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FROM GAMETE COLLECTION TO DATABASE DEVELOPMENT:
DEVELOPMENT OF A MODEL CRYOPRESERVED GERMPLASM REPOSITORY FOR
AQUATIC SPECIES WITH EMPHASIS ON STURGEON

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The School of Renewable Natural Resources

By
William Rittenhouse Wayman
B.S., Auburn University, 1991
M.S., Louisiana State University, 1996
August 2003

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Abstract

Development of a cryopreserved sperm repository could greatly benefit the study and recovery of endangered species. Currently, a national repository for endangered aquatic species does not exist. This dissertation addressed the development of methods for the creation of a model germplasm repository, which included methods to reduce bacterial contamination within sperm samples, verify sample identification, store sperm (refrigerated or cryopreserved), evaluate sperm quality before and after thawing, and maintain sample and broodstock information. Although these methods were developed for sturgeon, they could be adapted for any aquatic species.

Accurate species identification of sperm samples is essential for an archival repository. A polymerase chain reaction and restriction digest method was developed to identify three sturgeon species, and separate the remaining species into distinct groups. Bacterial contamination of sperm samples can lead to sample deterioration and transmission of pathogens. Twenty-seven species of bacteria from 15 genera were isolated from sperm samples of sturgeon in this study. Addition of antibiotics did not lengthen refrigerated storage time. Cryopreservation procedures developed for sturgeon sperm did not reduce bacterial numbers in thawed samples compared to pre-freeze numbers. Seminal plasma osmolality (100 mOsmol/kg) and sperm activation data (reduction in potassium ion concentration) were used to develop extenders. Hanks' balanced salt solution or modified Hanks' balanced salt solution (mHBSS) at 100 mOsmol/kg allowed refrigerated storage of sperm for as long as 21 d. Sperm were cryopreserved using mHBSS (100 mOsmol/kg), methanol (5% or 10%), a cooling rate of 22-24 °C/min, and 0.5-ml straws. A dual-staining flow cytometry technique was used to determine gamete quality in sperm samples before and after thawing. The procedure was validated by evaluating membrane integrity in

mixed populations of untreated cells and heat-treated membrane damaged cells. When used with thawed samples, whenever membrane integrity was correlated ($r^2 > 0.8068$) to percent fertilization and hatch, motility was also correlated ($r^2 > 0.7650$) to percent fertilization and hatch. A computer database was developed using Microsoft[®] Access to maintain inventory and quality control of sample information. The database is currently being used at the Warm Springs Fish Technology Center of the United States Fish and Wildlife Service.

Chapter 1

Foreword

There are nine species of sturgeon in North America. Shortnose sturgeon (*Acipenser brevirostrum*), pallid sturgeon (*Scaphirhynchus albus*), white sturgeon (*Acipenser transmontanus*), and Alabama sturgeon (*Scaphirhynchus suttkusi*) have been federally listed as endangered (U.S. Office of the Federal Register, 1967; 1990; 1994; 2000). The Gulf sturgeon (*Acipenser oxyrinchus desotoi*), a subspecies of the Atlantic sturgeon (*Acipenser oxyrinchus oxyrinchus*), has also been federally listed as threatened (U.S. Office of the Federal Register, 1991). The United States Fish and Wildlife Service (USFWS) has developed recovery plans for pallid sturgeon (U.S. Fish and Wildlife Service, 1993), shovelnose sturgeon (National Marine Fisheries Service, 1998), the Kootenai population of the white sturgeon (U.S. Fish and Wildlife Service, 1999) and Gulf sturgeon (U.S. Fish and Wildlife Service and Gulf Coast Marine Fisheries Commission, 1995). These plans call for research into methods for the spawning, culture, and rearing of these species, and for the establishment of refuge populations of broodstock. Cryopreservation of sturgeon sperm could be used as a tool in the conservation of genetic resources and the restoration of these threatened and endangered species.

Cryopreservation of sturgeon sperm offers many advantages for their induced spawning. Mature sturgeon can weigh from 4 kg to more than 800 kg and require large holding facilities. Sperm cryopreservation can be used to reduce the number of males maintained in hatcheries, allowing more resources to be devoted to female broodstock and offspring. Because a major obstacle in the induced spawning of sturgeon is asynchronous maturation of broodstock (DiLauro and Krise, 1994), cryopreserved sperm would facilitate induced spawning when eggs from a particular species were available. Cryopreserved sperm can also enhance genetic research

by providing a non-invasive source of DNA suitable for the development of genetic markers and the study of specific DNA sequences. Development of a repository of cryopreserved sturgeon sperm could therefore greatly benefit the study and recovery of threatened and endangered sturgeon species.

At present, national germplasm repositories do not exist for fish. This dissertation is based on the development of methods necessary for the creation of a germplasm repository for North American sturgeon species. This work is intended to provide a model for all aquatic species. There are certain protocols that are the foundations for the development of a successful repository. Chapters of this dissertation focus on these protocols and are organized in the order in which they would be encountered in the development of a repository (Figure 1-1), including the verification of species identification, screening of samples for bacterial contaminants, development of techniques for refrigerated storage and cryopreservation, and development of a database to store information pertaining to broodstocks and sperm samples.

The USFWS is charged with the preservation and recovery of threatened and endangered species. In the southeastern United States alone there are three endangered sturgeon species (shortnose sturgeon, pallid sturgeon, and Alabama sturgeon) and one threatened species (Gulf sturgeon). Thus the goal of the research presented in this dissertation is the creation of a regional or national repository for sturgeon sperm at the Warm Springs Fish Technology Center in Warm Springs, Georgia, a USFWS facility.

The specific objectives are as follows. *1) Develop methods to verify the field identification of species.* Different sturgeon species can occur in the same habitat and can be difficult to differentiate in the field. When sperm samples come into a cryopreservation facility the species identification needs to be verified before the samples can be entered into an archival repository.

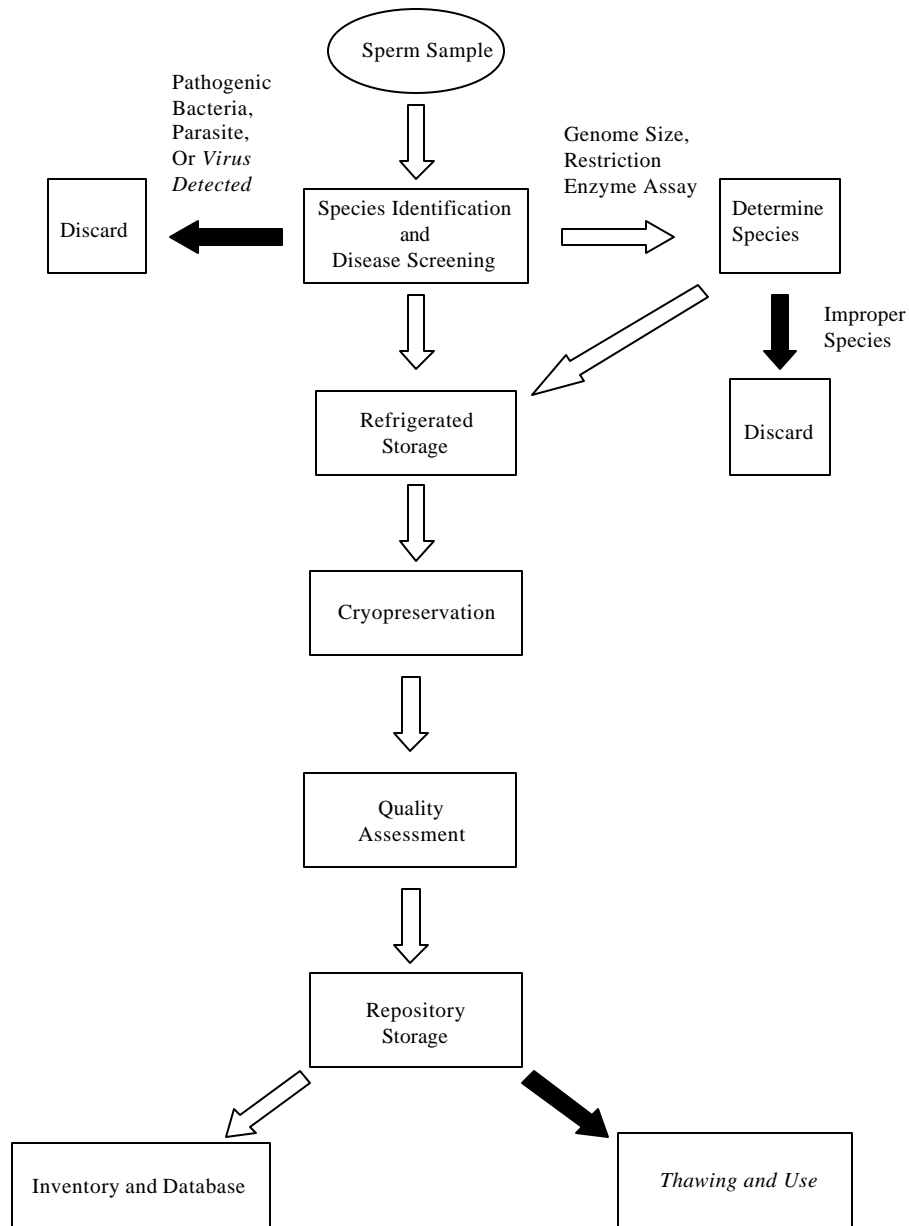


Figure 1-1. Components necessary for the development of a germplasm repository. Sperm samples enter the process at the top and proceed through a series of steps designed for quality assurance and quality control of the samples before they enter the repository. Black arrows represent processes in which sperm samples leave the repository. Items in italics were not specifically investigated in this dissertation.

2) *Determine the presence of bacterial contaminants.* Sperm samples are typically collected with non-sterile techniques, and bacterial contamination is likely. Delineating these bacteria is the first step in avoiding the transfer of bacterial pathogens by way of sperm samples. 3) *Develop refrigerated storage methods.* In order for sperm samples to be shipped from a collection location to a cryopreservation facility, the sperm must be stored on ice or refrigerated for a period of at least 24 hours without significant deterioration of the samples. 4) *Develop cryopreservation techniques.* There are limited reports on the cryopreservation of sturgeon sperm, with only two reports for North American species. Although methods for different species may be similar, differences exist and methods should be developed on a species-by-species basis. 5) *Develop methods for the objective determination of gamete quality.* Presently the most common technique for the evaluation of sperm quality is estimation of sperm motility. Although this technique is easy and rapid, it is also subjective and can be biased by the estimator. 6) *Develop a database.* A very important procedure in the development of a germplasm repository is the development of a database to store pertinent information.

The development of these procedures has allowed the creation of a working sturgeon sperm repository at the USFWS Warm Springs Fish Technology Center in Warm Springs, Georgia. At the time of this writing, the facility holds cryopreserved sperm from 15 species, including 7 sturgeon species. The samples include sperm from endangered shortnose sturgeon (from a captive broodstock used for research purposes), Alabama sturgeon (9 straws from the last captive sturgeon, which died in 2002), and pallid sturgeon (sperm from 22 males for use in restoration efforts), and from non-threatened lake sturgeon and shovelnose sturgeon. At the time of this writing (July 2003), a total of 3,952 straws (1,976 ml) are presently contained in the repository.

