ABSTRACT

Title of Document: THE EFFECT OF FREEZING RATE ON STRIPED BASS (*MORONE SAXATILIS*) SPERMATOZOA

Tyler Edward Frankel, Master of Science, 2013

Directed By: Dr. L. Curry Woods III

Department of Animal and Avian Sciences

Several studies have attempted to determine the optimal freezing rate for the cryopreservation of striped bass spermatozoa. In this study, the effects of freezing rate (-10, -15, -20 and -40^{o}C/min) on sperm quality was examined utilizing Sybr-14/Propidium Iodide staining to confirm membrane integrity, a luciferin-luciferase assay to estimate ATP concentration and a Hamilton-Thorne CEROS™ system to characterize sperm motion. Males (n=12) were sampled once a week for five weeks. Samples were extended, cryo-protected using a 7.5% (v/v) dimethyl sulfoxide solution, and frozen using a controlled-rate freezer. Samples were stored in liquid nitrogen for 49 days and re-evaluated post-thaw. Sperm cryopreserved at -40^{o}C per minute resulted in: total motility (10.06%), progressive motility (7.14%), ATP concentration (0.86 pmol/million cells), and sperm viability (56.5%); which were greater ($P < 0.05$) than the slower rates. Results demonstrate that -40^{o}C/minute was the optimal freezing rate among those tested for the cryopreservation of striped bass spermatozoa.

THE EFFECT OF FREEZING RATE ON STRIPED BASS (*MORONE SAXATILIS*) SPERMATOZOA

By

Tyler Edward Frankel

Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment of
the requirements for the degree of
Master of Science
2013

Advisory Committee:

Professor L. Curry Woods III, Chair/Advisor
Dr. Edward Orlando
Dr. Andrew Lazur

Dedication

Dedicated to my parents

Jack S. Frankel

and

Joan A. Frankel

Acknowledgements

Over the past two years, I have been the recipient of invaluable assistance and support from many people, and I would like to take the time to acknowledge them for all of their help and contributions.

A special thanks goes to my advisor, Dr. L. Curry Woods III, whose mentorship and guidance has helped me to thrive in the academic environment. His enthusiasm, thoughtfulness, and compassion have helped to kindle my interest in both research and academia. I have been both fortunate and honored to have him as a mentor. I would also like to acknowledge Daniel Theisen for all of his help during the experiment. I extend my gratitude to my other committee members, Drs. Edward Orlando and Andrew Lazur, for their time and consideration during my tenure as a Masters student.

I am extremely grateful for the assistance and expertise shared by Dr. Howard Guthrie and Glenn Welch, and thank them for all of their help with experimental design, sample preparation, and statistical analyses.

I wish to thank my parents for their unconditional love and support. Without their dedication and encouragement, this work would never have been possible.

Table of Contents

Chapter 1- Literature Review

Aquaculture: Challenges and Benefits

In the United States, the increased demand for seafood has stretched the resources of both commercial fishing and the aquaculture industry. More than 220 species of finfish and shellfish are now farmed for human consumption, with production rates of over 29 million tons per year (Naylor *et al.*, 2000). In contrast, wild capture rates of over 89 million tons per year have led to the overexploitation of over 70% of wild aquatic species harvested for the world's seafood market. To help cope with both the increased demand for consumable seafood and the detrimental effects that capture fishing has had on the world's aquatic population, interest in aquaculture has risen dramatically over a short period of time. Following this trend, the striped bass market experienced an approximate ten-fold increase in production from 1985 to 1995 (SBGA, 1998). Research performed by Lougheed and Nelson (2001) has shown that striped bass have become a major component in the growing U.S. aquaculture industry, with production rates of over 11.5 million pounds per year and an annual market value of over $27.8 million. However, striped bass production rates since 1995 have leveled off at approximately 12 million lbs/year (Carlberg *et al.*, 2000).

Striped Bass Overview

Populations of striped bass (*Morone saxatilis*, Moronidae) are found along the entirety of the Atlantic coast of the United States and are also present in coastal tributaries of the Gulf of Mexico from Florida to Louisiana (Brown, 1965). Smaller

populations have also been introduced to inland lakes and reservoirs of the United States as well as the Pacific coast, where they have taken up residence from Mexico to British Columbia. The largest natural production of coastal migratory striped bass along the Atlantic coast has most commonly occurred in the Chesapeake Bay, with smaller contributions made from the Delaware, Hudson River, Mid- and South Atlantic, as well as maritime provinces of Canada striped bass populations. Because of its consumer acceptance, high market price, palatability, and other favorable factors, striped bass and its related hybrids have become established in 48 of the 50 United States through the practices of aquaculture or the stocking of natural bodies of water. Striped bass have also been exported to China, France, Germany, Israel, Portugal, Russia, Taiwan, and Spain where, in some cases, populations have been established for aquaculture and commercial farming (Harrell and Webster, 1997; Liu *et al.*, 1998; Yan *et al.*, 2001; Woods, 2005; Jobling *et al.*, 2010).

Use of Purebred and Hybrid Crosses in Aquaculture

Hybrid crosses involving members of the Moronidae family are commonly utilized in aquaculture production (Woods, 2005), ranking fourth in value compared to other teleost species cultured in the United States (Carlberg *et al.*, 2000). Hybrids produced for commercial purposes are created utilizing *in vitro* fertilization methods, although it has been shown that the production of hybrid offspring through volitional tank spawning of female striped bass and male white bass is possible (Woods *et al.*, 1995). While there are many different hybridizations that have been successfully spawned, the most common hybrid utilized for aquaculture production is the

reciprocal cross or sunshine bass (Table 1.1). This hybrid cross is preferred for several reasons, including the relative ease of holding and spawning female white bass compared to female striped bass as well as the females from the other con-specifics within the genus used to create hybrid crosses (Kohler *et al.*, 1994; Smith *et al.*, 1996), the faster growth rates and lower feed conversion ratio of sunshine bass compared to both purebred and other hybrid species (Rudacille and Kohler, 2000), and that gametes are more difficult to collect from gravid wild female striped bass compared to female white bass (McGinty and Hodson, 2008). In addition, female white bass become sexually mature in a much shorter time period compared to striped bass, making them easier to maintain in captive broodstock populations compared to striped bass females. Production of other hybrid varieties, such as the palmetto and paradise bass, are limited and are mainly cultured for recreational fisheries (Harrell *et al.*, 1990).

Table 1.1-Common Hybrid *Morone* Crosses

Hybrid	***Female***	***Male***
Palmetto Bass	Striped Bass	White Bass
	Morone saxatilis	*Morone chrysops*
Sunshine Bass	White Bass	Striped Bass
	Morone chrysops	*Morone saxatilis*
Virginia Bass	Striped Bass	White Perch
	Morone saxatilis	*Morone Americana*
Maryland Bass	White Perch	Striped Bass
	Morone americana	*Morone saxatilis*
Paradise Bass	Striped Bass	Yellow Bass
	Morone saxatilis	*Morone mississippiensis*

From Harrell *et al.*, 1990.

Striped Bass Aquaculture Industry Concerns

Several industry concerns that contribute to the striped bass industry's lack of growth involve the paucity of available broodstock. To produce fingerlings, growers collect gametes from either wild or captive populations. Given the limited number of improved domesticated broodstock or even captive, acclimated broodstock, the industry's continued reliance on wild populations has contributed significantly to the lack of growth of the striped bass industry (Woods *et al.*, 1992). While both sources of gametes can result in the production of viable seedstock, they each also present a set of benefits and risks to the industry (Harrell *et al.*, 1990; Woods, 2001, Jobling *et al.*, 2010). For individuals that are collected and maintained from the wild, it is important to make a distinction between captive broodstocks (individuals who have been recently collected from the wild, maintained without selection, and acclimated to their captive environment) and domesticated broodstocks (individuals that have been genetically selected for desirable phenotypes). While domesticated broodstocks of striped bass have been successfully cultivated and maintained through academic or research efforts (Woods *et al.*, 1995; Woods, 2001; Garber and Sullivan, 2006; Fuller *et al.*, 2011), the industry currently has not elected to invest in or utilize domesticated, genetically improved broodstock primarily due to the risks and costs associated with the long-term care and husbandry of broodstock or the lack of technical expertise associated with inducing domestic animals to reproduce successfully in captivity (Woods *et al.*, 1992; Sullivan *et al.*, 1997). Studies involving the creation of genetically improved strains of domesticated striped bass (Fuller *et al.*, 2010; 2011)

have also been performed with some degree of success, but are not commonly available for commercial use.

When collected during the peak of the spawning season, gametes from wild individuals are usually of optimal quality, resulting in high fertilization and hatch rates. However, because striped bass and other moronids are seasonal, group-synchronous spawners (Sullivan *et al.*, 1997), gametes collected from wild individuals are not available year-round. Permits are required for the collection of wild fish, often restricting the number of individuals that growers are allowed to obtain during a single spawning season, thereby limiting the number of gametes they are able to collect and the number of fingerlings they are able to produce. One of the major disadvantages of using wild broodstock is the lack of genetic information available for any of the adults (and resultant gametes) used to create seedstock. Such information could be invaluable for improving production rates, allowing for the selection of individuals that exhibit desired phenotypes including: increased fecundity, larger body size, faster growth rates, and disease resistance. Finally, because the natural spawning seasons of the white bass (*M. chrysops*) and striped bass (*M. saxatilis*) only partially overlap, the production of popular hybrid crosses is often limited when dependent solely on the use of wild broodstock. These disadvantages can be partially mitigated through the development of captive populations.

The use of captive broodstock for seedstock production provides several advantages compared to the utilization of wild stocks. When kept in a photo-thermally controlled environment, growers are able to manipulate the spawning season of captive fish, allowing gametes to be produced outside of the natural

spawning season. Because the same individuals can be utilized over several years for spawning, the development of domesticated, genetically improved strains of striped bass becomes economically feasible. In addition, captive broodstock allow for striped bass production to occur in geographical areas where wild individuals may be difficult to obtain.

One of the major disadvantages associated with captive broodstock is the inherently large cost involved with the maintenance and husbandry of large, adult fish once they are collected from the wild. Populations that are being manipulated for spawning outside of the natural gametogenic cycle must be kept in strictly controlled photo-thermal conditions for gametogenesis to successfully occur, often in expensive indoor facilities with advanced lighting, heating and cooling systems. To help induce spawning and prolong the period of spermiation, gonadotropin releasing hormone agonist (GnRHa), kisspeptin, and other hormone injections are often utilized (Mylonas *et al.*, 1997, 1998; Beck *et al.*, 2012). While these treatments have been shown to effectively increase the quantity of semen and potentially extend the spawning period, gametes collected from these individuals may be of lower quality compared to those collected from wild individuals (Mylonas *et al.*, 1997).

Benefits of Cryopreservation

To meet the ever increasing demands for seafood, new techniques and methods are needed to help increase striped and hybrid bass production rates. One possible solution that can help mitigate the limited availability of improved broodstock is the use of cryopreserved, high quality gametes from selected individuals.

Storage of striped bass spermatozoa through cryopreservation provides several benefits for the striped bass industry. The cryopreservation and subsequent long-term storage of germplasm would allow growers to either alleviate or completely bypass many of the disadvantages associated with gamete collection from wild or genetically unimproved captive broodstock populations. The potential use of cryopreserved sperm spans multiple segments of the *Moronid* industry, as it can be used to help maintain genetic lines of striped bass as well as for the production of hybrids for the aquaculture industry.

Benefits of using cryopreserved striped bass sperm include:

1) Long-term storage of cryopreserved, high quality sperm, collected from males during the peak of the spawning season allows for gametes to be utilized in fertilizations far beyond the natural lifespan of the individual itself. This would lessen the number of requests for permits to collect from wild individuals and, in the case of captive fish, would allow for the collection and storage of large quantities of semen from a single proven individual. Frozen samples are easily transferrable, and would give growers in geographically isolated areas easy access to high quality sperm for seedstock production.
2) If collected and frozen in sufficient quantities, sperm from any given, "superior" individual male would be available for multiple generations of spawning, allowing for the collection of genetic information including fertility, hatch rate, viability and growth rate of the offspring. Using this data, growers would be able to create genetic improvement programs for both

purebred and hybrid striped bass through the selection and storage of gametes from phenotypically valuable individuals (Garber and Sullivan, 2006).

Cryopreserved sperm would allow for growers to continue maintaining and increasing their seedstock populations without the expensive proposition of maintaining male broodstock populations, and would allow them to spawn fish year-round irrespective of the natural spawning season.

Cryopreservation Methodology

The use of cooling agents (refrigeration, liquid nitrogen, etc.) to preserve non-germ cells at extremely low temperatures has been in development for the past half century (Gosden, 2011). When frozen and stored in liquid nitrogen (-198^{o} C), gametes are able to remain viable for several decades (Leung, 1991; Leibo and Songsasen, 2002). However, the ability of these gametes to successfully produce viable offspring through *in vivo* or *in vitro* fertilization is extremely variable, depending largely on the individual species being used, as well as the differences found between individuals of the same species. Cells undergoing the freezing process are exposed to a number of factors affecting sperm quality including intracellular and extracellular ice crystal formation, dehydration, cryoprotectant toxicity, chemo-osmotic changes, and physical/mechanical stress (Figure 1.1). If not taken into consideration, the accumulation of cryoinjuries over time can lead to the complete loss of viable cells (Figure 1.2).

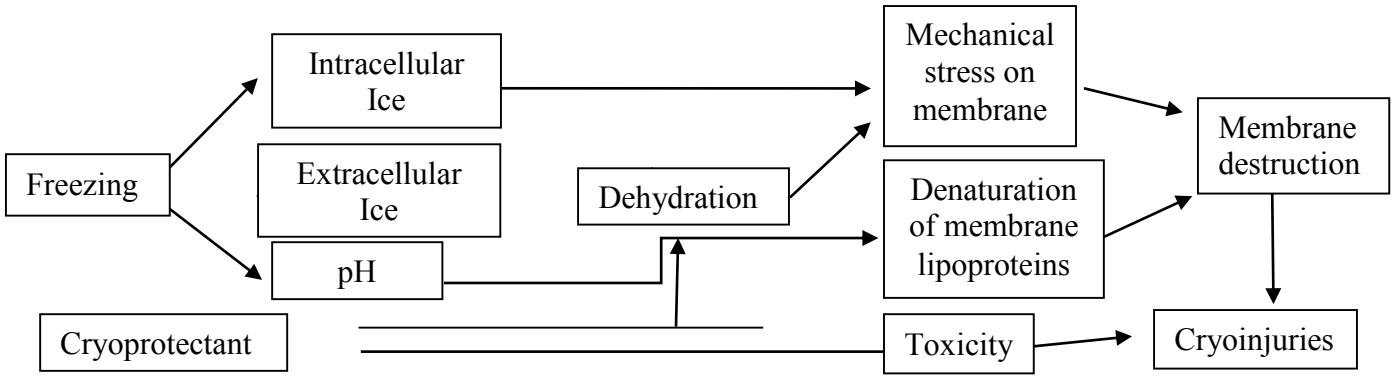


Figure 1.1- Cumulative effects of cryoinjuries during the freezing and cryoprotectant process (From Leung, 1991).

