striped bass showed a significant change during the spawning season (Figure 2.3). Week 3 was significantly lower than all but the 1<sup>st</sup> week while week 2 was significantly higher than all but the 4<sup>th</sup> and 6<sup>th</sup> weeks. Seasonal variation in the duration of sperm motility has been documented in other species as well. For example, rainbow trout sperm were found to have the highest the duration of motility at the peak of the spawning season which then gradually declined as the season progressed (Benau and Turner 1980).

The sperm density of striped bass semen showed no significant change during the spawning season. The average density from all six weeks was  $71.5 \pm 3.29 \times 10^9$  spermatozoa/mL. Variation in sperm density during the spawning season appears to be species specific. Species, such as *Oreochromis mossambicus*,

Figure 2.3: The duration of sperm motility in striped bass semen during the spawning season. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other at  $P < 0.05$ .





maintain constant sperm concentrations throughout the spawning season (Kruger et al. 1984), whereas other species, such as Atlantic salmon (Kazakov 1981), rainbow trout (Büyükhatoğlu and Holtz 1984), carp (Christ et al. 1996), and perch (Piironen and Hyvärinen 1983), have shown significant changes in sperm density during the spawning season.

### Chemical Characterization

The osmolality of the striped bass semen and seminal plasma changed significantly across the spawning season (Figures 2.4 and 2.5), whereas the osmolality of the blood plasma remained constant with an average value of  $339 \pm 1.3$  mmol/kg. Due to the viscosity of neat semen, most researchers measure

Figure 2.4: The osmolality of striped bass semen during the spawning season. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other at  $P < 0.05$ .

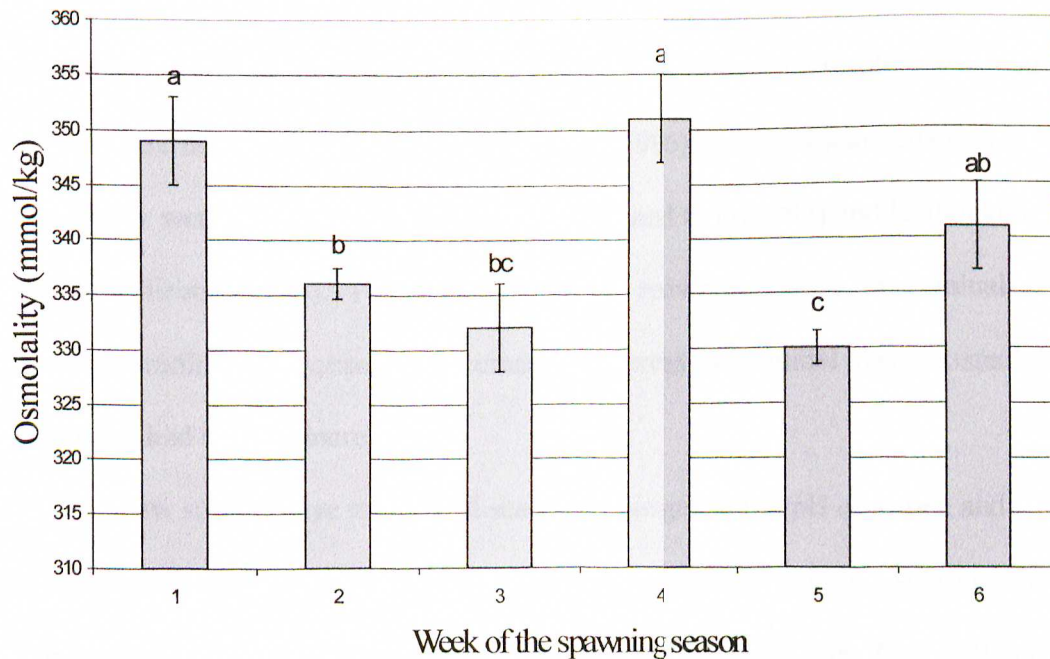
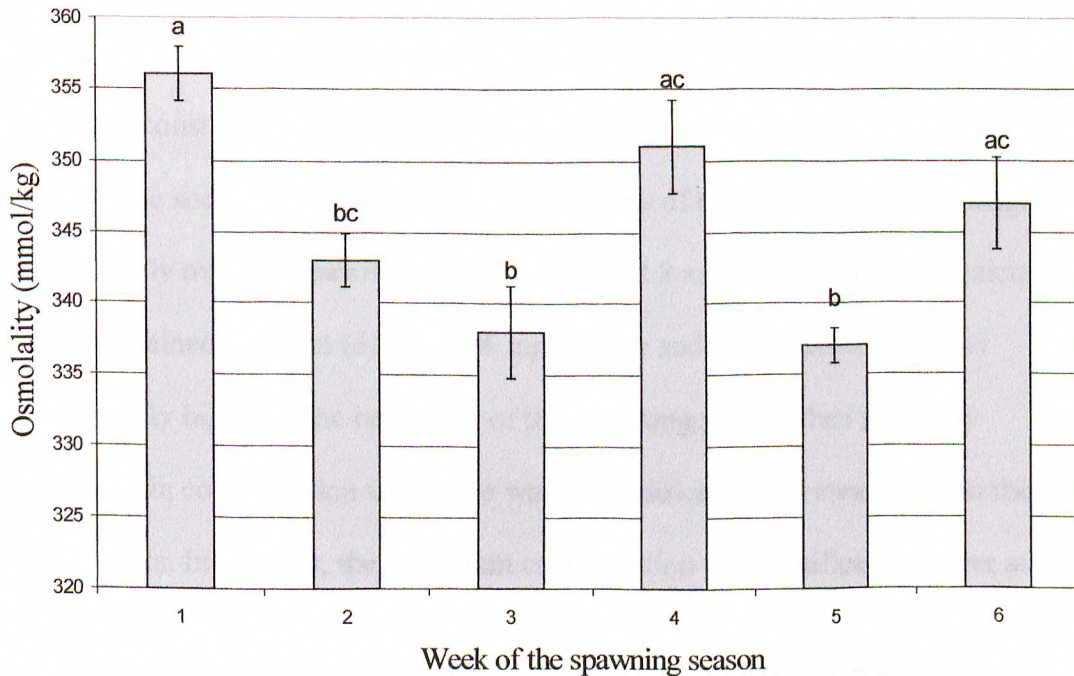


Figure 2.5: The osmolality of striped bass seminal plasma during the spawning season. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other at  $P < 0.05$ .



seminal plasma osmolality only. Seminal plasma osmolalities during the spawning season have been documented for several species with varying results. Seasonal effects were not observed in carp (Christ et al. 1996), but significant changes in osmolality were observed in ocean pout (Wang and Crim 1997) and landlocked salmon (Piironen 1985). Toward the end of the spawning season, the seminal plasma osmolality of ocean pout decreased, whereas the seminal plasma osmolality of landlocked salmon increased.

Few studies have monitored seasonal changes in the pH of semen and seminal plasma. Fluctuations that occur, if any, are usually within a small range. For example, the pH of ocean pout seminal plasma increases from 7.4 to 7.9 during

the spawning season (Wang and Crim 1997). The pH of striped bass semen and seminal plasma varied significantly across the spawning season but within a small range (Figures 2.6 and 2.7). The pH of the neat semen ranged from 7.7 to 8.2 and the pH of the seminal plasma ranged from 7.4 to 8.2. The blood plasma pH remained constant throughout the spawning season ( $7.6 \pm 0.04$ ).

The sodium and potassium concentrations of the seminal plasma changed significantly over the spawning season (Figures 2.8 and 2.9), whereas the calcium levels remained constant ( $61.4 \pm 1.36$  mg/L). The sodium concentration was significantly higher at the beginning of the spawning season, then gradually decreased in concentration until there was no significant difference between the last three weeks. In contrast, the potassium concentration was significantly lower at the

Figure 2.6: The pH of striped bass semen during the spawning season. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other at  $P < 0.05$ .

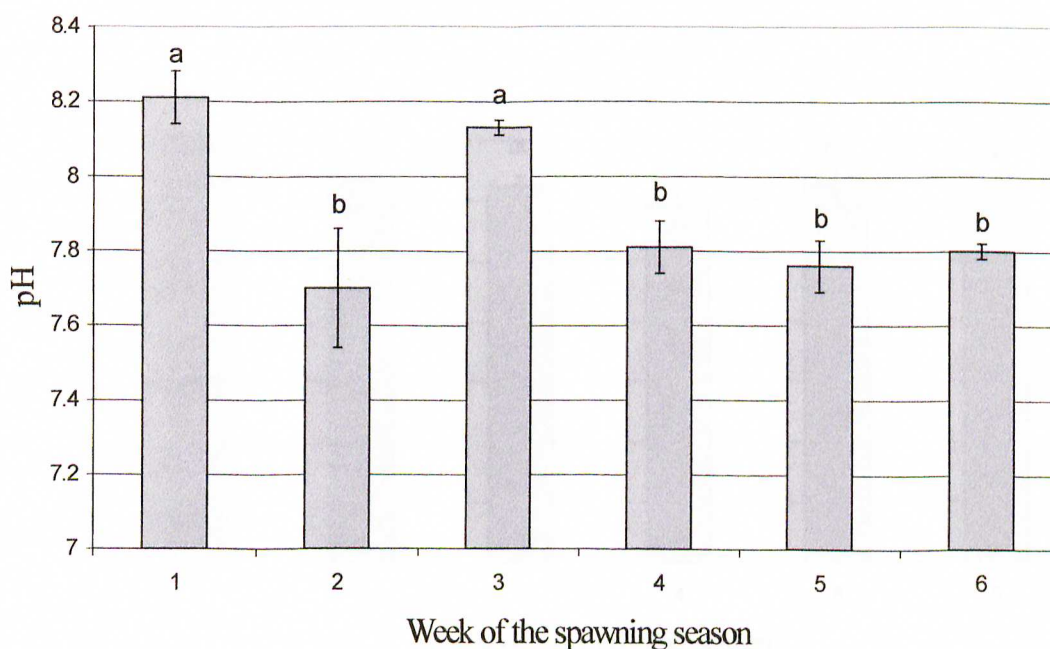




Figure 2.7: The pH of striped bass seminal plasma during the spawning season. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other at  $P < 0.05$ .

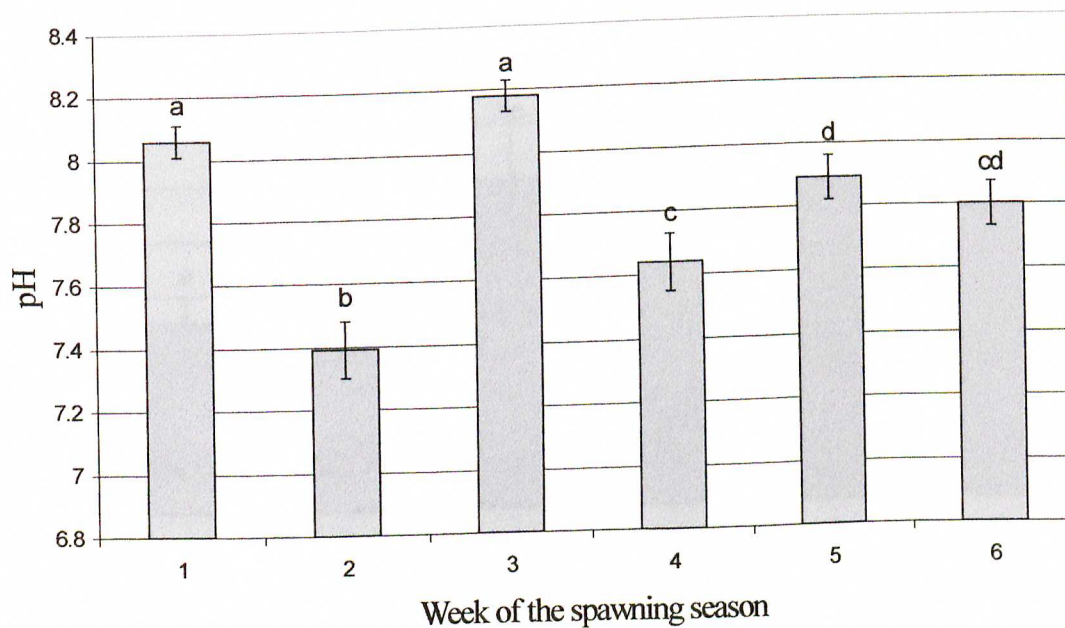


Figure 2.8: The sodium concentration of striped bass seminal plasma during the spawning season. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other at  $P < 0.05$ .

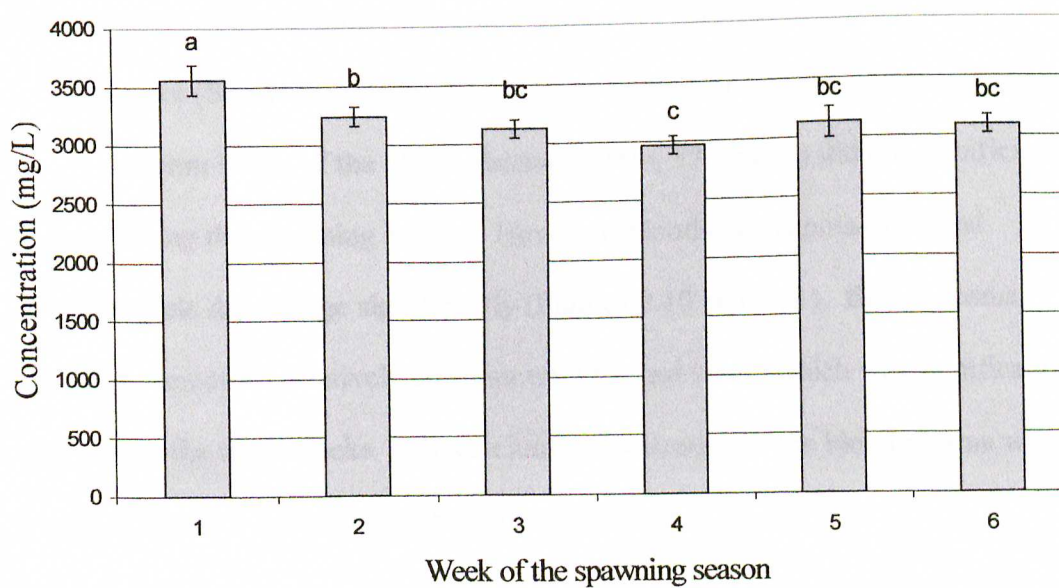
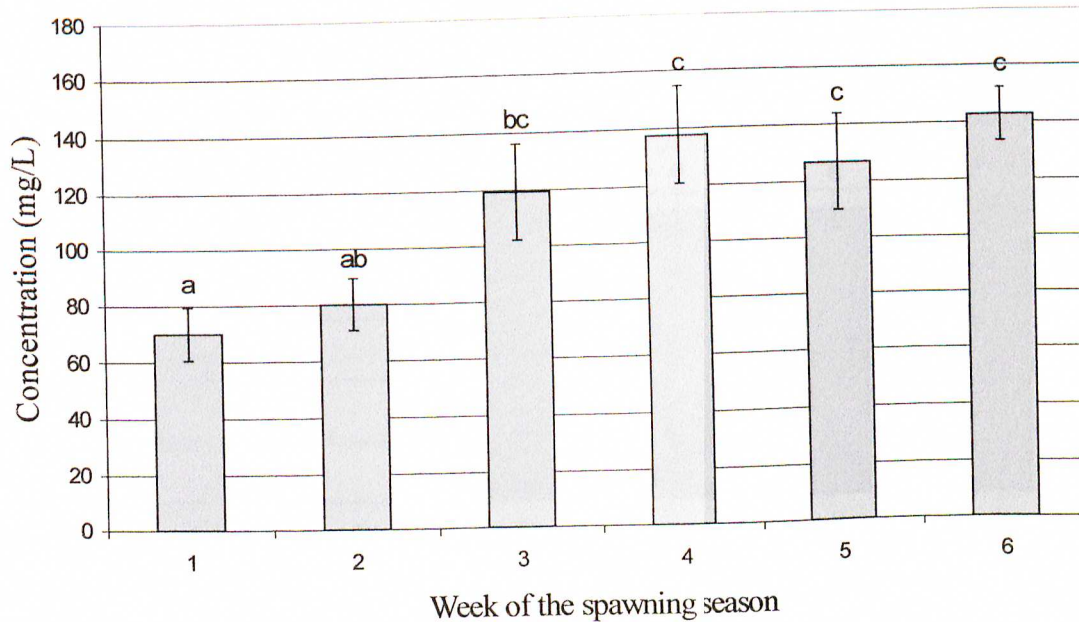


Figure 2.9: The potassium concentration of striped bass seminal plasma during the spawning season. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other at  $P < 0.05$ .



beginning of the season, then increased in concentration with no significant difference between the last four weeks. Similar variations were documented in the sodium levels of ocean pout (Wang and Crim 1997) and the potassium levels of rainbow trout (Sanchez-Rodriguez 1978) and landlocked salmon (Piironen 1985).

Sodium levels of the blood plasma ( $3690 \pm 37.6$  mg/L) did not significantly change during the spawning season. However, blood plasma potassium and calcium levels did change significantly (Figures 2.10 and 2.11). Blood plasma potassium remained relatively constant until the last week, which was significantly higher than the other weeks. The calcium concentration of the blood plasma was lowest in weeks 3 and 4 and highest in weeks 5 and 6.

Figure 2.10: The potassium concentration of striped bass blood plasma during the spawning season. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other at  $P < 0.05$ .

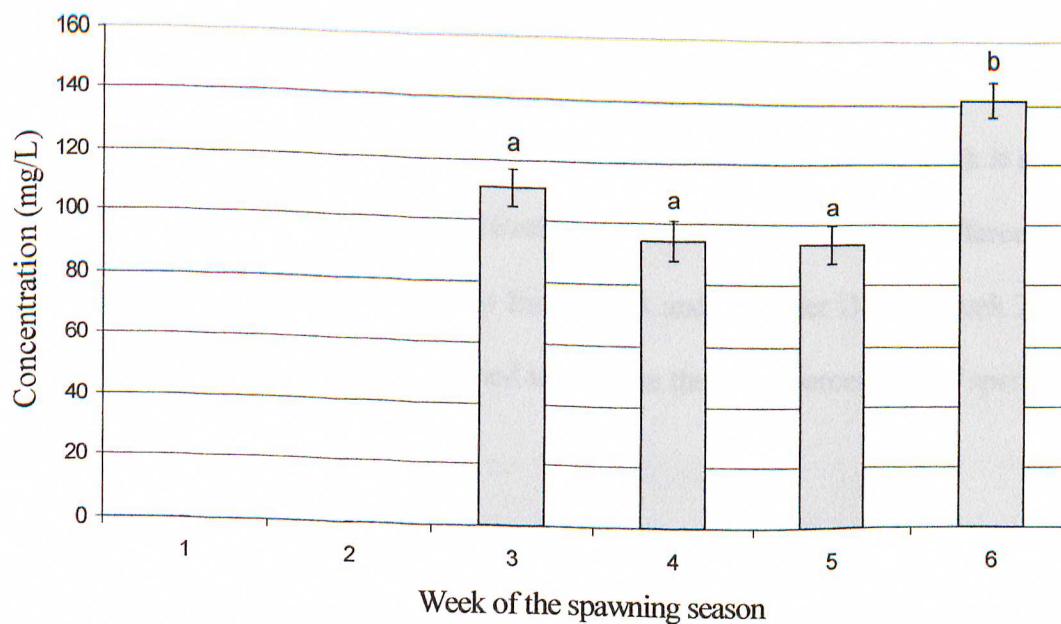
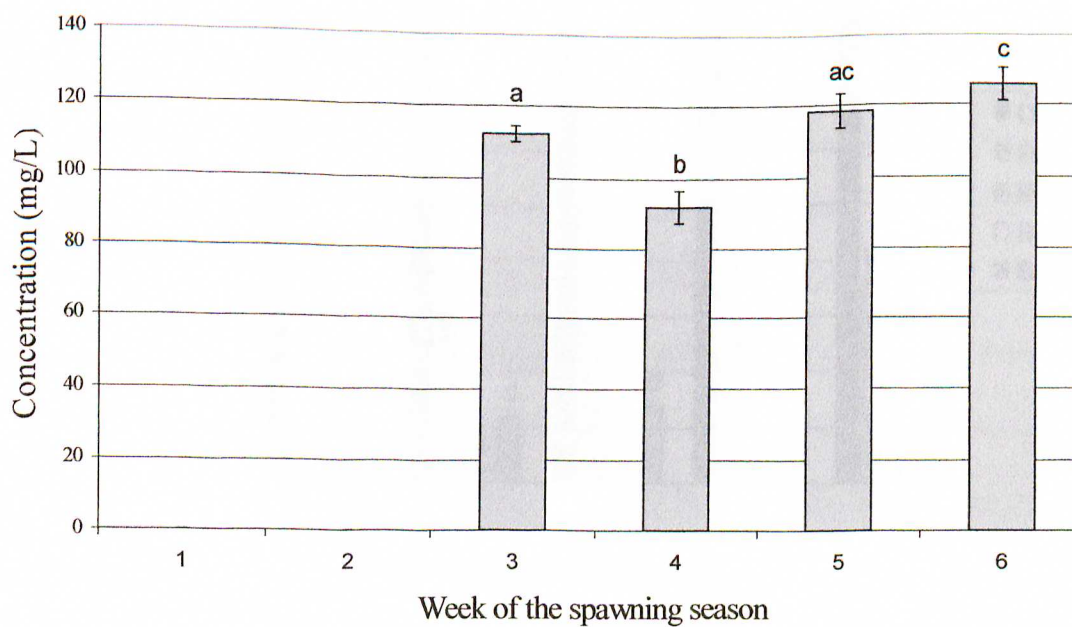


Figure 2.11: The calcium concentration of striped bass blood plasma during the spawning season. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other at  $P < 0.05$ .





## Extender Evaluation

Beginning in week 2, all neat semen samples were activated with DIUF and with each extender upon initial collection to determine what percentage of sperm were activated during the addition of the extender. The percentage of motile sperm and the duration of sperm motility obtained from these samples each week is shown in Figure 2.12 and Table 2.2, respectively. It is interesting to note the difference in the percentage of sperm activated by Extender A and Extender D from week 2 to week 3. Extenders C2 and C3 seemed to activate the same percentage of sperm each week.

Figure 2.12: The percentage of motile sperm obtained from neat striped bass semen samples when activated with DIUF and extenders. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other within each week at  $P < 0.05$ .

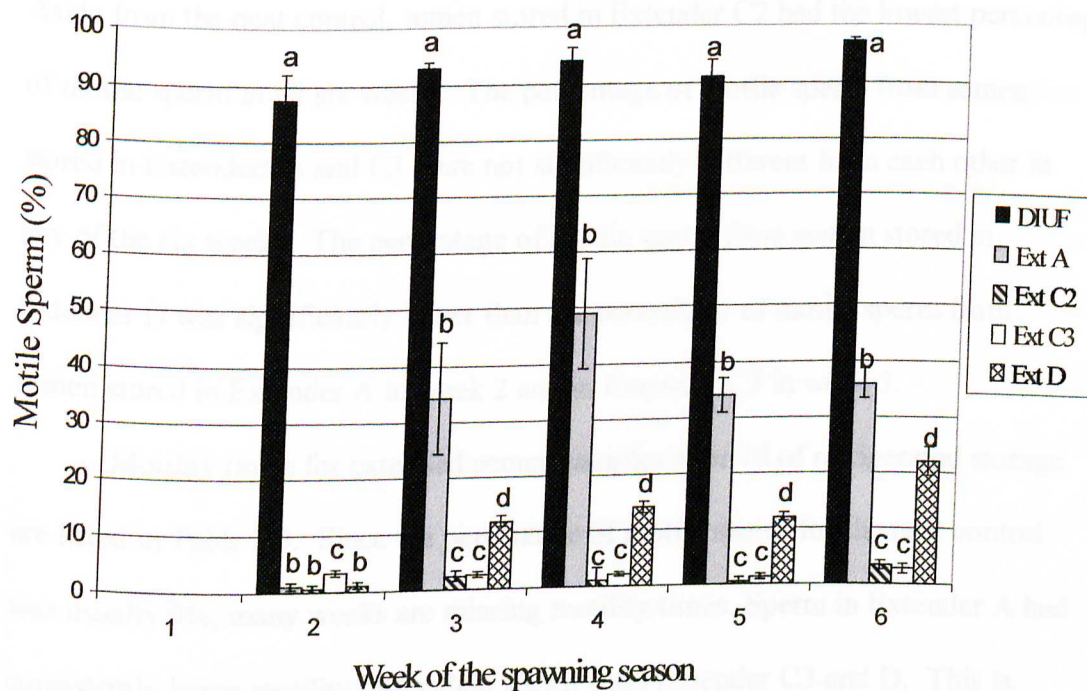


Table 2.2: The duration of sperm motility (s) in neat semen samples activated with DIUF and extenders.

Week	DIUF	Extender A	Extender C2	Extender C3	Extender D
1	--	--	--	--	--
2	31 <sup>a</sup>	8 <sup>bc</sup>	3 <sup>b</sup>	14 <sup>c</sup>	6 <sup>bc</sup>
3	25 <sup>a</sup>	45 <sup>b</sup>	12 <sup>c</sup>	18 <sup>ac</sup>	34 <sup>d</sup>
4	28 <sup>a</sup>	40 <sup>b</sup>	7 <sup>c</sup>	9 <sup>c</sup>	30 <sup>a</sup>
5	27 <sup>a</sup>	39 <sup>b</sup>	3 <sup>c</sup>	9 <sup>c</sup>	42 <sup>b</sup>
6	29 <sup>a</sup>	37 <sup>b</sup>	20 <sup>ac</sup>	19 <sup>c</sup>	36 <sup>b</sup>

After 1d of refrigerated storage, all extended samples contained a significantly higher percentage of motile sperm than the neat control (Figure 2.13). Aside from the neat control, semen stored in Extender C2 had the lowest percentage of motile sperm in all six weeks. The percentage of motile sperm from semen stored in Extenders A and C3 were not significantly different from each other in any of the six weeks. The percentage of motile sperm from semen stored in Extender D was significantly lower than the percentage of motile sperm from semen stored in Extender A in week 2 and in Extender C3 in week 3.