The facility has aided researchers across the nation in the conservation of genetic resources from declining populations of these sturgeon.

This dissertation was written in journal format for ease of future publication of the various chapters. All chapters were formatted to meet dissertation format and style criteria. At present, a manuscript entitled “Cryopreservation of Sperm from Pallid Sturgeon,” part of Chapter 5 “Development of cryopreservation techniques,” has been submitted for publication to the *Journal of Fish Biology*. Chapters 3, 4, 5, 6, 7, and 8 are also intended for submission to peer-reviewed journals. Additionally, information included in this dissertation has been presented at numerous scientific meetings (Table 1-1).

Table 1-1. Abstracts originating from this dissertation that were presented at various scientific meetings.

Date	Title	Conference	Location
1998	Development of methods for a sturgeon germplasm repository: blood collection	Louisiana and Mississippi Chapters of the American Fisheries Society	Bay St. Louis, MS
1998	On-site cryopreservation with nitrogen-vapor shipping dewars	World Aquaculture Society	Las Vegas, NV
2000	Refrigerated storage of shortnose sturgeon sperm	World Aquaculture Society	New Orleans, LA
2001	Cryopreservation of sperm of pallid sturgeon <i>Scaphirhynchus albus</i>	World Aquaculture Society *	Orlando, FL
2002	Cryopreservation of sperm of shovelnose sturgeon <i>Scaphirhynchus platyrhynchus</i>	World Aquaculture Society	San Diego, CA
2002	A chromatin structure assay for cryopreserved sperm from lake sturgeon (<i>Acipenser fulvescens</i>)	Louisiana and Mississippi Chapters of the American Fisheries Society	Biloxi, MS
2002	An assay for DNA integrity using sperm from lake sturgeon (<i>Acipenser fulvescens</i>)	Louisiana Academy of Sciences	Baton Rouge, LA
2003	Viability staining for quality assessment of cryopreserved sturgeon sperm	World Aquaculture Society	Louisville, KY

* Best Student Abstract Award

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Chapter 2

Introduction

The family Acipenseridae is an ancient family of bottom-feeding, freshwater and anadromous fishes. Fossil remains of Acipenser fishes dating back to the Upper Cretaceous period have been found in North America (Gardiner, 1984). Sturgeon have an elongated cylindrical body, which contains 5 rows of bony plates called scutes (Figure 2-1). Sturgeon have a single dorsal fin placed well back on the body, four barbels located anterior to protractile mouths, and a snout which varies in length according to species. The tail is heterocercal with the upper lobe longer than the lower lobe. Species range in color from dark brown to a pale grey, with some variability within species.



Figure 2-1. A typical sturgeon of the genus *Acipenser*. (Drawing courtesy of the National Marine Fisheries Service). Sturgeon are characterized by 5 rows of scutes, a heterocercal tail, a single dorsal fin, four barbels, and an extended snout.

The family comprises 23 living species, which are grouped into two subfamilies: Scaphirhynchinae, the shovelnosed sturgeons, and Acipenserinae, typical sturgeons. The subfamily Scaphirhynchinae comprises two genera, each with three species. Species of the genus *Psuedoscaphirhynchus* are found only in central Asia, and species of the genus *Scaphirhynchus* are found only in North America. The subfamily Acipenserinae has two genera, *Huso* found only in Europe and Asia and *Acipenser* found throughout the Northern Hemisphere. The genus *Huso* contains two species, and the genus *Acipenser* contains 17 species (Birstein and Bemis, 1997).

There are nine species (including 1 subspecies) of sturgeon in North America. These fishes occur throughout the major river systems of the United States (Figure 2-2). Shortnose sturgeon, *Acipenser brevirostrum*, and Atlantic sturgeon, *Acipenser oxyrinchus oxyrinchus*, occur along the Atlantic Coast. Shortnose sturgeon range from the St. John River in Canada to the St. John's River in Florida (Vladykov and Greeley, 1963). Atlantic sturgeon range from the St. Lawrence River in Canada to the St. Johns River in Florida (Waldman and Wirgin, 1998). Gulf sturgeon *Acipenser oxyrinchus desotoi*, a subspecies of the Atlantic sturgeon, occur along the Gulf of Mexico coast from the Mississippi delta in Louisiana eastward to Tampa Bay, Florida (Grunchy and Parker, 1980). Lake sturgeon *Acipenser fulvescens*, pallid sturgeon *Scaphirhynchus albus*, shovelnose sturgeon *S. platyrhynchus* and Alabama sturgeon *S. suttkusi* occur throughout the inland waters of the United States. Lake sturgeon has one of the largest ranges of any North American sturgeon, occurring throughout the Mississippi River drainage, the Great Lakes, and Hudson Bay (Etnier and Starnes, 1993). Pallid sturgeon occur in the Mississippi River system from the Gulf of Mexico to the mouth of the Missouri River, and in the entirety of the Missouri River system (Bailey and Cross, 1954). Shovelnose sturgeon occur in most of the larger rivers of

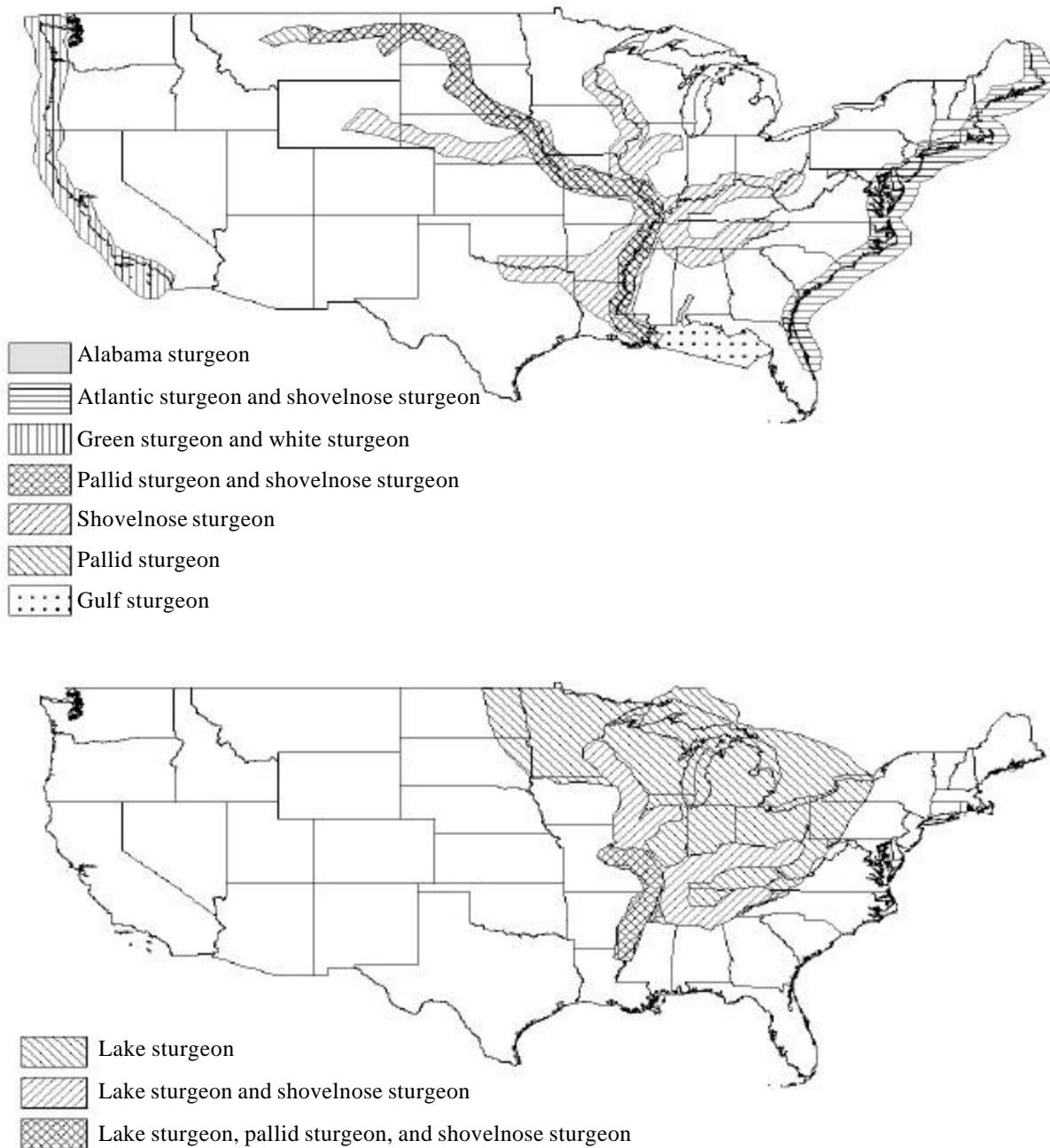


Figure 2-2. Ranges of North American species of sturgeon. Green sturgeon and white sturgeon occur along the Pacific coast. Atlantic sturgeon and shortnose sturgeon occur along the Atlantic coast. Gulf sturgeon occur in the Gulf of Mexico from the Mississippi River eastward to the Sewannee River, Florida. Lake sturgeon occur in the central U.S. from the Great Lakes to northeastern Louisiana. Shovelnose sturgeon occur throughout the central U.S. in every major river system. Pallid sturgeon occur in the Missouri River and in the Mississippi River below the mouth of the Missouri River. Alabama sturgeon only occur in a small section of the Mobile River.

the Mississippi River basin, and in the Rio Grande River (Bailey and Cross, 1954). Alabama sturgeon occur only in the Mobile River basin (Burke and Ramsey, 1995). White sturgeon *A. transmontanus* and green sturgeon *A. medirostrus* inhabit the waters of the Pacific coast. White sturgeon occur from the Aleutian Islands in Alaska to central California (U.S. Fish and Wildlife Service, 1999). Green sturgeon occur from the Aleutian Islands to Ensenada Mexico (Houston, 1988).

Sturgeon species are classified as freshwater or anadromous species. Lake sturgeon, pallid sturgeon, Alabama shovelnose sturgeon and shovelnose sturgeon spend their entire lives in freshwater. Atlantic sturgeon, Gulf sturgeon, shortnose sturgeon, white sturgeon and green sturgeon are anadromous and spend most of their lives in salt water and enter freshwater to spawn. All sturgeon species spawn in rivers over gravel to rocky formations. Gulf sturgeon begin to enter rivers when water temperatures rise above 16 °C, and return to the Gulf of Mexico when river temperatures begin to fall from 26 to 17 °C (Foster and Clugston, 1997). The spawning season of Atlantic sturgeon on the Hudson river occurs from late spring to early summer, with males entering the spawning grounds earlier than females (Van Eenennaam et al., 1996). Lake sturgeon in northern Ontario begin upstream migrations in January and continue until May, congregate on the spawning sites as water temperatures reach 8 to 10 °C, and disperse downstream when water temperatures reach 13 °C (McKinley et al., 1998). Shortnose sturgeon begin spawning when water temperatures rise above 9 °C (Hall et al., 1991; Kieffer and Kynard, 1996), and actively spawn for a period of only 5 to 8 d (Kieffer and Kynard, 1996).