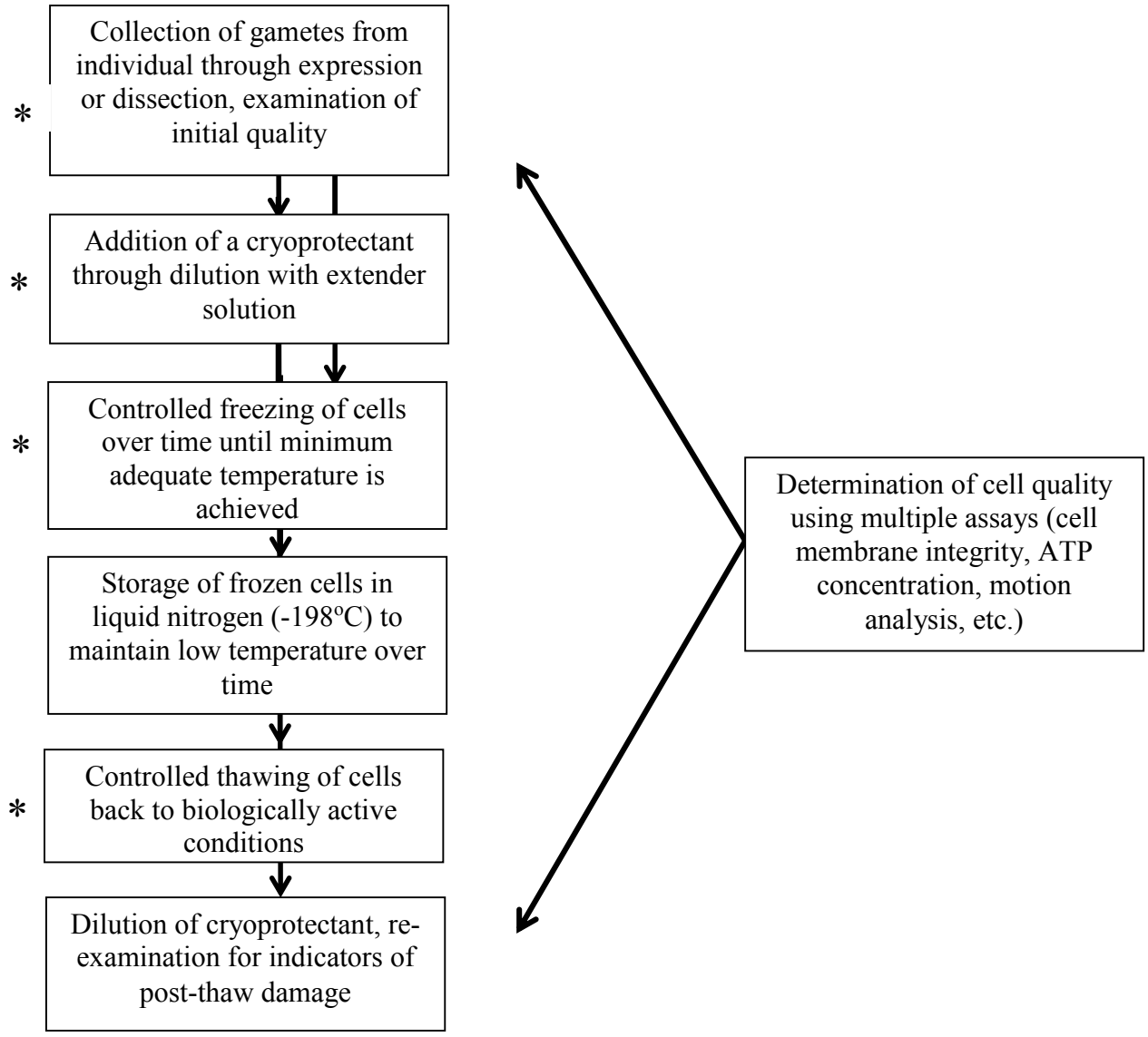


Figure 1.2- General overview of cryopreservation
* denotes procedure with potential for the occurrence of cryoinjury

Much of the research performed in the past few decades involves the reduction or elimination of these cryoinjuries though the use of cryoprotectants, extenders, and improved freezing protocols. There has also been a considerable amount of research that has examined ways to identify "high quality" semen samples from "low quality"

samples, in order to better understand the extent of the damage caused by cryopreservation, as well as to judge the effectiveness of methods designed to decrease the damage caused by cryoinjuries.

Cryoprotectants

To protect from the damages inflicted by the cryopreservation process, the use of a cryoprotectant is required to maintain viability during the freezing and thawing process. Cryoprotectants help to prevent enzyme denaturation, protein destabilization, and intra/inter-cellular ice crystal formation during the freezing and thawing process (Chao, 1991). Care must be taken when selecting a cryoprotectant to preserve fish semen, as the effectiveness of each appears to vary significantly among species. If not used carefully, damage from cryoprotectant use may occur due to toxicity or osmotic shock (Leung, 1991).

Cryoprotectants may be further sub-divided into non-permeant and permeant groups. Non-permeant substances such as sugars (trehalose, raffinose, and lactose), polyesters (polyvinyl pyrollidone), and amino acids (glycine, betaine, glutamine, and proline) are often used in conjunction with permeant cryoprotectants for preserving sperm. Non-permeant cryoprotectants function mainly through the increase of liquid-phase water in a sample at any given temperature, as well as decreasing the freezing point and increasing the surround solution's vitrification temperature (Leung, 1991). Some non-permeants, such as trehalose, act through interactions with membrane lipids and proteins, altering their phase transition behavior and hydration state, ultimately increasing the number of viable sperm post-thaw (Rudolph *et al.*, 1986;

Ahmad and Aksoy, 2012). The insertion of these non-permeant cryoprotectants into mammalian somatic cells via cell membrane pores formed by genetically engineered mutant *Staphylococcus aureus* alpha-hemolysin (Eroglu *et al.*, 2000) or by swelling activated channels (Shirakashi *et al.*, 2005) has been the focus of several studies. Because these substances are mostly non-toxic to cells, the possibility of utilizing their cryoprotective capabilities both inter- and intra-cellularly could provide a great advantage for those wishing to cryopreserve spermatozoa.

Permeant cryoprotectants, which include 1,2-propanediol, glycerol, DMSO, methanol, ethylene glycol, butanediol, and acetamide, are able to transverse the membrane-lipid bilayer and accumulate in cell cytoplasm. Glycerol, along with other permeant substances, acts mainly through the depression of freezing point (resulting in smaller ice crystal formation), in addition to the decrease of electrolyte concentrations as a sample goes through the process of freezing (Mazur, 1984). Although glycerol has been shown to be extremely effective in preserving sperm viability of many different mammalian species, it has seen limited success when applied to fish sperm as a cryoprotectant. Fish species that appear to respond positively to glycerol compared to other cryoprotectants for sperm include the bluefin tuna (Doi *et al.*, 1982), barramundi (Leung, 1987), yellowfin seabream (Gwo, 1994), and striped trumpeter (Ritar and Campet, 2000). Glycerol has been shown to negatively affect sperm viability and/or fertilization rates of many different species of fish, including the red drum (Robertson *et al.*, 1988), loach (Kopeika *et al.*, 2003), zebrafish (Harvey *et al.*, 1983), rainbow trout (Tekin *et al.*, 2007) and striped bass (Kerby, 1983). Glycerol has also been shown to negatively affect membrane

integrity, fluidity, and electrical capacitance (Parks and Graham, 1992). In many cases, the negative effects of glycerol cannot be mediated through the washing or dilution of treated cells (Harvey *et al.*, 1983).

Methanol has been found to be effective for the cryopreservation of tilapia spermatozoa (Chao *et al*, 1987) as well as for: Atlantic salmon (Jodun *et al*., 2007) spine foot fish (Chao, 1991), bitterling (Ohta *et al.*, 2001), European catfish (Ogier de Baulny *et al.*, 1999), and African catfish (Viveiros *et al.*, 2000). For channel catfish, sperm motility was maintained for up to 21 days with the addition of methanol at a 5% final (v/v) concentration (Christensen and Tiersh, 1996). For striped bass, it has been shown that, while the addition of methanol to semen samples at a concentration of 5% and 10% yielded high levels of sperm motility prior to cryopreservation, methanol is not able to preserve high levels of sperm motility post-thaw (He and Woods, 2003b).

Dimethyl-sulfoxide (DMSO) at a concentration of 7.5% was determined to be the optimal cryoprotectant for striped bass sperm and concentrations ranging from 2.5 to 10% (v/v) were found to have no effect on sperm membrane integrity after 10 minutes of exposure pre-thaw for striped bass sperm (He and Woods, 2004b). During the freezing process, it was found that preservation of membrane integrity increased concurrently with DMSO concentration when post-thaw samples were examined (Figures 1.3 and 1.4), although mitochondrial function was found to decrease with DMSO use as evidenced by a drop in ATP concentration (He and Woods, 2004b).

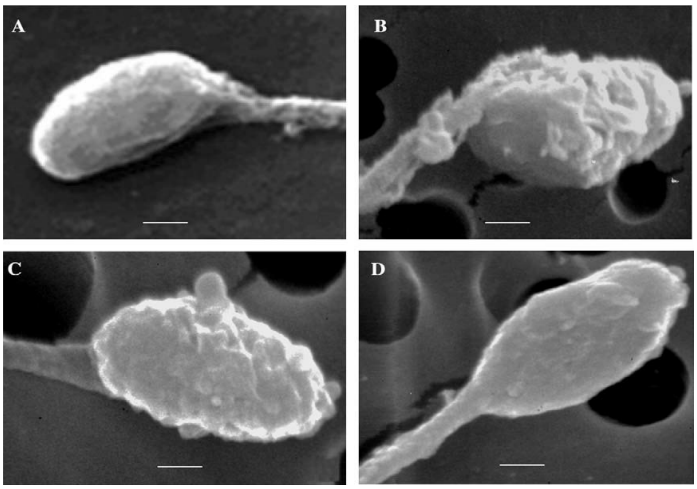


Figure 1.3- Ultrastructure of striped bass spermatozoa (40,000x). (A) fresh spermatozoa; (B) 2.5% Me_2SO post-thaw; (C) 5% Me_2SO post-thaw; and (D) 10% Me_2SO post-thaw. Bar=0.5 um. (From He and Woods, 2004b).

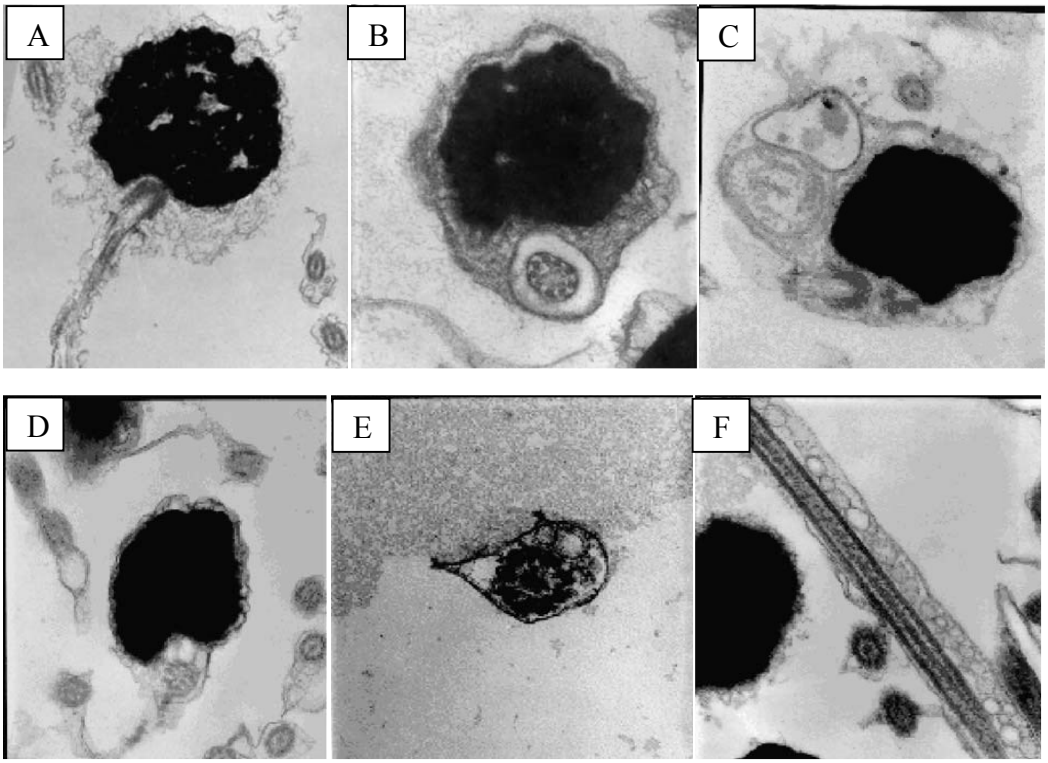


Figure 1.4- Transmission electron photomicrographs of post-thaw striped bass spermatozoa. (A) Spermatozoa cryopreserved with 2.5% DMSO (bar = 0.6 Am); (B) spermatozoa cryopreserved with 5% DMSO (bar = 0.4 Am); (C) spermatozoa cryopreserved with 10% DMSO (bar = 0.5 Am); (D) spermatozoa cryopreserved with 5% DMSO and 50 mM glycine (bar = 0.5 Am); (E) axoneme cross-section, spermatozoa cryopreserved with 2.5% DMSO (bar = 0.2 Am); (F) flagellum, spermatozoa cryopreserved with 5% DMSO (bar = 0.5 Am). (From He and Woods, 2004a).