Motility times for extended semen samples after 1d of refrigerated storage are listed in Table 2.3. Since the percentage of motile sperm for the neat control was usually 0%, many weeks are missing motility times. Sperm in Extender A had consistently lower motility times than sperm from Extender C3 and D. This is partly due to the “explosive” movement of sperm in Extender A as compared to the



Figure 2.13: The percentage of motile sperm in neat and extended striped bass semen samples after 1d of refrigerated storage. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other within each week at  $P < 0.05$ .

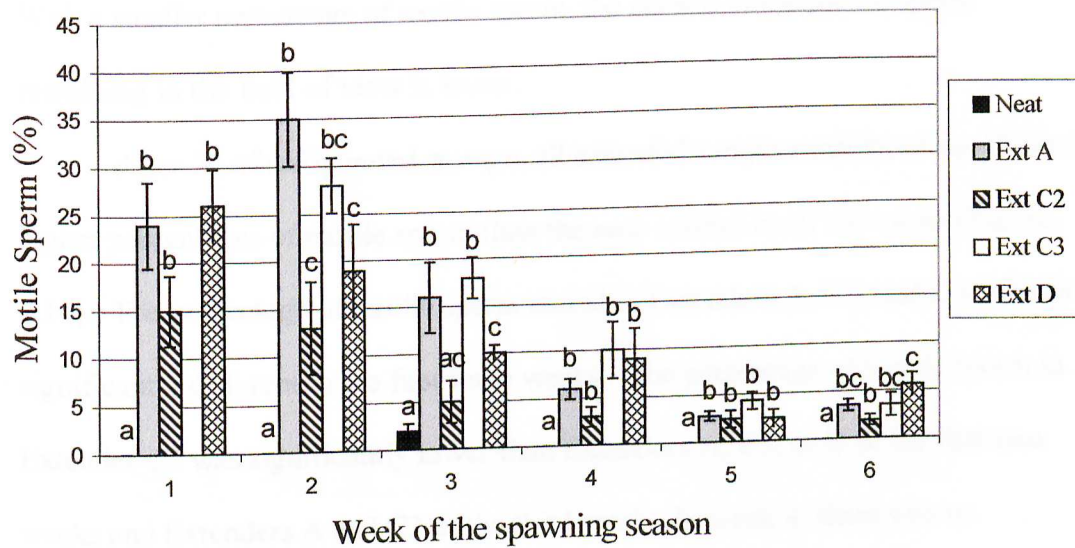


Table 2.3: The duration of sperm motility (s) of neat and extended semen samples after 1d of refrigerated storage.

Week	Neat	Extender A	Extender C2	Extender C3	Extender D
1	--	31 <sup>a</sup>	39 <sup>b</sup>	--	37 <sup>b</sup>
2	--	28 <sup>a</sup>	28 <sup>a</sup>	40 <sup>b</sup>	37 <sup>b</sup>
3	--	25 <sup>a</sup>	20 <sup>a</sup>	40 <sup>b</sup>	33 <sup>c</sup>
4	9 <sup>a</sup>	27 <sup>b</sup>	24 <sup>ab</sup>	40 <sup>c</sup>	30 <sup>b</sup>
5	--	15 <sup>a</sup>	14 <sup>a</sup>	25 <sup>b</sup>	14 <sup>a</sup>
6	--	18 <sup>a</sup>	8 <sup>b</sup>	22 <sup>a</sup>	23 <sup>a</sup>



slower movement of sperm from Extenders C3 and D. The percentage of motile sperm in all extended samples dropped significantly in the last two weeks. This is most likely due to the decreased percentage of motile sperm in the last two weeks. With a smaller percentage of motile sperm, the chance of the motile sperm remaining in the field of view is lower.

After 2d of refrigerated storage, all extended samples exhibited significantly higher percentages of motile sperm than the neat control in all six weeks (Figure 2.14). The percentage of motile sperm stored in Extenders A, C3, and D were not significantly different in the first three weeks. The percentage of motile sperm in Extender C2 was significantly lower than Extenders A, C3, or D in the first two weeks and Extenders A and C3 in the third week. In week 4, there was no difference in the percentage of motile sperm stored in any of the extended samples. In week 5, only samples extended in Extender A exhibited any motility and in week 6, the percentage of motile sperm from Extenders A and D was  $2.2 \pm 0.8$  and  $0.7 \pm 0.8\%$  respectively.

The duration of sperm motility after 2d of storage did not differ significantly during the six weeks in samples from Extenders A, C3, and D (Table 2.3). There was no "explosive" movement in any of the extended samples as the day before. Sperm movement in all neat and extended samples became progressively slower as the storage time increased.

After 7 days of refrigerated storage, only semen stored in Extender A exhibited any significant motility. The highest average percentage of motile sperm obtained in this extender was 4.3% in week 3. Likewise, motility times were only

Figure 2.14: The percentage of motile sperm in neat and extended striped bass semen samples after 2d of refrigerated storage. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other within each week at  $P < 0.05$ .

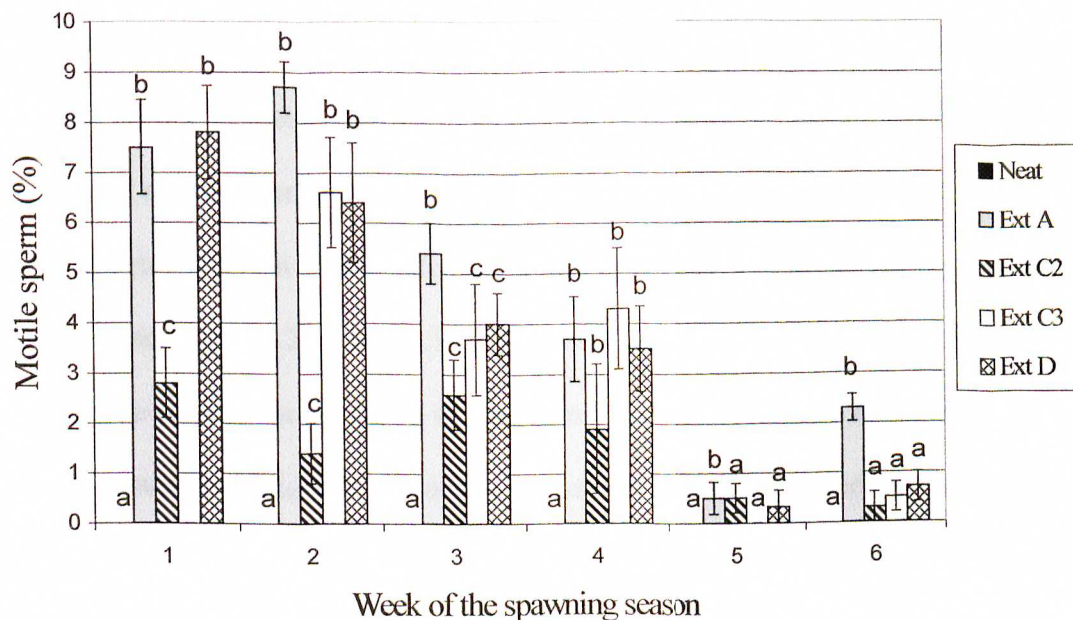


Table 2.4: The duration of sperm motility (s) of neat and extended semen samples after 2d of refrigerated storage.

Week	Neat	Extender A	Extender C2	Extender C3	Extender D
1	--	24 <sup>ab</sup>	15 <sup>a</sup>	--	26 <sup>b</sup>
2	--	25 <sup>a</sup>	7 <sup>b</sup>	26 <sup>a</sup>	28 <sup>a</sup>
3	--	24 <sup>a</sup>	10 <sup>b</sup>	22 <sup>ab</sup>	19 <sup>ab</sup>
4	--	17 <sup>a</sup>	7 <sup>b</sup>	16 <sup>ab</sup>	16 <sup>ab</sup>
5	--	9 <sup>a</sup>	3 <sup>a</sup>	--	4 <sup>a</sup>
6	--	15 <sup>a</sup>	2 <sup>b</sup>	4 <sup>b</sup>	6 <sup>b</sup>

obtained for sperm stored in Extender A. Average duration of motility ranged from 2 to 18 s.

From the extender evaluation, it would appear that there was a significant decrease in semen quality as the spawning season progressed. In this case, semen quality would be measured as the percentage of motile sperm obtained from the extended semen after a certain amount of storage time. The percentage of motile sperm obtained from all the extended samples decreased as the spawning season progressed (Figures 2.13 and 2.14). These results contradict the previous results in which the percentage of motile sperm from the freshly collected neat semen samples was found to increase as the spawning season progressed. Although the percentage of motile sperm from the neat semen was statistically found to change during the spawning season, the range was very small (92-97%). This indicates that the sperm motility of the neat semen may not be an accurate indicator of semen quality.

## CONCLUSIONS

The results of this experiment show that many of the physical and chemical characteristics of striped bass semen change significantly during the spawning season. Although the percentage of motile sperm and the duration of sperm motility obtained from freshly collected neat semen was found to statistically change during the spawning season, the range for these values was small (92-97% and 24-31s, respectively). Therefore, it is uncertain whether individual fish variation or the subjective method of measurement may have affected these results and possibly



conferred a significant change where there is none. A significant change was also found to occur in the osmolality and pH of striped bass semen and the osmolality, pH, and sodium and potassium concentrations of striped bass seminal plasma during the spawning season. However, these characteristics fluctuated from week to week and it is uncertain whether these characteristics naturally fluctuate, or if individual fish variation coupled with the small sample size may have obscured any detectable pattern.

Extender A, in comparison to Extender C2, Extender C3, and Extender D, proved to be the best extender for the short-term refrigerated storage of striped bass semen. There was no significant difference in the percentage of motile sperm obtained from semen stored in Extender A and Extender C3 after 1d of storage. However, during the last two weeks of the experiment, the percentage of motile sperm obtained from semen stored in Extender A was significantly higher than the percentage of motile sperm obtained from semen stored in Extender C3 after 2d of storage. Also, as in the previous experiment (Experiment 1), the semen samples stored in Extender A were the only samples exhibiting any sperm motility after 7d of storage.

## CHAPTER 3

The characterization of semen from individual striped bass across the spawning season and the analysis of four extenders for the short-term storage of striped bass semen.

## ABSTRACT

The physical and chemical characteristics of striped bass semen were measured weekly during the entire spawning season from six individual fish given GnRHa implants, six individual fish given sham implants, and six fish randomly drawn from the general population of captive and domesticated striped bass. No significant change in the percentage of motile sperm obtained from the repeatedly sampled individual striped bass was found to occur across the spawning season. However, the percentage of motile sperm obtained from the fish drawn from the general population did show a significant change. In all three groups of fish, the duration of sperm motility significantly increased and the sperm density significantly decreased in the middle of the spawning season. The pH of the semen and seminal plasma did not change in the GnRHa implanted fish, but the pH of the semen and seminal plasma from the sham implanted fish and the fish drawn from the general population fluctuated randomly during the spawning season. The osmolality of the semen and the seminal plasma and the sodium, potassium, and calcium concentrations of the seminal plasma were found to significantly change in all three groups of fish throughout the spawning season. The seminal plasma sodium was found to have a significant correlation of 0.52 with the semen osmolality and 0.37 with the sperm density.

Four experimental extenders were evaluated for use in the short-term refrigerated storage striped bass semen. The best extender from the two previous experiments, Extender A, and the extender currently used by many striped bass researchers, Extender D, were compared to two new extenders, Extender C4 and



Extender C5. Extenders C4 and C5 were found to be the best extenders for the short-term refrigerated storage striped bass semen. Semen stored in Extender C4 and Extender C5 contained a higher percentage of motile sperm than semen stored in Extender A or Extender D after 1, 2, and 7d of storage.

## INTRODUCTION

In the previous experiment, Experiment 2, several of the physical and chemical characteristics of striped bass semen were found to change over a six week period during the spawning season. However, many of the characteristics which were found to change during the spawning season (semen osmolality, seminal plasma osmolality, semen pH, and seminal plasma pH), fluctuated randomly from week to week. Since the fish sampled were randomly drawn from the population and weren't the same fish each week, the individual response for a given parameter may have clouded the observance of a specific pattern. Also, the six week sampling period did not encompass the entire spawning season and changes occurring at the beginning or end of the spawning season may not have been detected.

This experiment, Experiment 3, was designed to analyze the physical and chemical characteristics from twelve individual striped bass repetitively sampled across an entire spawning season. As a control, semen samples were also collected and analyzed weekly from six fish randomly drawn from a general population of captive and domesticated striped bass males maintained in a single 6m diameter tank. Multiple strippings of the same fish have decreased the total volume of semen in turbot (Suquet et al. 1994) and rainbow trout (Büyükhatoğlu and Holtz 1984). GnRHa implants have been used to increase semen volume in striped bass without affecting sperm density (Mylonas et al. 1998). Therefore, to ensure that an adequate amount of semen could be collected from the individual fish throughout the spawning season, six of the individual fish were given GnRHa implants that

maintained elevated plasma levels of GnRH $\alpha$  for at least two weeks (Woods and Sullivan 1993). For comparison, an additional six individual fish were given sham implants. In addition to the physical and chemical characterization of striped bass semen, two new extenders were compared to Extender A, the extender yielding the best results in Experiments 1 and 2, and Extender D, the extender currently used by most striped bass researchers.

## MATERIALS AND METHODS

### Animals

Twelve male striped bass from the captive population at Crane Aquaculture Facility were moved from a 6m diameter tank and randomly divided into two 3m diameter tanks supplied with flow-through ambient water from the Chesapeake Bay. The fish in one tank received GnRH $\alpha$  implants every other week across the spawning season and the fish in the other tank received sham implants. The GnRH $\alpha$  implants consisted of approximately 100  $\mu$ g of [D-Ala<sup>6</sup>-Pro<sup>9</sup>-Net]-LHRH (GnRH $\alpha$ ) contained in a matrix of cholesterol and cellulose (Woods and Sullivan 1993). The sham implants were identical to the GnRH $\alpha$  implants except they contained no GnRH $\alpha$ . A few striped bass were collected early in March to check for the onset of spermiation. Sample collection began when the first flowing male was found on March 30, 1999 and ended when semen could no longer be obtained from any of the fish on June 2, 1999. Semen samples were collected from each of the 12 fish once a week during this time period. Blood samples were collected once every two weeks. Semen samples were also collected from six fish randomly drawn from the



general population of captive and domesticated male striped bass each week. Every other week, both semen and blood samples were taken from the six randomly collected fish. These fish were not given any implants and were returned to their 6m diameter tank that was also supplied with flow-through ambient water from the Chesapeake Bay.

#### Sample Collection

The fish were anesthetized with quinaldine at a concentration of 70mg/L (Woods et al. 1992). The bladder was expressed and the urogenital vent was wiped clean and dry to prevent contamination by water, urine, and feces. Semen was expressed directly into sterile 50-mL conical tubes by applying gentle abdominal pressure. Minimal amounts of semen (3-6mL) were collected from the 12 individual fish to prevent unnecessary stress and possible physical injury to the fish. Larger amounts of semen were collected from the fish randomly drawn from the population since these fish were not under the stress of repeated sampling. Blood samples were drawn from the caudal vein using heparinized needles (21 gauge) and syringes (3cc). All samples were kept on ice or under refrigeration until further analysis could be performed.

#### Physical Characterization

The color and viscosity of semen collected was observed and recorded immediately after collection. The sperm was activated in replicate samples of three, using Fisher's deionized ultra-filtered water (DIUF) with an osmolality of 0 to 5

mmol/kg. The sperm activation was recorded on videotape using a Hitachi Model KP-140 video camera attached to a Zeiss Model D-7082 compound microscope at a magnification of 400x. The percentage of motile sperm and the amount of time the sperm remained motile were determined later from the videotapes. The percentage of motile sperm was estimated for all samples by the same observer. Duration of motility was timed beginning the instant the DIUF was mixed with the semen and ending when the majority (approximately 90%) of the sperm in the field of view had stopped moving. Spermatozoa that simply vibrated or did not show progressive forward movement were not considered motile. Duration of motility for each sample replicate was measured three times with a stopwatch and recorded to the nearest 1/100s. The eosin-nigrosin differential staining technique was not used for reasons explained in Chapter 2. Therefore, the percentage of live sperm was not measured in this experiment. Semen samples were diluted by a factor of 200 and sperm density was measured using a Makler Counting Chamber.

### Chemical Characterization

Semen samples were centrifuged at 14000 RPM for 30min and blood samples were centrifuged at 10000 RPM for 5 min. To keep the samples at 4°C while spinning, a Heraeus Model 400R refrigerated centrifuge was used. Blood and seminal plasma samples were removed and placed in 1.5mL snap-cap vials and stored on ice or under refrigeration until further analysis could be performed.

Neat semen, seminal plasma, and blood plasma were measured for osmolality (mmol/kg) and pH using a Wescor Model 5400 vapor pressure



Table 3.1: Extender Formulas for Experiment 3.<sup>1</sup>

Extender A <sup>2</sup>	Extender C4	Extender C5	Extender D <sup>3</sup>
100mg glucose	100mg glucose	100mg glucose	860mg NaCl
750mg NaCl	870mg NaCl	940mg NaCl	100mL water <sup>4</sup>
200mg NaHCO <sub>3</sub>	200mg NaHCO <sub>3</sub>	200mg NaHCO <sub>3</sub>	
40mg KCl	40mg KCl	40mg KCl	
100mL water <sup>4</sup>	100mL water <sup>4</sup>	100mL water <sup>4</sup>	
20mL egg yolk	20mL egg yolk	20mL egg yolk	
Osmolality:	Osmolality:	Osmolality:	Osmolality:
290 mmol/kg	310 mmol/kg	330 mmol/kg	270 mmol/kg
pH: 7.6	pH: 7.6	pH: 7.6	pH: 7.6

<sup>1</sup> All extenders were mixed and then pH adjusted with HCl or NaOH.

<sup>2</sup> Formula taken from Stein and Bayrle (1978).

<sup>3</sup> Formula taken from Brown and Mims (1995).

<sup>4</sup> Water used was Fisher's deionized ultra-filtered water (DIUF).

replicates of three. Sperm motility was recorded on videotape using a Hitachi Model KP-140 video camera attached to a Zeiss Model D-7082 compound microscope at a magnification of 400x. The percentage of motile sperm and the amount of time the sperm remained motile were determined later from the videotapes by the same procedures described under Physical Characterization.

#### Statistical analysis

Analysis of variance was used to identify significant differences for each of the physical and chemical characteristics of striped bass semen measured from the three groups of striped bass (the GnRHa implanted fish, the sham implanted fish, and the fish drawn from the general population). Since significant differences were



found to occur, each group was statistically analyzed separately. Individual semen characteristics were subjected to analysis of variance in order to determine if any changes occurred during the spawning season. Analysis of variance was also used to identify significant differences in the percentage of motile sperm and the duration of sperm motility obtained from the different extenders. The mixed model procedures from SAS version 6.12 statistical software were used (SAS 1996). Pairwise contrasts between weeks were made to detect significant differences at the 5% level. Significant correlations between variables were determined by Spearman's rank correlation procedure. Results unless otherwise noted, are reported as means  $\pm$  S.E.M.

## RESULTS AND DISCUSSION

### Physical Characterization

In all three groups of fish, the color of the semen remained white throughout the entire spawning season except for a few blood contaminated samples which had a pink hue. The viscosity of the semen was high during the first two and last two weeks of the spawning season and low during the weeks in between.

The average percentage of motile sperm obtained from the individual fish given GnRHa or sham implants did not change significantly during the spawning season with a mean value of  $94 \pm 0.4\%$ . Figures 3.1 and 3.2 track the percentage of motile sperm obtained weekly from each fish. The unusually low percentages obtained from fish #5 in week 7 and fish #9 in weeks 4 and 9 were removed from the statistical analysis since these samples were contaminated with urine and/or

Figure 3.1: The percentage of motile sperm from individual GnRHa implanted striped bass during the spawning season

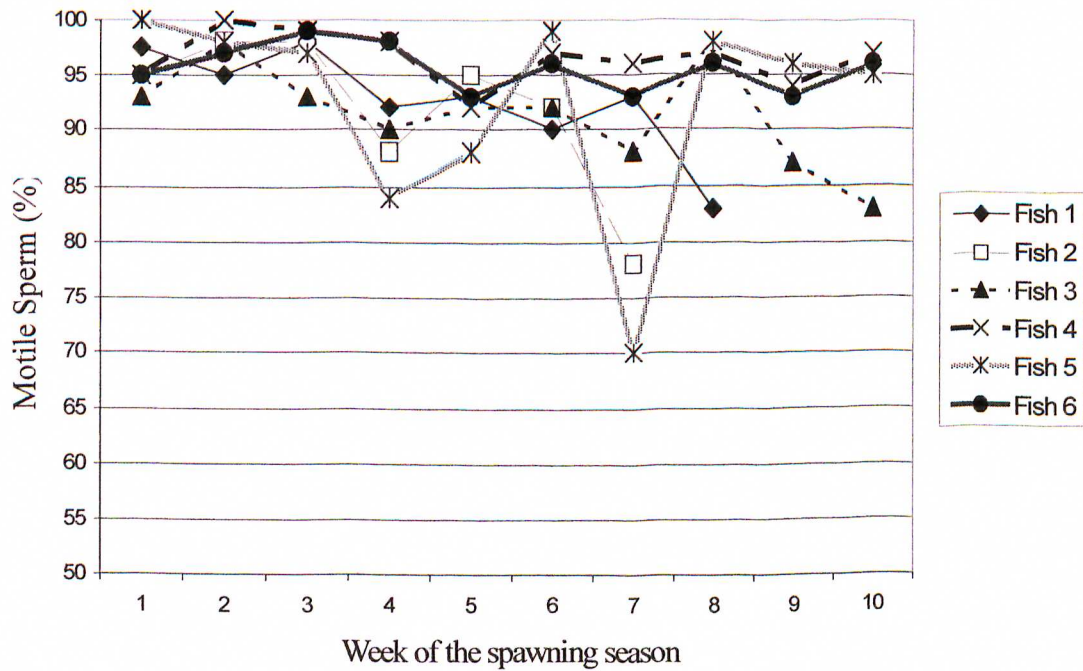
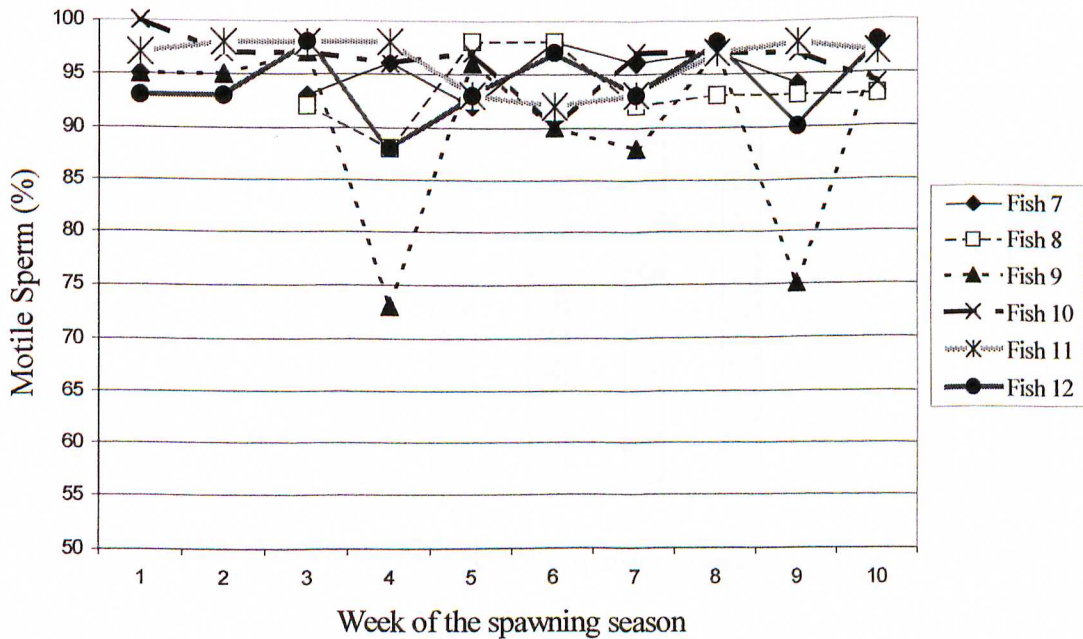


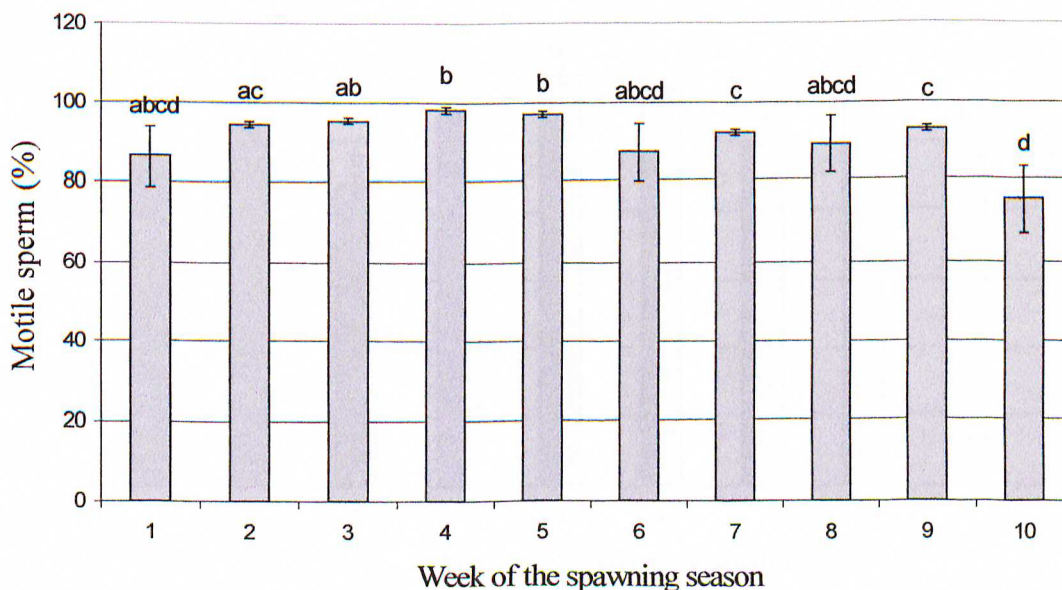
Figure 3.2: The percentage of motile sperm from individual sham implanted striped bass during the spawning season.



feces. Although the majority of the fish showed no change in the percentage of motile sperm, a few of the individuals showed a decline in motile sperm in the week before they ceased spermiation.