Sturgeon typically require more than 3 years to mature and begin spawning. A reasonable estimation of age at sexual maturity for Atlantic sturgeon may be 12 to 14 years for males and 14 to 18 years for females (Van Eenennaam and Doroshov, 1998). Female pallid sturgeon reach

sexual maturity at 9 to 12 years, and their first spawning may not occur until age 17 or older (Keenlyne and Jenkins, 1993). In the Saint John River, Canada, female shortnose sturgeon initially spawn at 18 years of age, and males reach maturity at 10 years of age (Dadswell, 1984). Minimal information is available on the periodicity of sturgeon spawning. Male shortnose sturgeon are capable of spawning for at least 3 years in a row (Kieffer and Kynard, 1996), however female shortnose sturgeon require a minimum duration of 3 years between spawning events (Dadswell, 1984). These two properties, age to maturity and spawning periodicity, combine to make sturgeon species vulnerable to population depletion due to over-fishing.

Overall, sturgeon are large and long-lived fishes. The largest North American species is the white sturgeon, which can grow to be over 600 cm (Carlander, 1969) and weigh up to 800 kg (Clemens and Wilby, 1961). White sturgeon more than 100 years old have been reported (Rien and Breamesderfer, 1994). Shortnose sturgeon, a medium-sized sturgeon, can attain lengths of 143 cm total length and weigh as much as 24 kg (Dadswell, 1979). Shortnose sturgeon in northern latitudes have a greater longevity than do southern fish. The oldest female shortnose sturgeon reported from the St. John River (Canada) was 67 years old, and the oldest male was 32 years old (Dadswell, 1979). The oldest shortnose sturgeon from the Altamaha River (Georgia) was 10 years old (Dadswell et al., 1984). The smallest of the North American species are the shovelnose sturgeon and Alabama sturgeon. The shovelnose sturgeon can grow to 85 cm (Carlander, 1969) and weigh as much as 4.5 kg (Lee, 1980). The oldest shovelnose sturgeon reported was 16 years (Morrow et al., 1998a). The Alabama sturgeon can grow to 72 cm (Williams and Clemmer, 1991), but there is little information reported for this species. These large sizes make the sturgeon a valuable fish for commercial fisheries.

A variety of valuable products can be obtained from sturgeon. The meat can be pickled, smoked or served freshly cooked. The eggs can be salted to produce caviar. Oil can be obtained from boiling the head, skin and backbone, and isinglass can be obtained from the swim bladder. Isinglass is used in jellies, wine and beer clarification, special cements and glues (Barkuloo, 1988). Currently the main products derived from sturgeon are meat and caviar.

Caviar has been consumed for hundreds of years. Although until the twentieth century, caviar was regarded as being of low value by the majority of people. For example, fishermen ate caviar instead of the more valuable meat (Saffron, 2002). Caviar was also provided to British soldiers (who would often pay for canned sardines instead of eating the caviar) in World War I, used as a free bar snack to encourage drinking, and used to season sauerkraut (Kurlansky, 2002). In the early twentieth century, caviar began to become more valued. From 1900 to 1915 caviar doubled in price as it began to be widely imported into Western Europe from Russia (Saffron, 2002). Between 1991 and 1997, the amount of caviar imported into the United States tripled from 32 to 95 tons annually (Saffron, 2002). As demand for caviar increased, so did its value. Today, caviar can cost as much as U.S. \$2,420 per kg (www.caviar-caviar.com). A single beluga sturgeon can yield as much as 409 kg of eggs (worth nearly U.S. \$ 1,000,000 at today's prices) (Saffron, 2002), however sturgeon that large are rarely encountered today. This high value has placed intense pressure on sturgeon fisheries. As the number of fish continues to decline and the demand for caviar grows, the price for caviar continues to increase, creating more pressure on the fishery.

The Soviet Union (USSR) was the dominant area for commercial sturgeon fishery production during the 1970s and 1980s. In 1988, statistics for the Soviet Union were included in the European statistics, which accounts for the large increase in production of European sturgeon

fisheries (Figure 2-3). Even these fisheries, which had been increasing until 1977, have had a dramatic decrease in production. Until the collapse of the Soviet Union, sturgeon fisheries in the Caspian Sea were strictly controlled by the Soviet government. The government controlled the numbers of fish caught, caviar processing, caviar exports, and even ran hatcheries to re-populate the fishery. When the Soviet Union fell, the sturgeon industries were taken over by private producers, and restrictions on fishing and exportation were removed. The unregulated fisheries were rapidly overfished. Official catches were reduced from about 30,000 tonnes in the late 1970s to less than 3,000 tonnes in the late 1990s. In fact, Russia and Kazakhstan were unable to meet their legal quota of fish in 2000 (www.cites.org).

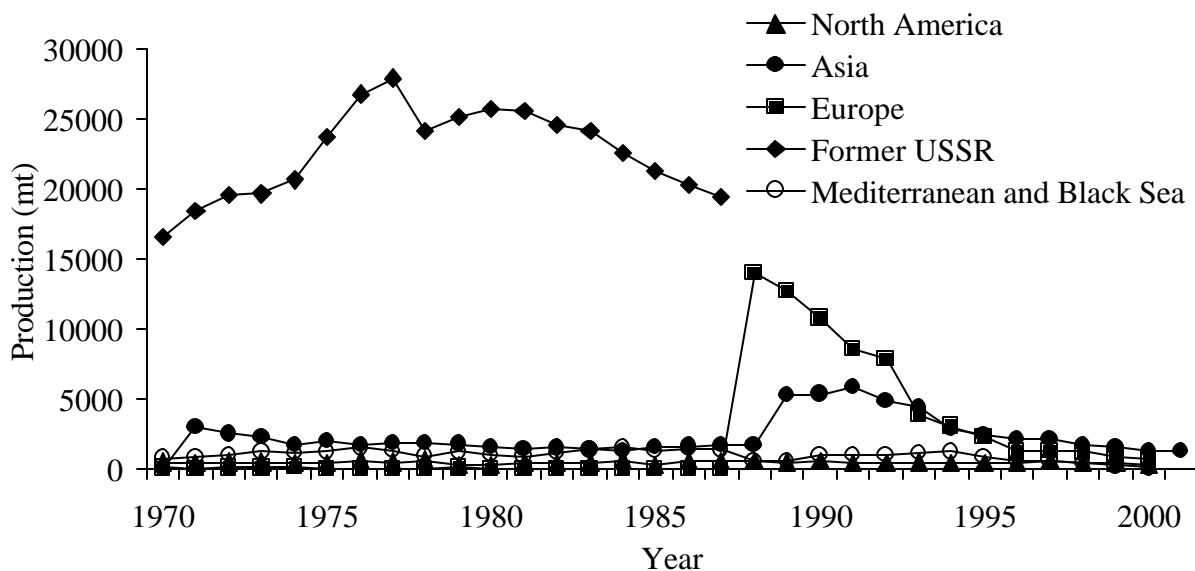


Figure 2-3. Commercial sturgeon fisheries production from 1970 to 2000 (FAO, 2000). Commercial harvest of sturgeon was dominated by the USSR prior to 1988. With the breakup of the USSR, former Soviet republics have been included within the European statistics, which accounts for the large increase in European production in 1988. Overall, sturgeon fisheries have experienced a major decline in the last 20 years.

Poaching has also become a major problem for sturgeon. The international demand for caviar in 1995 was estimated at 450 tonnes, which greatly exceeded the legal production of caviar from Russia and Iran of 228 tonnes (the two largest producers) (DeSalle and Birstein, 1996). Presently it is estimated that the illegal catch of sturgeon in the four republics of the former Soviet Union is 10-12 times the legal catch (www.cites.org). Illegal fishing is not limited to the former Soviet Union. From 1985-1990, an estimated 2,000 white sturgeon were poached by a group of poachers in Oregon to produce approximately 1,350 kg of caviar (Cohen, 1997). In order to reduce the illegal trade in sturgeon and sturgeon products, The Convention on International Trade in Endangered Species placed all sturgeon under Appendix II, requiring importation and export permits for all sturgeon products.

Sturgeon fisheries in the United States have also suffered through drastic declines over the past century. Prior to 1860, a commercial fishery for sturgeon in the United States did not exist (Harkness and Dymond, 1961). Typically the fish were considered a nuisance due to entanglement in nets and competition among bottom fishes. However, when the value of the smoked flesh and caviar became known, it did not take long for the fisheries to decline. Fisheries for Atlantic sturgeon peaked around 1890 at 3.3 million kg, however recent landings of Atlantic sturgeon have ranged from 100,000 to 130,000 kg (Smith, 1985). At the turn of the century, commercial landings of Gulf sturgeon in Florida were 160,000 kg (U.S. Commission of Fish and Fisheries, 1902). In 1976, the commercial landings of Gulf sturgeon in Florida accounted for only 2,761 kg (National Marine Fisheries Service, 1977). As worldwide commercial fisheries have declined over the last 20 years (Figure 2-4), aquaculture production has become more important.

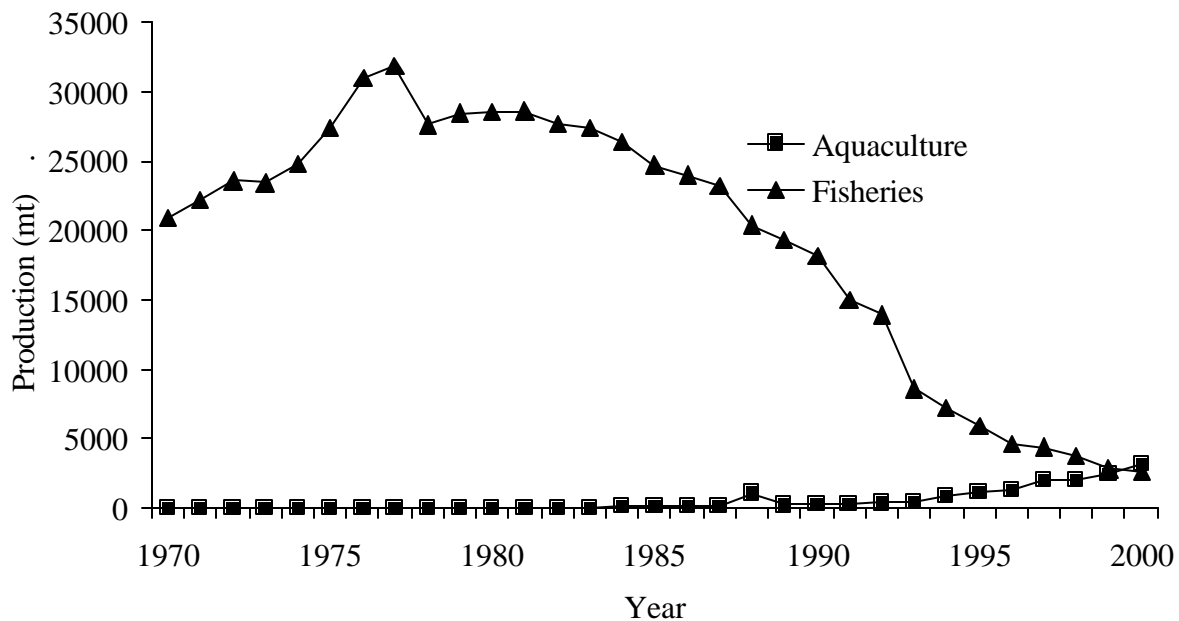


Figure 2-4. Worldwide production of sturgeon from 1970 to 2000 (FAO, 2000). Commercial sturgeon fisheries have drastically declined during the last 20 years. Due to this decline, the aquaculture production of sturgeon has seen an increase during the last 10 years in response to demand.