The addition of DMSO at various concentrations has also been shown to increase the number of motile spermatozoa in post-thaw samples in several studies, although DMSO was unable to preserve post-thaw motility at fresh semen levels for any of the concentrations tested (Jenkins and Woods, 2002b; He and Woods, 2002; 2004b). When added to cryopreserved samples, DMSO has also been shown to preserve fertilization capacity (Kerby, 1983; Jenkins and Woods, 2002b; He and Woods, 2004a). DMSO in combination with glycine applied to cryopreserved sperm has been shown to produce fertilization rates statistically similar to those obtained using fresh samples (He and Woods, 2003a). Based on past research examining motility, membrane integrity, and mitochondrial function, DMSO has been found to be one of the most effective cryoprotectants currently available for cryopreserving striped bass sperm, especially when combined with other non-permeable protectants such as glycine.

Extenders and Diluents

When used as a diluent for semen, extenders help to preserve the viability, motility and fertilization capacity of spermatozoa during short-term periods of storage. For teleost species, semen extenders serve several functions, including:

1- Prevention of sperm motility until activation and movement is desired,
2- Prevention of wasteful energy utilization and mitochondrial function due to unwanted activation of motility,
3- Prevention of cell membrane degradation due to processes such as perioxidation,

4- Prevention of cell membrane damage and death due to crenation and/or hypotension as a result of intra- and inter-cellular osmotic imbalances, and
5- Prevention of damage during cryopreservation through the addition of cryoprotectants

Because chemical and osmotic properties of semen vary widely from species to species, extenders must be carefully selected to be optimally effective. Various experiments examining extender formulas for effectiveness have been performed for each fish species whose sperm are being stored under hypothermal conditions including the striped bass (Jenkins and Woods, 2002a,b; He and Woods, 2003a,b, 2004a,b; Guthrie *et al.*, 2008, 2011).

It has been found that multiple factors influence the effectiveness of a particular extender formula concerning the retention of sperm quality, either during short term hypothermic storage or long term storage via cryopreservation. Early experiments examining the effects of various extender formulas on striped bass spermatozoa utilized a relatively simple solution, containing NaCl, KCl, $CaCl_2$, H2O, and NaH_2PO_4. Combined with cryoprotectants (fructose, lecithin, or mannitol with DMSO), semen was cryopreserved, stored long term, and utilized for successful fertilization of female striped bass eggs (Kerby, 1983). Later experiments utilized various extender formulas that also included components such as egg yolk, $NaHCO_3$, glucose, and KCl in varying concentrations (Jenkins and Woods, 2002a). Calcium and magnesium have been found to play an inhibitory function concerning striped bass sperm motility (He *et al.*, 2004), with a substantial drop in sperm motion when activated with deionized ultra-filtered water (DIUF) solutions containing Ca^{2+} or

Mg^{2+} ion concentrations of 5mM or greater. Calcium was unable to halt sperm motility when added to samples that have already been activated using calcium-free distilled water, indicating a possible link between initiation of sperm motility and voltage gated channels (He *et al.*, 2004).

Osmolality has been shown to have a great effect on an extender's ability to preserve the motility, mitochondrial function, and viability of striped bass spermatozoa. When osmolality was adjusted using sodium chloride, both hypotonic and isotonic solutions relative to striped bass seminal plasma (350mOs/kg) still allowed for some levels of sperm activation. Only hypertonic solutions with salt concentrations of at least 600mOs/kg were able to completely hold cells in quiescence. When examined over a period of 30 minutes, the 500mOs/mg extender was best able to preserve sperm motility, and during freezing experiments, allowed for the highest retention of post-thaw motility (He and Woods, 2003b). This effect was also seen in another study, where axoneme motility of de-membranated sperm were inhibited in the presence of hypertonic extenders (He *et al.*, 2004). Although activation of sperm motility could be avoided with these hypertonic solutions; they have also been shown to cause severe cellular dehydration during longer periods of storage, with detrimental effects to both membrane integrity and cell membrane viability after approximately 30-45 minutes of equilibration. In contrast, isotonic solutions have been shown to retain significantly higher levels of membrane integrity (79.9%) and ATP concentration (2.94 pmol/ 10^6 cells) after a 20 minute period of time (Guthrie *et al.*, 2008). Even though they are not able to hold cells quiescent, isotonic solutions are able to provide better results for cells that are being held for

longer periods of time under refrigeration without the intention of cryopreservation (He and Woods, 2003b).

Sperm are able to remain viable in a broad range of pH. High levels of motility were expressed by cells activated with de-ionized water at a pH between 6.1 to 8.1. Above or below this range, motility was slightly decreased, with a precipitous drop off below 5.0 and above 8.5 (He *et al.*, 2004). Like osmolality, results suggest that a pH close to that of striped bass seminal plasma seems to be best for extending striped bass semen samples.

The work mentioned above has provided an excellent basis from which an extender can be formulated that is able to better preserve striped bass spermatozoa in terms of motility, mitochondrial function (ATP), and cell membrane viability. To date, the most effective striped bass extender (SBE) has been developed (Table 1.2) and published by the Woods' aquaculture laboratory at the University of Maryland - Crane Aquaculture Facility (Woods *et al.*, 2009).

Table 1.2- Woods' Striped Bass Extender (SBE)

Component	***Concentration***
NaCl	*680mg/1400mg**
KCl	40mg
$NaHCO_3$	200mg
Glucose	100mg
Glycine	75mM
De-ionized Ultra Filtered Water (DIUF)	100mL
*DMSO	*7.5% (v:v)*

Concentrations listed above result in an osmolality of 350mOs/kg at a pH of 7.5.
* Denotes modifications/additives for use in cryopreservation.

Freezing Rate

The rate at which sperm are cooled during cryopreservation has been shown to play an important role in preserving the viability, motility, and integrity of those cells. Because sperm vary so widely in morphology from species to species, the rate at which each is frozen must be chosen with care to achieve the best results. Determination of an optimal freezing rate ensures that cells undergoing cryopreservation are frozen fast enough to prevent or reduce exposure to harmful concentrations of solutes, yet slow enough to allow the escape of water from the cell, minimizing damage from ice crystal formation (Leung, 1991).

Multiple studies have been performed, prior to my research, to determine the optimal freezing rate for striped bass. In each study, semen samples were frozen at various rates ranging from -10 to -40^{o}C/minute, stored for a period of time under

liquid nitrogen, and then examined post-thaw for motility using *non*-computer assisted sperm analysis methods. Unfortunately, results from these studies have not been concordant, and recommendations have historically ranged from -16^{0}C/minute to -40^{o}C/minute (Jenkins and Woods 2002b; He and Woods, 2004a; Thirumala *et al.* 2006). As the most recent publication, Thirumala *et al.* utilized a cell water transport model to predict that a slower optimal freezing range of -14 to -20^{o}C/minute for striped bass sperm cryopreserved with DMSO. This prediction was then tested empirically, examining the effects of three cooling rates (-4, -16, and -40^{o}C/minute) on sperm motility. While the use of various water transport models have been successful in predicting the optimal freezing rate for other species (Thirumala *et al.*, 2003, 2005), the methodology and conclusions of this particular study were extremely flawed. By using a novel protocol to cryopreserve only one of the experimental rates (-16^{o}C/minute), it would be impossible to compare or contrast the data obtained for all three freezing rates accurately. Results from this study were also inconsistent, as the authors found extremely low levels of motility (a maximum average of 16%) or a complete absence of motility post-thaw for all of the rates tested, and no significant differences between any of the treatment rates. In spite of these issues, a recommendation of -16^{o}C/minute was still made and published (Thirumala *et al.*, 2006). Because the freezing rate plays such a critical role in sperm cryopreservation, it is extremely important that the correct recommendation be made, using multiple methods of assessment to delineate between "good" and "poor" quality semen samples post-thaw.

Sperm Quality Assessment

Sperm undergoing short term storage via refrigeration or long term storage via cryopreservation suffer from various forms of damage, each of which can have a profound impact on fertilization. Much of the work mentioned above has aimed to ameliorate or eliminate the amount of damage caused to various components of the cell (axoneme, cell membrane, mitochondria, etc.). Because fertilization is potentially dependent on multiple, positive sperm characteristics (motility, cell membrane integrity, ATP concentration, etc.) to successfully transfer its genetic information to the egg, it is important that studies examining the optimal conditions under which sperm are cryopreserved and stored employ multiple methods of evaluation.

Mitochondrial Function- ATP Assays

Compared to mammalian spermatozoa, striped bass sperm are motile for a relatively short period of time (30-45 seconds). When compared to other teleost species, striped bass sperm appear to have much lower levels of ATP available for use in motility (Perchec *et al.*, 1995; Christen *et al.*, 2005). Commensurate with these observations, the use of electron microscopy has revealed the presence of only two mitochondria contained within striped bass sperm (Figure 1.4). When de-membranated, striped bass sperm are unable to utilize exogenous ATP for motion (He *et al.*, 2004), and are also unable to manufacture new ATP via mitochondrial function or oxidative phosphorylation (Guthrie *et al.*, 2008). Quantification of ATP in striped

bass sperm has been performed in several studies using multiple assay techniques (He and Woods, 2004b; Guthrie *et al.*, 2011). The most recent technique utilizes the chemiluminescent luciferin-luciferase reaction to quantify the amount of ATP in a given sample. This assay has been well documented and verified over the past three decades (Spielmann *et al.*, 1981; Kiesslich *et al.*, 2003), and has been utilized successfully to determine somatic and gametic tissue ATP concentrations in several different species, such as the mouse (Allen *et al.*, 2002), zebrafish (Jagadeeswaran *et al.*, 1999), Atlantic sturgeon (Dorsey *et al.*, 2011), and bovine (Rieger, 1997). When added to a tissue sample, luciferin reacts with ATP to form luciferyl adenylate. Luciferyl adenylate is then oxidized, causing the formation of H_2O, CO_2, oxyluciferin, and light. Converted to a logarithmic scale, light intensity is directly correlated to ATP concentration, most commonly quantified as picomoles per million cells. Because striped bass sperm have such limited amounts of energy for motility and are seemingly unable to utilize or produce new ATP from endogenous sources, ATP quantification is an extremely relevant method to compare and contrast semen samples for differences in quality.

Membrane Integrity- Sybr-14 / Propidium Iodide Assay

Morbidity exhibited by sperm during storage and cryopreservation can be partly attributed to damage to the sperm membrane. Much of the past work examining osmolality, pH, ions, along with the concentration of extenders and cryoprotectants, has been in part to prevent perturbations and damage (oncosis, crenation, etc.) to the cell membrane. Sperm membranes are composed largely of phospholipids (65-70%),

which allow for a large degree of membrane fluidity, as well as sterols which help to provide stabilization as the membrane undergoes shifts from fluid to gel phases (Holt, 2000). When spermatozoa are frozen, rapid fluid to gel phase transitions occur as temperature decreases, causing membrane destabilization that leaves cells more vulnerable to damage from ice crystal formation and the effects of cold shock (Parks and Lynch, 1992; Drobnis *et al.*, 1993). During the thawing process, cell membranes are also subjected to osmotic shock, as rapid changes in osmotic concentrations occur both inter- and extra-cellularly.

To determine the extent of damage caused by hypothermic storage or freezing, an assay can be performed utilizing two fluorescent dyes. Sybr-14 is a membrane-permeant nucleic acid stain that fluoresces green when added to a cell sample. In contrast, propidium iodide (PI) is a membrane-impermeant nucleic acid stain that fluoresces red when in contact with cells' DNA which can only occur when cells exhibit compromised membrane integrity. When added in conjunction to a semen sample, the Sybr14-PI combination stain allows for a differentiation between cells that have intact membranes (i.e. "live" cells that fluoresce green) and cells that have compromised membranes (i.e. "dead" or "moribund" cells that fluoresce red). A fluorescence activated cell sorter (FACS) can then be utilized to obtain accurate quantification of the number of Sybr-14 and/or PI positive cells. This assay has been used successfully to examine differences in sperm quality from a number of fish species including: the European catfish (Ogier de Baulny *et al.*, 1999), Nile tilapia (Segovia *et al.*, 2000), Atlantic cod (DeGraff and Berlinsky, 2004), haddock (DeGraff and Berlinsky, 2004), Brazilian flounder (Lanes *et al.*, 2008), Atlantic sturgeon

(Dorsey *et al.*, 2011) and striped bass (He *et al.*, 2004; Guthrie *et al.*, 2008, 2011). The use of Sybr14 and PI is a excellent method to evaluate effectiveness of experimental freezing rates in preventing damage to sperm membranes during the freezing and thawing processes.

Sperm Motion Characteristics- Computer Assisted Sperm Analysis (CASA)

Motility is perhaps the most common method used to evaluate sperm quality and has been correlated to fertilization capacity in various species such as the common carp (Linhart *et al.*, 2000), rainbow trout (Lahnsteiner *et al.*, 1998), and summer flounder (Brown *et al.*, 2012). Historically, motion analysis has been performed by an observer activating an aliquot of sperm and estimating the percentage of moving cells in a single field of view. To increase the accuracy, video recordings of the cell movement were often utilized to allow the observer to examine the same sample multiple times. These estimations, while often fairly accurate, are subject to some degree of error depending upon the observer's experience and skill level.

The utilization of a computer assisted sperm analysis (CASA) system allows for the collection of unbiased data on sperm motion characteristics. These characteristics include not only total numbers of moving cells, but secondary characteristics such as velocity, beat frequency, direction of movement (i.e. straight vs curvilinear) along with other specific measurements unobtainable by non-CASA methods. While primarily used for mammalian research, CASA units have successfully been used to examine sperm motion characteristics of several fish species (Table 1.3) such as the

Summer flounder (Brown *et al.*, 2012), African catfish (Rurangwa *et al.*, 2001), common carp (Christ *et al.*, 1996), red seabream (Liu *et al.*, 2007), Atlantic sturgeon (Dorsey *et al.*, 2011) and European sea bass (Felip *et al.*, 2006). Multiple publications have also suggested that CASA provides a simple and quick quantitative assessment of fish sperm quality and in some cases has been correlated to fertilization capacity (Kime *et al.*, 2001; Fauvel *et al.*, 2010). Because CASA has never been used previously to examine the motion characteristics of striped bass, this study is the first to provide quantitative data on multiple motion characteristics for both fresh and post-thaw striped bass sperm. The results obtained from these analyses will provide yet another method of objectively and quantitatively differentiating between striped bass sperm quality.