The percentage of motile sperm obtained from fish randomly drawn from the population showed a significant change during the spawning season. As in the second year's experiment, no clear pattern was observed (Figure 3.3). The highest values were obtained in weeks 3-5 and the lowest values were obtained in weeks 1, 6, 8, and 10. This random pattern may be attributed to individual fish beginning and ending spermiation at different times.

Figure 3.3: The change in motile sperm obtained from neat semen samples in striped bass randomly drawn from the general population during the spawning season. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other at  $P < 0.05$ .

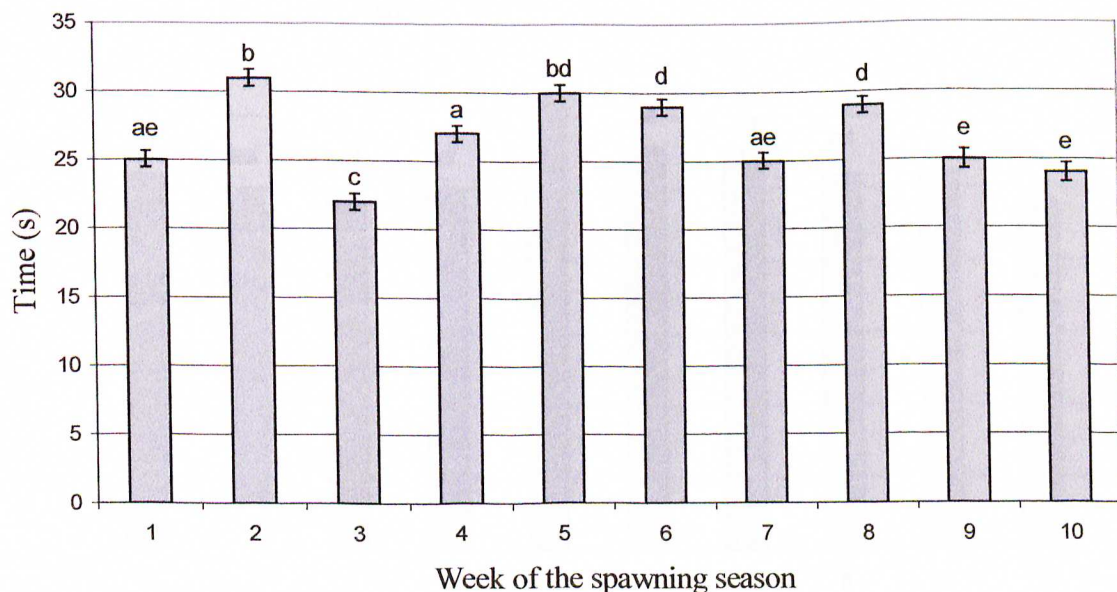




By week 11, most of the fish were dry and samples of less than 1mL could only be obtained from 3 fish. The percentage of motile sperm obtained from these samples was very low ranging from less than 1% to 10%. In a commercial facility, fish yielding such small volumes of semen would be considered dry and therefore such low percentages of motile sperm would not normally be encountered. Week 11 was therefore dropped from the statistical analysis.

The fish implanted with GnRH $\alpha$  showed a significantly longer duration of sperm motility in weeks 2, 6, and 8 as well as week 5 (Figure 3.4). With the exception of weeks 6 and 10, the weeks following hormone implantation always showed a significantly longer duration of sperm motility than the preceding week in which the hormone was given. This increase in the duration of sperm motility may

Figure 3.4: The change in duration of sperm motility obtained from neat semen samples in individual GnRH $\alpha$  implanted striped bass during the spawning season. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other at  $P < 0.05$ .



be due to the higher levels of circulating hormone 7 days after implantation as opposed to 14 days after implantation. Several studies have evaluated the effect of GnRHa on semen quality (Mylonas et al. 1997, Clearwater and Crim 1995, Sorbera et al. 1995), but rarely is the duration of sperm motility used as a factor in evaluating semen quality. One study found the duration of sperm motility in European catfish given GnRHa implants to gradually decrease on a daily basis with a significant decrease by day 7 post-implantation (Linhart and Billard 1994).

The duration of sperm motility in the fish given sham implants and the fish randomly drawn from the population increased significantly in the middle of the spawning season (week 5), then gradually decreased as the season progressed (Figures 3.5 and 3.6). Similar results have been observed in sea bass (Sorbera et al.

Figure 3.5: The change in duration of sperm motility obtained from neat semen samples in individual sham implanted striped bass during the spawning season. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other at  $P < 0.05$ .

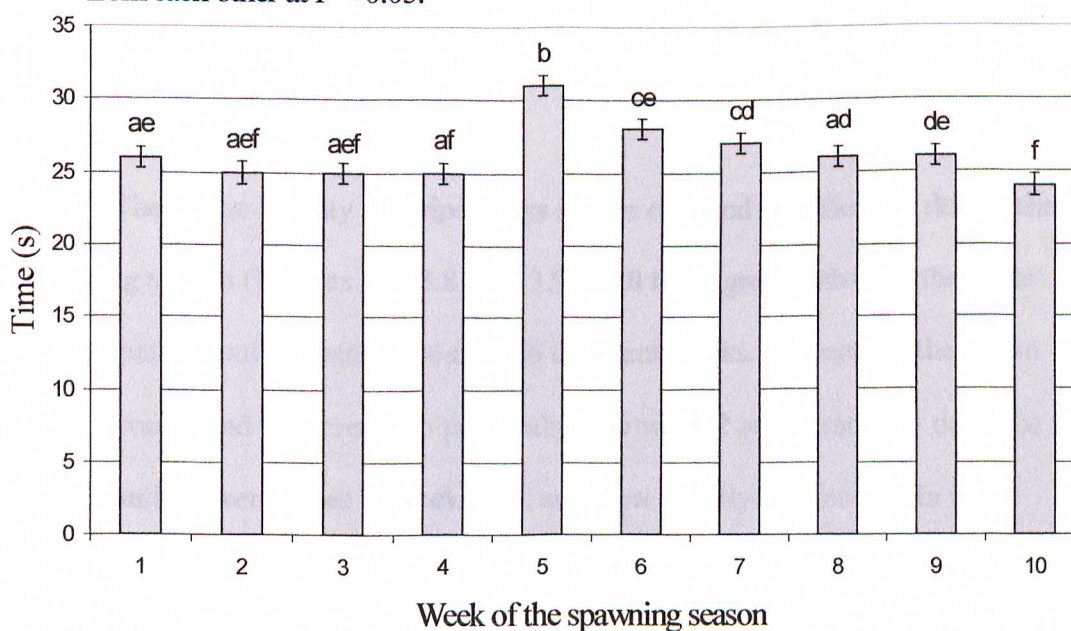
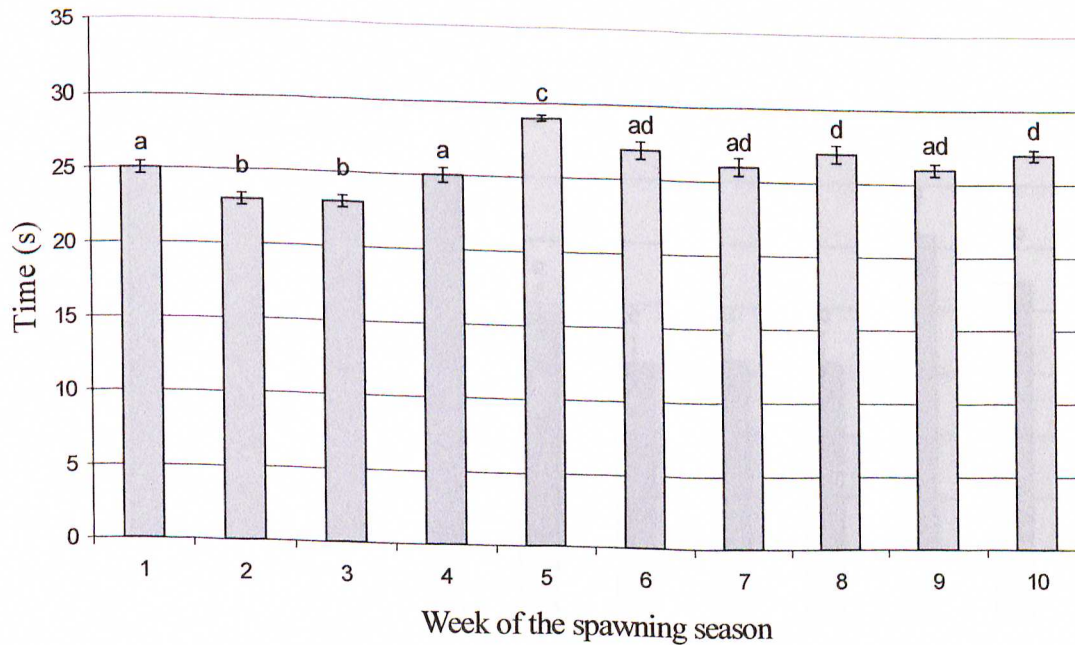




Figure 3.6: The change in the duration of sperm motility obtained from neat semen samples in striped bass randomly drawn from the general population during the spawning season.



1996) and pike (Suquet et al. 1998). The duration of sperm motility in the rainbow trout, brook trout, and brown trout was also found to be highest at the peak of the spawning season and then gradually decline as the season progressed (Benau and Turner 1980).

The sperm density of striped bass semen changed significantly during the spawning season (Figures 3.7, 3.8, and 3.9). All three groups showed the same general pattern but the peaks occurred in different weeks. In general, the sperm density was found to increase to peak values by week 2 or 4, gradually decrease to significantly lower values in weeks 6-8, and then slightly increase again in the weeks 9-10 of the spawning season. These results differ from those of Experiment 2 in which the sperm density showed no significant change during the spawning



Figure 3.7: The change in sperm density in individual GnRHa implanted striped bass during the spawning season. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other at  $P < 0.05$ .

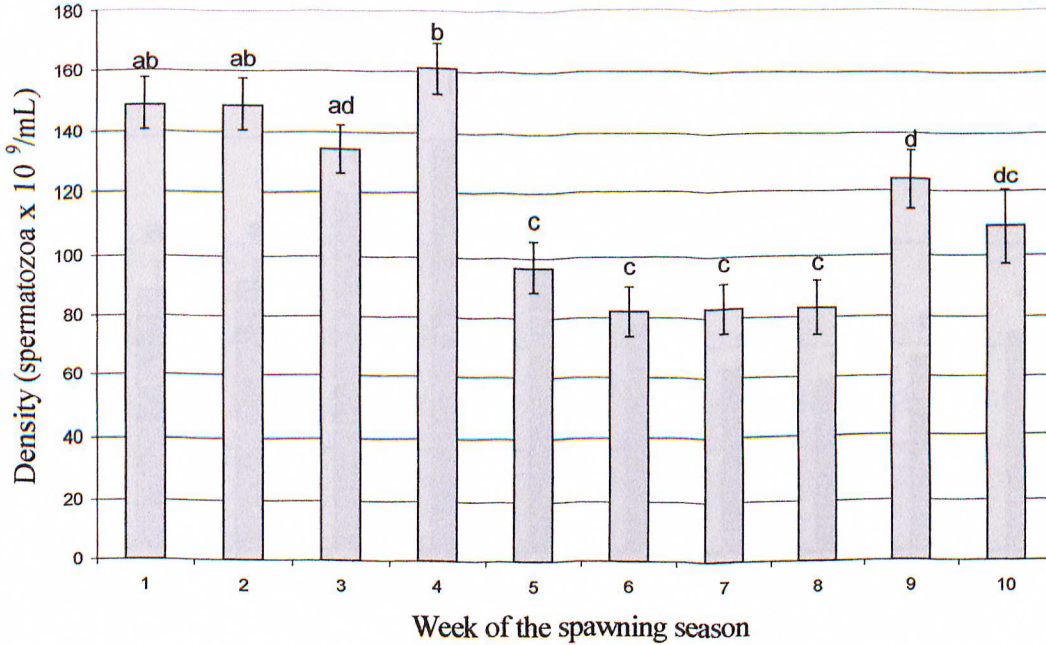


Figure 3.8: The change in sperm density in individual sham implanted striped bass during the spawning season. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other at  $P < 0.05$ .

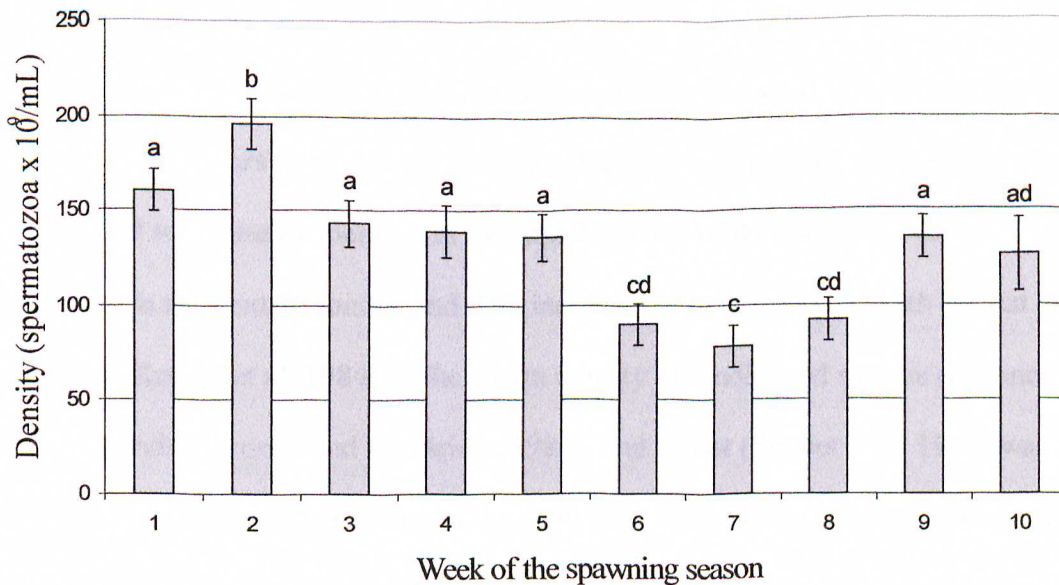
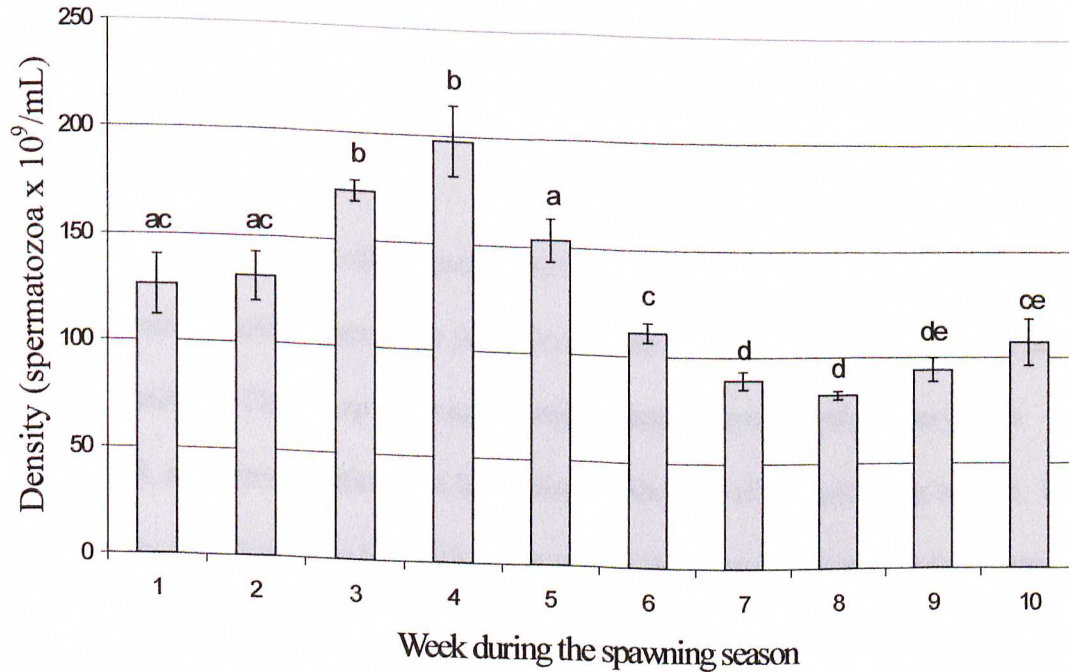


Figure 3.9: The change in sperm density in striped bass randomly drawn from the general population during the spawning season. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other at  $P < 0.05$ .



season. However, the sampling period in the second year did not encompass the entire spawning season and six weeks may have been too short a time to see a significant change.

Seasonal variation in sperm density has been observed in several teleost species and appears to be species specific. The sperm density of the common carp was found to gradually increase in the first 2 months of the spawning season, decrease in the next 2 months, and then increase again in the last month (Christ et al. 1996, Kruger et al. 1984). The sperm density of landlocked salmon (Piironen 1985), perch (Piironen and Hyvärinen 1983), and turbot (Suquet et al. 1998) was found to be low at the beginning of the spawning season, then steadily increase to a high at the end of the season. Atlantic salmon's sperm density was found to be

lowest at the beginning and end of the spawning season and highest mid-season (Kazakov 1981). Büyükhatipoglu and Holtz (1984) found the sperm density of rainbow trout to decrease at the end of the spawning season, whereas Piironen and Hyvärinen (1983) found the sperm density of rainbow trout to be low in the beginning of the spawning season, then increase and stabilize until the end of the season.

Sperm density is often used as an indicator of semen quality. A higher sperm density could increase the possible number of eggs a given male might be able to fertilize. The sharp decrease in striped bass sperm density observed in weeks 6-8, may have marked the beginning of the end of the spawning season. The high sperm densities seen in perch at the end of their spawning season have been attributed to the re-absorption of seminal plasma (Piironen and Hyvärinen 1983). Although the density of striped bass sperm increased again during the last 2 weeks of the spawning season, it did not reach the levels achieved at the beginning of the season. The viscosity of the striped bass semen also increased in the last two weeks, therefore it is uncertain whether the elevated sperm density is due to increased sperm production or re-absorption of seminal fluids.

#### Chemical Characterization

As in the previous experiment (Experiment 2), the osmolality of the semen changed significantly during the spawning season. No clear trend or pattern was determined from the results obtained in Experiment 2, but trends were observed during this experiment. The semen osmolality in fish randomly drawn from the



general population was high in the first 2 to 3 weeks, gradually declined mid-season, and then increased again at the end of the season (Figure 3.10). Figures 3.11 and 3.12 track the seasonal change of the semen osmolality in individual fish given GnRHa and sham implants, respectively. In the middle of the spawning season, the semen osmolality in an individual fish tended to rise and fall on a weekly basis but remained within 320–350mmol/kg. Due to the viscosity of neat semen, most researchers measure the osmolality of the seminal plasma instead. Therefore, no studies measuring neat semen osmolality could be found in the literature for comparison with the data obtained in this experiment.

Figure 3.10: The change in the semen osmolality in striped bass randomly drawn from the general population during the spawning season. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other at  $P < 0.05$ .

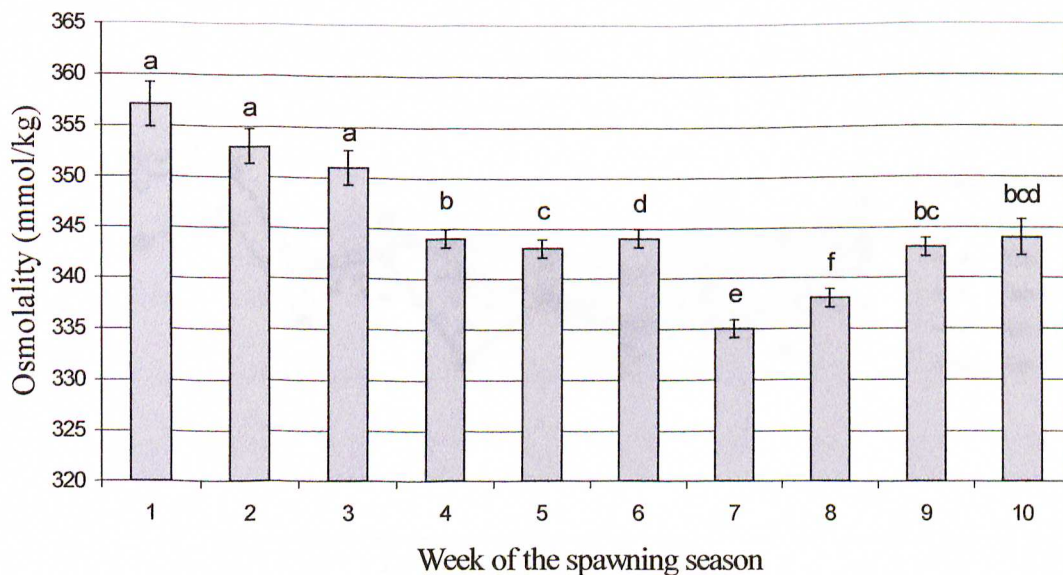


Figure 3.11: The change in semen osmolality during the spawning season of individual striped bass given GnRHa implants.

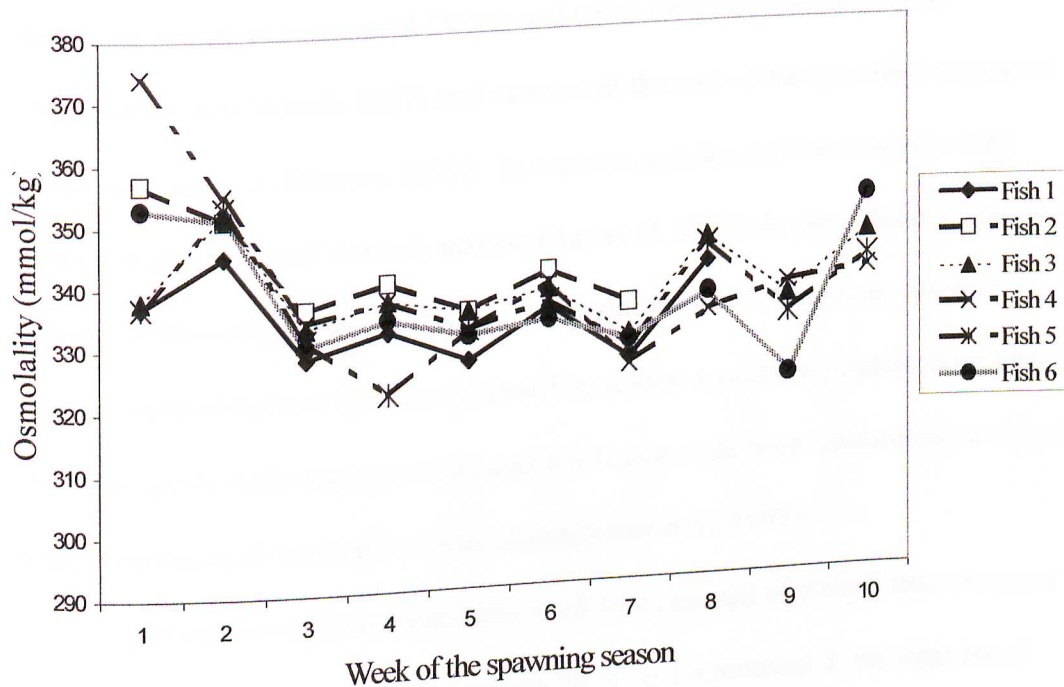
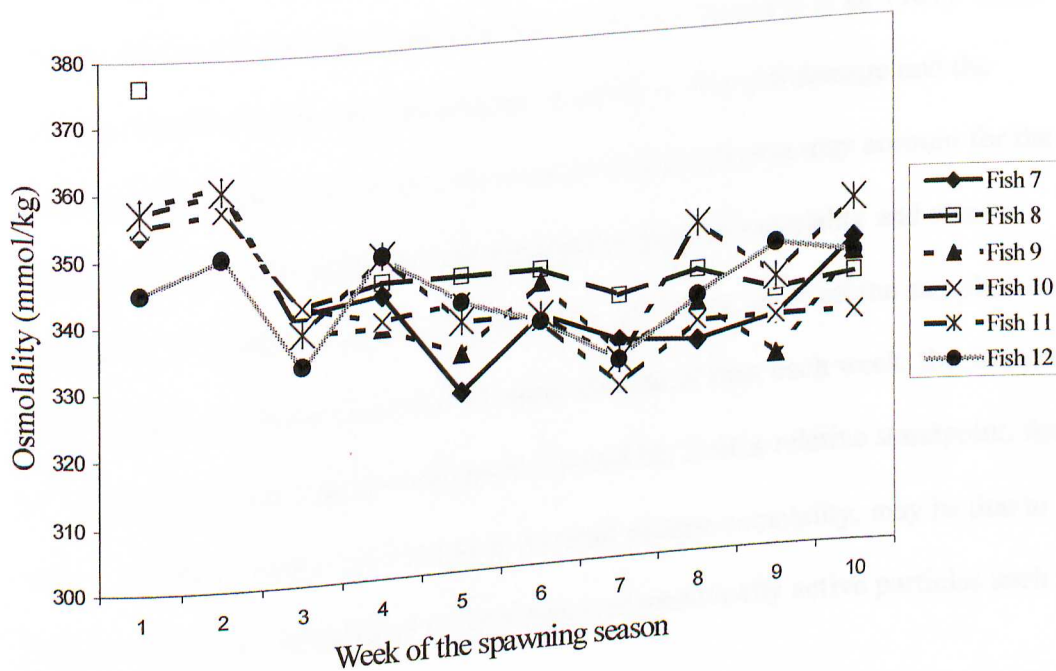


Figure 3.12: The change in semen osmolality during the spawning season of individual striped bass given sham implants.