Commercial aquaculture of sturgeon species has historically been a small industry. However the decline of commercial fisheries has allowed aquaculture production to become profitable as the price for sturgeon products has increased. Development of numerous technologies have also fueled the advancement of the sturgeon culture (Monaco and Doroshov, 1983). In 2000, aquaculture production of sturgeon exceeded fishery production for the first time (Figure 2-4). Europe has dominated the aquaculture industry for sturgeon, but with the recent development of white sturgeon aquaculture in the United States, North American production is increasing (Figure 2-5). A limiting factor in the development of United States sturgeon aquaculture is the endangered or threatened status of many of the North American species.

Shortnose sturgeon were originally listed as an endangered species in 1967 (U. S. Office of the Federal Register, 1967). Their decline is attributed to pollution and overfishing, which

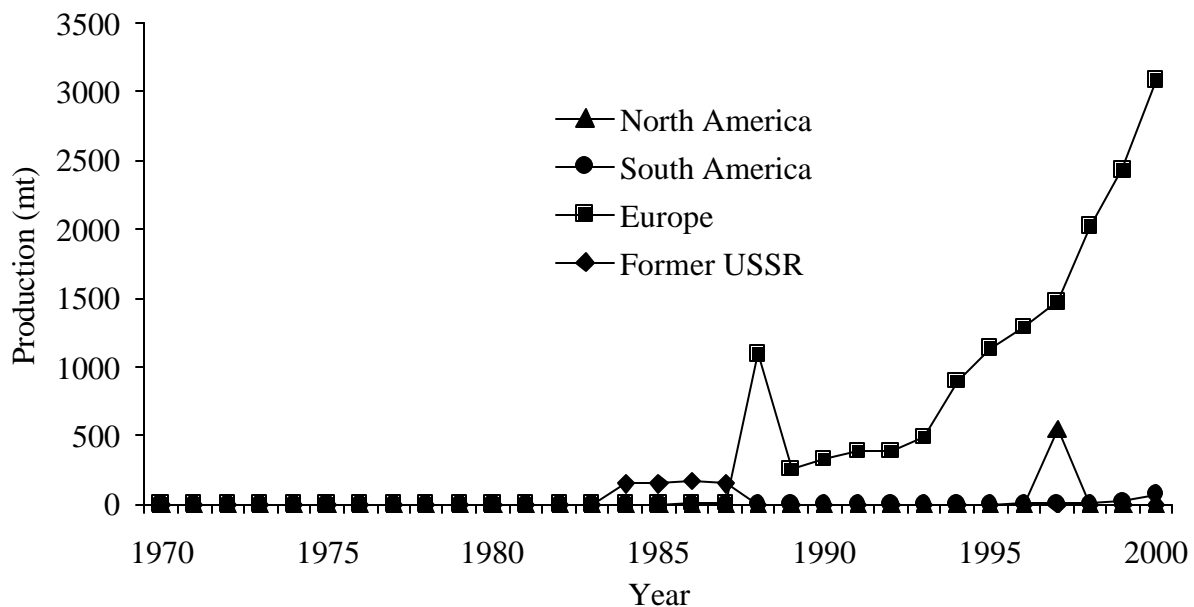


Figure 2-5. Sturgeon aquaculture production from 1970 to 2000 (FAO, 2000). Sturgeon aquaculture was nearly non-existent prior to 1985. As commercial fisheries collapsed, aquaculture production increased to fill the void. Europe has been the leading aquaculture producer of sturgeon. The United States has recently begun aquaculture production of white sturgeon.

includes unintentional capture (bycatch) in shad and Atlantic sturgeon fisheries (National Marine Fisheries Service, 1998). Shortnose sturgeon from different river systems are considered as distinct population segments, in that the populations are reproductively isolated (Kynard, 1997). The loss of a single population of shortnose sturgeon could result in the loss of unique genetic information that is critical to the survival and recovery of the species (National Marine Fisheries Service, 1998).

The pallid sturgeon was federally listed as an endangered species in 1990 (U. S. Office of the Federal Register, 1990). Hybrids between pallid sturgeon and shovelnose sturgeon have been collected, and if the hybrids were fertile, could be a threat to the survival of the species through genetic mixing (introgression) (Carlson et al., 1985). It has also been hypothesized that the

continued hybridization between pallid and shovelnose sturgeon will cause the loss of genetic diversity among these species (Simons et al., 2001).

The Gulf sturgeon was federally listed throughout its range as a threatened species in 1991 (U. S. Office of the Federal Register, 1991). Their decline is attributed to overfishing (Barkuloo, 1988), blockage of spawning migration routes (Morrow et al., 1998b; Wooley and Crateau, 1985), and deterioration of water quality (Bateman and Brim, 1994). The Gulf sturgeon Recovery Plan calls for the maintenance of the genetic integrity and diversity of wild and hatchery stocks (U. S. Fish and Wildlife Service and Gulf States Marine Fisheries Commission, 1995).

The Kootenai River population of white sturgeon was federally listed as endangered in 1994 (U. S. Office of the Federal Register, 1994). The population became isolated by changes in the river basin during the last ice age (~10,000 years ago). Genetic analysis has indicated that the Kootenai population is a unique interbreeding stock with lower genetic diversity than white sturgeon from the lower Columbia River (Setter and Brannon, 1990). Human activities along the river have changed the spawning, egg incubation and larval rearing habitats, contributing to a lack of recruitment since the mid-1960s (U. S. Fish and Wildlife Service, 1999). The United States Fish and Wildlife Service Recovery Plan calls for the use of conservation aquaculture aimed at conserving the genetic diversity of the remaining population.

The Alabama sturgeon was federally listed as an endangered species in 2000 (U. S. Office of the Federal Register, 2000). Of the 1,641 km of historic habitat, recent confirmed captures indicate that the Alabama sturgeon is now restricted to a 557 km stretch of river in the Mobile drainage (Burke and Ramsey, 1995). Currently there are no Alabama sturgeon in captivity, and efforts to collect more fish have been unsuccessful.

The threatened or endangered status of many of the North American sturgeon has led to research into conservation methods for these fishes. Sperm cryopreservation can assist in this effort by preserving the genetic material of declining species or stocks and by allowing greater flexibility with induced spawning in conservation aquaculture programs.

The first published report of cryopreservation of fish sperm was by Blaxter (1953). The study was done on spring and autumn spawning herring, and allowed the crossing of these stocks that would not ordinarily occur. A review (Figiel and Tiersch, 1998) estimated the number of published reports at 185, including abstracts, conference proceedings, technical reports, book chapters, and journal articles. Interest in cryopreservation has been increasing recently. Thirty-seven percent of these reports were published between 1990 and 1996. The majority of cryopreservation research has been on the family Salmonidae, with 67 reports published on 12 different species. The majority of this salmonid research was reviewed by Scott and Baynes (1980) and Billard (1992).

Research into the cryopreservation of sturgeon sperm has received far less attention than cryopreservation of salmonid sperm, and the majority of this research was done in the former Soviet Union (Table 2-1). Much of this information is presented in Russian and is difficult to obtain. There is only one peer-reviewed publication of the cryopreservation of sturgeon sperm from the United States. Post-thaw motility characteristics have been reported for lake sturgeon sperm (Ciereszko et al., 1996). Further research is needed to develop successful cryopreservation methods for North American sturgeon species.

Cryopreservation is the freezing and thawing of biological material such that it remains viable. Theoretically once frozen and stored in liquid nitrogen, material will remain viable indefinitely (Leung and Jamieson, 1991). Cryoprotectants are chemicals that are used to help

Table 2-1. Studies on the cryopreservation of sturgeon sperm. All were performed in the former Soviet Union except Ciereszko et al., 1996, Tsvetkova et al., 1996 and Jähnichen et al., 1999. Reports in Russian were summarized by Mims et al. (2000).

Species	Cryoprotectant	Post-thaw motility (%)	Fertilization (%)	Citation	Language
<i>Huso huso</i>	Glycerol	50-80%	<1%	Burtsev and Serebryakova, 1969	Russian
	Dimethyl sulfoxide	--	43%	Andreev et al., 1996	Russian
<i>H. dauricus</i>	Glycerol	10%	<1%	Burtsev and Serebryakova, 1969	Russian
<i>Acipenser ruthenus</i>	Glycerol	10%	<1%	Burtsev and Serebryakova, 1969	Russian
	Dimethyl sulfoxide	15%	23%	Tsvetkova et al., 1996	English
	Ethylene glycol	10-27%	72-78%	Jähnichen et al., 1999	English
<i>H. huso x A. ruthenus</i>	Glycerol	40%	<1%	Burtsev and Serebryakova, 1969	Russian
<i>A. gueldenstaedti</i>	Glycerol	--	--	Kasimov et al., 1974	Russian
	Dimethyl sulfoxide	50-60%	--	Pushkar et al., 1979	Russian
<i>A. stellatus</i>	Glycerol	40%	35%	Kasimov et al., 1974	Russian
	Dimethyl sulfoxide	--	63%	Pushkar et al., 1979	Russian
	Dimethyl sulfoxide	30-40%	--	Drokin and Kopeika, 1996	English
<i>A. baeri</i>		23%	53%	Tsvetkova et al., 1996	English
	Dimethyl sulfoxide	30-40%	--	Drokin and Kopeika, 1996	English
<i>A. medirostris micadoi</i>	Dimethyl sulfoxide	20%	--	Drokin et al., 1991	Russian
<i>A. medirostris micadoi</i>	Dimethyl sulfoxide	20%	--	Drokin et al., 1993	English
<i>A. nudiventris</i>	Dimethyl sulfoxide	10-90%	--	Cherepanov et al., 1993	English
<i>A. fulvescens</i>	Dimethyl sulfoxide	14%	--	Ciereszko et al., 1996	English
<i>A. mikadoi</i>	Dimethyl sulfoxide	15-20%	--	Drokin and Kopeika, 1996	English

cells survive the cryopreservation process. There are two kinds of cryoprotectants, permeating and non-permeating. Permeating cryoprotectants can cross the cell membrane, and are believed to aid the cell by raising the internal osmotic pressure, preventing dehydration and internal cellular ice formation. Non-permeating cryoprotectants cannot cross the cell membrane, and are believed to aid the cells by attachment to or coating of the membrane to protect it from extracellular ice formation. Cryoprotectants are often toxic to cells, so care must be taken to not kill the cells prior to freezing. Extender composition (typically saline solutions used to maintain sperm in a quiescent state thereby prolonging storage time), freezing rate, and thawing rates are also variables that affect the cryopreservation process.