Table 1.3

Species	***P. dentatus***	***C. gariepinus***	***H. hippoglossus***	***P. major***	***A. oxyrinchus***	***D. labrax***	***O. eperlanus***
MOT (%)	49.5	98.5	46.5	64.7	90.0	55.0	53.7
VCL (µm/sec)	115.1	56.0	99.6	-	207.0	131.0	79.9
BCF	-	-	-	-	-	5.0	-
LIN	86.5	-	81.5	-	-	-	40.6
STR (%)	-	-	-	-	-	-	-
VAP (µm/sec)	104.3	46.0	-	113.1	-	-	55.7
VSL (µm/sec)	115.1	38.0	82.3	-	-	46.2	38.7
Source	Brown *et al.*, 2012	Rurangwa *et al.*, 2001	Ottesen *et al.*, 2009	Liu *et al.*, 2007	Dorsey *et al.*, 2011	Felip *et al.*, 2006	Krol *et al.*, 2009

CASA motion analysis characteristics for several species assembled from multiple publications.

Chapter 2- The Effects Of Freezing On Striped Bass (*Morone Saxatilis*) Spermatozoa[1]

[1]T.E. Frankel, D.D. Theisen, H.D. Guthrie, G.R. Welch, L.C., Woods III. 2013. The effect of freezing rate on the quality of striped bass sperm. Theriogenology. 79(6): 940-945.

Abstract

Several studies have been conducted in an attempt to determine the optimal freezing rate for the cryopreservation of striped bass (*Morone saxatilis*) spermatozoa. In this study, the effects of freezing rate (-10, -15, -20 and -40ºC/min) on gamete quality was examined, utilizing Sybr-14 and propidium iodide to confirm viability (sperm membrane integrity), ATP concentration using a luciferin-luciferase bioluminescence assay and a Hamilton-Thorne CEROS™ system to characterize striped bass sperm motion. Adult male striped bass (n=12) were sampled once a wk for five weeks. Collected samples were extended, cryo-protected using a 7.5% (v/v) dimethyl sulfoxide (DMSO) final concentration solution and frozen using a Planer Kryosave controlled-rate freezer. The samples were stored in liquid nitrogen for 49 d, after which sperm quality was re-evaluated post-thaw, utilizing the same methods. Sperm cryopreserved at -40ºC/min resulted in means for total motility (10.06%), progressive motility (7.14%), ATP concentration (0.86 pmol/million cells), and sperm viability (56.5%) that were greater ($P < 0.05$) than the lower cooling rates. These results demonstrate that -40ºC/min was the optimal freezing rate among those tested for the cryopreservation of striped bass sperm.

Introduction

Hybrid crosses with members of the teleost family Moronidae are widely utilized in fishery management and aquaculture. The most common cross used for commercial purposes involves *in vitro* fertilization of eggs obtained from white bass (*Morone chrysops*) females with sperm expressed from striped bass (*Morone saxatilis*) males. Hybrid progeny exhibit several highly desirable traits from an industry perspective, including faster growth, hardiness and adaptability to environmental conditions and stressors (Harrell *et al.*, 1990). Although there is currently a lack of fundamental knowledge regarding the molecular basis of hybrid vigor; there have been promising results with recurrent selection of *Morone* broodstock, based on the performance of their progeny (Garber and Sullivan, 2006).

To create this hybridization, semen is collected from spermiating striped bass males during the spring spawning season, which may often be prolonged in male striped bass by the administration of a gonadotropin-releasing hormone agonist (GnRHa) to induce spermiation and increase milt production (Woods and Sullivan, 1993) or with the human chorionic gonadotropin product Chorulon® as it has recently been approved by the U.S. Food and Drug Administration as a spawning aid for all brood fish (Jobling *et al.*, 2010). More than 220 species of finfish and shellfish are now farmed, with production rates of over 29 million tons (Naylor *et al.*, 2000). As part of the total production rate, the striped bass aquaculture industry has been estimated to be as high as fifth in volume and fourth in value of all food fish grown in the U.S. (Carlberg *et al.*, 2000). To increase production rates, one of the major needs cited is the efficient production and survival of larval fish in order to ensure that

adequate numbers of fingerlings are available on a year-round basis to producers (Carlberg *et al.*, 2000; Woods, 2005). The striped bass industry is very dependent upon wild individuals for gametes, creating a challenge for the development of domesticated striped bass and white bass brood stock (Woods, 2005) coupled with a genetic improvement program (Jobling *et al.*, 2010). Cryopreservation is commonly used to preserve semen samples for long-term storage and for use with asynchronous and geographically isolated spawning populations, such as the white bass and striped bass (Harrell *et al.*, 1990). To date, successful fertilization of *Morone* eggs has been achieved in several studies utilizing cryopreserved striped bass sperm (Kerby, 1983; Kerby *et al.*, 1985; He and Woods, 2004a).

Multiple experiments have been conducted in an attempt to determine the optimal freezing rate for the cryopreservation of striped bass semen (He and Woods, 2004a; Jenkins and Woods, 2002b; Thirumala *et al.*, 2006), examining a range of freezing rates from -10 to -40^{o}C/min.

Our study was designed to confirm the optimum freezing rate for striped bass sperm and to quantify additional information on the effects of cryopreservation on cell viability, motion and energetics (ATP content).

Materials and Methods

Research Facilities

This research was performed at the University of Maryland Crane Aquaculture Facility (UMCAF) in College Park, Maryland in addition to the Beltsville Agricultural Research Center (BARC) located in Beltsville, Maryland.

Care and Housing

The fish utilized in this study were housed in a single recirculating aquaculture system (RAS). The system was composed of four ten foot 2,000-gallon tanks equipped with a fluidized biofilter, drumfilter, oxygen cone, foam fractionator, ozone system and photo-thermal controls. Temperatures ranged from 9^{o}C during the wintering period to 18^{o}C during the spawning season. A water replenishment tank was used to maintain tank volumes over time, as well as salinity (5-6ppt) and calcium (150-200ppm). Tests for ammonia (<.05mg/L), nitrite (<.025mg/L), and nitrate (<75mg/L) were performed weekly to ensure optimum water quality. Oxygen levels (7-10ppm) and pH (7.75-8.3) were monitored daily.

Animals

A population of full sibling, eight year old striped bass males was used for this experiment. Males were assessed for good body condition and spermiation prior to the start of the spawning season (Hodson *et al.*, 1999). Each fish was previously

tagged with a subcutaneous passive integrated transponder (PIT) tag for identification.

Anesthetization

Water from the recirculating aquaculture system was placed into a 300L plastic tank and aerated to maintain adequate oxygen levels. Tricane Methanesulphonate (MS-222) was added to a concentration of 100 mg/L, and pH adjusted to 8.0 using sodium bicarbonate. Fish were placed into the anesthetic bath until total loss of equilibrium was achieved, along with a slow but steady opercular rate. After anesthetization, fish were removed from the bath and semen was collected using the procedure outlined below.

Semen Collection

Eight year-old striped bass males (n=12) were selected from a population maintained under computerized photo-thermal control (Version 2.72 Grafix Eye Lutron GRX-PRG) at the University of Maryland's Crane Aquaculture Facility. Four different individuals were sampled each day, three days a week for five weeks to ensure that all twelve individuals were sampled. After reaching the appropriate level of anesthetization, fish were removed from the bath and manually restrained. To prevent contamination, slight pressure was applied to the urogenital vent to expel urine, and then the vent was wiped clean with absorbent wipes. Each semen sample (~5 mL) was then expressed into sterile, 50 mL conical tubes and immediately placed

over ice. One mL of semen was then removed from each sample and used to determine baseline sperm concentration (total number of sperm/mL), viability, ATP and motion characteristics prior to freezing.

Extender, Cryoprotectant and Cryopreservation

Freshly collected samples were extended 1:3 (v:v) using our lab's striped bass extender (SBE) (Table 1.2) modified to an osmolality of 550 mmol/L using NaCl. Hyperosmotic extenders have been shown to improve post-thaw quality of cryopreserved striped bass sperm samples when quickly frozen following dilution (Woods *et al.*, 2009; He and Woods, 2003b). Dimethyl sulfoxide was then added as the cryoprotectant to obtain a final concentration of 7.5% (v/v), which has been shown to help preserve sperm plasma membranes and protect mitochondrial function during the freezing process (He and Woods, 2004b). Following a 10 minute equilibration period, a set of six 250 µL volume aliquots from a single individual were pipetted into 500 µL Cassou straws and immediately heat sealed. The set of six straws was then placed into a programmable freezer (Planer Kryosave-Model KS30) and frozen at a rate of -10, -15, -20 or -40^{o}C/minute. Once the straw's core temperature of -120^{o}C was reached (as determined by a thermister placed into an extra straw containing the treatment mixture), the straws were removed from the freezer and immediately placed into dewars containing liquid nitrogen for storage. The initial freezing rate used was rotated for each day of each sampling week to account for potential differences in the time samples were frozen from the time they were collected. In all cases, semen samples were frozen in no longer than 10 minutes

after being exposed to extender and cryoprotectant; equivalent to the time it takes to load and seal the straws following extension. Samples were stored for 49 days under liquid nitrogen, after which they were thawed for 6 seconds in a 40°C water bath and re-evaluated for post-thaw viability, ATP, and motion characteristics.

Concentration, viability and motion analysis

Concentration

To determine initial sperm concentrations of the freshly collected semen, 10 µL of neat semen was added to 3990µL of de-ionized ultra-filtered water (DIUF) in a 5 mL conical tube to activate the sperm (1:400 (v:v) dilution). In order to obtain accurate readings, sperm were held in DIUF for one minute until all movement had ceased. After gently homogenizing the sample, 15 µL of the diluted sample was placed onto a Makler counting chamber (Sefi Medical Instruments, Haifa, Israel) and analyzed utilizing a Hamilton-Thorne CEROS™ computer assisted sperm analysis system (CASA). Cell counts performed by the CEROS unit were validated using a Makler counting chamber (Appendix A). Cell concentration estimations were then utilized for both viability and ATP assays.

Viability

Striped bass sperm were examined for viability using a LIVE/DEAD sperm viability kit (Invitrogen (L-7011)) both before and after freezing. The same procedure was utilized for both fresh and post-thaw samples. For the post-thaw

samples, Cassou straws were placed in a 40ºC water bath for 6 seconds, after which the sample was expelled into a test tube and immediately diluted with SBE (osmolality of 350 mmol/kg) to create a sperm concentration of $6.4x10^6$ cells/mL. To each straw's diluted semen, 9.6μM of propidium iodide and 0.14 μM Sybr-14 molecular stains were added. The mixture was incubated for 5 minutes on ice, after which it was run on a BD FACSVerse cytometer for analysis via FACSuite software. For the purposes of this study, cells that fluoresced both green and red were considered moribund but counted as "dead", since a red fluorescence signal can only be indicated by sperm whose membranes are compromised.

ATP

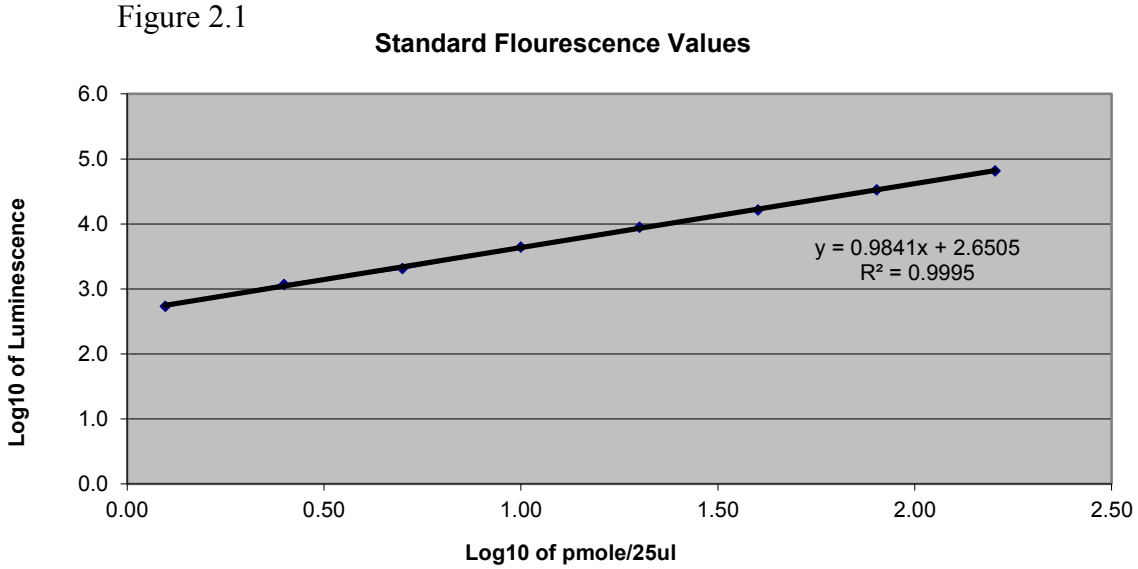
Adenosine 5′-triphosphate (ATP) concentration for each sample was determined with a firefly-luciferase bioluminescence procedure, using an ATP assay mix purchased from Sigma-Aldrich. The same procedure was utilized for both fresh and post-thaw samples. For the post-thaw samples, Cassou straws were placed in a 40ºC water bath for 6 seconds, after which the sample was expelled into a test tube in preparation for analysis.

ATP standard prep

To each well of a white-bottomed, 96 well micro-titer plate, 50 μL of DIUF was first added. Nine ATP standards (0.625, 1.25, 2.5, 5, 10, 20, 40, 80 pmol/ mL) were created from an ATP disodium salt hydrate stock solution (Sigma-Aldrich (FLAAS-1VL)) and 25 μL of each standard was added to wells 1 to 27 in triplicate.

Fluorescence values from these standards were then used to create a standard curve from which sample ATP concentrations could be estimated (Figure 2.1). A new set of standards was run for each plate of samples to account for any changes in environmental or handling conditions.