Seasonal changes in seminal plasma osmolality appear to be species specific. The seminal plasma osmolality was found to decrease at the end of the spawning season in ocean pout (Wang and Crim 1997) and rainbow trout (Munkittrick and Moccia 1987) and increase at the end of the spawning season in landlocked salmon (Piironen 1985). In contrast, species such as common carp (Christ et al. 1996) and Atlantic salmon (Aas et al. 1991) do not exhibit a seasonal change in seminal plasma osmolality. Although seasonal changes in seminal plasma osmolality have been documented in several species, a positive correlation between seminal plasma osmolality and fertilization has been demonstrated only in a few species, such as rainbow trout (Lahnsteiner et al. 1997).

The seminal plasma osmolality in all three groups of striped bass changed significantly during the spawning season but as in Experiment 2, no clear trend could be observed (Figures 3.13, 3.14, and 3.15). The concentration of various chemicals in brown trout and rainbow trout seminal plasma have been found to change due to cell damage caused by centrifugation (Schmehl et al. 1987). Such changes would alter the seminal plasma osmolality. The cell damage and the consequent spillage of the inner cell contents into the plasma may account for the large differences occurring between the seminal plasma osmolality and semen osmolality in weeks 4, 5, and 9 (Figure 3.16). However, since all the samples were centrifuged at the same speed for the same amount of time each week, the amount of cell damage occurring in each sample should be, from a relative standpoint, the same. Therefore, these fluctuations in seminal plasma osmolality, may be due to fluctuations in the intracellular concentration of osmotically active particles such as



Figure 3.13: The change in seminal plasma osmolality in individual GnRH $\alpha$  implanted striped bass during the spawning season. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other at  $P < 0.05$ .

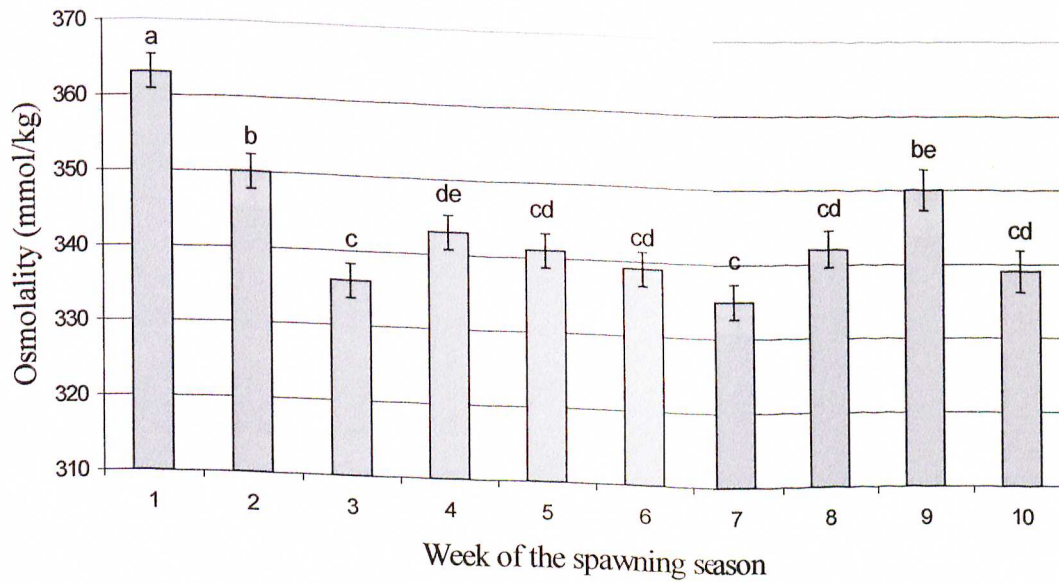


Figure 3.14: The change in seminal plasma osmolality in individual sham implanted striped bass during the spawning season. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other at  $P < 0.05$ .

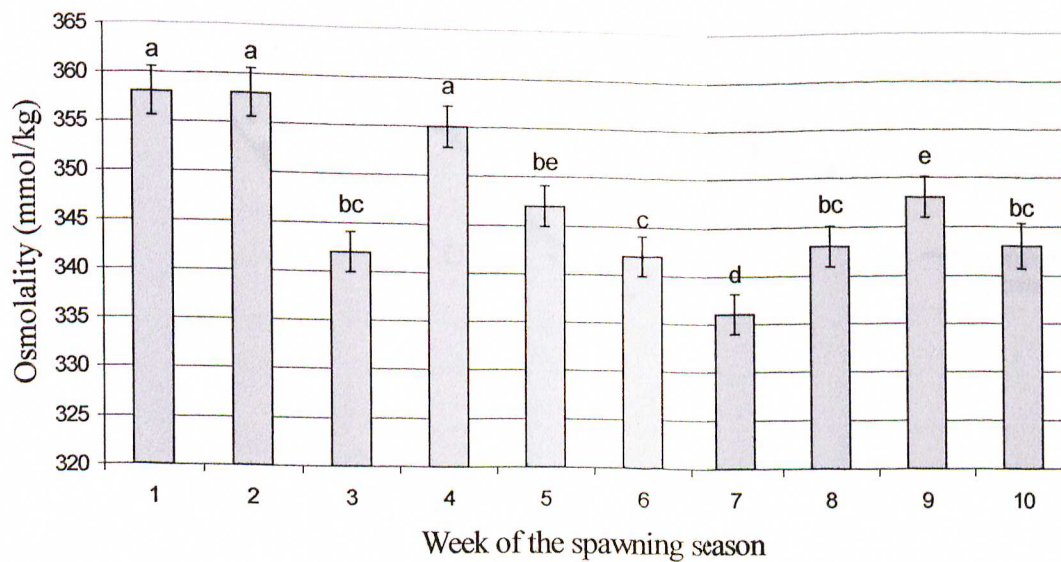


Figure 3.15: The change in the seminal plasma osmolality in striped bass randomly drawn from the general population during the spawning season. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other at  $P < 0.05$ .

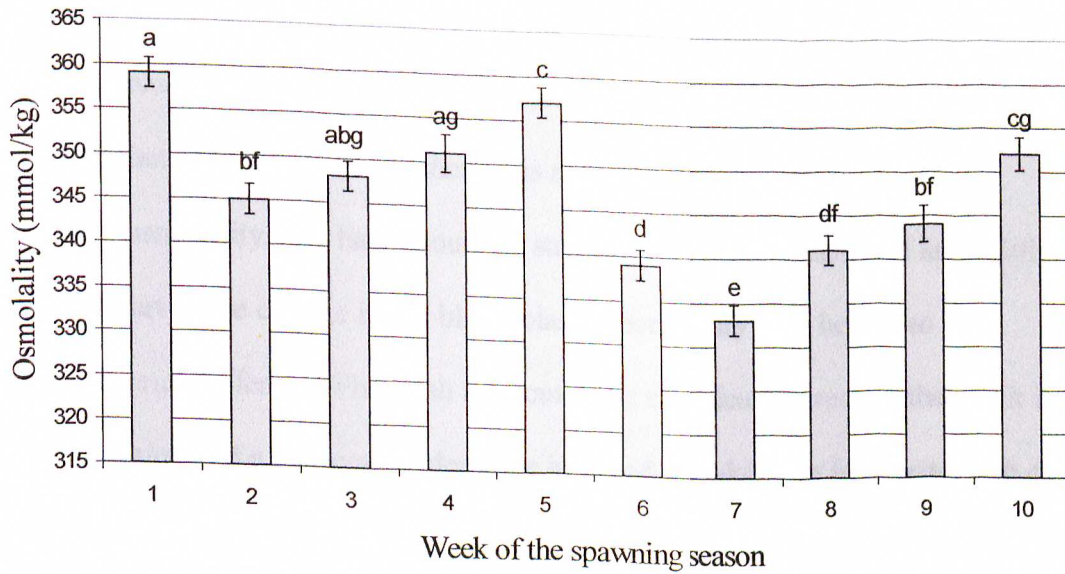
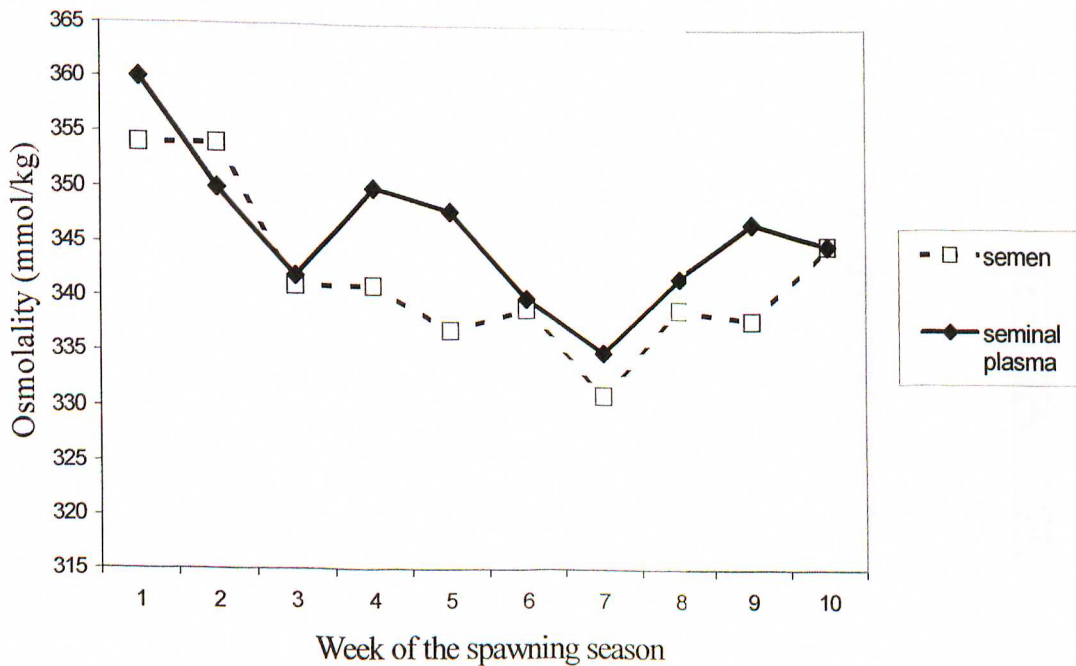


Figure 3.16: The change in striped bass semen and seminal plasma osmolality during the spawning season.



sodium, potassium, and calcium which are released into the plasma when the cell membrane is compromised.

The osmolality of the blood plasma in all three groups of striped bass changed significantly during the spawning season (Figures 3.17, 3.18, and 3.19). Striped bass have been shown to be extremely sensitive to stress. One experiment showed that after 90s of stress, there was a significant increase in the lactate, cortisol, osmolarity, and haematocrit of striped bass blood (McDonald and Milligan 1997). Part of the change in the blood plasma osmolality may be due to environmental effects. When fish are acutely or chronically stressed, the result is hemodilution and a consequent decrease in blood osmolality in freshwater fish or hemoconcentration and a consequent increase in blood osmolality in saltwater fish

Figure 3.17: The change in blood plasma osmolality in individual GnRH $\alpha$  implanted striped bass during the spawning season. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other at  $P < 0.05$ .

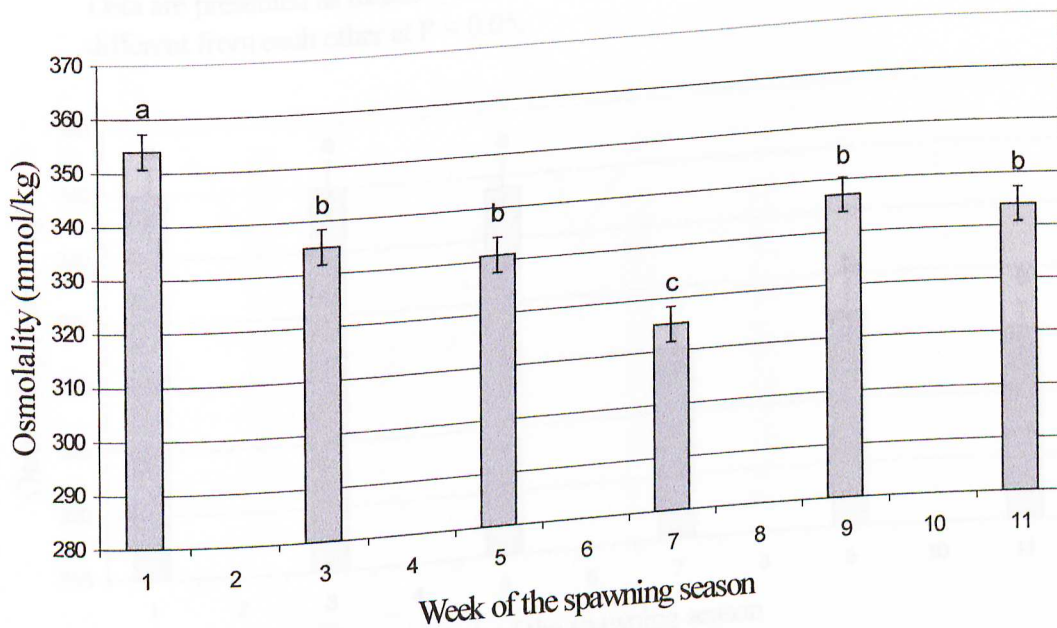




Figure 3.18: The change in blood plasma osmolality in individual sham implanted striped bass during the spawning season. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other at  $P < 0.05$ .

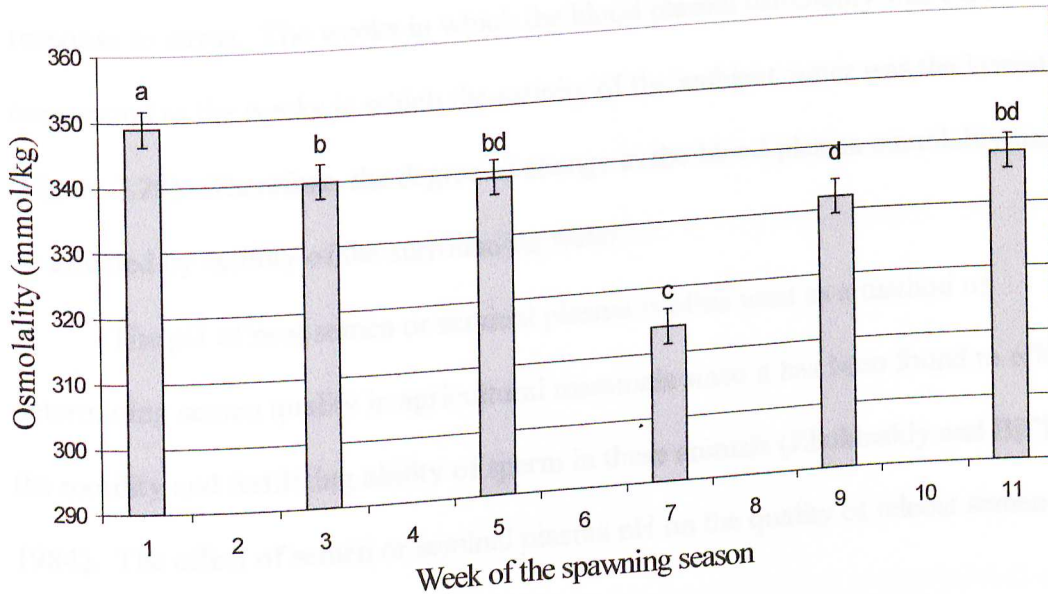
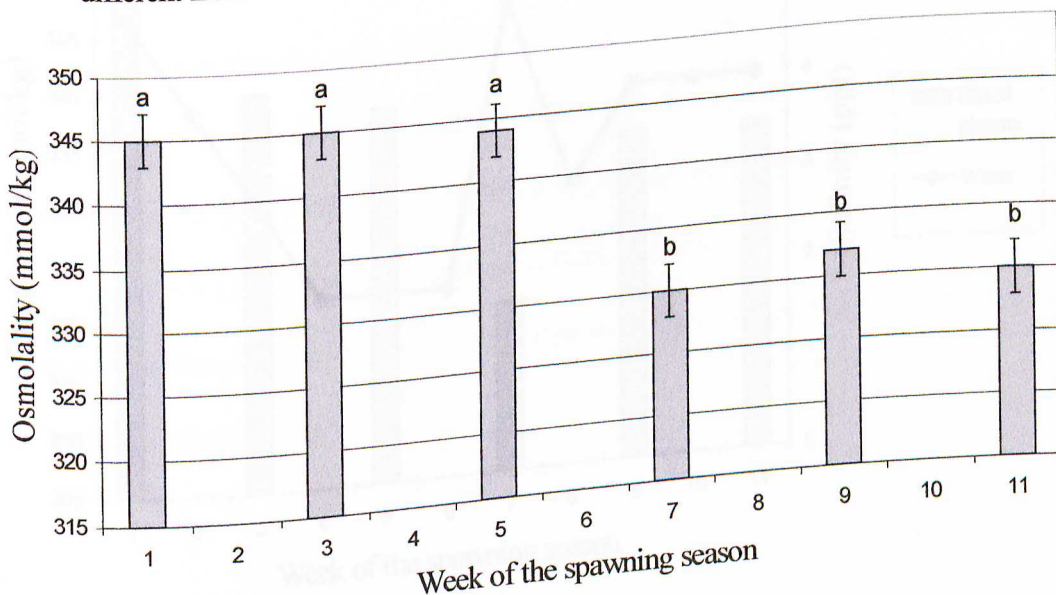


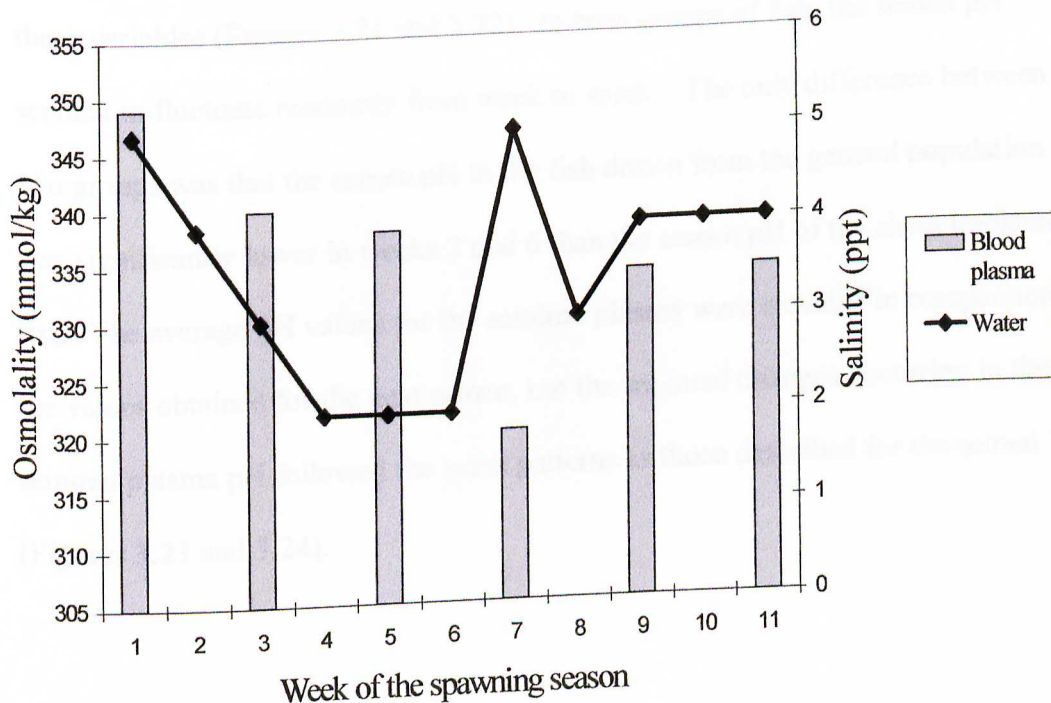
Figure 3.19: The change in the blood plasma osmolality in striped bass randomly drawn from the general population during the spawning season. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other at  $P < 0.05$ .



(Wedemeyer et al. 1990). The salinity of the ambient water fluctuated between 1 and 4ppt during the spawning season. Therefore, the change observed in the striped bass blood plasma osmolality is most likely due to hemodilution occurring in response to stress. The weeks in which the blood plasma osmolality was the lowest correspond to the weeks in which the salinity of the ambient water was the lowest (Figure 3.20). Therefore, the degree of change in the blood plasma osmolality may be affected by salinity of the surrounding water.

The pH of neat semen or seminal plasma is often used as a method of determining semen quality in agricultural mammals since it has been found to effect the motility and fertilizing ability of sperm in these animals (Zhukinskiy and Bil'ko 1984). The effect of semen or seminal plasma pH on the quality of teleost semen

Figure 3.20: The change in the salinity of the ambient water and the osmolality of striped bass blood plasma during the spawning season.



has been studied in only a few species. Sperm motility in the rainbow trout was shown to be inhibited when the pH of the surrounding media fell below 7.5 (Baynes et al. 1981). However, the pH of rainbow trout seminal plasma was found to be greater than 7.5, thus indicating that the seminal plasma contains something else to inhibit sperm motility (Billard and Cosson 1992). Nevertheless, the pH of rainbow trout seminal plasma has been found to have a positive correlation with fertilization rate (Lahnsteiner et al. 1998), indicating that semen or seminal plasma pH can be used as a valid indicator of semen quality in some teleosts.

No significant difference was found to occur in the semen and seminal plasma pH of the GnRH $\alpha$  implanted fish across the spawning season. The average values were  $7.9 \pm .01$  and  $8.1 \pm 0.02$ , respectively. GnRH $\alpha$  may have had some role in maintaining the semen and seminal plasma pH since the sham implanted fish and the fish drawn from the general population showed a significant change in both of these variables (Figures 3.21 and 3.22). In both groups of fish, the semen pH seemed to fluctuate randomly from week to week. The only difference between the two groups was that the semen pH in the fish drawn from the general population was significantly lower in weeks 2 and 6 than the semen pH of the sham implanted fish. The average pH values for the seminal plasma were elevated in comparison to the values obtained for the neat semen, but the seasonal changes occurring in the seminal plasma pH followed the same patterns as those described for the semen (Figures 3.23 and 3.24).



Figure 3.21: The change in semen pH in individual sham implanted striped bass during the spawning season. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other at  $P < 0.05$ .

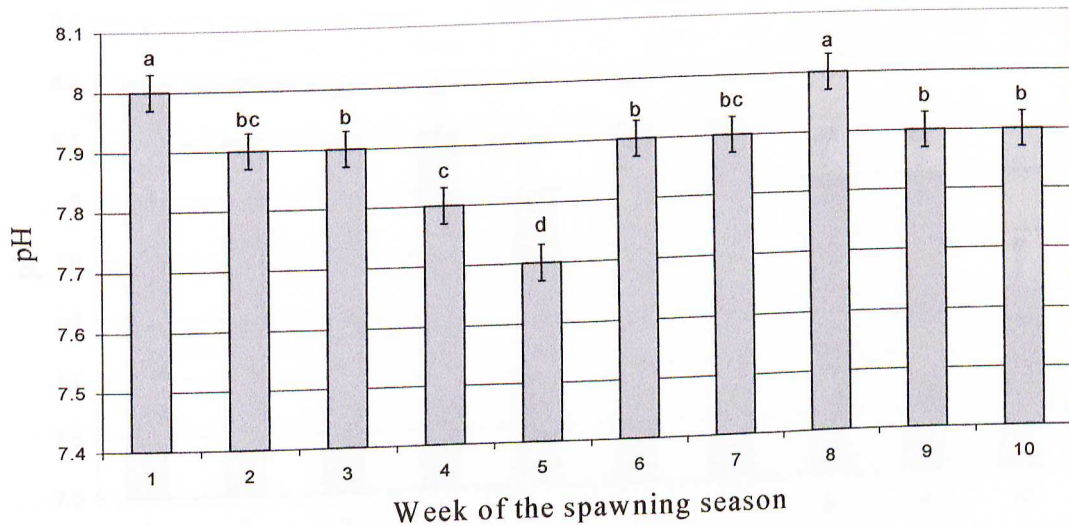


Figure 3.22: The change in semen pH in striped bass randomly drawn from the general population during the spawning season. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other at  $P < 0.05$ .