Certain aspects of sturgeon reproductive physiology are different from typical teleost fishes, which may contribute to the variety of methods used for the cryopreservation of sturgeon sperm. Typically fish eggs are encased in an impenetrable layer called the chorion, which helps to prevent polyspermy (fertilization of a single egg by more than one sperm) by allowing sperm access to the egg through a single opening (micropyle). Sturgeon eggs possess a chorion, but instead of a single micropyle the eggs have 3 to 52 micropyles (Cherr and Clark, 1982; Dettlaff et al., 1993). It is hypothesized that the multiple micropyles allow low concentrations of sperm to produce high rates of fertilization. Furthermore, if the acrosome reaction (a reaction that causes development of a filament on the heads of sturgeon sperm which assists in penetration of the egg) must occur within the micropyle, numerous micropyles allow for successful fertilization to occur even if sperm with reacted acrosomes enter a micropyle (Cherr and Clark, 1985b). Polyspermy is rare in sturgeon eggs, possibly due a rapid reaction that seals the remaining micropyle openings after fertilization (Cherr and Clark, 1985b).

Sturgeon sperm cells are composed of an acrosome, a head, a midpiece, and a flagellum. The main body of sturgeon sperm cells, which includes the acrosome, head, and midpiece, is long, cylindrical and radially symmetrical, however there are differences among species (Figure 2-6). For example, sperm heads of lake sturgeon are nearly three times as long and twice as wide as those of Atlantic sturgeon (DiLauro et al., 1998).

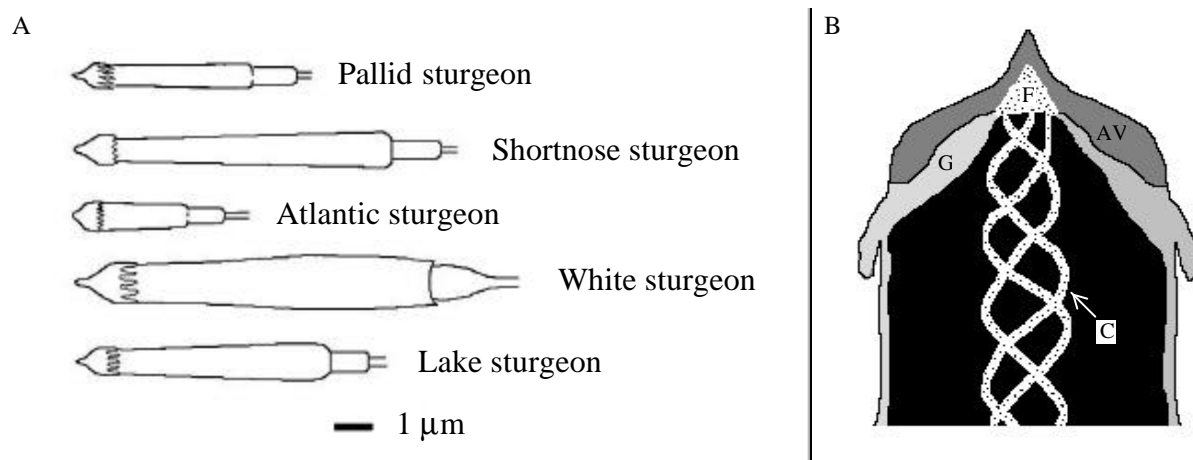


Figure 2-6. Sturgeon sperm cell morphology (adapted from DiLauro et al., 2001 and Cherr and Clark, 1984). Sturgeon sperm are variable in size and are cylindrical in shape (Panel A). Unlike teleost sperm, sturgeon sperm possess acrosomes (Panel B). Below the acrosomal vesicle (AV) is the subacrosomal area, which is composed of filamentous (F) and granular material (G). Channels (C) of filamentous material extend into the nucleus.

The acrosome is a cap-like structure that covers the anterior portion of the sperm head. The acrosome in sturgeon contains projections that extend down along the sides of the nucleus (DiLauro et al., 1998). The main function of the sperm acrosome in aquatic species is the lysis of the jelly coat layer, the outer-most layer surrounding the egg (Baccetti, 1979), however this does not appear to be the case in sturgeon. The jelly coat layer of sturgeon is adhesive and functions to attach the egg to the substrate (Figure 2-7). The jelly coat layer of white sturgeon eggs does not induce acrosome reactions in white sturgeon sperm or lake sturgeon sperm (Cherr

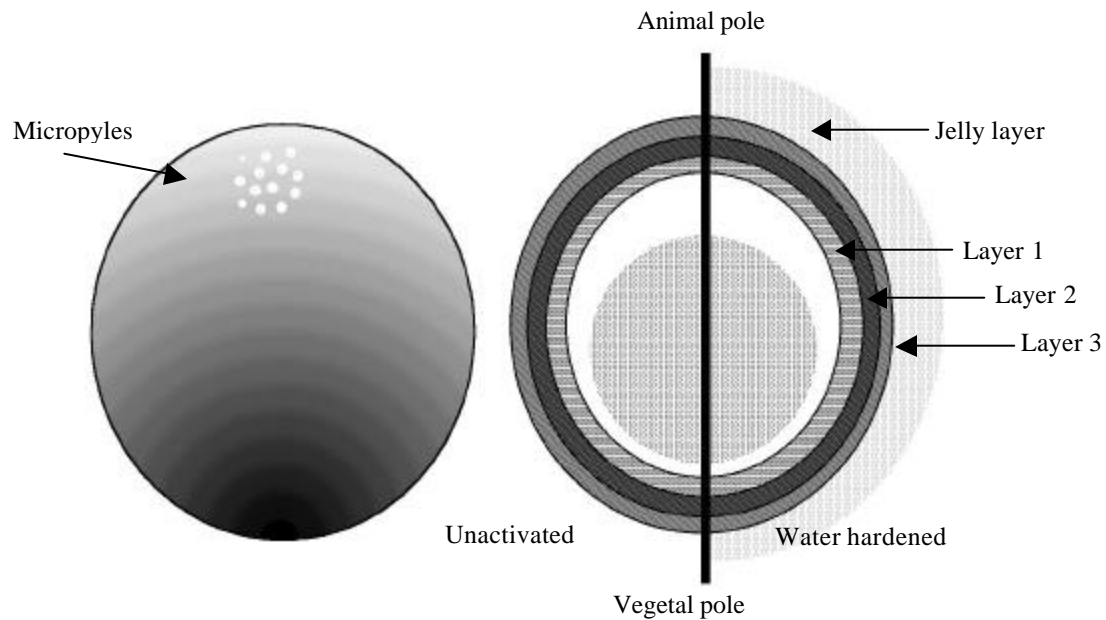


Figure 2-7. Sturgeon egg morphology prior to and after exposure to freshwater (adapted from Cherr and Clark, 1985b). Sturgeon eggs are surrounded by an envelope composed of 4 layers and has multiple micropyles. The outermost layer produces the jelly layer when exposed to freshwater. The other 3 layers are similar to the chorion of teleostean fishes and help to protect the egg.

and Clark, 1985a). A glycoprotein component of the egg envelope of white sturgeon induces the acrosome reaction in white sturgeon sperm, and is species-specific, in that it will not induce an acrosome reaction in lake sturgeon sperm (Cherr and Clark, 1985a).

Within the acrosome is an area referred to as the sub-acrosomal region (Figure 2-6), which is composed of actin protein and can be found in two forms, granular and fibrous (Baccetti, 1979). Actin filaments and granular material were found within the acrosome of white sturgeon (Cherr and Clark, 1984), however only granular material was found in Atlantic sturgeon sperm acrosomes (DiLauro et al., 1998). The sub-acrosomal region is responsible for the creation of the acrosomal process, which is used to help the sperm traverse the micropyle and fertilize the

egg. The variation among the sperm of sturgeon species may necessitate the development of cryopreservation procedures for sturgeon sperm on a species-by-species basis.

The purpose of this research was to assist in the development of a cryopreserved sperm repository for North American sturgeon species at the USFWS Warm Springs Fish Technology Center. In addition, the components outlined in this dissertation are intended to provide the framework for development of cryopreserved sperm repositories at other locations for all species of fish. The main components of a sperm repository, identified in this dissertation, are: 1) proper sample identification; 2) elimination or minimization of disease agent transfer; 3) techniques for refrigerated storage of sperm; 4) techniques for cryopreservation of sperm; 5) evaluation of sperm quality before and after thawing; and 6) incorporation of all known information regarding samples and broodstocks into a database.

Chapter 3, “Development of Methods to Verify the Field Identifications of Species,” focuses on methods that can be used to verify species identification for sperm samples. Sturgeon species are similar in appearance and in many cases are sympatric (occur together in the same river system). Proper species identification is critical for archival storage of cryopreserved samples. A polymerase chain reaction (PCR) and restriction digest method is illustrated in Chapter 3 as a possible method to aid in the proper identification of species. The PCR was used to amplify a specific segment of the cytochrome b gene found in mitochondrial DNA, which allows the DNA to be visualized through staining. Restriction enzymes were then used to cut the DNA segment at specific locations based on a series of specific base-pair sequences. Species-specific differences in the base-pairs at these sites allow the differentiation of the species. These differences can be visualized by electrophoresis of the digested DNA on an agarose gel, staining

the DNA with ethidium bromide, and viewing the DNA under ultraviolet light. These techniques were chosen because they are relatively simple, inexpensive, quick and reliable.

Transfer of bacterial diseases among fish stocks and species has come under intense scrutiny (Ganzhorn et al., 1994). The cryopreservation of sperm from wild fishes and the subsequent shipping of frozen sperm within and among nations involve the risk of transporting bacterial pathogens to new areas. In an effort to reduce this risk, Chapter 4, “Bacterial Contamination of Sperm Samples,” focused on identifying common bacterial contaminants of sturgeon sperm, and on methods to reduce their transmission. Sixteen genera of bacteria, including known pathogens, were isolated from sturgeon sperm samples. The Chapter includes information on the effect of antibiotics on refrigerated storage of sperm and its fertilizing ability, and the effect of sperm cryopreservation procedures on bacterial survival.