Figure 2.1



ATP Standard	*Log_{10} of pmole/25uL*	*Log_{10} of Luminescence*
80	0.097	2.73
40	0.398	3.07
20	0.699	3.31
10	1.000	3.64
5	1.301	3.95
2.5	1.602	4.21
1.25	1.903	4.53
0.625	2.204	4.82

Sample prep

A sample aliquot equivalent to $40x10^6$ sperm/mL was first created using an ATP assay mix dilution buffer (Sigma-Aldrich (FLAAB-1VL)) as a diluent. Immediately after diluting, 10 µL of Phosphatase Inhibitor Cocktail (Sigma-Aldrich (P5726-1VL)) consisting of sodium vanadate, sodium molybdate, sodium tartrate and imidazole was added. Addition of an inhibitor cocktail has been shown to help preserve ATP normally dephosphorylized by acid, alkaline and tyrosine phosphatases (Meynard *et al.*, 2009). The inhibited samples were then allowed to rest at room temperature for 30 min and subsequently placed into a -80^{o}C freezer for storage until all samples were collected and prepared for the five week duration. The frozen samples were then placed in a boiling water bath for 10 minutes. After boiling, the samples were centrifuged for 5 minutes at 23x100 g, and the resulting supernatants removed into separate 1.5 mL snap-cap vials. 25 µL of each was then added in triplicate to the remaining empty wells. To each well containing a standard or sample, 100 µL of working assay mixture (consisting of ATP assay mix and ATP assay mix dilution buffer) was added to initiate the luciferin-luciferase reaction. The entire plate was then analyzed using a Tecan® SpectraFlour bioluminescence plate reader. Samples were analyzed within 5 min after the working assay mixture was added to each well. The standard curve was calculated from the fluorescence readings and the mean luminescence for each sample was converted to $\log_{10}$ for determination of ATP concentration in pmol/million cells (Long and Guthrie, 2006).

Motion analysis

For motion analysis, 15 µL of DIUF was first added to a Makler counting chamber (Sefi Medical Instruments, Haifa, Israel) and inserted onto a Hamilton-Thorne Bionomic® temperature controlled stage mounted onto a negative phase contrast Olympus CX41 microscope. The DIUF was allowed to chill down to a temperature of 4ºC $\pm$ 2ºC. The addition of sperm to DIUF results in simultaneous activation and using the Hamilton-Thorne CEROS™ (version 12, Hamilton-Thorne, Beverly, Maine) CASA, allowed for rapid tracking of sperm motion. After the Makler reached temperature, an undiluted fresh or post-thaw semen sample containing between 350 to 500 sperm was added to the Makler chamber and the contents quickly mixed. Sperm activity was then immediately recorded and analyzed using a CEROS™ CASA. For each sample, a single 0.5 second acquisition was made within 5 seconds of activation. Parameters measured included total motility; progressive motility, defined as cells exhibiting a path velocity (VAP) greater than 80 µm/s and straightness (STR) greater than 80%), and curvilinear velocity (VCL) measured in µm/s. Percent linearity (LIN), % straightness (STR), path velocity (VAP) in µm/s and progressive velocity (VSL) in µm/s were calculated for all sperm in the field of view for the 0.5 second period (Table 2.1). Image capture settings were adjusted to a frames per second (fps) rate of 60hz and the number of frames adjusted to 30. Because striped bass sperm are physically smaller relative to mammalian species, the minimum cell size was adjusted to 2 pix with the minimum contrast adjusted to 45.

Table 2.1

Total Motility (%)	Percentage of cells moving in frame
Progressive Motility (%)	Percentage of total cells with minimum path velocity (VAP), straightness (STR), and curvilinear velocity (VCL) as defined by user
Smoothed Path Velocity (VAP)	Time/average of sperm head along its spatial average trajectory
Track Velocity (VCL)	Time/average velocity of a sperm head along its actual curvilinear trajectory
Straight Line Velocity (VSL)	Time/average velocity of a sperm head along the straight line between its first detected position and its last position
Beat Cross Frequency (BCF)	The time-average rate at which the curvilinear sperm trajectory crosses its average path trajectory
Linearity (LIN)	The linearity of the curvilinear trajectory LIN=(VSL/VCL) x 100
Straightness (STR)	Linearity of the spatial average path STR=(VSL/VAP) x 100

Statistical analysis

Data analysis was performed using SAS 9.2 (SAS Institute, Cary, NC). A repeated measures, mixed-model analysis of variance was utilized, with the p value for significance set at 0.05 (Little *et al.*, 1996). Homogeneity of treatment variance and normal distribution of residual errors were both examined prior to the selection of covariance structure. An LSMEANS statement using the DIFF option was used for comparison of least squares means. For the statistical model freezing rate was considered a fixed effect, while the population of individuals used for the duration of the experiment was considered a random effect.

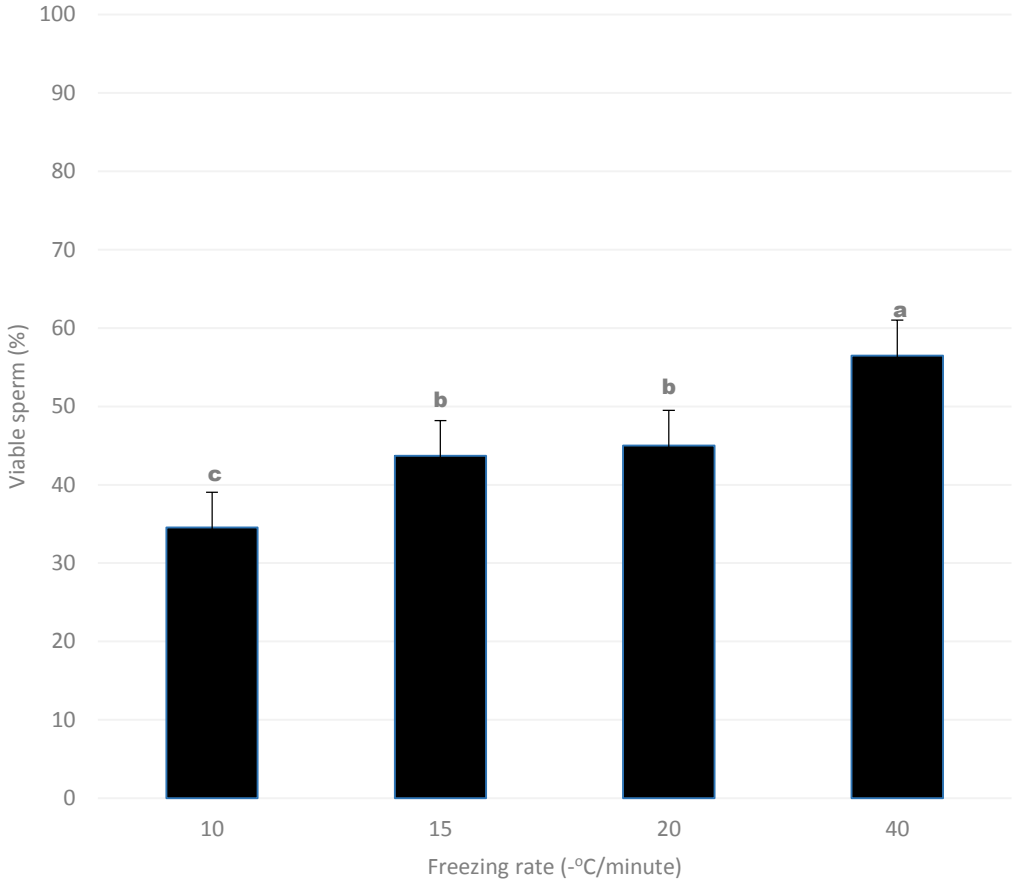
Results

Viability

The mean percentages of viable striped bass sperm over the course of the five week spawning period were significantly higher in treatments utilizing the faster freezing rates (Figure 2.2). An average of 96.60 $\pm$ 0.10% SEM was obtained for the fresh data over the same time period (not shown). The -40ºC/min freezing rate was found to exhibit the greatest mean percentage of viable sperm (56.50 $\pm$ 0.10% SEM) post thaw and was significantly different from all other freezing rates. In contrast, the -10ºC/min freezing rate was found to exhibit the lowest mean percentage of sperm with intact cell membranes (34.54 $\pm$ 0.10% SEM). Mean viability values obtained from the intermediate rates of -15ºC/min and -20ºC/min were better than the slower rate and not statistically different from one another (Figure 2.2).

Figure 2.2

The Effects of Freezing Rate on Sperm Viability Over Five Weeks



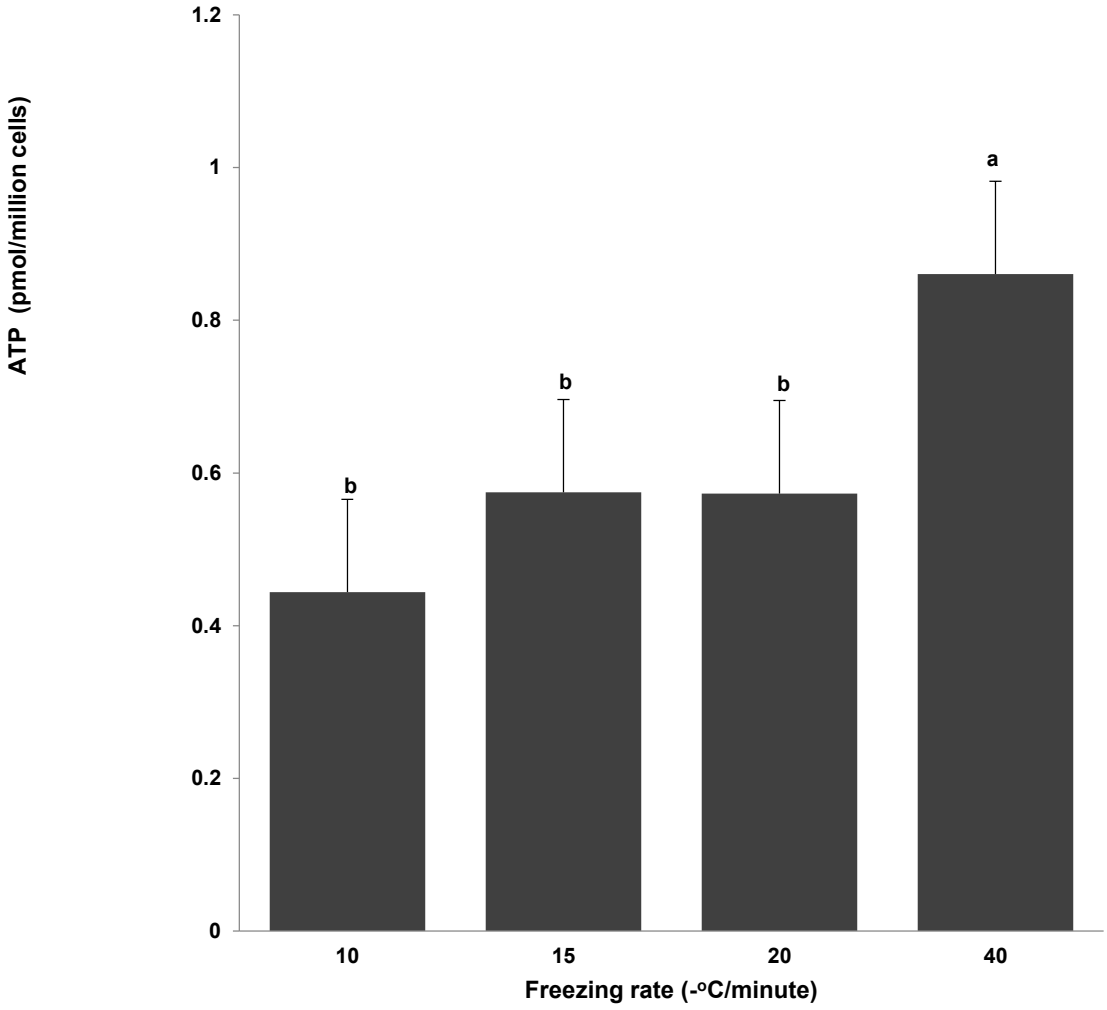
Mean percentages of viable post-thaw striped bass male (n=12) sperm exhibiting green fluorescence (maximum absorption at 488nm and emission at 518nm) when cryopreserved at different freezing rates over the five week spawning period. Bars represent SEM. [a-c]Means without a common superscript differed (P<.05).

ATP

Adenosine triphosphate concentration in striped bass sperm was determined for both freshly collected and post-thaw samples over the final three weeks of our five week spawning period (Figure 2.3) when semen samples were at or approaching peak quality and concentration for the entire population. Fresh samples had an average ATP concentration of 1.18 $\pm$ 0.007 SEM pico moles per million cells (not shown). Sperm cryopreserved using the fastest freezing rate we examined of -40^{o}C/min, contained significantly higher levels of ATP (0.86 pmol/million cells $\pm$ 0.007 SEM) than the other freezing rates. All other freezing rates were found to be insignificantly different from one another. (Figure 2.3)

Figure 2.3

The Effects of Freezing Rate on ATP Concentration



Mean ATP concentration of post-thaw striped bass male (n=12) sperm (pmol/million sperm) when cryopreserved at different freezing rates over a three week period. Bars represent SEM. [a-c]Means without a common superscript differed (P<.05).

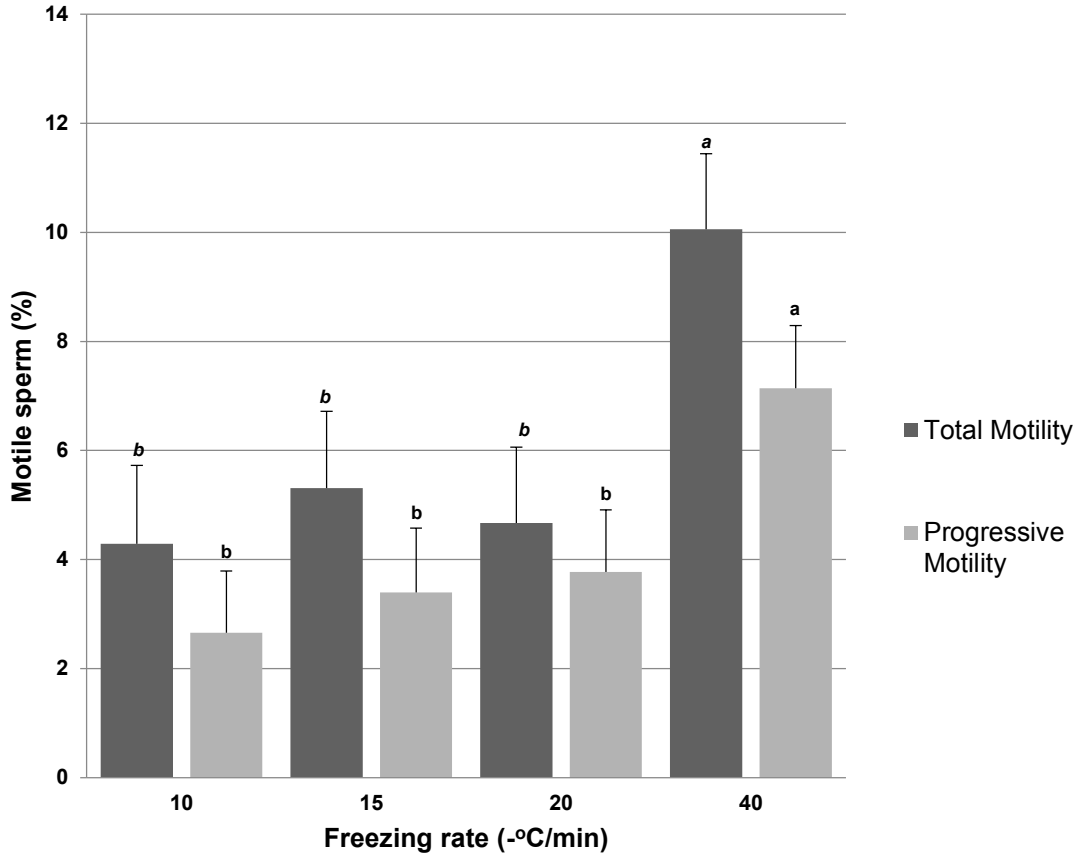
Motion analysis

For each freezing rate, total motility for every post-thaw sperm sample was greater than the corresponding sample's estimate of progressive motility (Figure 2.4). An average total motility of 54.64 $\pm$ 0.085% SEM and progressive motility of 42.81 $\pm$ 0.085% SEM was obtained for fresh samples (not shown). For both total and progressive motility, post-thaw sperm that were frozen using the more rapid -40^{o}C/min rate exhibited significantly higher mean percentages of motile sperm, 10.06 $\pm$ 0.085% SEM and 7.14 $\pm$ 0.069% SEM respectively, than sperm frozen at any of the three slower rates. Total and progressive sperm motility, when frozen at the three slower rates, were not significantly different from one another. (Figure 2.4).