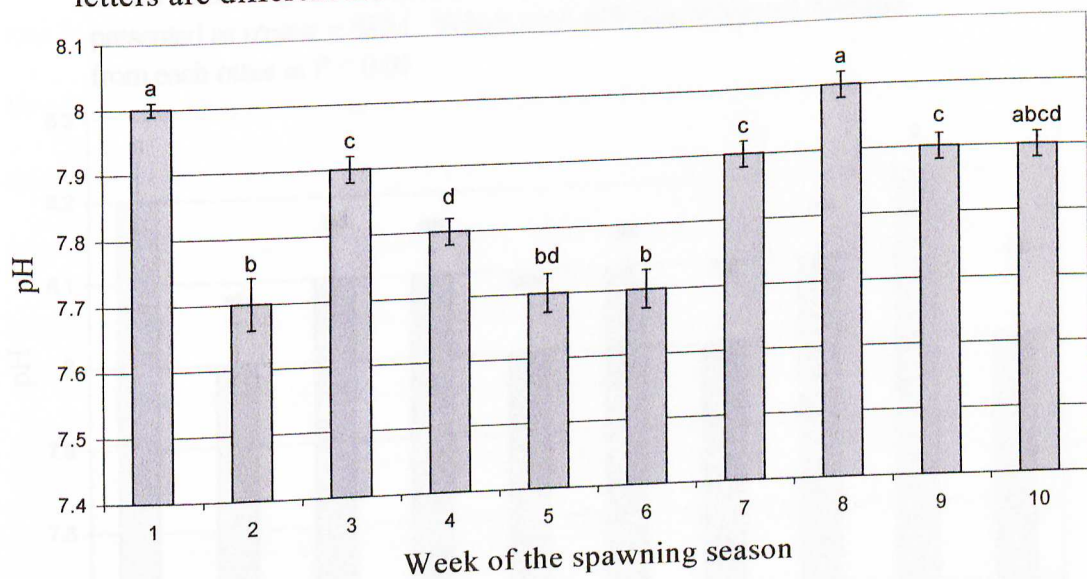


Figure 3.23: The change in seminal plasma pH in individual sham implanted striped bass during the spawning season. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other at  $P < 0.05$ .

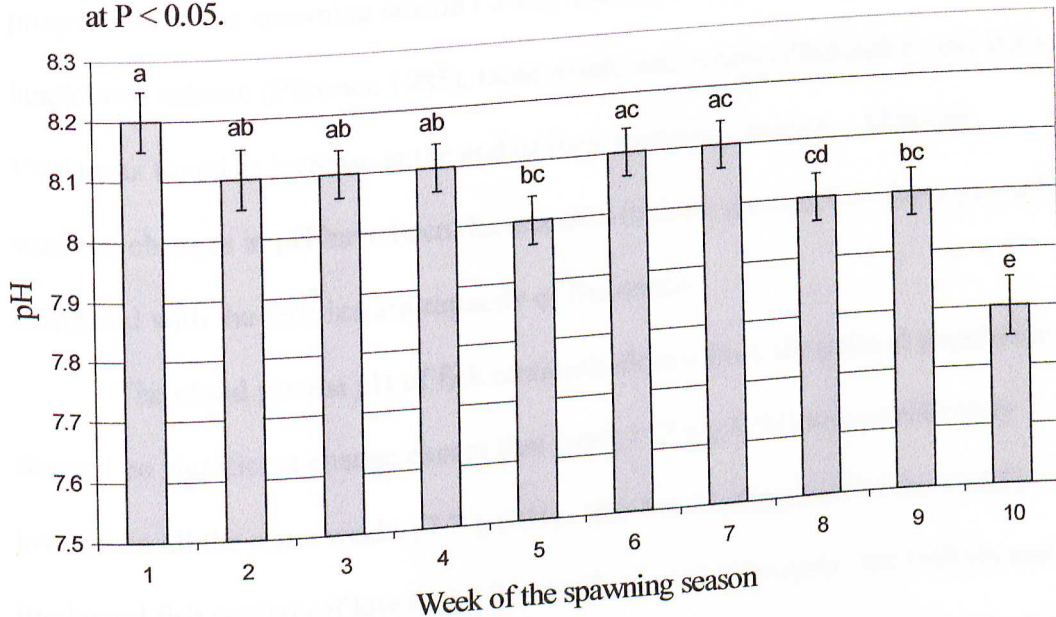
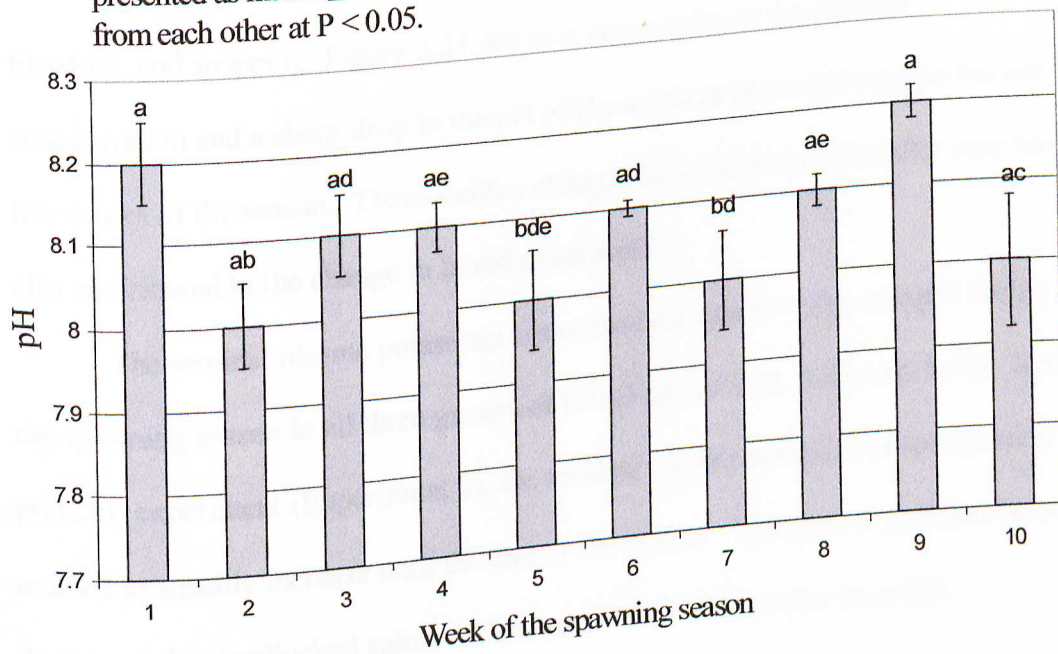


Figure 3.24: The change in seminal plasma pH in striped bass randomly drawn from the general population during the spawning season. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other at  $P < 0.05$ .





Seasonal changes in seminal pH have been documented in only a few species. The pH of the seminal plasma in ocean pout was found to increase with the progression of the spawning season (Wang and Crim 1997). The pH of semen from landlocked salmon (Piironen 1985), taran roach, and bream (Zhukinskiy and Bil'ko 1984) was found to increase at the end of their spawning seasons. Although seasonal changes in pH have been documented in these species, they have yet to be correlated with the fertilization capacity of the semen.

The blood plasma pH of fish randomly drawn from the general population showed no significant change except that week 1 ( $7.6 \pm 0.03$ ) was significantly lower than all the other weeks ( $7.7 \pm 0.01$ ). The blood plasma pH of the GnRH $\alpha$  implanted fish started off low in the first week as well. However, the GnRH $\alpha$  and sham implanted fish showed a significant drop in blood plasma pH at the end of the spawning season that was not observed in the fish from the population (Figures 3.25 and 3.26). This drop may have been due to the effects of weekly netting, handling, bleeding, and stripping. Figure 3.27 shows a sharp spike in the calcium concentration and a sharp drop in the pH of the ambient water occurring in the last few weeks of the season. These sudden changes in ambient water quality may have also contributed to the change in blood plasma pH.

The seminal plasma potassium concentration significantly changed during the spawning season in all three groups of fish (Figures 3.28, 3.29, and 3.30). In the previous experiment (Experiment 2), the seminal plasma potassium concentration seemed to steadily increase until the end of the season. This pattern has also been documented in landlocked salmon (Piironen 1985). The results from this



Figure 3.25: The change in blood plasma pH in individual GnRHa implanted striped bass during the spawning season. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other at  $P < 0.05$ .

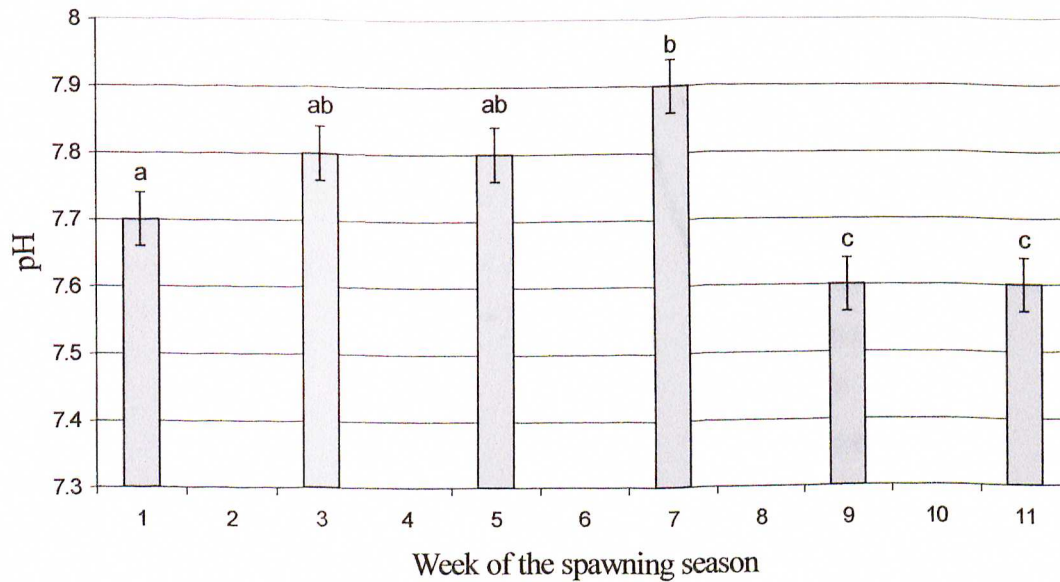


Figure 3.26: The change in blood plasma pH in individual sham implanted striped bass during the spawning season. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other at  $P < 0.05$ .

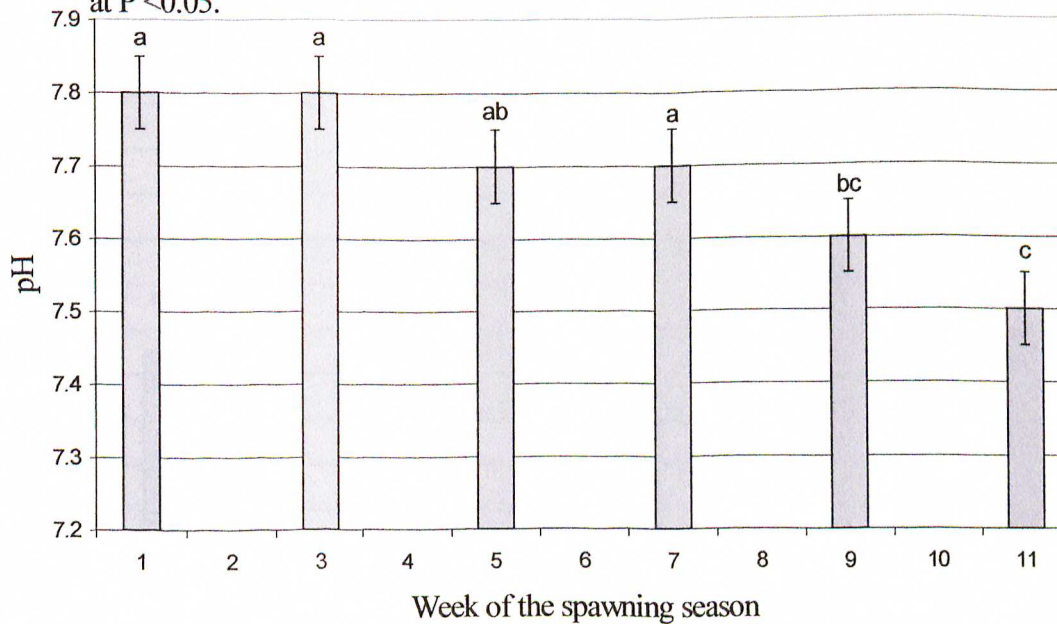


Figure 3.27: The calcium concentration and pH of the ambient water during the spawning season

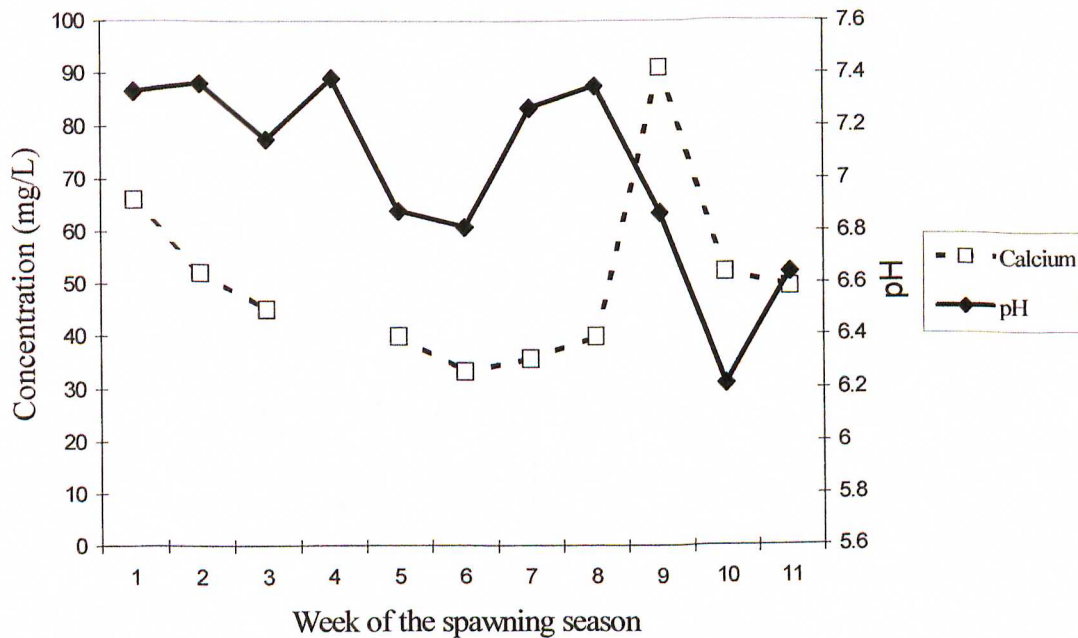


Figure 3.28: The change in the seminal plasma potassium concentration in individual GnRHa implanted striped bass during the spawning season. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other at  $P < 0.05$ .

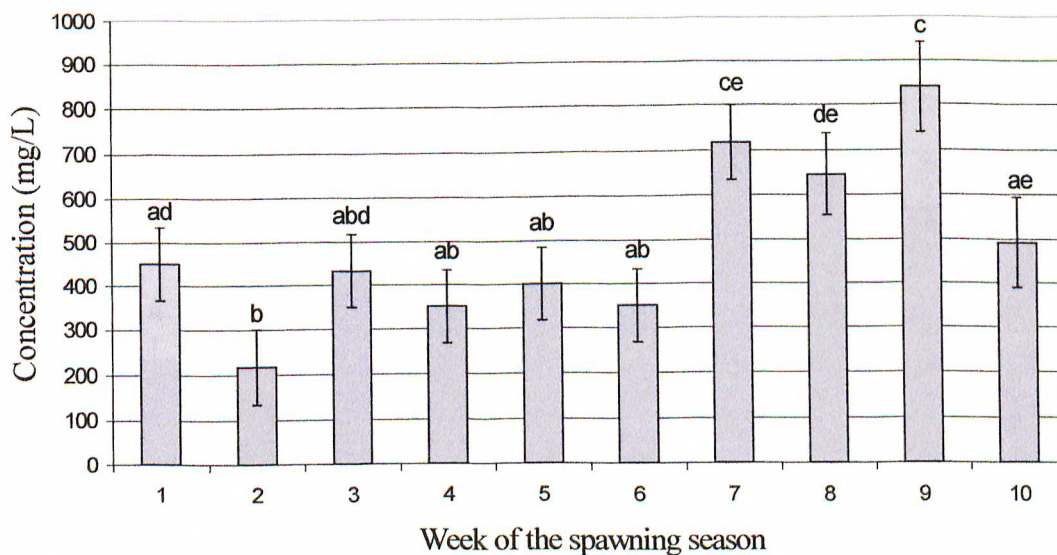


Figure 3.29: The change in the seminal plasma potassium concentration in individual sham implanted striped bass during the spawning season. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other at  $P < 0.05$ .

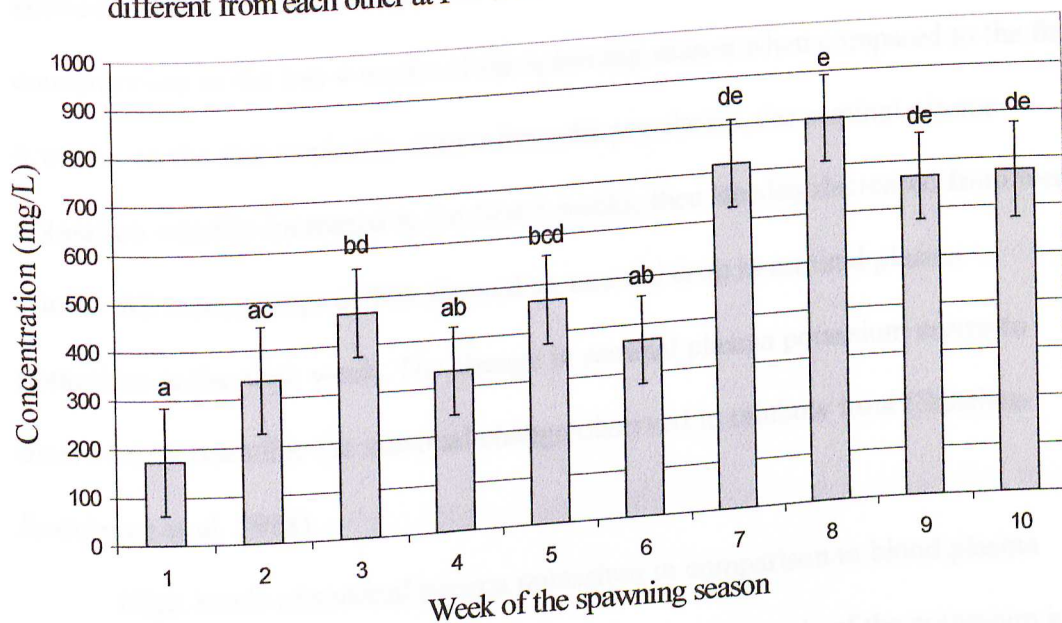
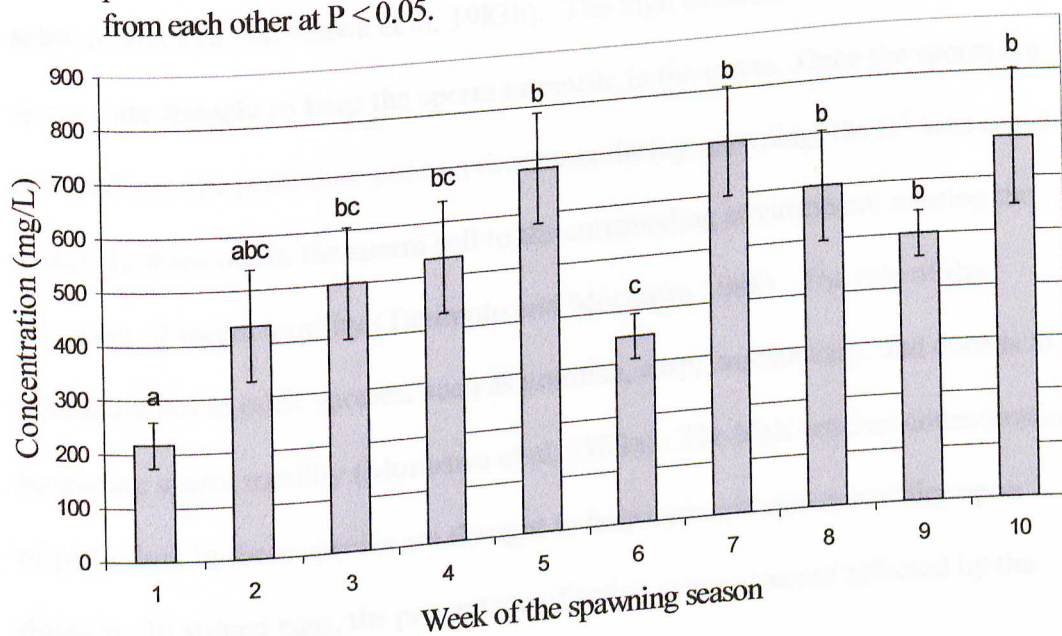


Figure 3.30: The change in the seminal plasma potassium concentration in striped bass randomly drawn from the general population. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other at  $P < 0.05$ .





experiment show no clear pattern in the change of seminal plasma potassium. The GnRHa and sham implanted fish showed random highs and lows on a weekly basis. However, there is a definite increase in the overall seminal plasma potassium concentration in the last 4 weeks of the spawning season when compared to the first 6 weeks. In the fish randomly drawn from the population, the seminal plasma potassium steadily increased in the first 5 weeks, then steadily decreased from week 7 to 9. All three groups of fish showed an unusual drop in seminal plasma potassium in the sixth week. The change in seminal plasma potassium seems to most closely resemble the seasonal change observed in rainbow trout (Sanchez-Rodriguez et al. 1978).

High levels of seminal plasma potassium in comparison to blood plasma potassium have been found in several species of fish. The role of the potassium ion in the seminal plasma appears to be species specific. Potassium has been found to suppress sperm motility in several species of fish such as rainbow trout, masu salmon, and ayu (Morisawa et al. 1983b). The high seminal concentrations in these species are thought to keep the sperm immotile in the testes. Once the sperm are released into the potassium poor environment during spawning, the  $K^+$  ions move passively from inside the sperm cell to the surrounding environment causing the initiation of sperm motility (Tanimoto and Morisawa 1988). The role of the potassium ion in other species, such as goldfish, carp, crucian carp, and dace is to accelerate sperm motility (Morisawa et al. 1983a). The high seminal concentrations of potassium in these species are thought to help maintain sperm motility upon dilution. In striped bass, the percentage of active sperm was not affected by the

Figure 3.31: The change in the seminal plasma sodium concentration in individual GnRH $\alpha$  implanted striped bass during the spawning season. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other at  $P < 0.05$ .

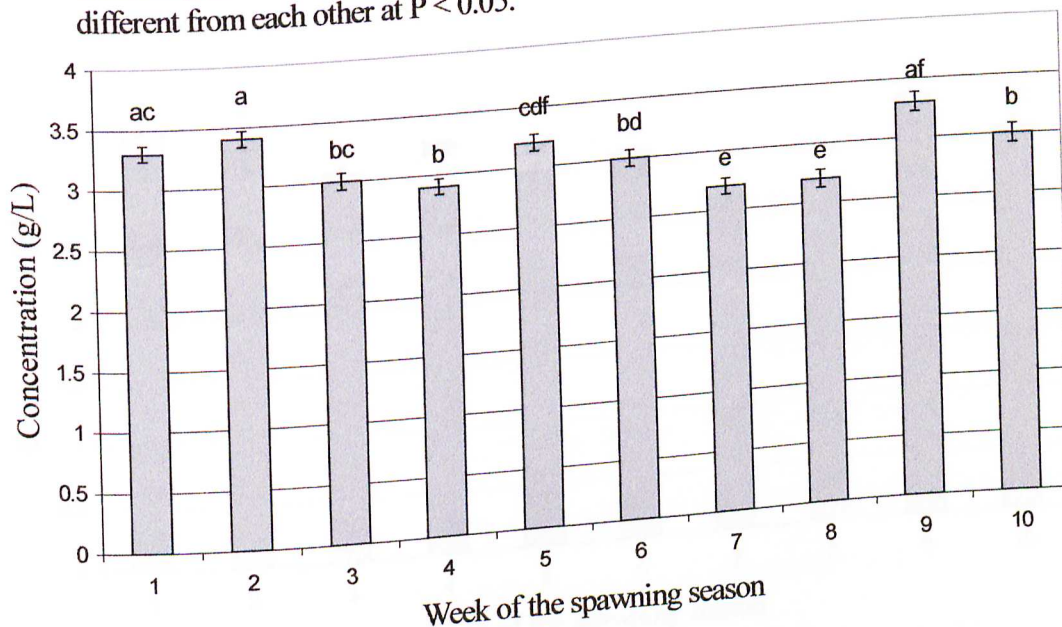


Figure 3.32: The change in the seminal plasma sodium concentration in individual sham implanted striped bass during the spawning season. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other at  $P < 0.05$ .

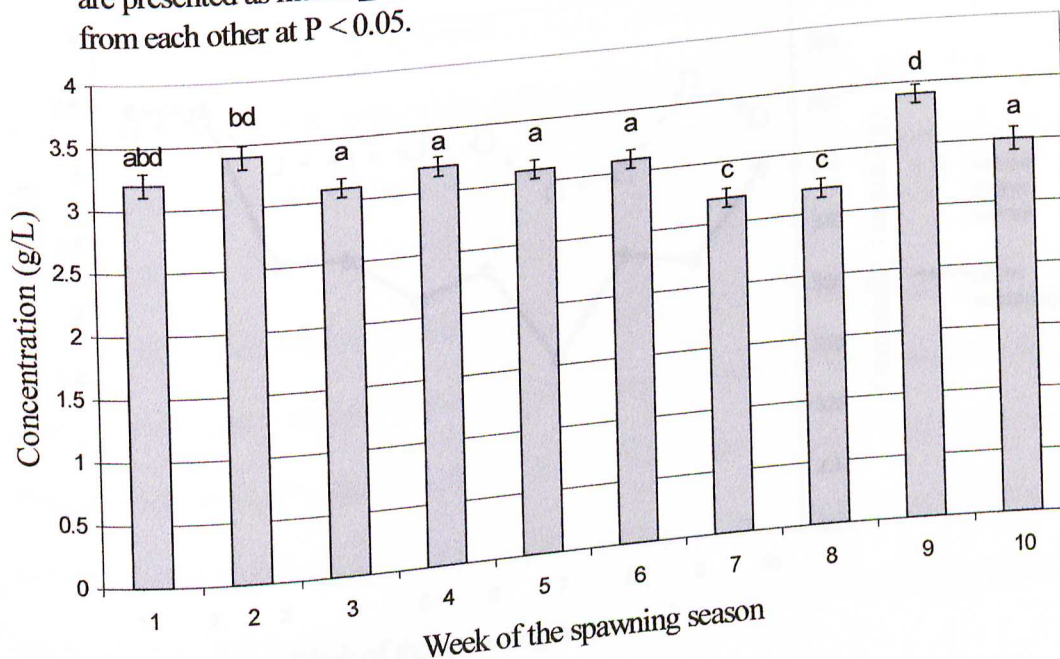




Figure 3.33: The change in the seminal plasma sodium concentration in striped bass randomly drawn from the general population during the spawning season. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other at  $P < 0.05$ .