Chapter 5, “Development of Refrigerated Storage Methods,” examines methods for the non-frozen storage of sturgeon sperm. The ability to maintain viable sperm in a refrigerated condition is necessary for practical function of a cryopreserved sperm repository. Refrigerated storage allows sperm to be collected from distant locations and shipped to a centralized cryopreservation facility. This allows one facility to centralize the equipment and techniques necessary for cryopreservation, while receiving sperm from other locations that possess expertise and facilities necessary for handling and spawning of broodstock. As part of this work, studies evaluated the osmolality and chemical composition of sturgeon seminal and blood plasma. The Chapter investigated varied concentrations of ionic and non-ionic solutions for the ability to activate sturgeon sperm motility. Extenders for refrigerated storage were developed based on the seminal plasma osmolality and chemical composition, as well as the solutions that inhibited

sperm activation. Extenders formulated from this research were used to retain sperm motility during refrigerated storage for as long as 21 d.

Methods for the cryopreservation of sturgeon sperm are discussed in Chapter 6, “Development of Cryopreservation Techniques.” Cryopreservation is a process of freezing biological material in which the material is still viable upon thawing. Cryopreserved sperm can allow the conservation of genetic material from declining stocks of endangered species, as well as benefit commercial aquaculture by providing sperm when spawning females are available. The Chapter discusses the effects of various cryoprotectants on equilibration motility (i.e., toxicity of the chemicals), post-thaw motility, and fertilization rates. Six cooling rates (from 5 to 40 °C/min) in a controlled-rate freezer and a field method of freezing in a nitrogen-vapor shipping dewar were evaluated for their effects on cryopreservation. Also, differences in the effects of extenders on cryopreservation of sturgeon sperm are briefly discussed.

The ability to determine the quality of cryopreserved samples without the necessity of fertilization trials, would be of benefit to a repository. Chapter 7, “Development of Methods for the Objective Determination of Sperm Quality,” evaluates an alternative method to motility estimation for sperm quality assessment. The most common method employed for the determination of sperm quality is motility estimation. Typically this method is fast and fairly accurate, but because the method is subjective, technical bias is involved in the estimation. Also in some instances, motility has not been correlated to fertility of thawed sperm (Stein and Bayrle, 1978). A dual-staining technique (SYBR-14 and propidium iodide) was evaluated for its ability to accurately identify membrane integrity, and to determine if membrane integrity was correlated with fertilizing ability. The use of flow cytometry produced a fast (300 cells/sec), quantitative method to evaluate 10,000 cells per sample.

Inventory control and maintenance of information regarding cryopreserved samples is critical to the operation of a sperm repository. Chapter 8, "Development of a Model Cryopreserved Sperm Repository Database for Fishes," illustrates a model database that was developed for storage of information for sperm samples held in a germplasm repository for aquatic species at Warm Springs Fish Technology Center. The database was developed using a commercially available software, Microsoft Access, and includes sections for sample identification and information, fish information, and feedback from users of thawed samples. Currently the database is being used to maintain a five dewar repository containing samples from more than 15 species of fishes (7 species of sturgeon), including four endangered species, and 3,952 straws.

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Chapter 3

Development of Methods to Verify the Field Identifications of Species.

Introduction

The pallid sturgeon *Scaphirhynchus albus* and shovelnose sturgeon *S. platyrhynchus* occur in the same river systems and overlap in size and appearance in the southern portions of their range (Snyder, 2002). The method used to distinguish the two species is a composite of 14 measurements (Bailey and Cross, 1954). The pallid sturgeon and shovelnose sturgeon are closely related and only slight genetic differences can be detected between the species (Phelps and Allendorf, 1983; Campton et al., 2000; Simons et al., 2001). The Alabama sturgeon *S. suttkusi* is also closely related to the shovelnose sturgeon, and due to their similar appearances was likely misidentified as a shovelnose sturgeon in 1953 (Chermock, 1955). The Alabama sturgeon was not recognized as a separate species until 1991 (Williams and Clemmer, 1991).

Species of sturgeon whose ranges do not overlap can also be difficult to differentiate. The Atlantic sturgeon *Acipenser oxyrinchus oxyrinchus* occurs along the Atlantic coast from the St. Lawrence River in Canada to the St. Johns River in Florida (Waldman and Wirgin, 1998). The Gulf sturgeon *A. o. desotoi* occurs along the Gulf coast from Tampa Bay to the mouth of the Mississippi River (Grunchy and Parker, 1980), and is a subspecies of the Atlantic sturgeon (Vladykov, 1955). Other than distribution, the only character found to be diagnostic in identifying the two sub-species was relative spleen length, although this character was found to overlap (Wooley, 1985).

The use of cryopreserved sperm from clearly identified species will assist recovery efforts for these and other threatened and endangered species (U. S. Fish and Wildlife Service, 1993). The correct identification of the species from which sperm samples are collected is essential for an

archival repository. Typically sperm samples sent to a cryopreservation facility should be accompanied with documentation of sample information, however the facility should have independent methods of verifying the species identification.

A similar problem exists during the inspection of caviar for importation into the United States. Caviar can only be imported into the United States by way of a permit from the Convention on International Trade in Endangered Species (CITES). In order to guarantee that the caviar imported was the same as listed on the permit, the United States Fish and Wildlife Service's Forensics Laboratory developed a method using the polymerase chain reaction and DNA sequencing to identify the species origin of the caviar (adapted from Wolf et al., 1999). The application of this technique for use with sturgeon species of North America could provide a useful method for species verification of sperm samples.

In order to ensure proper identification of samples placed in a cryopreserved sperm repository, this study was designed to evaluate two methods of species verification, other than phenotype. The specific objectives were to: 1) evaluate methods for the handling of sturgeon blood for analysis by flow cytometry; 2) determine if genome size analysis by flow cytometry could distinguish pallid sturgeon from shovelnose sturgeon; and 3) determine if a restriction enzyme assay developed for the identification of caviar samples could be used to differentiate sperm of shortnose sturgeon *Acipenser brevirostrum*, Atlantic sturgeon *A. oxyrinchus*, Gulf sturgeon *A. o. desotoi*, lake sturgeon *A. fulvescens*, white sturgeon *A. transmontanus*, pallid sturgeon *Scaphirhynchus albus*, and shovelnose sturgeon *S. platorynchus*.

Material and Methods

Blood Collection

Blood samples (2 ml) were collected (Standard Operating Procedure 1, Appendix A) (SOP) from pallid sturgeon ($n = 5$) held at United States Fish and Wildlife Service Natchitoches National Fish Hatchery (NNFH) in Natchitoches, Louisiana ($44^{\circ} 93' N$, $93^{\circ} 05' W$), and transported at $4^{\circ} C$ to the Louisiana State University Agricultural Center Aquaculture Research Station (ARS), Baton Rouge, Louisiana, ($30^{\circ} 22' N$, $91^{\circ} 10' W$). In order to verify that blood-handling techniques would not affect genome size values, blood was stored at 3 concentrations of anticoagulant and genome size values analyzed daily. Blood was transferred to 1.5-ml tubes containing acid citrate dextrose anticoagulant (ACD) (Catalog # 364606, Becton Dickinson Vacutainer Systems, Franklin Lakes, New Jersey) resulting in a final ACD concentration of 10, 25, and 50%. The samples were refrigerated and analyzed by flow cytometry daily up to 4 days for changes in apparent genome size (described below).

Blood Storage

Blood samples (2 ml) were collected (SOP 1, Appendix A) from shovelnose sturgeon ($n = 23$) held at NNFH, and transported at $4^{\circ} C$ to the ARS. To determine the effect of refrigerated storage on apparent genome size values, samples were refrigerated and analyzed by flow cytometry daily from day 0 to day 4 and then on day 7, 8 and 11 for changes in apparent genome size (described below).

Genome Size

Blood samples were collected (SOP1, Appendix A) and analyzed within 24 hr. Blood of pallid sturgeon or shovelnose sturgeon (0.5 µl) was suspended in 0.5 ml of propidium iodide (PI) solution (SOP 2, Appendix A) (Crissman and Steinkamp, 1973). Blood of channel catfish *Ictalurus punctatus* (0.5 µl) was added to each sample and used as an internal standard (Tiersch et al., 1989). Channel catfish cells have a genome size of 2.0 pg of DNA (Tiersch et al., 1989). The samples were incubated for 10 min in the dark, and analyzed by flow cytometry (FACSCalibur[®], Becton Dickinson Immunocytometry Systems, BDIS, San Jose, California). A minimum of 10,000 cells was counted for each sample. After 10 samples of each sturgeon species, three samples each of channel catfish (0.5 µl) and human blood (60 µl), channel catfish (0.5 µl) and female chicken *Gallus domesticus* blood (10 µl), and channel catfish (0.5 µl) and male chicken blood (10 µl) were analyzed to maintain calibration of the flow cytometer.

Genome size for the sturgeon samples was calculated according to Tiersch et al. (1989). The formula used was:

$$\text{pg DNA} = 7.0 (X/S)(S/H)$$

Where pg DNA is the mass of DNA per cell in picograms, X is the fractional mode channel (FMC) of the fluorescence peak for the sturgeon sample, S is the FMC of the fluorescence peak for channel catfish blood, and H is the FMC of the fluorescence peak for the human blood.

Restriction Enzyme Assay

Total DNA was extracted using a QIAamp[®] DNA blood mini kit (Cat # 51104, Qiagen Inc., Valencia, California) from frozen sturgeon blood and sperm samples. The blood and body fluid spin protocol (supplied with the kit) was used, with the exception that 25 µl of fish blood or

sperm and 175 µl of molecular grade water were used instead of 200 µl of human blood. The protocol is designed to extract DNA from human blood samples in which only the white blood cells are nucleated. However, fish red blood cells are nucleated, and the use of higher amounts of fish blood or sperm caused the columns to become clogged with excess DNA.

A 240-bp fragment of the cytochrome b gene was amplified by the polymerase chain reaction (PCR) from the extracted DNA. Primers L14851-13 and H15125 (Table 3-1) (Fain et al., 2000) were used for the amplification. The PCR procedure using these primers was optimized using the Failsafe PCR Premix Selection Kit (Epicentre Technologies, Madison, Wisconsin). The PCR amplifications were performed in 50-µl reaction volumes and contained 0.5 µl of template DNA, 0.5 µl of each primer, 0.5 µl of enzyme mix, 25 µl of premix H, and 23.0 µl of molecular grade water (Geno Technology Inc., St. Louis, Missouri). The amplifications were conducted in a thermal cycler (Amplitrone II, Thermolyne, Dubuque, Iowa) using a program of 30 cycles of denaturing for 30 sec at 96 °C, primer annealing for 30 sec at 55 °C, and extension for 90 sec at 72 °C. A negative control (deionized water substituted for the template DNA) was included in each amplification set.

Table 3-1. Nucleotide sequences of the primers used for amplification of a 240-bp fragment of the cytochrome b gene from mtDNA. The primers were adapted from Fain et al. (2000).