Mean post-thaw sperm STR were significantly higher than the average STR for their corresponding fresh sample values (92.05 $\pm$ 0.028% SEM), regardless of the freezing rate used to cryopreserve striped bass sperm. The highest mean VAP (85.78 $\pm$ 0.197 SEM), VCL (93.130 $\pm$ 0.148 SEM) and VSL (81.12 $\pm$ 0.204 SEM) for post-thaw samples were obtained from the -40^{o}C/min freezing rate, although they were not statistically different from the values obtained with the -15^{o}C/minute rate.

Figure 2.4

The Effects of Freezing Rate on Striped Bass Sperm Motility



Mean percentage total and progressive motility estimates for post-thaw striped bass males (n=12) sperm when cryopreserved at different freezing rates over a five week spawning period. Progressive motility is defined as cells exhibiting VAP greater than 80 μm/s and straightness greater than 80%. Bars represent SEM. [a-c] Means without a common superscript differed (P<.05).

Discussion

Motility, viability, and ATP quantification are some of the most widely used standards to determine sperm quality, and have been correlated with fertilization success for multiple species. For the purpose of this study, an "optimal" freezing rate was defined as the rate that produced: the highest percentage total and progressive motility; the highest percentage viability; and the highest levels of ATP concentration in post-thaw striped bass sperm. Because the programmable freezer utilized for this study was only able to create consistent, repeatable freezing rates up to -40ºC/min, we were not able to examine the effects of faster freezing rates. Our results demonstrate that when using the well-documented, preferred cryoprotectant DMSO along with appropriate isosmotic extenders for striped bass semen, a freezing rate of -40ºC/min is the optimal freezing rate for the cryopreservation of striped bass sperm of those we tested.

For viability, an increasing percentage of striped bass sperm with intact cell membranes was observed as the freezing rate increased, with the highest membrane integrity levels obtained from the -40ºC/min freezing rate. Because the freezing process has been previously shown to cause varying degrees of damage to sperm membranes (Gwo and Arnold, 1992), axonemes (Yao *et al.*, 2000) and mitochondria (Conget *et al.*, 1996) due to osmotic changes and ice crystal formation, it is vital for the optimal freezing rate to demonstrate the capacity to produce sperm with intact membranes.

Post-thaw sperm ATP concentrations in species such as the sea bass Dicentrarchus labrax (Zilli *et al.*, 2004) and carp (Perchec *et al.*, 1995) have been

positively correlated with sperm motility and fertilization capacity. We determined that sperm from our striped bass males, when cryopreserved using the -40°C/min rate had significantly higher levels of ATP post-thaw. Preservation of these limited ATP resources is paramount due to several limiting factors unique to striped bass sperm. Compared to mammalian and other teleost species, striped bass sperm contain a relatively small amount of ATP, commensurate with the presence of only two mitochondria (He and Woods, 2004b). In addition, striped bass sperm appear to be unable to both replenish endogenous energy and to utilize exogenous energy sources for motion, as demonstrated by a previous study in which demembranated striped bass sperm incubated with excess exogenous ATP were not able to maintain normal duration of motility (Guthrie *et al.*, 2008). This is in contrast to a study performed using demembranated rainbow trout sperm, where duration of motility was increased (from 30 seconds to roughly 20 minutes) with the addition of ATP to the extender solution (Saudrais *et al.*, 1998). These differences highlight not only the inability for striped bass sperm to create new ATP via mitochondrial function and oxidative phosphorylation (He *et al.*, 2004) once activated, but to date, have also not been shown to be able to actively transport or utilize in any measurable way the energy substrates, when they have been provided through extenders or cryomedia to the cells before or after freezing.

This experiment was the first to utilize a CEROS™ CASA system, not only to quantify striped bass sperm motion characteristics post-thaw in a controlled and repeatable manner, but to use the data in a comparative method to evaluate the best freezing rates for cryopreservation of striped bass sperm. Both total and progressive

motilities were significantly higher in samples frozen using the -40ºC/min rate than all other rates. As striped bass sperm frozen at the -40ºC/min rate had the highest ATP concentrations and the highest levels of cell membrane integrity, it is reasonable to infer that they should also have had the highest percentages of motile sperm. Although there was a significant statistical difference between the motility data obtained post-thaw from the -40ºC/min rate and other freezing rates, all of the samples were very low (<10%) when compared to the corresponding fresh samples.

An unusually high degree of straightness (%) was observed from both fresh and post-thaw striped bass sperm samples, surpassing many different species such as the stallion (Blach *et al.*, 1989), turkey (King *et al.*, 2000) and even in other teleost species such as the Siberian sturgeon (Sieczyñski *et al.*, 2012) and Chinook salmon (Rosengrave *et al.*, 2008). However, it was not possible to determine whether freezing rate had an effect on this particular motion analysis characteristic in striped bass sperm. The use of CASA to determine motility may provide an explanation for the differences found between the results published by earlier sources (He and Woods, 2004a,b; Jenkins and Woods, 2002b; Thirumala *et al.*, 2006), although a direct comparison is difficult due to the much smaller concentration of cells needed for CASA analysis compared to previous methods (Appendix B).

In previous studies, motility was determined using human visual recognition. The CASA system allows for the collection of unbiased, controlled data, as well as multiple other characteristics (i.e. VSL, VCL, STR, VAP, etc.) that we have been previously unable to quantify. Because successful fertilization may require multiple, positive sperm characteristics (motility, cell membrane integrity, ATP concentration,

etc.) in order to successfully transfer its genetic information to the egg, it is important that studies examining the optimal conditions under which sperm are cryopreserved and stored use multiple methods of evaluation. Interestingly, when striped bass sperm have been cryopreserved in the presence of DMSO and at freezing rates of -10, -20, -30, and -40 ^{o}C/min, the faster rate of -40^{o}C/min provided significantly higher post-thaw motility than the slower rates tested and when used in fertilization trials, provided a fertilization rate that was not statistically different from the fresh semen controls (He and Woods, 2004a).

The results of previous studies examining the effects of freezing rates on striped bass sperm have not been concordant, with recommendations of both slower and faster freezing rates found in various publications (He and Woods, 2004a,b; Jenkins and Woods, 2002b; Thirumala *et al.*, 2006). While our results of -40^{o}C/min were comparable to those obtained by He and Woods (2004a), their study was limited by a pooled sample of three individuals that were only sampled over the course of a single week during the spawning season. In addition, sperm quality was based solely on ATP concentration and measurements of motility obtained without the use of a computer assisted sperm analysis (CASA) unit. The current study was performed to re-affirm the optimal freezing rate, utilizing multiple assay techniques to further define and delineate differences in sperm quality of striped bass sperm samples collected from a substantially larger sample size (n=12) over an extended period of time (5 weeks).

Chapter 3- Concluding Summary

Due to the extensive effort put forth by members of the Woods lab over the past decade, an effective protocol for cryopreserving striped bass semen has been developed. Research focusing on the effects of various ion concentrations, extender formulas, osmolalities, cryoprotectants, sperm morphology and physiology have all helped to elucidate the negative effects brought about by the freezing and thawing processes. While many publications have agreed that a rate of -40ºC/minute is optimal for the cryopreservation of striped bass sperm, a more recent publication has suggested a slower optimal freezing rate of -16ºC/minute. Because the rate at which striped bass spermatozoa are frozen has been shown to play a pivotal role in the degree of cryoinjuries sustained during cooling, the present study was undertaken to examine the optimal freezing rate for striped bass spermatozoa, using multiple methods to compare and contrast the effects of each freezing rate.

A freezing rate of -40ºC/minute was shown to preserve the highest levels of membrane integrity, motility, and ATP concentration when compared to the -10, -15, and -20ºC/minute rates examined. While other studies have shown that the use of slower cooling rates allow for the maintenance of osmotic equilibrium, mortality is observed due to dehydration and subsequent crenation during the freezing process as water exits from the cell. Although the maintenance of osmotic balance does help to reduce the size of the ice crystals that form, it has also been shown that slowly cooled intracellular water does not undergo total or partial vitrification, increasing the frequency of ice crystal formation (Leung, 1991). Slower freezing rates may also negatively impact sperm health by increasing the amount of time that metabolically active cells are exposed to toxic cryoprotectants such as DMSO. In contrast, faster

cooling rates do not allow for water to leave the cell as quickly during the freezing process, and under extreme cooling conditions, partial or total vitrification of intracellular water is observed. The retention of water becomes extremely detrimental to spermatozoa during the thawing process, as large intracellular ice crystals are allowed to form as a result of devitrification and re-crystallization (Leung, 1991). Based on this information, it is expected that an optimal freezing rate should be slow enough to allow for the loss of water during the freezing process, yet rapid enough to cause the partial vitrification of any intracellular water that remains to minimize ice crystal formation.

The results from this study suggest that the slower rates of -10, -15 and -20°C/minute are not sufficient to prevent ice crystallization and do not allow for partial vitrification to occur, resulting in a significant decrease in cell membrane integrity, ATP concentration, and motility when examined post-thaw. Furthermore, the -40°C/minute rate was sufficient to prevent lethality as a result of devitrification and recrystallization (indicative of an overly rapid freezing rate), in addition to cell dehydration and crenation as a result of water loss (indicative of an inappropriately slow freezing rate). While controlled freezing rates greater than -40°C/minute may provide even greater sperm quality post-thaw, studies examining striped bass spermatozoa subsequent to freezing by direct contact with liquid nitrogen (-198°C) or by floating in liquid nitrogen vapor (Woods, unpublished data) have resulted in extremely poor motility for post thaw samples, indicating that there is a limit to how rapidly striped bass sperm can be frozen before sperm quality is compromised.

It is important to note that assay results obtained from all post-thaw samples were significantly lower in quality compared to those obtained from fresh samples. Because of the extreme changes in osmotic balance, ion concentrations, and membrane fluidity, the observed decrease in quality due to freezing is to be expected, even with the use of methods to ameliorate the effects of cryoinjuries. Indeed, the observed changes in motility, viability and ATP concentration were consistent with the results published by previous studies examining the differences between pre- and post-thaw semen samples (Guthrie *et al.*, 2008; Guthrie *et al.*, 2011; He and Woods, 2003b; He and Woods 2004b; Jenkins and Woods, 2002b). Sperm whose membranes have been compromised would also allow for the loss of intracellular ATP into the environment, explaining the relatively similar decreases observed from both the Sybr-PI and luciferin-luciferase assay results. Both the loss of cell membrane integrity and ATP can negatively impact the motion capabilities of sperm, explaining the post-thaw losses of both total and progressive motility, which was greater than the percentage losses for both viability and ATP concentration.

Differences observed between pre- and post- thaw samples may also be attributed to the application of DMSO as a cryoprotectant. When used at appropriate concentrations, DMSO is able to prevent much of the losses in viability, ATP concentration, and motility that normally occurs during the freezing process (He and Woods, 2004b), while still allowing for fertilization at rates not statistically different from fresh semen samples (Jenkins and Woods, 2002b; He and Woods, 2004a). However, previous studies have also shown that striped bass sperm are extremely sensitive to DMSO over time, with an approximate 40% loss of motility after only 30

minutes of exposure to a 5% v/v concentration (He and Woods, 2003b). In addition, it is often difficult to create a completely homogenous mixture when sperm are added to a cryoprotectant solution due to the viscosity of fresh semen. Micro-environments formed as a result of this heterogeneity can cause varying degrees of exposure of spermatozoa to DMSO, possibly resulting in a negative impact on total post thaw sample quality. As such, the development of new protocols that take advantage of non-toxic cryoprotectants such as trehalose (Rudolph *et al.*, 1986; Ahmad and Aksoy, 2012) are essential for the continuing improvement of striped bass semen cryopreservation.

By decreasing the reliance on both wild and captive populations, the use of cryopreservation for long term storage of gametes holds great potential for improving the striped bass industry. Spermatozoa that have been frozen and stored in liquid nitrogen can be utilized months, years, or even decades later for successful fertilization, allowing for gametes from males exhibiting advantageous phenotypes to be utilized well beyond the individual's lifespan. By selectively preserving gametes from these individuals, gene banking would allow for the industry to gradually improve the quality of their seedstock over time. This would also allow for year round production of both striped bass and hybrid striped bass fingerlings with the use of phase-adjusted females when striped bass are normally not in spawning condition, or for fingerling production in areas where gametes are not naturally available due to geographic restriction. Populations of genetically improved striped bass have already been in development in the United States, although they are not yet available for public use (Garber and Sullivan, 2006; Fuller *et al.*, 2010, 2011).

The availability of a reliable, repeatable cryopreservation protocol combining an appropriate extender formula, cryoprotectant, and optimal freezing rate has set the stage for the industry to benefit, both in terms of production efficiency and economic growth. While striped bass culturists are slow to utilize the potential benefits of cryopreservation, I believe its use will become increasingly important as the growing human population places increasing demands upon the aquaculture industry.