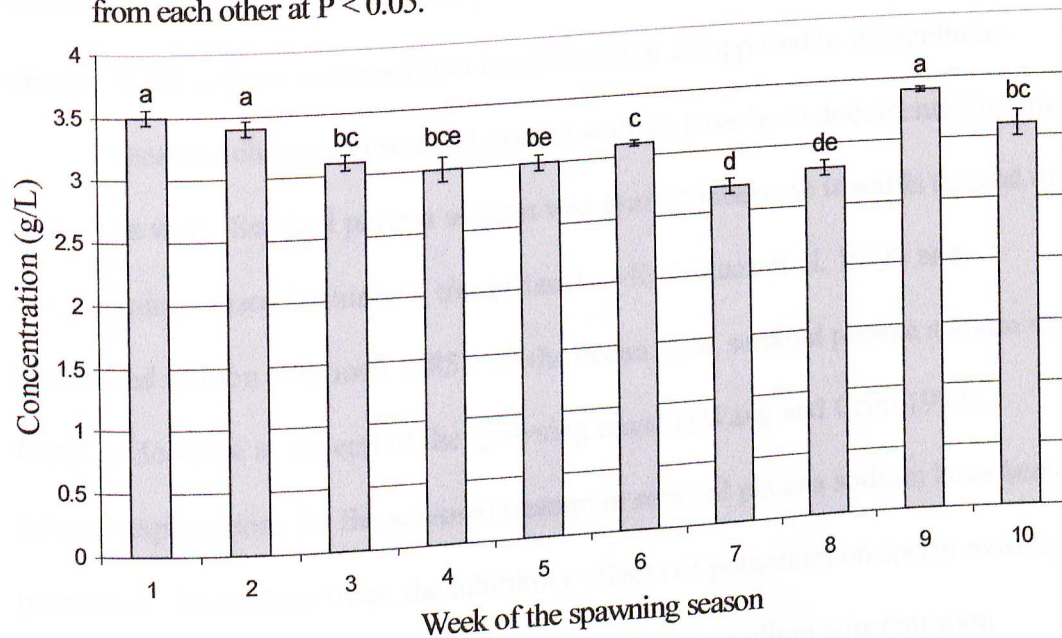
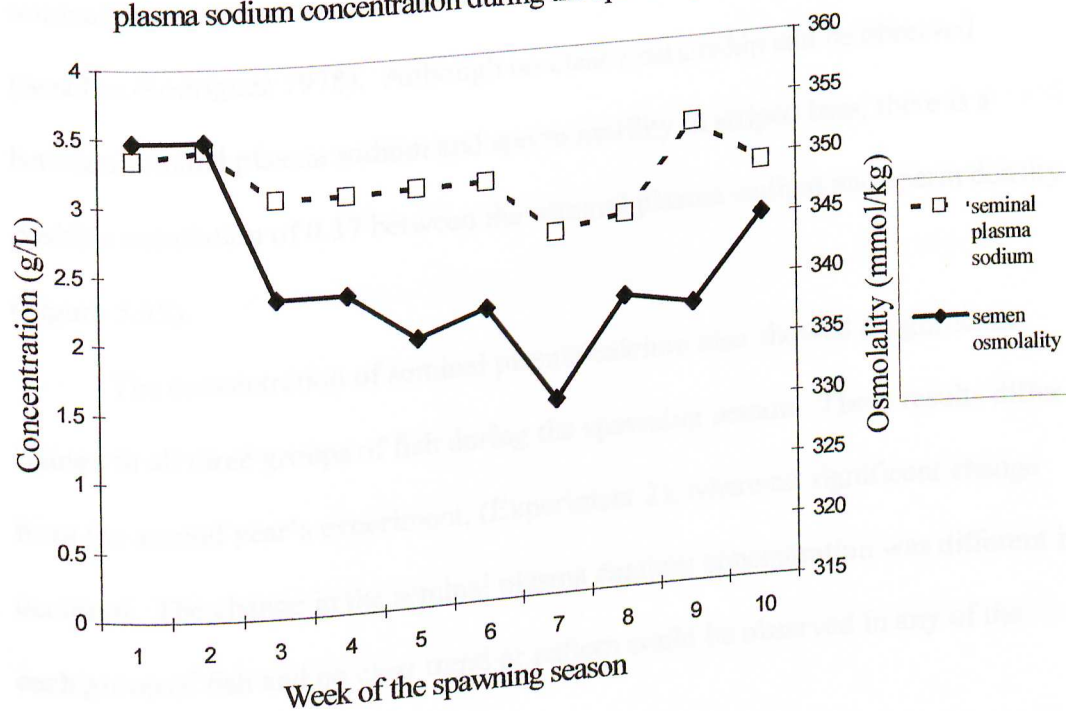


Figure 3.34: The change in striped bass semen osmolality and seminal plasma sodium concentration during the spawning season.



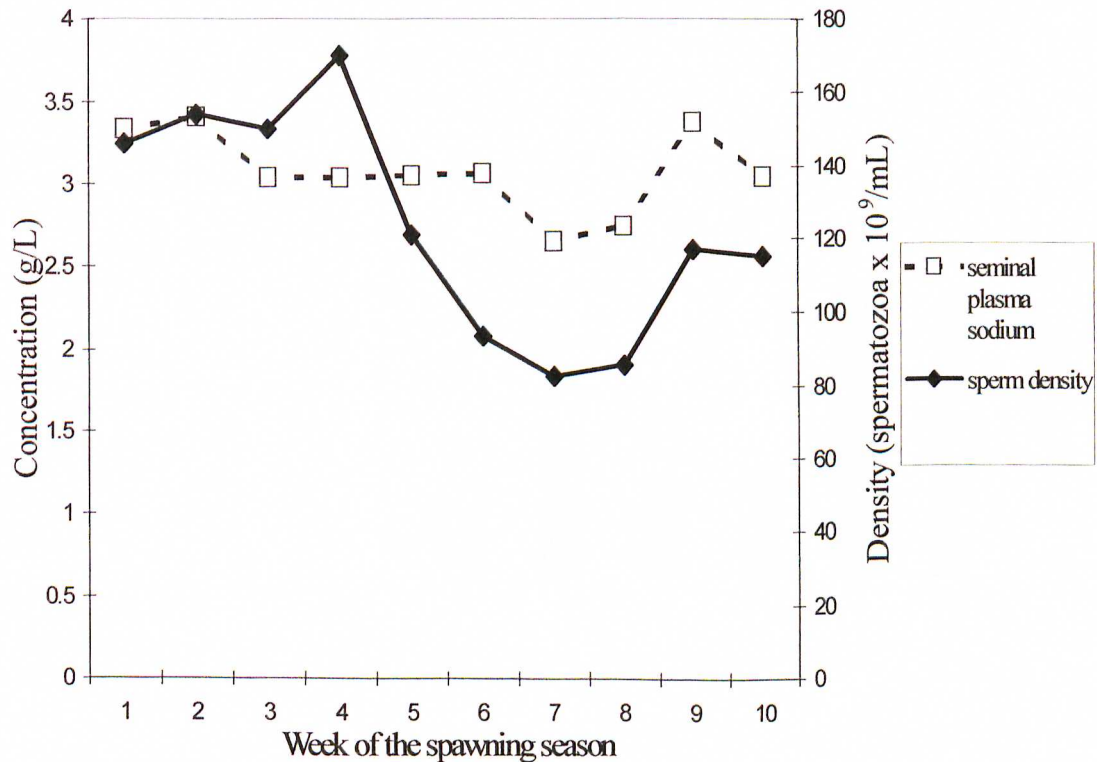


has a higher correlation with the change in semen osmolality (0.52) instead of the seminal plasma osmolality (0.45), which would support the theory of cell damage occurring during the centrifugation process. This would also indicate that the change in the sodium concentration is extracellular as opposed to intracellular.

Seasonal changes in seminal plasma sodium have been documented in other species as well. Seminal plasma sodium was found to increase towards the end of the spawning season in rainbow trout (Sanchez-Rodriguez et al. 1978) and landlocked salmon (Piironen 1985). In the ocean pout, seminal plasma sodium was found to decrease at the end of the spawning season (Wang and Crim 1997). Several explanations for the seasonal change in seminal plasma sodium have been postulated. In rainbow trout, the inhibitory effects of potassium on sperm motility were counteracted to a certain extent by increasing the sodium concentration (Morisawa et al 1983a). Also, a rise in the sodium concentration of rainbow trout seminal plasma was correlated with elevated sperm production and plasma t-GTH (Sanchez-Rodriguez 1978). Although no clear relationship can be observed between seminal plasma sodium and sperm motility in striped bass, there is a positive correlation of 0.37 between the seminal plasma sodium and sperm density (Figure 3.35).

The concentration of seminal plasma calcium also showed a significant change in all three groups of fish during the spawning season. These results differ from the second year's experiment, (Experiment 2), where no significant change occurred. The change in the seminal plasma calcium concentration was different in each group of fish and no clear trend or pattern could be observed in any of the

Figure 3.35: The change in striped bass sperm density and seminal plasma sodium concentration during the spawning season.



groups. In the GnRH $\alpha$  implanted fish (Figure 3.36), the seminal plasma calcium concentration increased in weeks 3 and 4, decreased over weeks 5 through 7, increased in week 8, decreased sharply in week 9, and then increased again in week 10. In the sham implanted fish (Figure 3.37), no significant change occurred in the seminal plasma calcium concentration during the first six weeks. However, a significant decrease in the calcium concentration occurred in weeks 7 and 8 followed by a slight increase in weeks 9 and 10. In the fish drawn randomly from the general population (Figure 3.38), the seminal plasma calcium concentration significantly decreased from week 1 to week 3, then increased again by weeks 4 and 5, and then stabilized with no significant differences occurring in weeks 6 through 8.

Figure 3.36: The change in the seminal plasma calcium concentration in individual GnRHa implanted striped bass during the spawning season. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other at  $P < 0.05$ .

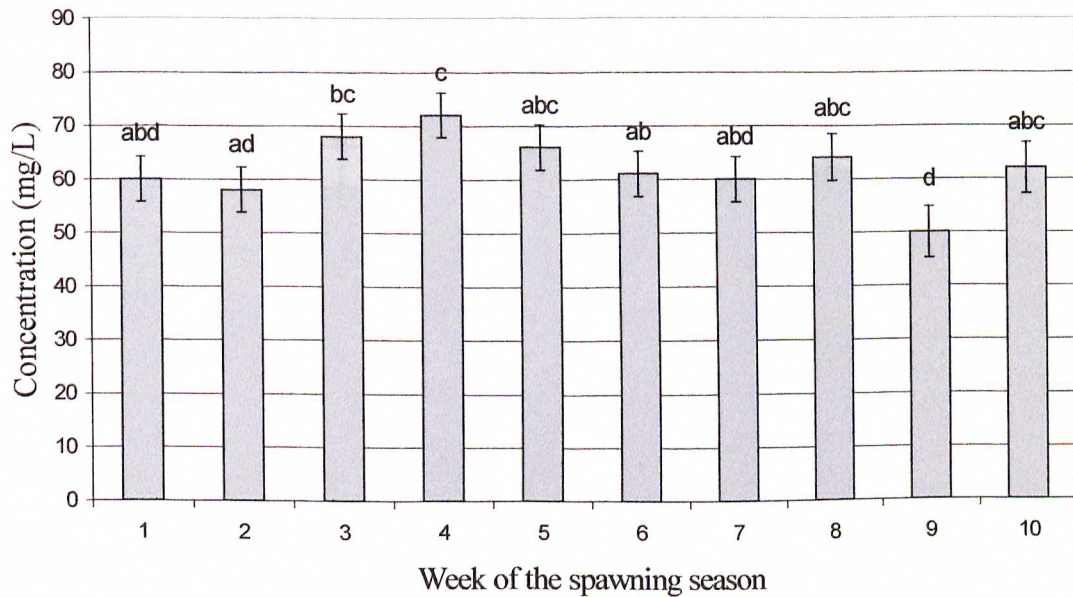


Figure 3.37: The change in the seminal plasma calcium concentration in individual sham implanted striped bass during the spawning season. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other at  $P < 0.05$ .

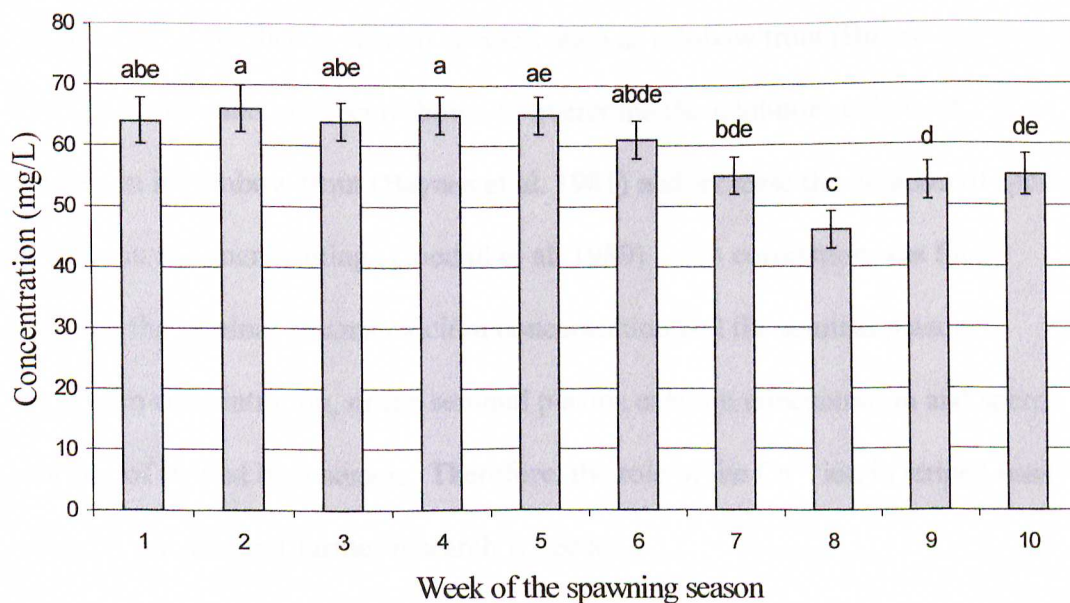
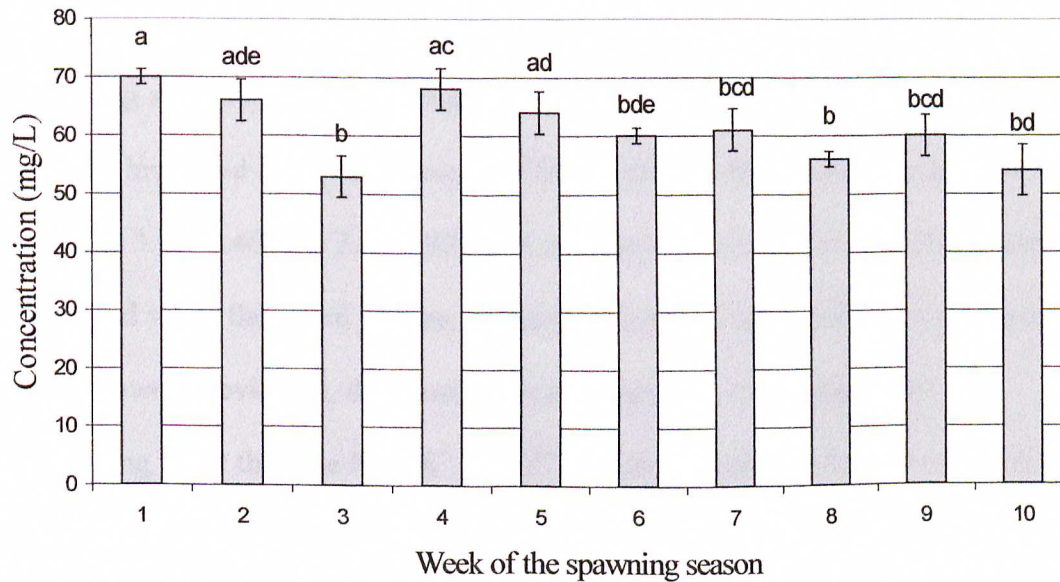




Figure 3.38: The change in the seminal plasma calcium concentration in striped bass randomly drawn from the general population during the spawning season. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other at  $P < 0.05$ .



Seasonal changes in seminal plasma calcium have only been documented in a few species such as carp (Kruger et al. 1984). External calcium is necessary to initiate sperm motility in several species, such as rainbow trout (Billard and Cosson 1992). Calcium has also been shown to overcome the inhibitory effects of potassium in rainbow trout (Baynes et al. 1981) and increase the duration of sperm motility in summer whiting (Goodall et al. 1989). No correlation was found between the seminal plasma calcium concentration and the seminal plasma potassium concentration, or the seminal plasma calcium concentration and sperm motility of striped bass semen. Therefore, the role of the  $\text{Ca}^{2+}$  ion in striped bass semen is unclear and further research is needed.

No significant change occurred in the potassium concentration of striped bass blood plasma during the spawning season ( $129 \pm 5.3$  mg/L). The blood plasma sodium concentration also showed no change during the spawning season except that week 11 ( $3533 \pm 32.3$  mg/L) was significantly lower than the rest of the spawning season ( $3843 \pm 28.8$  mg/L). The blood plasma calcium concentration steadily increased in all three groups of fish as the spawning season progressed (Figures 3.39, 3.40, and 3.41). Why the calcium concentration of the blood plasma increased while the blood plasma osmolality decreased during the spawning season is unknown. Obviously, the decrease in blood plasma osmolality is due to something other than the  $\text{Na}^+$ ,  $\text{K}^+$ , or  $\text{Ca}^{2+}$  ion concentrations. Further research is needed to explain these changes.

Figure 3.39: The change in the blood plasma calcium concentration in individual GnRH $\alpha$  implanted striped bass during the spawning season. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other at  $P < 0.05$ .

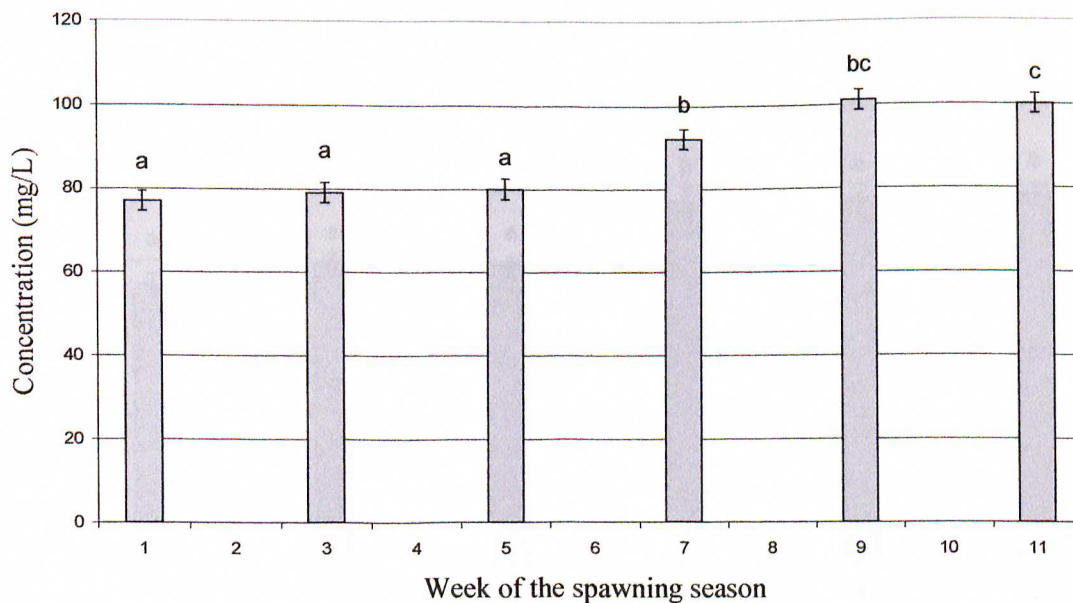


Figure 3.40: The change in the blood plasma calcium concentration in individual sham implanted striped bass during the spawning season. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other at  $P < 0.05$ .

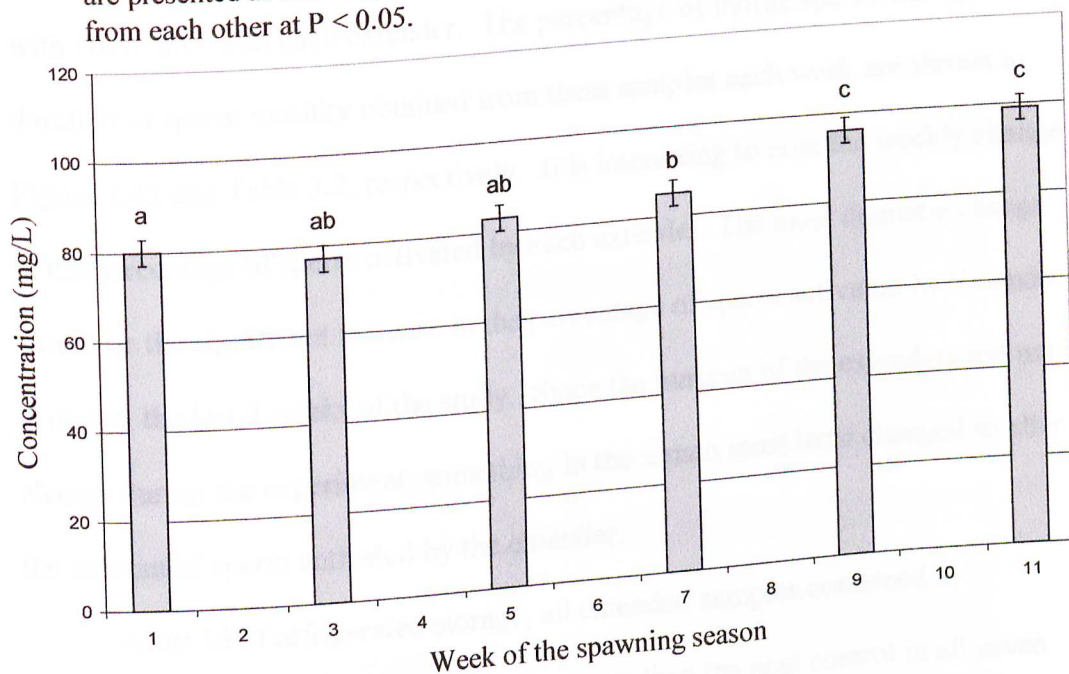
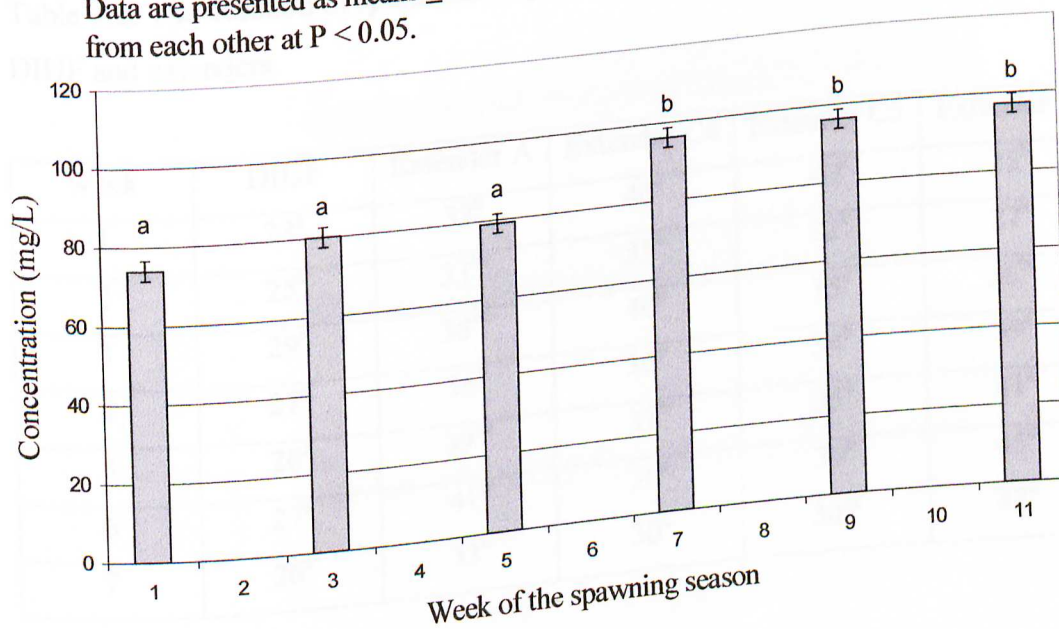


Figure 3.41: The change in the blood plasma calcium concentration in striped bass randomly drawn from the general population during the spawning season. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other at  $P < 0.05$ .