Name	Sequence
L14851-13	5' – GTGTGATGAAATTTGGCTC – 3'
H15125	5' – AGTACATATCCTACGAAGGC – 3'

Restriction digest assays of amplified DNA were used to differentiate the sturgeon species. Three restriction enzymes (*Mse* I, *Hae* III, and *Rsa* I; New England Biolabs, Beverly, Massachusetts) were used simultaneously in each reaction to cut the DNA. The reaction mixtures contained 2 µl of New England Biolabs buffer 2 (supplied with the restriction enzymes), 2 µl of bovine serum albumin (supplied with the restriction enzymes), 3 µl molecular grade water, and a µl of each restriction enzyme. The mixtures were incubated for 1 hr at 37 °C. After incubation the mixtures were analyzed on a 4% agarose gel (A-2790, Sigma Chemical Company, St. Louis, Missouri) in 1% tris-borate-ethylenediaminetetraacetic acid buffer (T-3913, Sigma Chemical Company). Gels were electrophoresed for 2.5 hr at 70 volts, and stained for 15 min in 5 µg/ml of ethidium bromide (Intermountain Scientific Corporation, Kaysville, Utah) and destained in distilled water for 45 min. Restriction fragments were visualized and photographed (Polaroid 667 film, Polaroid Corporation, Cambridge, Massachusetts) on a transilluminator using ultraviolet light (FBTI-88; Fisher Scientific, Pittsburgh, Pennsylvania).

Statistical Analysis

The main effects of ACD concentration and storage time on apparent genome size were determined using a 3 (ACD concentration) x 3 (storage time) factorial analysis of variance (ANOVA) (NCSS 2000, Number Cruncher Statistical Systems, Kaysville, Utah). Differences in apparent genome size when stored in 10% ACD were determined using a one-way ANOVA. Means were separated at the $P = 0.05$ level using a Tukey-Kramer multiple comparison test.

Results

Blood Collection

There were significant differences in apparent genome size due to time of storage ($P < 0.0001$) and ACD concentration ($P = 0.0254$) for pallid sturgeon blood. After 3 d of storage, blood stored in 50% ACD had significantly higher ($P < 0.0500$) genome size values (3.957 pg) than did blood stored in 25% ACD (3.931 pg). The genome size value for blood stored in 10% ACD (3.941 pg) was similar ($P > 0.0500$) to that of blood stored in 25% or 50% ACD.

Blood Storage

There were significant differences ($P = 0.0001$) among the apparent genome size of stored shovelnose sturgeon blood over time. Blood stored for longer than 2 d had a significantly higher value ($P < 0.05$) for genome size (≥ 3.980 pg) than did blood stored for shorter periods (≤ 3.918 pg).

Genome Size

There was no significant difference ($P = 0.9333$) between the genome sizes of pallid sturgeon (3.909 ± 0.019 pg) (mean \pm SD) and shovelnose sturgeon (3.894 ± 0.021 pg). The genome size estimated by flow cytometry for the reference standards was 2.0 ± 0.01 pg for channel catfish, 2.5 ± 0.01 pg for female chicken, and 2.6 ± 0.02 pg for male chicken, which agree with values reported by Tiersch et al. (1989).

Restriction Enzyme Assay

The assay was used to differentiate the sturgeon samples into 5 groups (Figure 3-1). Lake sturgeon *A. fulvescens*, shortnose sturgeon *A. brevirostrum*, and white sturgeon *A. transmontanus* had unique restriction fragments. Atlantic sturgeon and Gulf sturgeon had identically sized restriction fragments, and pallid sturgeon and shovelnose sturgeon also had identically sized restriction fragments (Figure 3-1).



Figure 3-1. Restriction enzyme analysis of seven North American sturgeon species. A section of the cytochrome b gene was amplified by the polymerase chain reaction and cut with the restriction enzymes *Hae* III, *Mse* I and *Rsa* I. The restriction fragments were electrophoresed on a 4% agarose gel at 70 volts for 2.5 hr. The size standard is a 20-bp ladder.

Discussion

The taxonomic status of pallid sturgeon and shovelnose sturgeon has been debated since the original description of pallid sturgeon in 1905 (Forbes and Richardson, 1905). The species are morphometrically distinct as adults (Bailey and Cross, 1954), but show little or no genetic differentiation. Pallid sturgeon and shovelnose sturgeon had identical protein electrophoretic patterns (Phelps and Allendorf, 1983) and low levels of DNA sequence divergence in the

mitochondrial control region (2.06%, less than typically observed between species) (Campton et al., 2000) and cytochrome-b gene (0-11%) (Simons et al., 2001). The purpose of the current study was to determine if genome analysis by flow cytometry could provide a method to differentiate these species. However, there was no difference between the genome size values of pallid sturgeon and shovelnose sturgeon in this study. Flow cytometry has been used to determine sex in birds (Nakamura et al., 1990) and mammals (Elias et al., 1988; Kent et al., 1988) due to differences in size of the sex chromosomes, with the ability to discriminate a difference of as little as 0.01 pg. Flow cytometry has also been used to identify and distinguish channel catfish, blue catfish *Ictalurus furcatus*, flathead catfish *Pylodictus olivaris* or black bullhead catfish *Ameiurus melas* parental stocks and hybrids, which differed in genome size by as little as 0.04 pg of DNA (Tiersch and Goudie, 1993).

The failure of flow cytometry to distinguish a difference in the genome size of the two species in the current study may have been caused by improper assignment of species. The samples used in this study were collected from the Atchafalaya River in Louisiana. Pallid sturgeon and shovelnose sturgeon in this part of their ranges are similar morphometrically, and can be difficult to identify (Snyder, 2002). The samples may have also included hybrid sturgeon, which may have caused a convergence of the genome values. Possible pallid and shovelnose hybrid sturgeon have been captured in the Mississippi River (Bailey and Cross, 1954; Carlson et al., 1985). However, the pallid sturgeon and shovelnose sturgeon used in the current study were identified by a member of the pallid sturgeon recovery team (Bobby Reed, Louisiana Department of Wildlife and Fisheries), using outer barbel placement relative to inner barbels, outer barbel length as compared to inner barbel length, and presence or absence of scutes on the belly. These characteristics were identified by Bailey and Cross (1954) as being diagnostic for these species.

Our estimate of genome size for shovelnose sturgeon (3.894 pg) differs from two previously reported estimates. One estimate of genome size by flow cytometry for shovelnose sturgeon was 4.73 pg of DNA (Blacklidge and Bidwell, 1993). The samples used in that study were shipped (24 hr) and stored for 24-48 hr prior to analysis. In the current study blood samples stored for longer than 2 d had a higher apparent genome size (+ 0.18 pg) than when originally collected. Also no mention was made of the concentration of ACD used when blood was collected in that study. High concentrations (50%) of ACD in the current study also produced larger genome size values after 2 d of storage. The concentration of ACD and the storage time prior to analysis in the 1993 study could have resulted in elevated genome size values. A second report, using microdensitometry of Feulgen-stained cells estimated genome size for shovelnose sturgeon to be 3.6 pg of DNA (Ohno et al., 1969). There are no published reports of the genome size of pallid sturgeon.

Although not specifically designed to differentiate the North American sturgeon species, the restriction digest of the cytochrome b gene was able to differentiate shortnose sturgeon, lake sturgeon, and white sturgeon. Atlantic sturgeon and Gulf sturgeon could not be differentiated from each other, but could be isolated from the other sturgeon species. The Gulf sturgeon is a sub-species of the Atlantic sturgeon. The two sub-species show very little mtDNA genotypic diversity (0.670 overall) (Bowen and Avise, 1990), however, a study identified a polymorphic site within the mtDNA control region that is fixed in either sub-species (Ong et al., 1996). The addition of the primers identified in the 1996 study and a restriction enzyme (HpyCH4V, New England Biolabs) would allow the differentiation of these closely related sub-species.

The protocol used in the current study could not differentiate species from the genus *Scaphirhynchus*. The three species are closely related and exhibit very low mitochondrial

diversity (Campton et al., 2000; Simons et al., 2001). A recent study has determined a genetic marker that may be used to distinguish among Alabama sturgeon, pallid sturgeon and shovelnose sturgeon (Campton et al., 2000). The alteration of our current protocol by using different primers to evaluate different gene sequences and restriction enzymes may allow the differentiation of these species.

The visual identification of sturgeon species can be difficult. The inclusion of cryopreserved samples in a repository for use with endangered species must be based on proper identification. The methods discussed in this study were able to identify shortnose sturgeon, lake sturgeon, and white sturgeon, and to separate pallid sturgeon, shovelnose sturgeon, and Alabama sturgeon into a group, and Atlantic sturgeon and Gulf sturgeon into a different group. The method could not differentiate Atlantic sturgeon from Gulf sturgeon or pallid sturgeon, shovelnose sturgeon, and Alabama sturgeon from each other. Future work should focus on the differentiation of these species through the addition of new primer sets and restriction enzymes.

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Chapter 4

Bacterial Contamination of Sperm Samples

Introduction

The introduction of disease agents through the transfer of fishes is a well-documented problem (Kohler and Courtenay, 1986). Fish pathogens that were once endemic to small areas or populations have been transported worldwide, causing disease in wild and cultured stocks of fishes. For example, furunculosis, a disease caused by the bacteria *Aeromonas salmonicida*, was initially native to rainbow trout *Oncorhynchus mykiss* of western North America, but from introduction of rainbow trout throughout the world, is now considered to be present wherever salmonids are present (Welcomme, 1984). At least forty-eight species of parasites have been documented to occur outside their native areas due to the transportation of infected fishes (Hoffman, 1970). The transfer of pathogens through the shipment of fish gametes is also a potential problem that has not received considerable attention.

The recent surge in development of cryopreservation techniques for aquatic species has focused attention on the need for the consideration of pathogen transfer through the use of preserved samples (Jenkins, 2000). Recommendations and guidelines exist for the importation and transportation of gametes from aquatic species (e.g. United States Fish and Wildlife Service, 1995; Office International des Epizooties, 1997), however these recommendations typically address screening for a particular list of pathogens and are typically designed for fertilized eggs. The transfer of pathogens through the use of sperm or cryopreserved sperm samples has received limited attention (Tiersch and Jenkins, 2003).

Certain pathogens are capable of being transferred vertically (transmitted from parent to offspring) through the sperm of infected fishes (Mulcahy and Pascho, 1984; Nusbaum, 1987;

Saint-Jean et al., 1992). The white sturgeon iridovirus (Hedrick et al., 1990) can be vertically transmitted (Georgiadis et al., 2001). Several other viral diseases have been identified that affect sturgeon (Hedrick et al., 1985; Hedrick et al., 1991; Watson et al., 1995), and vertical transmission is suspected to occur (Watson et al., 1995). The development of cryopreservation procedures to allow sperm to survive freezing and thawing processes, likely would allow bacteria and viruses to survive as well.