Appendices

Appendix A- CEROS Sperm Density (cells/mL) Validation

Because striped bass spermatozoa are physically smaller compared to many other vertebrate species and have not previously been analyzed using CASA, it was necessary to determine if the Hamilton-Thorne CEROS analysis system was capable of providing accurate, repeatable estimates of sperm density for a given sample. The use of the CEROS unit for density estimates would serve as a much more efficient method of estimation compared to traditional methods that count cells individually, by eye within a grid system, with the aid of a hemocytometer or Makler® [sperm] Counting Chamber.

Materials and Methods

Three Cassou straws containing cryopreserved striped bass semen samples from 2007 were removed from liquid nitrogen dewars and immediately thawed in a 40ºC water bath for seven seconds, after which they were placed into separate watch glasses over ice. From each post-thaw sample, 5uL of semen was extended 1:10 (v:v) with SBE350 and gently homogenized for 30 seconds. A 15uL aliquot was then placed onto a Makler counting chamber, and the sample density estimated using the gridded coverslip. This same sample was then re-analyzed using a H-T CEROS unit. Four replicates for each straw were performed to account for sample heterogeneity. Density estimates from both the Makler and the CEROS unit were then compared using a Student's T-test. Results are presented below.

Results

STRAW 1		*STRAW 2*		*STRAW 3*	
Replicate 1		**Replicate 1**		**Replicate 1**	
Makler Chamber Count	**CEROS Count**	**Makler Chamber Count**	**CEROS Count**	**Makler Chamber Count**	**CEROS Count**
$99x10^6$	$96.3x10^6$	$119x10^6$	$121.9x10^6$	$145x10^6$	$143x10^6$
Replicate 2		**Replicate 2**		**Replicate 2**	
Makler Chamber Count	**CEROS Count**	**Makler Chamber Count**	**CEROS Count**	**Makler Chamber Count**	**CEROS Count**
$86x10^6$	$84.4x10^6$	$209x10^6$	$199.5x10^6$	$167x10^6$	$170.1x10^6$
Replicate 3		**Replicate 3**		**Replicate 3**	
Makler Chamber Count	**CEROS Count**	**Makler Chamber Count**	**CEROS Count**	**Makler Chamber Count**	**CEROS Count**
$129x10^6$	$130.4x10^6$	$228x10^6$	$217.4x10^6$	$103x10^6$	$107x10^6$
Replicate 4		**Replicate 4**		**Replicate 4**	
Makler Chamber Count	**CEROS Count**	**Makler Chamber Count**	**CEROS Count**	**Makler Chamber Count**	**CEROS Count**
$132x10^6$	$131.3x10^6$	$95x10^6$	$97.2x10^6$	$198x10^6$	$196x10^6$
Average		**Average**		**Average**	
$111.5x10^6$	$110.6x10^6$	$162.75x10^6$	$159x10^6$	$153.25x10^6$	$154.025x10^6$
T-Test (a=.05)	0.3764	**T-Test (a=.05)**	0.3794	**T-Test (a=.05)**	0.6636

Combined Density Estimate Averages	
Makler Chamber Count	**CEROS Count**
$142.5x10^6$	$141.208x10^6$
T-test-Overall (a=.05)	
0.4312	

Discussion

Based on the results, there were no statistical differences ($p > .05$) between the sperm density estimates provided by the H-T CEROS unit or the Makler counting chamber. As such, the CEROS unit is an appropriate system for providing accurate and repeatable estimates of sperm densities for striped bass semen samples. Because

sperm densities were extremely variable, even when taken from the same sample, it is suggested that multiple acquisitions must be taken and averaged in order to ensure the best possible estimates of cell density for a specific sample.

Appendix B- Computer Assisted Motion Analysis Characteristics Of Fresh Striped Bass Spermatozoa

Motion Characterization of Fresh Striped Bass Spermatozoa Over A Five Week Period

	Density	***Total***	***Progressive***	***Track Speed***	***Beat Freq***	***LIN***	***STR***	***Prog Velocity***	***Path Velocity***
	(x10 ^9)	**Motility (%)**	**Motility (%)**	**um/s**	**Hz**	**%**	**%**	**um/s**	**um/s**
Wk 1	84.85	47.17	38.33	128.90	30.13	83.92	93.25	108.28	116.96
Wk 2	94.75	64.33	50.00	149.65	30.75	82.00	90.67	123.19	135.17
Wk 3	94.68	56.89	46.33	140.56	31.12	82.67	92.22	117.63	126.82
Wk 4	104.50	53.42	40.42	137.67	29.75	81.00	92.00	112.44	121.83
Wk 5	111.70	52.75	40.75	145.04	28.37	83.08	92.17	121.16	130.88
Average	98.28	54.81	43.00	140.35	29.97	82.53	92.05	116.48	126.31

Data was collected from 8 year old striped bass males (n=12) once a week over the course of a five week period. Males were anesthetized using MS-222, the urogenital vent wiped clean of urine and feces to prevent contamination, and the samples collected into sterile Falcon tubes and immediately stored on ice. An aliquot equivalent to 350-500 sperm was added to 15uL of DIUF and analyzed on the CEROS unit within 15 minutes of collection.

References

Ahmad, E. and Aksoy, M. 2012. Trehalose as a cryoprotective agent for the sperm cells: a mini review. Animal Health, Prod. And Hyg. 1(2): 123-129.

Allen, D., Lännergren, J., Westerblad, H. 2002. Intracellular ATP measured with luciferin/luciferase in isolated single mouse skeletal muscle fibres. Pflügers Archiv. 443(5-6): 836-842.

Beck, B.H., Fuller, S.A., Peatman, E., McEntire, M.E., Darwish, A., Freeman, D.W. 2012. Chronic exogenous kisspeptin administration accelerates gonadal development in basses of the genus Morone. Comparative biochemistry and physiology. Part A, Molecular & integrative physiology. 162(3): 265.

Blach E.L., Amann R.P., Bowen R.A., Frantz D. 1989. Changes in quality of stallion spermatozoa during cryopreservation: plasma membrane integrity and motion characteristics. Theriogenology. 31: 283-98.

Brown, B.E. 1965. Meristic counts of striped bass from Alabama. Transactions of the American Fisheries Society. 94(3): 278-279.

Brown, R.T., Colburn, H.R., Berlinsky, D.L., Ziolkowski, A., Nardi, G.C. 2012. Swimming characteristics and fertilizing capacity of meiogynogenetic summer flounder, *Paralichthys dentatus*, spermatozoa. Journal of the World Aquaculture Society. 43(6): 869-877.

Carlberg J.M., Van Olst J.C., Massingill, .M.J. 2000. Hybrid striped bass: an important fish in US aquaculture. Aqua Mag. 26: 26–38.

Chao, N.H. 1991. Fish sperm cryopreservation in Taiwan: Technology advancement and extension efforts. In Paper on International Symposium on Reproductive Biology in Aquaculture. Dept. of Aquaculture, Taiwan Fisheries Res. Ins., Taiwan (p. 31).

Chao, N.H., Chao, W.C., Liu, K.C., Liao, I.C. 1987. The properties of tilapia sperm and its cryopreservation. Fish Biology. 30: 107-118.

Christ, S.A., Toth, G.P., McCarthy, H.W., Torsella, J.A., Smith, M.K. 1996. Monthly variation in sperm motility in common carp assessed using computer-assisted sperm analysis (CASA). Journal of fish biology. 48(6): 1210-1222.

Christen, R., Gatti, J., Billard, R. 2005. The transient movement of trout sperm is related to changes in the concentration of ATP following the activation of the flagellar movement. European Journal of Biochemistry. 166(3): 667-671.

Christensen, J.M. and Tiersch, T.R. 1996. Refrigerated storage of channel catfish sperm. Journal of the World Aquaculture Society. 27(3): 340-346.

Conget P., Fernandez, M., Herrera, G., Minguell, J.J. 1996. Cryopreservation of rainbow trout (*Oncorhynchus mykiss*) spermatozoa using programmable freezing. Aquaculture. 143: 319- 29.

DeGraaf, J.D. and Berlinsky, D.L. 2004. Cryogenic and refrigerated storage of Atlantic cod (*Gadus morhua*) and haddock (*Melanogrammus aeglefinus*) spermatozoa. Aquaculture. 234(1): 527-540.

Doi, M., Hoshino, T., Taki, Y., Ogasawara, Y. 1982. Activity of the sperm of the bluefin tuna *Thunnus thynnus* under fresh and preserved conditions. Bulletin of the Japanese Society of Scientific Fisheries. 48: 495-498.

Dorsey, K.M., Guthrie, H.D., Welch, G.R., Mohler, J., Theisen, D.D., Siewerdt, F., Vinyard, B., Woods III, L.C. 2011. Quality assessment of wild Atlantic sturgeon semen under conditions of short-term storage. North American Journal of Aquaculture. 73(4): 418-425.

Drobnis, E.Z., Crowe, L.M., Berger, T., Anchordoguy, T.J., Overstreet, J.W., Crowe, J.H. 1993. Cold shock damage is due to lipid phase transitions in cell membranes: a demonstration using sperm as a model. Journal of Experimental Zoology. 265(4): 432-437.

Eroglu, A., Russo, M.J., Bieganski, R., Fowler, A., Cheley, S., Bayley, H., Toner, M. 2000. Intracellular trehalose improves the survival of cryopreserved mammalian cells. Nature biotechnology. 18(2): 163-167.

Fauvel, C., Suquet, M., Cosson, J. 2010. Evaluation of fish sperm quality. Journal of Applied Ichthyology. 26(5): 636-643.

Felip, A., Zanuy, S., Carrillo, M. 2006. Comparative analysis of growth performance and sperm motility between precocious and non-precocious males in the European sea bass (*Dicentrarchus labrax, L.*). Aquaculture. 256(1): 570-578.

Fuller, S.A., Beck, B.H., Farmer, B.D., Mcentire, M.E., Freeman, D.W. 2011. Quantitative genetics and differential performance and gene expression of half-sib families of hybrid striped bass in communal ponds. Journal of Animal Science. 89: 274.

Fuller, S.A., McEntire, M., Ludwig, G.M. 2010. Development and testing of a pedigree-marking system using visible implant elastomer tags for selective

improvement in Morone breeding programs. Aquaculture Research. 41(8): 1250-1254.

Garber, A.F. and Sullivan, C.V. 2006. Selective breeding for the hybrid striped bass (*Morone chrysops*, Rafinesque× *M. saxatilis*, Walbaum) industry: status and perspectives. Aquaculture Research. 37(4): 319-338.

Gosden, R. 2011. Cryopreservation: a cold look at technology for fertility preservation. Fertility and sterility. 96(2): 264-268.

Guthrie, H.D., Welch, G.R., Theisen, D.D., Woods III, L.C. 2011. Effects of hypothermic storage on intracellular calcium, reactive oxygen species formation, mitochondrial function, motility, and viability in striped bass (*Morone saxatilis*) sperm. Theriogenology. 75: 951-961.

Guthrie, H.D., Woods III, L.C., Long, J.A., Welch, G.C. 2008. Effects of osmolality on inner mitochondrial transmembrane potential and ATP content in spermatozoa recovered from the testes of striped bass (*Morone saxatilis*). Theriogenology. 69: 1007-1012.

Gwo, J. and Arnold, C.R. 1992. Cryopreservation of atlantic croaker spermatozoa: evaluation of morphological changes. J. Exp Zool. 264: 444-53.

Gwo, J.C. 1994. Cryopreservation of yellowfin seabream (*Acanthopagrus latus*) spermatozoa. Theriogenology. 41(5): 989-1004.

Harrell, R.M. and Webster, D.W. 1997. An overview of Morone culture: Developments in Aquaculture and Fisheries Science. 30: 1-10.

Harrell, R.M., Kerby, J.H., Smith, T.I.J., and Stevens, R.E. 1990. Striped bass and striped bass hybrid culture: the next twenty-five years. In: Harrell, R.M., Kerby, J.H.,

Minton, R.V., editors. Culture and propagation of striped bass and its hybrids. Bethesda, MD: American Fisheries Society. p. 253–61.

Harvey, B., Kelley, R.N., Ashwood-Smith, M.J. 1983. Permeability of intact and dechorionated zebra fish embryos to glycerol and dimethyl sulfoxide. Cryobiology. 20(4): 432-439.

He S, Jenkins K, Woods LC. 2004. Activation of sperm motility in striped bass via a cAMP-independent pathway. Theriogenology. 61: 1487-98.

He, S. and Woods III, L.C. 2003a. Effects of glycine and alanine on short-term storage and cryopreservation of striped bass (*Morone saxatilis*) spermatozoa. Cyrobiology. 46: 17-25.

He, S. and Woods III, L.C. 2003b. The effects of osmolality, cryoprotectant and equilibration time on striped bass sperm motility. Journal of the World Aquaculture Society. 34: 255-265

He, S. and Woods III, L.C. 2004a. Changes in motility, ultrastructure, and fertilization capacity of striped bass *Morone saxatilis* spermatozoa following cryopreservation. Aquaculture. 236: 667-687.

He, S. and Woods III, L.C. 2004b. Effects of dimethyl sulfoxide and glycine on cryopreservation induced damage of plasma membranes and mitochondria to striped bass (*Morone saxatilis*) Sperm. Cryobiology. 48: 254-262.

Hodson, R.G., Clark, R.W., Hopper, M.S., McGinty, A.S., Weber, G.M., Sullivan, C.V. 1999. Reproduction of domesticated striped bass: commercial mass production of fingerlings. UJNR Aquaculture. 28: 23-32.

Holt, W.V. 2000. Basic aspects of frozen storage of semen. Animal Reproduction Science. 62(1): 3-22.

Jagadeeswaran, P., Sheehan, J.P., Craig, F.E., Troyer, D. 1999. Identification and characterization of zebrafish thrombocytes. British journal of haematology. 107(4): 731-738.

Jenkins-Keeran, K. and Woods III, L.C. 2002a. An evaluation of extenders for the short-term storage of striped bass milt. North American Journal of Aquaculture. 64: 248-256.

Jenkins-Keeran, K. and Woods III, L.C. 2002b. The cryopreservation of striped bass semen. Journal of the World Aquaculture Society. 33: 70-77.

Jodun, W.A., King, K., Farrell, P., Wayman, W. 2007. Methanol and egg yolk as cryoprotectants for atlantic salmon spermatozoa. North American Journal of Aquaculture. 69(1): 36-40.