## Extender Evaluation

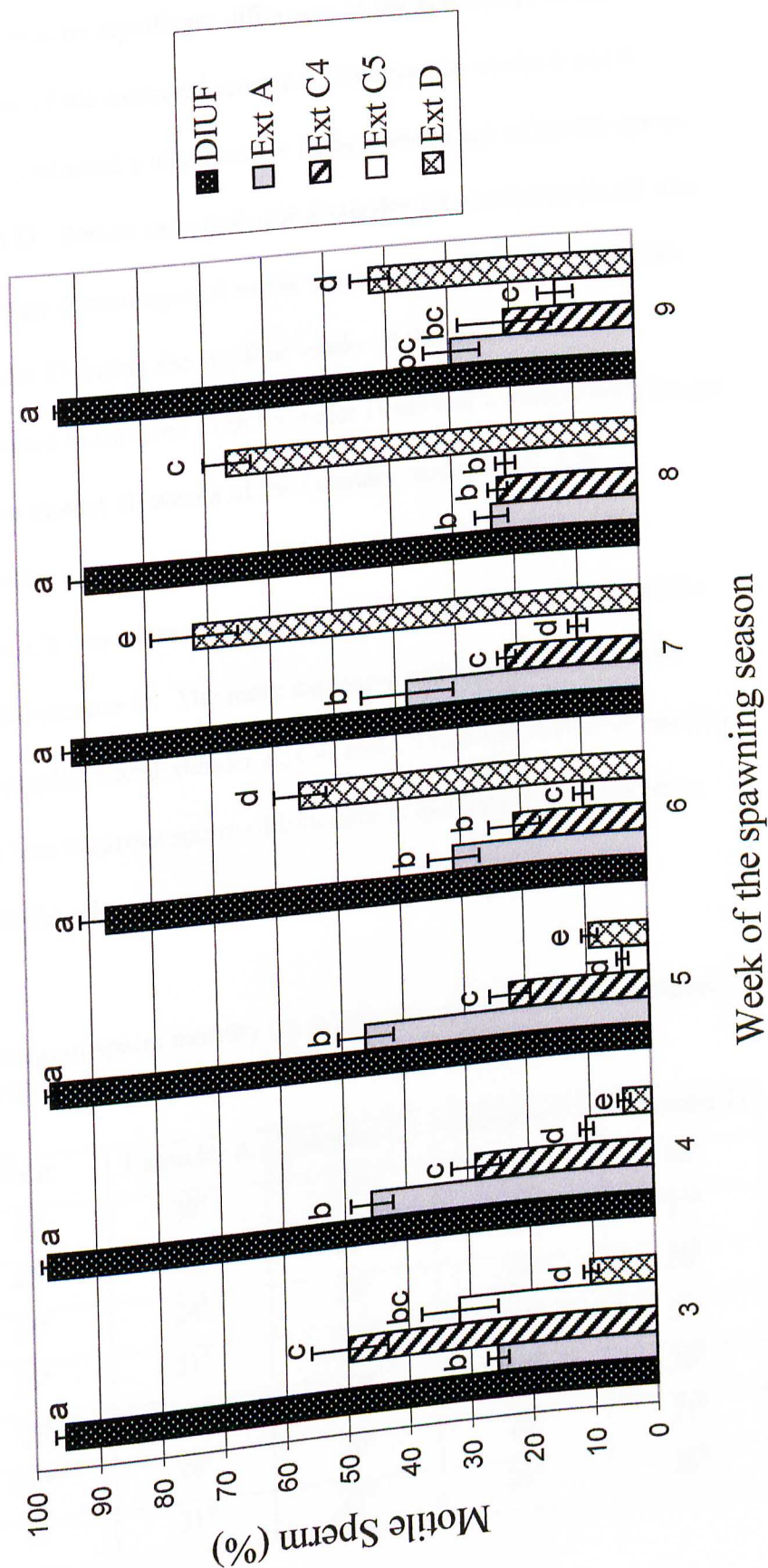
Upon initial collection of the semen, all neat semen samples were activated with DIUF and with each extender. The percentage of motile sperm and the duration of sperm motility obtained from these samples each week are shown in Figure 3.42 and Table 3.2, respectively. It is interesting to note the weekly change in the percentage of sperm activated by each extender. The most dramatic change would be the significant increase in the percentage of sperm activated by Extender D during the last 4 weeks of the study. Since the makeup of the extenders did not change during the experiment, something in the semen must have changed to alter the amount of sperm activated by the extender.

After 1d of refrigerated storage, all extended samples contained a significantly higher percentage of motile sperm than the neat control in all seven weeks of the extender study (Figure 3.43). Except for weeks 4 and 6 of the

Table 3.2: The duration of sperm motility (s) in neat semen samples activated with DIUF and extenders.

Week	DIUF	Extender A	Extender C4	Extender C5	Extender D
1	23 <sup>a</sup>	37 <sup>b</sup>	40 <sup>c</sup>	40 <sup>c</sup>	35 <sup>b</sup>
2	25 <sup>a</sup>	33 <sup>bc</sup>	35 <sup>c</sup>	32 <sup>b</sup>	27 <sup>a</sup>
3	29 <sup>a</sup>	38 <sup>bc</sup>	40 <sup>b</sup>	35 <sup>c</sup>	36 <sup>bc</sup>
4	27 <sup>a</sup>	36 <sup>b</sup>	35 <sup>b</sup>	34 <sup>b</sup>	39 <sup>c</sup>
5	26 <sup>a</sup>	39 <sup>bc</sup>	37 <sup>b</sup>	35 <sup>b</sup>	41 <sup>c</sup>
6	27 <sup>a</sup>	41 <sup>b</sup>	36 <sup>c</sup>	39 <sup>b</sup>	45 <sup>d</sup>
7	26 <sup>a</sup>	33 <sup>b</sup>	30 <sup>c</sup>	30 <sup>bc</sup>	42 <sup>d</sup>

Figure 3.42: The percentage of motile sperm in neat striped bass semen samples activated with DIUF and extenders. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other within each week at  $P < 0.05$ .





extender study, there was no significant difference in the percentage of motile sperm contained in any of the extended samples. However, in weeks 4 and 6, Extenders C4 and C5 contained a significantly higher percentage of motile sperm than Extenders A and D. Semen extended with Extender C4 and Extender C5 also contained higher average percentages of motile sperm than semen extended with Extender A or Extender D during the last four weeks of the study.

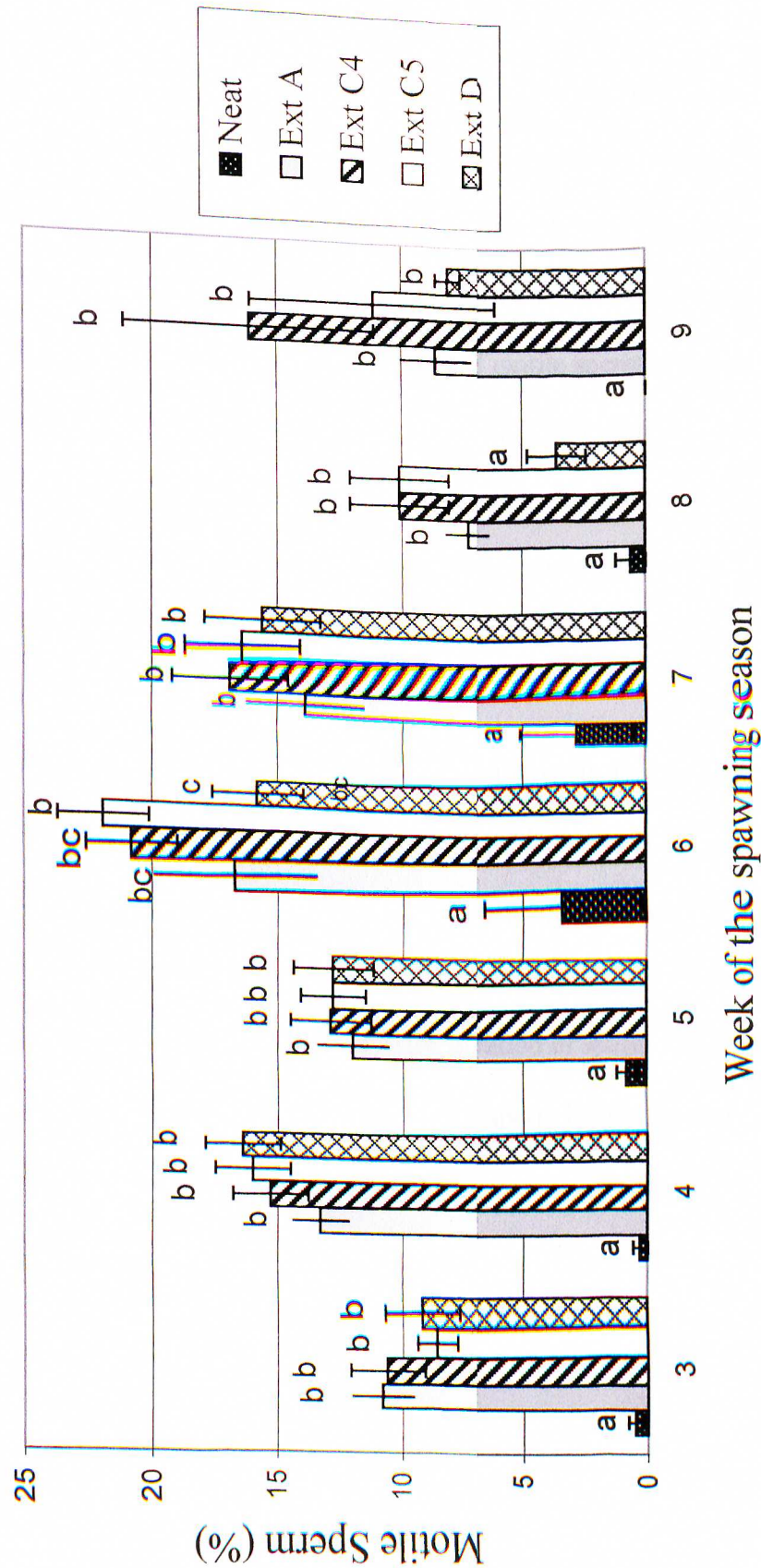
Sperm contained in samples from Extender D showed a significantly longer duration of motility in almost all weeks of the extender study (Table 3.3). However, sperm activity in the neat semen and in the semen extended with Extenders A, C4, and C5 was more vigorous when compared to the activity of the sperm extended with Extender D. The more explosive activity exhibited by the sperm in samples extended with Extender A, C4, and C5 resulted in shorter motility durations, while the less vigorous sperm of Extender D had longer duration times when compared with the other extenders.

Table 3.3: The duration of sperm motility (s) in neat and extended semen samples after 1d of refrigerated storage.

Week	Neat	Extender A	Extender C4	Extender C5	Extender D
1	16 <sup>a</sup>	20 <sup>a</sup>	19 <sup>a</sup>	19 <sup>a</sup>	20 <sup>a</sup>
2	22 <sup>a</sup>	23 <sup>a</sup>	23 <sup>a</sup>	23 <sup>a</sup>	27 <sup>b</sup>
3	24 <sup>a</sup>	24 <sup>a</sup>	24 <sup>a</sup>	24 <sup>a</sup>	28 <sup>b</sup>
4	23 <sup>a</sup>	21 <sup>b</sup>	21 <sup>ab</sup>	22 <sup>ab</sup>	27 <sup>c</sup>
5	25 <sup>a</sup>	22 <sup>b</sup>	24 <sup>ab</sup>	22 <sup>b</sup>	26 <sup>a</sup>
6	21 <sup>ab</sup>	20 <sup>a</sup>	20 <sup>a</sup>	20 <sup>a</sup>	21 <sup>b</sup>
7	--	21 <sup>a</sup>	21 <sup>a</sup>	21 <sup>a</sup>	26 <sup>b</sup>



Figure 3.43: The percentage of motile sperm in neat and extended striped bass semen samples after 1d of refrigerated storage. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other within each week at  $P < 0.05$ .



After 2d of refrigerated storage, all extended samples in all weeks of the extender study contained a significantly higher percentage of motile sperm than the neat control (Figure 3.44). The percentage of motile sperm in samples extended with Extender C4 and Extender C5 was significantly higher than the percentage of motile sperm obtained from samples extended with Extender D in all weeks except week 2. The only significant difference in the percentage of motile sperm obtained from samples extended with Extender A and samples extended with Extender C4 or Extender C5 occurred in week 6. However, the average percentage of motile sperm obtained from samples extended with Extender A was always lower than the average percentage of motile sperm obtained from samples extended with Extender C4 and/or Extender C5.

After 2d of refrigerated storage, sperm contained in samples from Extender D showed a significantly longer duration of motility in almost all weeks of the extender study (Table 3.4). Again, sperm activity in the semen extended with Extenders A, C4, and C5 was more vigorous when compared to the activity of the sperm extended with Extender D. This difference in the duration of sperm motility could also be the result of delayed activation, i.e. individual sperm beginning and ending motility at different times thereby extending the total time measured for duration of motility in a sample.



Figure 3.44: The percentage of motile sperm in neat and extended striped bass semen samples after 2d of refrigerated storage. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other within each week at  $P < 0.05$ .

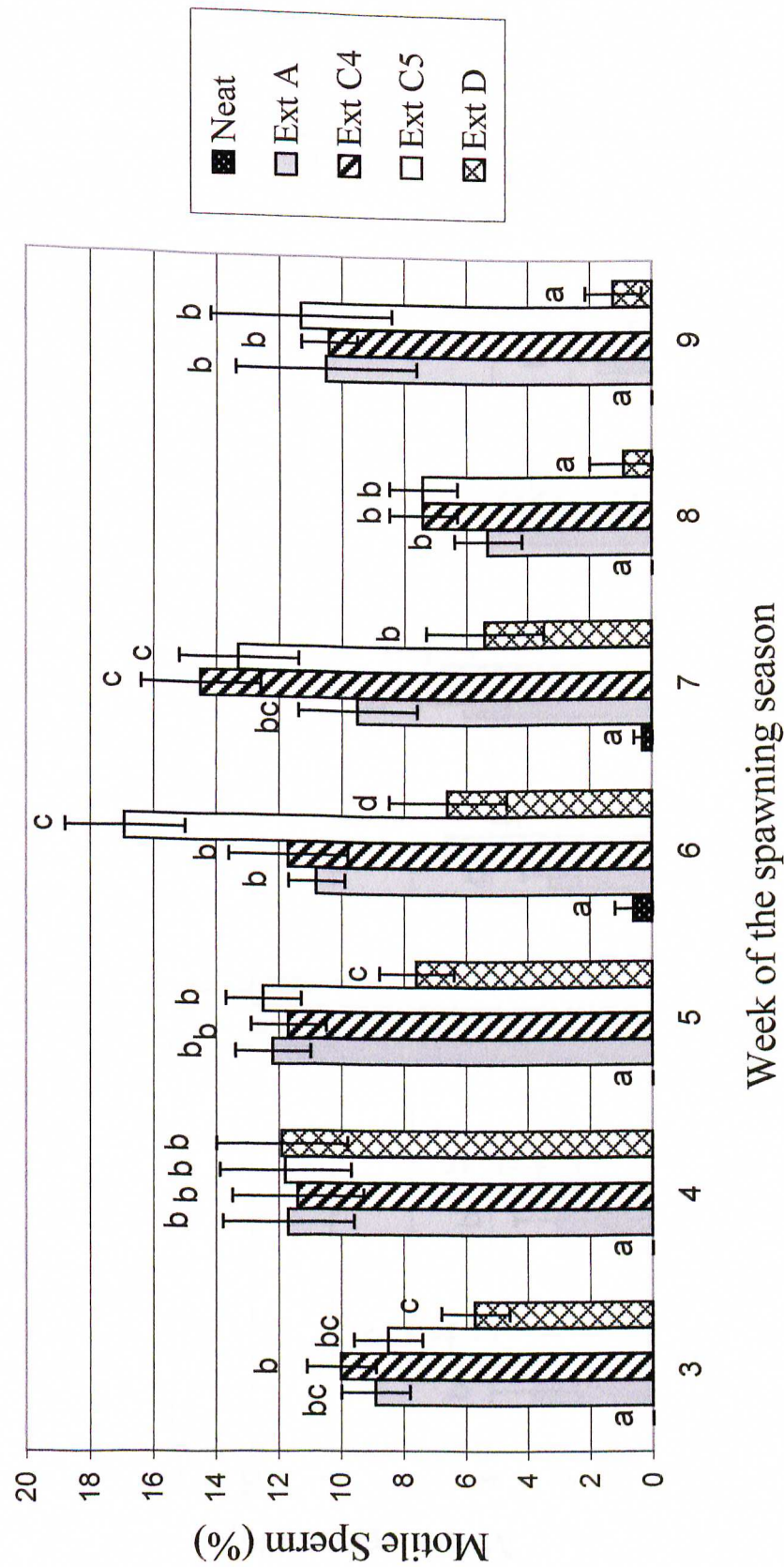




Figure 3.45: The percentage of motile sperm in neat and extended striped bass semen samples after 7d of refrigerated storage. Data are presented as means  $\pm$  SEM. Values with different letters are different from each other within each week at  $P < 0.05$ .

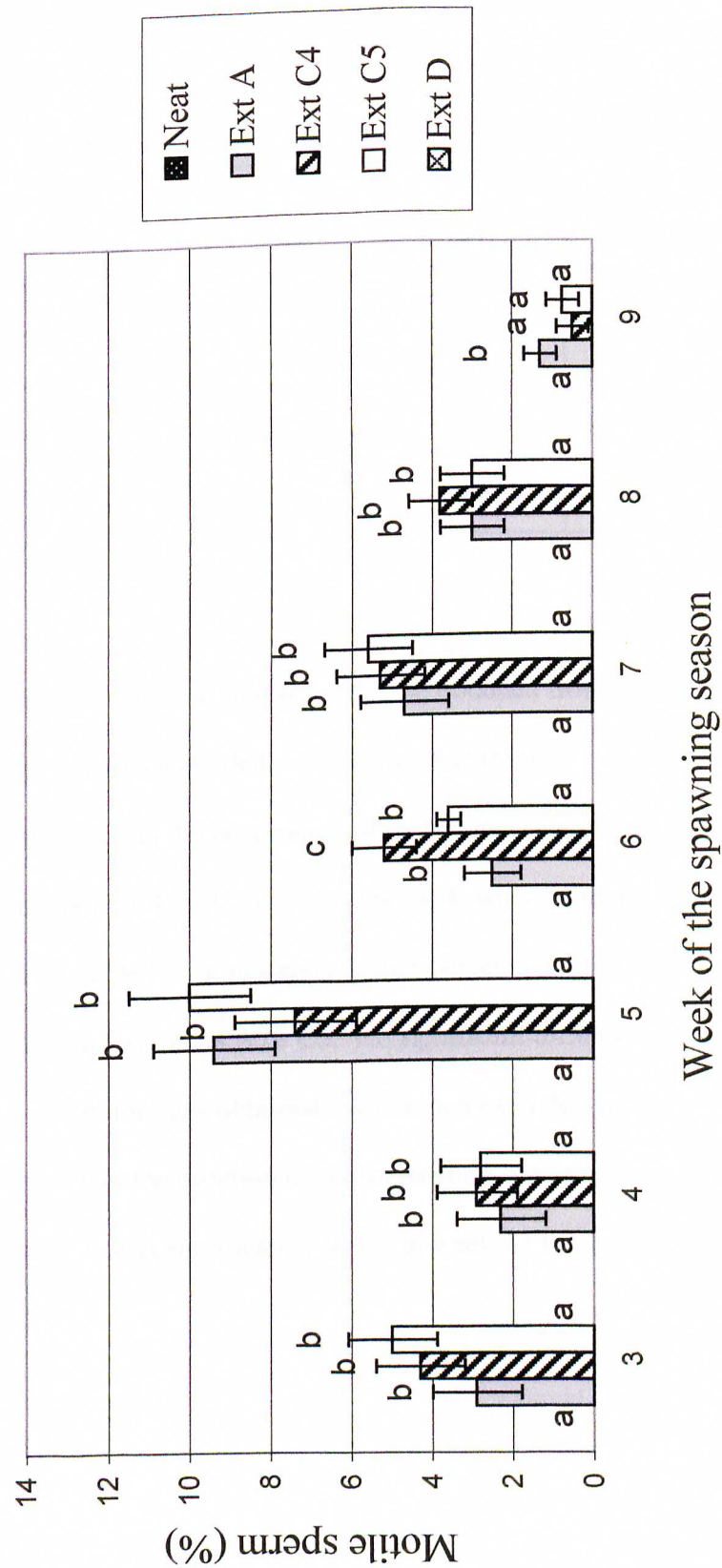


Table 3.4: The duration of sperm motility (s) in neat and extended semen samples after 2d of refrigerated storage.

Week	Neat	Extender A	Extender C4	Extender C5	Extender D
1	--	21 <sup>a</sup>	21 <sup>a</sup>	21 <sup>a</sup>	22 <sup>a</sup>
2	--	25 <sup>a</sup>	24 <sup>a</sup>	24 <sup>a</sup>	34 <sup>b</sup>
3	--	26 <sup>a</sup>	25 <sup>a</sup>	25 <sup>a</sup>	33 <sup>b</sup>
4	26 <sup>ab</sup>	23 <sup>b</sup>	23 <sup>ab</sup>	24 <sup>ab</sup>	30 <sup>ac</sup>
5	--	24 <sup>a</sup>	24 <sup>a</sup>	25 <sup>a</sup>	34 <sup>b</sup>
6	--	22 <sup>a</sup>	22 <sup>a</sup>	23 <sup>a</sup>	27 <sup>b</sup>
7	--	24 <sup>a</sup>	23 <sup>a</sup>	24 <sup>a</sup>	27 <sup>a</sup>

After 7d of refrigerated storage, no motility could be obtained from the neat semen samples or the semen samples extended with Extender D (Figure 3.45). There was no significant difference in the percentage of motile sperm obtained from semen extended with Extenders A, C4, or C5 except in week 4, where semen extended with Extender C4 contained a significantly higher percentage of motile sperm than semen extended with Extenders A or C5. No significant difference occurred in the duration of sperm motility obtained from semen extended with Extenders A, C4, or C5 except that the duration of sperm motility obtained from semen extended with Extender A was significantly lower in week 4 (Table 3.5).

Table 3.5: The duration of sperm motility (s) in neat and extended semen samples after 7d of refrigerated storage.

Week	Neat	Extender A	Extender C4	Extender C5	Extender D
1	--	21 <sup>a</sup>	20 <sup>a</sup>	22 <sup>a</sup>	--
2	--	26 <sup>a</sup>	29 <sup>a</sup>	27 <sup>a</sup>	--
3	--	21 <sup>a</sup>	22 <sup>a</sup>	22 <sup>a</sup>	--
4	--	21 <sup>a</sup>	23 <sup>b</sup>	23 <sup>b</sup>	--
5	--	25 <sup>a</sup>	26 <sup>a</sup>	24 <sup>a</sup>	--
6	--	20 <sup>a</sup>	21 <sup>a</sup>	19 <sup>a</sup>	--
7	--	21 <sup>a</sup>	19 <sup>a</sup>	18 <sup>a</sup>	--

## CONCLUSIONS

The results of this experiment show that many of the physical and chemical characteristics of striped bass semen change significantly during the spawning season. These results, for the most part, are in agreement with the results of the previous experiment (Experiment 2). In Experiment 2, the mean percentage of motile sperm obtained from fish randomly drawn from the general population was found to change during the spawning season. However, the results of this experiment showed no significant difference in the percentage of motile sperm contained in semen collected from individual fish during the spawning season. Therefore, sperm motility may not be the best method of estimating semen quality in striped bass.

In Experiment 2, a general pattern or trend could not be observed in many of the semen characteristics that were found to change. The results of this experiment showed that the semen osmolality, semen viscosity, and seminal plasma sodium



concentration were highest at the beginning and end of the spawning season.

During the last half of the spawning season, the sperm density was found to decrease and the seminal plasma potassium concentration was found to increase.

Also, the seminal plasma sodium concentration was found to have a positive correlation with both semen osmolality (0.52) and sperm density (0.37).

GnRHa was found to have some effect on semen quality. The duration of sperm motility in the fish given GnRHa was found to increase 7d post-implantation. Also, the pH of the semen and seminal plasma from these fish showed no significant change during the spawning season whereas the pH of the semen and seminal plasma from the fish not given any hormones fluctuated from week to week.

Extenders C4 and C5 proved to be the best extenders for the short-term refrigerated storage of striped bass semen. For the majority of the extender study, sperm stored in these two extenders maintained the highest percentage of motile sperm after 1, 2, and 7d of storage. Although sperm stored in Extender D showed a significantly longer duration of motility, the sperm motility in semen extended with Extender C4 and C5 was more vigorous. In all of the extended samples, the amount of explosive movement exhibited by the sperm was inversely proportional to storage time.

## CHAPTER 4

### The cryopreservation of striped bass semen.

## ABSTRACT

Three trials were performed to determine the best methods for cryopreserving striped bass semen. In the first trial, the highest post-thaw motility was obtained from cryopreserved semen thawed in a 25°C water bath as opposed to thawing by air. In the second trial, semen samples cryopreserved at a freezing rate of -40°C/min with 5% dimethyl-sulfoxide (DMSO) as the cryoprotectant exhibited the highest post-thaw motility. In the third trial, semen samples were cryopreserved with 5%DMSO at -40°C/min using three different extenders. The samples were thawed in a 25°C water bath and tested for motility and fertilizing ability. Semen cryopreserved in Extender D yielded the highest percentage of motile sperm and fertilized eggs post-thaw.



## INTRODUCTION

Cryopreservation of spermatozoa is a widely used practice in animal husbandry that is now being intensively studied for use in aquaculture. Successful cryopreservation techniques are needed to enhance and broaden the seasonal period of seedstock production as well as to establish gene banks. Gene banks would allow commercial production facilities to reduce the number of male broodstock maintained on site, expedite shipping of sperm to other facilities, preserve superior genomes (Kerby 1983), increase the genetic diversity of a population (Piironen 1993), and possibly aid in the conservation of important genetic information and/or the protection of threatened and endangered species (Gwo et al. 1991).

The striped bass industry is one of the fastest growing segments of finfish aquaculture in the United States (SBGA 1998). The major limitation to this growth is the industry's reliance on wild broodstock for seedstock. To overcome this, domesticated broodstocks and methods for their controlled reproduction must be developed (Smith 1989, Harrell et al. 1990). Although progress has been made (Woods et al. 1999), researchers are still trying to find solutions to many of the reproductive problems associated with striped bass held in captivity. The main problems faced by striped bass hatcheries are differing spawning times between fish of different sex, species, and geographical location and the inability to spawn fish year round. Such problems make the striped bass an excellent candidate for cryopreservation research.

There has been very little research on the cryopreservation of striped bass semen. The only published works to date are two papers, both by Kerby (1983,

1984). Kerby had limited success in cryopreserving striped bass sperm. Most of his samples exhibited no motility and there was a wide range of variability between individual samples in each trial (from 0 to 88% fertilization). However, Kerby did find that between glycerol, methanol, and dimethyl sulfoxide (DMSO), DMSO was the only cryoprotectant that yielded any motility or fertilization post-thaw.

In Experiment 4, three trials were performed on the cryopreservation of striped bass. Four extenders, three DMSO concentrations, three freezing rates, and two thawing rates were examined for their effectiveness in generating the highest rates of post-thaw sperm motility and fertilization.

## MATERIALS AND METHODS

### Trial 1 – June 3, 1997

Semen was collected from six striped bass at the end of the spawning season. The neat semen was activated in replicate samples of three, using Fisher's deionized ultra-filtered water (DIUF) with an osmolality of 0 to 5 mmol/kg. The sperm motility was recorded on videotape using a Hitachi Model KP-140 video camera attached to a Zeiss Model D-7082 compound microscope at a magnification of 400x. Only samples exhibiting motility  $\geq 90\%$  were used. Semen from each fish was extended with Extender A and Extender C in a 2:1 ratio of extender:semen. The formulas for the extenders are listed in Table 4.1. Samples from each fish and extender were divided into 3 aliquots of 2mL each. An additional 4mL of the appropriate extender containing either 5, 7.5, or 10% dimethyl-sulfoxide (DMSO)



was then added to each aliquot. The final dilution of extender+cryoprotectant:semen was 9:1.

The extended samples with cryoprotectant were placed in aliquots of 0.35mL into 0.5mL straws and sealed. The straws were color coded according to the extender, DMSO concentration, and freezing rate used. The total time the extended semen and cryoprotectant were allowed to equilibrate was 45 minutes (the amount of time it took to fill the straws) before freezing. Eight straws from each extender and cryoprotectant combination were frozen at a rate of  $-30^{\circ}\text{C}/\text{min}$  or  $-40^{\circ}\text{C}/\text{min}$  using a Planer Kryosave Model KS30 controlled rate freezer. Once the temperature of the samples reached  $-120^{\circ}\text{C}$ , the samples were plunged directly into liquid nitrogen.

After 30d of storage, straws from each extender, cryoprotectant, and freezing rate combination were thawed by air or water bath and the semen was activated in replicate samples of three, using DIUF. Thawing by air simply consisted of placing the straw on the countertop and allowing the sample to come to room temperature ( $25^{\circ}\text{C}$ ) by exposure to the air. Thawing by water bath consisted of plunging the sample into a  $25^{\circ}\text{C}$  water bath for approximately 12s. As with the neat semen, sperm motility was recorded on videotape using a Hitachi Model KP-140 video camera attached to a Zeiss Model D-7082 compound microscope at a magnification of 400x. The percentage of motile sperm and the amount of time the sperm remained motile were determined later from the videotapes. The percentage of motile sperm was estimated for all samples by the same observer to avoid bias. Duration of motility was timed beginning the instant the DIUF was mixed with the



semen and ending when the majority (approximately 90%) of the sperm in the field of view had stopped moving. Spermatozoa that simply vibrated or did not show progressive forward movement were not considered motile. Duration of motility for each straw was measured three times with a digital stopwatch and recorded to the nearest 1/100s.

#### Trial 2 – April 28, 1998

In 1998, the second year, two different extenders, two different DMSO concentrations, and three different freezing rates for cryopreserving striped bass semen were tested. Semen was pooled from six striped bass in the middle of the spawning season. Semen quality was estimated by motility and only samples with motility  $\geq 90\%$  were used. Motility for the pooled neat semen was recorded on videotape and the percentage of motile sperm and the duration of sperm motility were measured using the same procedures as those described in Trial 1. Half of the neat semen was extended with Extender A and half with Extender D in a 2:1 ratio of extender:semen. The formulas for the extenders are listed in Table 4.1. The semen extended with Extender A was divided into thirds and the semen extended with Extender D was divided in half. DMSO was added in concentrations of 5 and 7.5% to a portion of semen from each extender. No DMSO was added to the third portion of semen extended with Extender A. This was to allow for the opportunity to evaluate the egg yolk contained in Extender A as a cryoprotectant.

The extended samples with cryoprotectant were added in aliquots of 0.35mL into 0.5mL straws and sealed. The straws were color coded according to the

extender, DMSO concentration, and freezing rate used. The total time the extended semen and cryoprotectant were allowed to equilibrate was 36 minutes (the amount of time it took to fill the straws) before freezing. Ten straws from each extender and cryoprotectant combination were frozen at a rate of  $-30^{\circ}\text{C}/\text{min}$ ,  $-40^{\circ}\text{C}/\text{min}$ , or plunged directly into liquid nitrogen. To obtain freezing rates of  $-30^{\circ}\text{C}/\text{min}$  and  $-40^{\circ}\text{C}/\text{min}$ , a Planer Kryosave Model KS30 controlled rate freezer was used to freeze the samples to  $-120^{\circ}\text{C}$ . The samples were then plunged directly into liquid nitrogen.

After 1, 7, and 30 days of storage, three straws from each extender, cryoprotectant, and freezing rate combination were thawed in a  $25^{\circ}\text{C}$  water bath and activated with DIUF. Motility for each sample was recorded on videotape and the percentage of motile sperm and the duration of sperm motility were measured using the same procedures as those described in Trial 1.

#### Trial 3 – April 13, 1999

Semen was collected from six striped bass in the beginning of the spawning season. As in Trials 1 and 2, only semen samples with motility  $\geq 90\%$  were used. Motility for the neat semen was recorded on videotape and the percentage of motile sperm and the duration of sperm motility were measured using the same procedures as those described in Trial 1. Semen from each fish was divided into three portions of 1.5 mL each and extended with Extender A, Extender C4, or Extender D in a 2:1 ratio of extender:semen. The formulas for the extenders are listed in Table 4.1. An



additional 3mL of the appropriate extender containing 5% DMSO was then added to each aliquot. The final dilution of semen:extender+cryoprotectant was 1:4.

The extended samples with cryoprotectant were added in aliquots of 0.25mL into 0.5mL straws and sealed. The straws were color coded according to the fish and extender. The total time the extended semen and cryoprotectant were allowed to equilibrate before freezing was 25min. This was the maximum amount of time needed to fill all the straws for one fish. If the straws for a fish were filled before 25min, the straws were kept on ice for the remainder of the equilibration time and then were frozen. Straws were frozen at a rate of  $-40^{\circ}\text{C}/\text{min}$  to a temperature of  $-120^{\circ}\text{C}$  using a Planer Kryosave Model KS30 controlled rate freezer. The samples were then plunged directly into liquid nitrogen.

Straws from each fish and extender were thawed in a  $25^{\circ}\text{C}$  water bath and activated with DIUF. Motility for each sample was recorded on videotape and the percentage of motile sperm and duration of sperm motility were measured using the same procedures as those described in Trial 1.

The six fish from which sperm was cryopreserved were moved to individual 2m diameter tanks with recirculating water. Mature female striped bass were anesthetized in a 70mg/L quinaldine bath (Woods et al. 1992) and carefully catheterized to collect a sample of their eggs. The eggs were observed at magnification of 40x with a Nikon Model SMZ-10 dissecting microscope to determine their stage of development. The stage of egg maturation was determined according to the photos and descriptions outlined by Rees and Harrell (1990). Six females with eggs staged at 10-h from ovulation were injected with human



chorionic gonadotropin (hCG) and placed in a 2m tank containing a male striped bass. Beginning 24h after the hCG injection, egg samples were periodically collected by catheterization of the ovary from each female to determine the estimated time of ovulation. At ovulation, each female was removed from the tank and anesthetized. The urogenital vent was wiped clean and dry and the eggs were stripped from the female into a clean, dry pan. The male striped bass from the same tank was then removed, anesthetized, and semen was collected into a separate container in the same manner as the female.

The freshly collected eggs and neat milt were immediately taken to the lab. Samples of 900 eggs (1mL) each were allocated into four 300mL glass dishes labeled Neat, Extender A, Extender C4, and Extender D. Eighteen straws (six from each extender) of cryopreserved semen from the same fish that the neat semen was collected from, were thawed in a 25°C water bath for approximately 12 seconds. Immediately after thawing, the cryopreserved semen was placed directly on top of the eggs in the dish with the appropriate extender label. To insure that each dish contained approximately the same sperm/egg ratio, only 0.1mL of neat semen was placed directly on top of the eggs in the dish labeled "neat". Hatchery water was then added to each dish and the eggs and semen were gently swirled together. After 5 to 10 minutes, the eggs and semen from each dish were poured into labeled beakers containing 600mL of hatchery water. The beakers were maintained at a temperature of  $24 \pm 1^{\circ}\text{C}$  and supplied with oxygen. After 12h, all the eggs were observed under at a magnification of 40x with a Nikon Model SMZ-10 dissecting

Table 4.1: Extender Formulas for Experiment 4.<sup>1</sup>

Extender A <sup>2</sup>	Extender C	Extender C4	Extender D <sup>3</sup>
100mg glucose	10g glucose	100mg glucose	860mg NaCl
750mg NaCl	1000mg NaCl	870mg NaCl	100mL water <sup>4</sup>
200mg NaHCO <sub>3</sub>	130mg KHCO <sub>3</sub>	200mgNaHCO <sub>3</sub>	
40mg KCl	200mL water <sup>4</sup>	40mg KCl	
100mL water <sup>4</sup>	40mL egg yolk	100mL water <sup>4</sup>	
20mL egg yolk		20mL egg yolk	
Osmolality:	Osmolality:	Osmolality:	Osmolality:
290 mmol/kg	390 mmol/kg	310 mmol/kg	270 mmol/kg
Trial 1 pH: 6.0	pH: 4.0	pH: 7.6	pH: 7.6
Trial 2 & 3 pH:			
7.6			

<sup>1</sup> Extenders in trials 2 and 3 were pH adjusted with HCl or NaOH.

<sup>2</sup> Formula taken from Stein and Bayrle (1978).

<sup>3</sup> Formula taken from Brown and Mims (1995).

<sup>4</sup> Water used was Fisher's deionized ultra-filtered water (DIUF).

microscope for the presence of a germ ring to determine the percentage that were fertilized.

### Statistical Analysis

Analysis of variance was used to detect significant differences in the percentage of motile sperm, the duration of sperm motility, and/or the number of fertilized eggs obtained from cryopreserved semen undergoing different treatments (i.e. the different extenders, cryoprotectant concentrations, freezing rates, and thawing rates). The mixed model procedures from SAS version 6.12 statistical

software were used (SAS 1996). Significant differences were detected at the 5% level. Results, unless otherwise noted, are reported as means  $\pm$  S.E.M.

## RESULTS AND DISCUSSION

### Trial 1

Cryopreserved semen thawed in a 25°C water bath yielded a significantly higher percentage of motile sperm than cryopreserved semen thawed by air. In fact, no post-thaw motility was obtained from the cryopreserved semen thawed by air. This is most likely due to the faster warming rate associated with the water bath. Faster warming rates reduce the degree of recrystallization and hence, cell damage (Leung 1991). Faster warming rates have been shown to yield higher post-thaw motility in semen from *Sarotherodon mossambicus* (Harvey 1983) and higher post-thaw fertility in semen from rainbow trout (Wheeler and Thorgaard 1991).

No significant differences were observed between the DMSO concentrations or the freezing rates. However, the highest percentage of motile sperm was obtained from semen cryopreserved with 7.5% DMSO. Previous research on the cryopreservation of striped bass semen found 5% DMSO to yield higher fertilization percentages in comparison to 7.5 and 10% DMSO (Kerby 1983). Therefore, the performance of 5 and 7.5% DMSO was reevaluated in Trial 2. No significant difference was found in the post-thaw motility obtained from semen frozen at a rate of -30°C/min or -40°C/min. Therefore these freezing rates were also reevaluated in Trial 2.



Semen cryopreserved in Extender C yielded a significantly higher percentage of motile sperm than semen cryopreserved in Extender A. However, the difference between Extender A and Extender C was very small ( $0.1 \pm 0.4\%$  and  $1.6 \pm 0.4\%$ , respectively). In the short-term refrigerated storage trial of Experiment 1, the semen stored in Extender A yielded a significantly higher percentage of motile sperm than the semen stored in Extender C. This difference was much greater than the difference observed between the two extenders in the cryopreservation trial. Since it was believed that an extender yielding the best results during short-term refrigerated storage would also yield the best results during cryopreservation, Extender A was included in the second cryopreservation trial.

As in Kerby's experiment (1983), very little post-thaw sperm motility was obtained from any of the cryopreserved samples (range 0-15%). This may be because the semen was collected at the end of the spawning season and may have been of poor quality. Although the percentage of motile sperm obtained from the neat semen samples before cryopreservation was high ( $>90\%$ ), previous experiments (Experiments 2 and 3), showed that the percentage of motile sperm is usually between 80-100% throughout the spawning season and may not be a good indicator of semen quality.

## Trial 2

Semen samples cryopreserved in Extender A and Extender D exhibited no significant difference in the post-thaw motility after 1, 7, and 30d of storage in

liquid nitrogen. Therefore, these two extenders were carried over to the third and final trial to test their performance in relation to Extender C4.

Semen samples that were cryopreserved with egg yolk as the only cryoprotectant, exhibited no post-thaw motility and were therefore removed from the statistical analysis. After 1d of storage, semen samples cryopreserved with 5% DMSO exhibited a significantly higher percentage of motile sperm than semen samples cryopreserved with 7.5% DMSO. No significant differences were observed between the two cryoprotectant concentrations after 7 and 30d of storage. However, the average percentage of motile sperm obtained post-thaw was always higher in the samples cryopreserved with 5% DMSO (Table 4.2). No significant difference occurred in the duration of sperm motility obtained from semen cryopreserved with 5 or 7.5% DMSO after 1, 7, or 30d of storage. These results are in agreement with the previous experiments on the cryopreservation of striped bass semen reported by Kerby (1983).

Semen samples that were plunged directly into liquid nitrogen exhibited no post-thaw motility and were therefore removed from the statistical analysis. Many of the straws were damaged from the temperature shock associated with the direct plunge. When the data was statistically analyzed with the number of days in storage as a co-variable, semen samples frozen at a rate of  $-40^{\circ}\text{C}/\text{min}$  showed no significant difference in post-thaw motility from semen samples frozen at a rate of  $-30^{\circ}\text{C}/\text{min}$ . However, when the data from all three storage times were combined, semen samples frozen at  $-40^{\circ}\text{C}/\text{min}$  yielded a significantly higher percentage of motile sperm and duration of sperm motility than semen samples frozen at

Table 4.2: The percentage of motile sperm and the duration of sperm motility obtained from cryopreserved semen in Trial 2.

Treatment	Percentage of motile sperm (%)			Duration of sperm motility (s)		
	1	7	30	1	7	30
Days of storage						
Extender A	$0.6 \pm 0.1$	$1.3 \pm 0.9$	$0.9 \pm 0.5$	$15 \pm 2.1$	$17 \pm 2.1$	$15 \pm 2.4$
Extender D	$0.7 \pm 0.1$	$1.1 \pm 0.9$	$1.1 \pm 0.5$	$14 \pm 2.0$	$15 \pm 2.1$	$14 \pm 2.0$
5% DMSO	$1.3 \pm 0.2$	$2.1 \pm 1.3$	$1.3 \pm 0.6$	$14 \pm 1.2$	$16 \pm 1.7$	$14 \pm 2.0$
7.5% DMSO	0	$0.3 \pm 0.3$	$0.7 \pm 0.6$	--	$14 \pm 3.0$	$15 \pm 2.4$
-30°C /min	$0.7 \pm 1.6$	$0.4 \pm 2.6$	$0.3 \pm 3.0$	$12 \pm 0.3$	$14 \pm 1.1$	$10 \pm 2.0$
-40°C /min	$2.7 \pm 1.6$	$6.0 \pm 2.6$	$8.0 \pm 3.0$	$16 \pm 0.3$	$18 \pm 1.1$	$16 \pm 1.0$

-30°C/min. The number of samples from each individual storage time may have been too small to allow the detection of a significant difference.

### Trial 3

Semen samples cryopreserved with Extender D contained a significantly higher percentage of motile sperm post-thaw than semen samples cryopreserved with Extender A or Extender C4 (Table 4.3). However, there was no significant difference in the duration of motility obtained from semen cryopreserved with the different extenders.



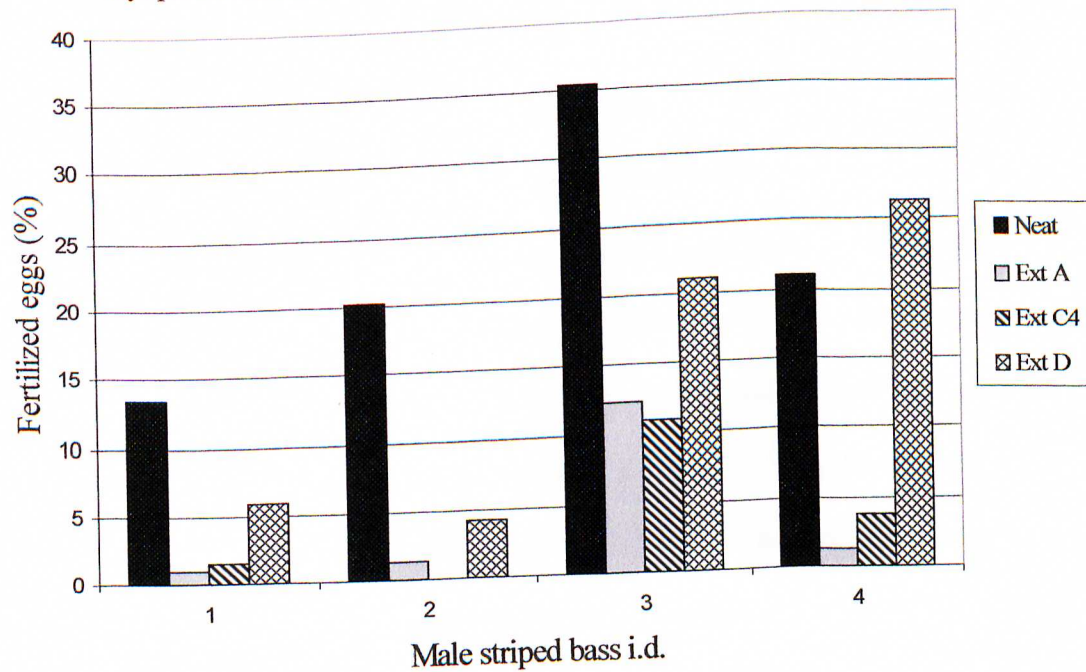
Freshly collected semen fertilized a significantly higher percentage of eggs than any of the cryopreserved semen samples (Figure 4.1). However, in Fish #4, the semen cryopreserved with Extender D yielded a higher percentage of fertilized eggs than the freshly collected semen. From the cryopreserved samples, semen frozen in Extender D yielded a significantly higher percentage of fertilized eggs than semen frozen in Extender A or Extender C4. Semen cryopreserved with Extender A and Extender C4 showed no significant difference in the percentage of motile sperm or fertilized eggs obtained.

In Experiment 3, semen extended with Extender A and Extender C4 contained a significantly higher percentage of motile sperm during short-term refrigerated storage than semen extended with Extender D. However, the results of Experiment 4 - Trial 3 clearly show that the highest percentage of motile sperm and fertilized eggs were obtained from semen cryopreserved with Extender D rather than Extender A or Extender C4. These results demonstrate that the best extender for the short-term storage of striped bass semen may not be the best extender for the cryopreservation of striped bass semen.

Table 4.3: The percentage of motile sperm and the duration of sperm motility obtained from cryopreserved striped bass semen in Trial 3.

Extender	Percentage of motile sperm (%)	Duration of sperm motility (s)
A	$2.5 \pm 3.1$	$11 \pm 1.2$
C4	$1.2 \pm 3.1$	$17 \pm 1.2$
D	$15.0 \pm 3.1$	$15 \pm 0.6$

Figure 4.1: The percentage of fertilized eggs obtained from neat and cryopreserved striped bass semen.



### CONCLUSIONS

From the three trials performed, the best results, in terms of sperm motility and fertilizing ability, were obtained from cryopreserving striped bass semen in Extender D with 5%DMSO at a rate of  $-40^{\circ}\text{C}/\text{min}$  and thawing the semen in a  $25^{\circ}\text{C}$  water bath. Further research is still needed to determine the optimum dilution ratio of extender+cryoprotectant:semen and the optimum equilibration time for the extended semen and cryoprotectant.



## CONCLUSIONS

### Characterization of Striped Bass Semen

The major objective of this research was to characterize the physical and chemical properties of striped bass semen. In the first experiment, the physical and chemical properties of striped bass semen were measured near the peak of the spawning season from captive and domesticated striped bass of three different age groups and filial generations. No significant differences were found to occur in any of the semen characteristics between fish of different age, size, or filial generation. However, these results are based on striped bass aged 5, 6, and 8-10 years. Vuthiphandchai and Zohar (1999) reported significant differences to occur in the sperm quality of striped bass aged 1, 3, and 14 years.

In the second experiment, the physical and chemical characteristics of striped bass semen were measured weekly for six weeks during the spawning season. Seasonal variations were found to occur in the percentage of motile sperm, duration of sperm motility, viscosity, osmolality and pH of the semen and the osmolality, pH, and sodium and potassium concentrations of the seminal plasma. These characteristics appeared to fluctuate randomly and it was uncertain whether variation between individual fish may have obscured the detection of a general pattern or trend.

In the third experiment, the physical and chemical characteristics of striped bass semen were measured weekly throughout the entire spawning season from six individual fish given GnRHa implants and six individual fish given sham implants. Semen samples were also collected and measured weekly from six fish randomly



drawn from the general population of captive and domesticated striped bass. The results showed that the percentage of motile sperm in semen collected from the individual striped bass did not change significantly during the spawning season, and therefore sperm motility may not be an accurate indicator of semen quality. Semen osmolality and viscosity and the seminal plasma sodium concentration were found to be highest at the beginning and end of the spawning season. The sperm density was found to significantly decrease during the last half of the spawning season whereas the seminal plasma potassium concentration significantly increased. Also, the seminal plasma sodium concentration was found to have a positive correlation with both semen osmolality (0.52) and sperm density (0.37).

In conclusion, variation in the semen characteristics would indicate a variation in the semen quality. The percentage of motile sperm is used most often to estimate semen quality. However, since the sperm motility in semen from the individual striped bass did not change significantly during the spawning season, some other variable must be used to estimate semen quality in striped bass. Other characteristics such as sperm density (Rana 1995), the seminal plasma pH, and the seminal plasma sodium concentration (Lahnsteiner et al. 1998) have been used to estimate semen quality in other species. Seminal plasma pH would not be an accurate indicator of striped bass semen quality since GnRHa, which is often used to spawn striped bass, was found to effect seminal plasma pH. The sperm density and seminal plasma sodium concentration of striped bass were both found to vary during the spawning season. Measurement of the seminal plasma sodium is a time-consuming process, but sperm density can be measured quickly and easily. Sperm

density would be the best choice for measuring semen quality but fertilization trials would have to be performed to determine whether it is an accurate indicator of striped bass semen quality.

#### Short-term Storage of Striped Bass Semen

The second objective of this research was to identify or develop the best extender for the short-term storage of striped bass semen. Over the course of three years and three experiments, eight different extenders were evaluated. Extenders C4 and C5 proved superior in terms of maintaining the highest percentage of motile sperm after 1, 2, and 7d of refrigerated storage.

Although the duration of sperm motility obtained from semen stored in Extender D was significantly longer than the duration of motility obtained from semen stored in Extenders C4 and C5, the duration of motility was not considered an accurate method of measuring extender performance. The semen stored in Extender D did not exhibit the explosive or vigorous activity that the samples with the shorter duration of sperm motility exhibited. Also, it could not be determined if the longer duration of motility was actually due to the sperm remaining motile for longer, or if individual sperm in the sample were activating at different times, thereby extending the total time measured for duration of motility.

#### Cryopreservation of Striped Bass Semen

The third and final objective of this research was to begin the development of a successful procedure for the cryopreservation of striped bass semen. Three

cryopreservation trials were performed to evaluate the effect of different extenders, cryoprotectant concentrations, freezing rates, and thawing methods on the post-thaw motility and fertilizing ability of striped bass semen. Semen that was cryopreserved at a rate of  $-40^{\circ}\text{C}/\text{min}$  in Extender D with 5% dimethyl-sulfoxide (DMSO) and thawed in a  $25^{\circ}\text{C}$  water bath yielded the highest post-thaw motility and fertilizing ability. Although this protocol for cryopreserving striped bass semen proved successful, further research is needed to improve these results. Future areas to research might be 1) to determine the optimum equilibration time for the extended striped bass semen and the cryoprotectant, 2) to determine the optimum dilution ratio of extender+cryoprotectant:semen, and 3) to compare thawing in a  $25^{\circ}\text{C}$  water bath with thawing in a water bath of a higher temperature.



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