Jobling M, Peruzzi S, Woods C. 2010. The temperate basses (Family: Moronidae). In: Le Francois N, Jobling M, Carter C, Blier P, editors. Finfish Aquaculture Diversification, CABI, Oxfordshire, UK. 337-60.

Kerby, J.H., Bayless, J.D., Harrell, R.M. 1985. Growth, survival, and harvest of striped bass produced with cryopreserved spermatozoa. Trans Am Fish Soc. 114: 761-65.

Kerby, J.H. 1983. Cryogenic preservation of sperm from striped bass. Transactions of the American Fisheries Society. 112(1): 86-94.

Kiesslich, T., Benno Oberdanner, C., Krammer, B., Plaetzer, K. 2003. Fast and reliable determination of intracellular ATP from cells cultured in 96-well microplates. Journal of biochemical and biophysical methods. 57(3): 247-251.

Kime, D.E., Van Look, K.J., McAllister, B.G., Huyskens, G., Rurangwa, E., Ollevier, F. 2001. Computer-assisted sperm analysis (CASA) as a tool for monitoring sperm quality in fish. Comparative biochemistry and physiology. Toxicology & pharmacology: CBP. 130(4): 425.

King, L.M., Holsberger, D.R., Donoghue, A.M. 2000. Correlation of CASA velocity and linearity parameters with sperm mobility phenotype in turkeys. Int J Androl. 21: 65-71.

Kohler, C.C., Sheehan, R.J., Habicht, C., Malison, J.A., Kayes, T.B. 1994. Habituation to captivity and controlled spawning of white bass. Transactions of the American Fisheries Society. 123(6): 964-974.

Kopeika, J., Kopeika, E., Zhang, T., Rawson, D.M. 2003. Studies on the toxicity of dimethyl sulfoxide, ethylene glycol, methanol and glycerol to loach (*Misgurnus fossilis*) sperm and the effect on subsequent embryo development. Cryoletters. 24(6): 365-374.

Krol, J., Kowalski, R.K., Hliwa, P., Dietrich, G.J., Stabiński, R., Ciereszko, A. 2009. The effects of commercial preparations containing two different GnRH analogues and dopamine antagonists on spermiation and sperm characteristics in the European smelt *Osmerus eperlanus*. Aquaculture. 286(3): 328-331.

Lahnsteiner, F., Berger, B., Weismann, T., Patzner, R. A. 1998. Determination of semen quality of the rainbow trout, *Oncorhynchus mykiss*, by sperm motility, seminal plasma parameters, and spermatozoal metabolism. Aquaculture. 163(1): 163-181.

Lanes, C.F.C., Okamoto, M., Cavalcanti, P.V., Collares, T., Campos, V.F., Deschamps, J.C., Sampaio, L.A. 2008. Cryopreservation of Brazilian flounder (*Paralichthys orbignyanus*) sperm. Aquaculture. 275(1): 361-365.

Leibo, S.P., and Songsasen, N. 2002. Cryopreservation of gametes and embryos of non-domestic species. Theriogenology. 57(1): 303-326.

Leung, L.K.P. 1987. Cryopreservation of spermatozoa of the barramundi, *Lates calcarifer*. Aquaculture. 64: 243-247.

Leung, L.K.P. 1991. Principles of biological cryopreservation. Jamieson. Fish evolution: evidence from spermatozoa. 231-244.

Linhart, O., Rodina, M., Cosson, J. 2000. Cryopreservation of sperm in common carp *Cyprinus carpio* sperm motility and hatching success of embryos. Cryobiology. 41(3): 241-250.

Little R.C., Milliken, G.A., Stroup, W.W., Wolfinger, R.D. 1996. SAS system for mixed models. Cary, NC: SAS Institute Inc.

Liu, F.G., Cheng, S.C., Chen, H.C. 1998. Induced spawning and larval rearing of domestic hybrid striped bass (*Morone saxatilis* x *M. chrysops*) in Taiwan. The Israeli journal of aquaculture-Bamidgeh. 50(3): 111-127.

Liu, Q.H., Li, J., Xiao, Z.Z., Ding, F.H., Yu, D.D., Xu, X.Z. 2007. Use of computer-assisted sperm analysis (CASA) to evaluate the quality of cryopreserved sperm in red seabream (*Pagrus major*). Aquaculture. 263(1): 20-25.

Long, J.A. and Guthrie, H.D. 2006. Validation of a rapid, large-scale assay to quantify ATP concentration in spermatozoa. Theriogenology. 65: 1620-30.

Lougheed, M. and Nelson, B. 2001. Hybrid Striped Bass Production, Markets and Marketing. Burdick Center for Cooperatives, North Dakota State University. Undated.

Mazur, P. 1984. Freezing of living cells: mechanisms and implications. American Journal of Physiology-Cell Physiology. 247(3): C125-C142.

McGinty, A.S. and Hodson, R.G. 2008. Hybrid striped bass: hatchery phase. Southern Regional Aquaculture Center.

Meynard, D., Kautz, L., Darnaud, V., Canonne-Hergaux, F., Coppin, H., Roth, M.P. 2009. Lack of the bone morphogenetic protein BMP6 induces massive iron overload. Nat Genet. 41: 478-81.

Mylonas, C.C., Scott, A.P., Vermeirssen, E.L., Zohar, Y. 1997. Changes in plasma gonadotropin II and sex steroid hormones, and sperm production of striped bass after treatment with controlled-release gonadotropin-releasing hormone agonist-delivery systems. Biology of reproduction. 57(3): 669-675.

Mylonas, C.C., Zohar, Y., Woods, L.C., Thomas, P., Schulz, R.W. 1998. Hormone profiles of captive striped bass *Morone saxatilis* during spermiation, and long-term enhancement of milt production. Journal of the World Aquaculture society. 29(4): 379-392.

Naylor, R.L., Goldburg, R.J., Primavera, J.H., Kautsky, N., Beveridge, M.C.M., Clay, J. 2000. Effect of aquaculture on world fish supplies. Nature. 405: 1017–24.

Ogier de Baulny, B., Labbe, C., Maisse, G. 1999. Membrane integrity, mitochondrial activity, ATP content, and motility of the European catfish (*Silarus glanis*) testicular spermatozoa after freezing with different cryoprotectant. Cryobiology. 39: 177-184.

Ohta, H., Kawamura, K., Unuma, T., Takegoshi, Y. 2001. Cryopreservation of the sperm of the Japanese bitterling. Fish Biology. 58: 670-681.

Ottesen, O.H., Babiak, I., Dahle, G. 2009. Sperm competition and fertilization success of Atlantic halibut (*Hippoglossus hippoglossus*, L.). Aquaculture. 286(3): 240-245.

Parks, J.E. and Lynch, D.V. 1992. Lipid composition and thermotropic phase behavior of boar, bull, stallion, and rooster sperm membranes. Cryobiology. 29(2): 255-266.

Parks, J.E., and Graham, J.K. 1992. Effects of cryopreservation procedures on sperm membranes. Theriogenology. 38(2): 209-222.

Perchec, G., Jeulin, C., Cosson, J., Andre, F., Billard, R. 1995. Relationship between sperm ATP content and motility of carp spermatozoa. Int J Androl. 21: 65-71.

Rieger, D. 1997. Batch analysis of the ATP content of bovine sperm, oocytes, and early embryos using a scintillation counter to measure the chemiluminescence produced by the luciferin–luciferase reaction. Analytical biochemistry. 246(1): 67-70.

Ritar, A. J. and Campet, M. 2000. Sperm survival during short-term storage and after cryopreservation of semen from striped trumpeter (*Latris lineata*). Theriogenology. 54(3): 467-480.

Robertson, S.M., Lawrence, A.L., Neill, W.H., Arnold, C.R., McCarty, G. 1988. Toxicity of the cryoprotectants glycerol, dimethyl sulfoxide, ethylene glycol,

methanol, sucrose, and sea salt solutions to the embryos of red drum. The Progressive Fish-Culturist. 50(3): 148-154.

Rosengrave, P., Gemmell, N.J., Metcalf, V., McBride, K., Montgomerie, R. 2008. A mechanism for cryptic female choice in chinook salmon. Behav Eco. 19: 1179-85.

Rudacille, J.B. and Kohler, C.C. 2000. Aquaculture performance comparison of sunshine bass, palmetto bass, and white bass. North American Journal of Aquaculture. 62(2): 114-124.

Rudolph, A.S., Crowe, J.H., Crowe, L.M. 1986. Effects of three stabilizing agents—proline, betaine, and trehalose—on membrane phospholipids. Archives of Biochemistry and Biophysics. 245(1): 134-143.

Rurangwa, E., Volckaert, F.A.M., Huyskens, G., Kime, D.E., Ollevier, F. 2001. Quality control of refrigerated and cryopreserved semen using computer-assisted sperm analysis (CASA), viable staining and standardized fertilization in African catfish (*Clariasgariepinus*). Theriogenology. 55(3): 751-769.

Saudrais, C., Fierville, F., Loir, M., Le Rumeur, E., Cibert, C., Cosson, J. 1998. The use of phosphocreatine plus ADP as energy source for motility of membrane-deprived trout spermatozoa. Cell Motil Cytoskeleton. 41: 91-106.

Segovia, M., Jenkins, J.A., Paniagua-Chavez, C., Tiersch, T.R. 2000. Flow cytometric evaluation of antibiotic effects on viability and mitochondrial function of refrigerated spermatozoa of Nile tilapia. Theriogenology. 53(7): 1489-1499.

Shirakashi, R., Reuss, R., Ludwig, J., Ehrhart, F., Zimmermann, U.L.R.I.C.H., and Sukhorukov, V.L. 2005. Introduction of lyo- and cryoprotective carbohydrates into

mammalian cells through swelling-activated channels. Transactions of the Japan Society of Mechanical Engineers. 17(3): 901-907.

Sieczyñski, P., Glogowski, J., Cejko, B.I., Grygoruk, C. 2012. Characteristics of siberian sturgeon and sterlet sperm motility parameters compared using CASA. Arch Pol Fish. 20: 137-43.

Smith, T.I., Jenkins, W.E., Heyward, L.D. 1996. Production and extended spawning of cultured white bass broodstock. The Progressive fish-culturist. 58(2): 85-91.

Spielmann, H., Jacob-Müller, U., Schulz, P. 1981. Simple assay of 0.1–1.0 pmol of ATP, ADP, and AMP in single somatic cells using purified luciferin luciferase. Analytical biochemistry. 113(1): 172-178.

Striped Bass Growers Association (SBGA). 1998. Results from the 1997 Striped Bass Growers Association producers survey. Aquaculture Magazine. 24(5): 25-27.

Sullivan, C.V., Berlinsky, D.L., Hodson, R.G. 1997. Reproduction. In: Harrell, R.M. (ed.). Striped Bass and Other Morone Culture. Elsevier Science, Amsterdam, p. 11-73.

Tekin, N., Secer, S., Akcay, E., Bozkurt, Y., Kayam, S. 2007. Effects of glycerol additions on post-thaw fertility of frozen rainbow trout sperm, with an emphasis on interaction between extender and cryoprotectant. Journal of Applied Ichthyology. 23(1): 60-63.

Thirumala, S., Ferrer, M.S., Al-Jarrah, A., Eilts, B.E., Paccamonti, D.L., Devireddy, R.V. 2003. Cryopreservation of canine spermatozoa: theoretical prediction of optimal cooling rates in the presence and absence of cryoprotective agents. Cryobiology. 47: 109–24.

Thirumala, S., Huang, C., Dong, Q., Tiersch, T.R., Devireddy, R.V. 2005. A theoretically estimated optimal cooling rate for the cryopreservation of sperm from a live-bearing fish, the green swordtail *Xiphophorus helleri*. Theriogenology. 63: 2395–415.

Thirumala, S., Campbell, W.T., Vicknair, M.R., Tiersch, T.R., Devireddy, R.V. 2006. Freezing response and optimal cooling rates for cryopreserving sperm cells of striped bass, *Morone saxatilis*. Theriogenology. 66: 964–73.

Viveiros, A.T.M., So, N., Komen, J. 2000. Sperm cryopreservation of African catfish, *Clarias gariepinus*: cryoprotectants, freezing rates and sperm: egg dilution ratio. Theriogenology. 54: 1395-1408.

Woods III, L.C. 2001. Domestication and strain evaluation of striped bass (*Morone saxatilis*). Aquaculture. 202(3): 343-350.

Woods III, L.C. 2005. Striped bass and hybrid striped bass culture. 339-353 In A.M. Kelly and J. Silverstein (eds.). Aquaculture in the 21ST Century. Fish Culture Section, American Fisheries Society, Bethesda, MD. 339-353.

Woods III, L.C., Bennett, R.O., Sullivan, C.V. 1992. Reproduction of a domestic striped bass brood stock. The Progressive Fish-Culturist. 54(3): 184-188.

Woods III, L.C., Ely, B., Leclerc, G., Harrell, R.M. 1995. Evidence for genetic purity of captive and domestic striped bass broodstocks. Aquaculture. 137(1): 41-44.

Woods III, L.C., He, S., Jenkins, K.J. 2009. Cryopreservation of striped bass *Morone saxatilis* spermatozoa. In: Cabrita, E., Robles, V., Herraez, P, editors. Methods in reproductive aquaculture: marine and freshwater species. Boca Raton, FL: CRC Press. p. 422–3.

Woods III, L.C., Kohler, C.C., Sheehan, R.J., Sullivan, C.V. 1995. Volitional tank spawning of female striped bass with male white bass produces hybrid offspring. Transactions of the American Fisheries Society. 124(4): 628-632.

Woods, L.C. and Sullivan, C. 1993. Reproduction of striped bass, *Morone saxatilis* (Walbaum) broodstock: monitoring maturation and hormonal induction of spawning. Aquaculture Res. 24: 211-22.

Yan, X., Zhenyu, L., Gregg, W. P., Dianmo, L. 2001. Invasive species in China—an overview. Biodiversity & Conservation. 10(8): 1317-41.

Yao, Z., Crim, L.W., Richardson, G.F., Emerson, C.J. 2000. Motility, fertility and ultrastructural changes of ocean pout (*Macrozoarces americanus*) sperm after cryopreservation. Aquaculture. 181: 361-75.

Zilli, L., Schiavone, R., Zonno, V., Storelli, C., Vilella, S. 2004. Adenosine triphosphate concentration and β-D-glucuronidase activity as indicators of sea bass semen quality. Biol Repro. 70: 1679-84.