The American Type Culture Collection (www.atcc.org) recommends the long-term storage of bacteria by adding 10% glycerol or 5% DMSO as a cryoprotectant and plunging the mixture into liquid nitrogen. Cryopreservation protocols for sperm also include the use of cryoprotectants and storage in liquid nitrogen, so it is therefore not surprising that bacteria can survive these freezing procedures. For example, the bacteria *Mycoplasma meleagridis* suffers little or no loss of viability when cryopreserved using a protocol designed for turkey sperm (Ferrier et al., 1982). However, there are no reports evaluating the survival of bacteria through cryopreservation protocols for fish sperm. Additionally, contaminated liquid nitrogen in which cryopreserved samples are stored can be a vector for the transfer of pathogens to other preserved samples (Bielanski et al., 2000).

Along with the possible transfer of pathogens, bacterial contamination has been shown to cause reductions in fish sperm motility and storage time (e.g., Jenkins and Tiersch, 1997). This problem has received ample attention in bovine (Shin et al., 1988) and equine sperm (Jasko et al., 1993), however it has received limited attention in aquatic species. Bacterial contamination of sperm has been shown to reduce fertility rates of Atlantic salmon *Salmo salar* (Stoss and Refstie, 1983). Motility and fertility were also reduced in contaminated sperm samples of common carp *Cyprinus carpio* (Saad et al., 1988). The use of antibiotics for the reduction of bacterial

contamination and to increase storage time in sperm of aquatic species needs further investigation.

Antibiotics are naturally produced chemicals that kill or inhibit the growth of cells (Todar, 2003). There are five primary modes of action of antibiotics: cell-wall synthesis inhibitors, cell membrane inhibitors, protein synthesis inhibitors, nucleic acid synthesis inhibitors, and competitive inhibitors (Todar, 2003). Antibiotics that are commonly used in the treatment of fish diseases are typically cell-wall synthesis inhibitors and protein synthesis inhibitors. Cell-wall synthesis inhibitors can be used to selectively target bacteria due to the lack of cell walls in eukaryotic cells. Penicillin is a common example. Protein synthesis inhibitors commonly target a component of protein synthesis (the 70s ribosome), which allow them to selectively target bacteria, and stop the production of proteins. Oxytetracycline and gentamicin are common antibiotics that use the protein synthesis inhibitor mode of action. Enrofloxacin is a commonly used synthetic antibacterial compound with a mode of action through nucleic acid synthesis inhibition. Enrofloxacin works by inhibiting the DNA gyrase enzyme, causing a blockage of DNA replication and fragments of DNA to be lost (summarized in Sarkozy, 2001). Differences in the structure of eukaryotic DNA gyrase allows for the bacterial selectivity of enrofloxacin (Walsh, 2000). The selectivity attributes of these antibiotics should allow their inclusion into sperm extenders to reduce bacterial numbers without damaging sperm.

The elimination or reduction of bacteria within cryopreserved samples is important to aid in the reduction of pathogen transfers and help retain viable samples. As more cryopreservation protocols are developed, there is need to incorporate methods into these protocols for the reduction of pathogen transfer. The specific objectives of this study were to: 1) characterize bacteria commonly found as contaminants in sturgeon sperm; 2) determine the effects of

antibiotics on refrigerated storage and fertilizing ability of sperm, and 3) determine the effects of a standard cryopreservation protocol on bacterial survival.

Materials and Methods

Lake Sturgeon

Sperm were collected according to SOP 5 (Appendix A) from 8 males collected from the Black River, Michigan (45° 24' N, 84° 24' W). Bacterial isolates were cultured from the sperm samples (SOP 4, Appendix A), and identified to at least the level of genus.

Shortnose Sturgeon

Sperm Collection:

Sperm were collected according to SOP 5 (Appendix A) from 5 males at the Bears Bluff National Fish Hatchery (United States Fish and Wildlife Service, Wadmalaw Island, South Carolina, 32° 39' N, 80° 15' W). Motility was determined for each sample (SOP 3, Appendix A), and only samples with motilities $\geq 90\%$ were used in studies.

Storage Study:

Sperm samples from 5 males were used in this study. Three antibiotics were tested for the ability to prolong the refrigerated storage time of sperm samples. Each antibiotic was mixed in a commercially available modified Hanks' balanced salt solution (mHBSS; H-4385, Sigma Corporation, St. Louis, Missouri) (SOP 6, Appendix A) that was diluted to an osmolality of 100 mOsmol/kg (Model 5520, Wescor Incorporated, Logan Utah). Gentamicin (Catalog # 15750-060, Lot # 1060070, Gibco BRL, Grand Island, New York) was used at concentrations of 2, 10,

and 20 µg/ml. Enrofloxacin (Baytril, Lot # 267140, Bayer, Shawnee Mission, Kansas) was used at concentrations of 1, 5, and 10 µg/ml. Oxytetracycline (Maxim 200, Lot # 9030308, Phoenix Pharmaceuticals Inc., St. Joseph, Missouri) was used at concentrations of 6, 30, and 60 µg/ml. Each sperm sample was diluted 1:4 (v:v; sperm:extender) in each of the antibiotic solutions. The motility of each sample was determined daily (SOP 3, Appendix A). After 21 d of refrigerated storage, the remaining samples with progressive motility were used in a fertilization and hatch trials according to SOP 9 and SOP 10 (Appendix A). Fresh sperm were used in fertilization trials as a control for egg quality. In an effort to equalize the quantity of sperm per treatment, 100 µl of sperm were used for controls and 500 µl of extended sperm (1:4, v:v) for each treatment.

Pallid Sturgeon

Sperm Collection and Bacterial Identifications

Sperm were collected according to SOP 5 (Appendix A) from 3 males at the Garrison Dam National Fish Hatchery (GDNFH) (U. S. Fish and Wildlife Service, Riverdale, North Dakota, 47° 29' N, 101° 25' W). Motility was determined for each sample (SOP 3, Appendix A), and only samples with motilities $\geq 85\%$ were used in studies. Bacterial isolates were cultured from the sperm samples (SOP 4, Appendix A), and identified to at least the level of genus.

Storage Study

Sperm samples from 3 males were used in this study. Three antibiotics were tested for the ability to prolong refrigerated storage of sperm samples. Each antibiotic was prepared as described above. The antibiotics used were gentamicin, enrofloxacin, and oxytetracycline. Each

antibiotic was used at concentrations of 2, 10, or 20 µg/ml. Each sperm sample was diluted 1:4 (v:v; sperm:extender) in each of the antibiotic solutions. The motility of each sample was determined daily (SOP 3, Appendix A). Bacterial isolates were obtained from the sperm samples (SOP 4, Appendix A), and identified to at least the level of genus.

Shovelnose Sturgeon

Sperm Collection and Bacterial Identification

Sperm were collected from 20 males caught from the Missouri River (47° 37' N, 108° 37' W) above Fort Peck Dam and held at the Charles M. Russell National Wildlife Refuge, U. S. Fish and Wildlife Service, Lewistown, Montana (47° 35' N, 108° 43' W). A sterile swab was dipped into each sperm sample and bacterial isolates were prepared and identified as described in SOP 4 (Appendix A).

Cryopreservation Protocol Effects on Bacterial Survival

Pure cultures from 8 bacterial samples were used in a study to determine the effects of cryopreservation on bacteria associated with sturgeon sperm. The isolates were passed twice on TSA slants prior to use in this study. Two-ml aliquots of mHBSS were inoculated with isolates from each bacterial sample, and incubated aerobically for 24 hr in a 22 °C incubator. Methanol was used as a cryoprotectant and was added to the bacterial samples to a final concentration of 5%, and allowed to equilibrate (the time between addition of the cryoprotectants and the beginning of the freezing process) for 3 min. The samples were frozen according to the procedure used for the cryopreservation of sturgeon sperm (SOP 12, Appendix A). The cooling rate was 31 °C/min. After 2 hr the samples were submerged in liquid nitrogen.

Serial dilutions of the initial mHBSS-bacterial mixtures were made to determine the number of colony forming units (CFU) /ml per sample prior to freezing. Dilutions were made in sterile filtered (0.2- μ m syringe filters) mHBSS. Each dilution (0.1 ml) was plated on brain heart infusion agar plates (Product # 241830, Difco), and incubated for 48 hr in a 22 °C incubator. Individual colonies were counted for each plate, and the number of CFU/ml was determined from the lowest dilution in which individual colonies could be counted.

The frozen samples were maintained in liquid nitrogen for 5 d before thawing for 9 sec in a 40 °C water bath. The straws were wiped dry, and the ends cut. The bacterial samples were collected in 1.5-ml centrifuge tubes. Serial dilutions were made from each sample to determine the numbers of CFU/ml per sample, which were compared to replicates before freezing. The effects of the cryopreservation process on bacterial survival were determined.

Statistical Analysis

A repeated measures analysis of variance (SAS, SAS Institute Inc., Cary, NC) was used to determine time by extender interaction effects in the refrigerated storage studies for the shortnose sturgeon and pallid sturgeon. A Tukey's Studentized range test was used to determine the differences between the mean percent motility for each extender at each time point. Due to a lack of replication for samples within the shortnose sturgeon refrigerated sperm fertilization trial, percent fertilization and percent hatch rates were not analyzed statistically. A paired t-test for means (Excel 2000, Microsoft Corporation, Redmond, WA) was used to determine differences between CFU/ml counts prior to freezing and after thawing for bacteria cryopreserved with the sturgeon sperm method.

Results

Lake Sturgeon

Bacterial Identifications

Bacterial identifications included 9 species representing 5 genera (Table 4-1). Four of those species (*Aeromonas hydrophila*, *A. sobria*, *Micrococcus luteus*, *Shewanella putrefaciens*) are known pathogens of fish.

Shortnose Sturgeon

Storage Study

There was a significant decline ($P < 0.0001$) in motility of samples over time. However there was no antibiotic by time interaction ($P = 0.0655$). All treated samples had motilities similar to the controls throughout the study (Figure 4-1). Samples retained motility for a maximum of 23 d. The motility of treated sperm for remaining samples was $15 \pm 5\%$ (\pm SD) after 21 d of refrigerated storage (Table 4-1). All refrigerated samples used in the study were capable of fertilizing eggs (range 1 - 81%) and producing embryos (range 1 - 45%). Due to the low sample sizes per treatment (1-2 replicates), results were not compared statistically.

Pallid Sturgeon

Bacterial Identifications

Bacterial identifications included 8 genera and 3 isolates that were not identified (Table 4-2). Three of the isolates identified came from genera that are known to be common environmental bacteria, and considered to be non-pathogenic (*Acinetobacter*, *Micrococcus*, and *Alkaligenes*).

Table 4-1. Mean values (\pm SD) for percent motility, percent fertilization, and percent hatch for shortnose sturgeon sperm exposed to three concentrations of three antibiotics for 21 d. Sperm (100 μ l of fresh sperm or 500 μ l of treated sperm) were used to fertilize 100-200 eggs; n = number of replicates per treatment. The eggs were incubated at 16 °C. After 6 hr, ~100 eggs were removed and preserved in 10% buffered formalin for determination of fertilization percentage at the 4-cell stage. The remaining eggs were incubated until hatch. Fresh sperm was used as a control for evaluation of egg quality.

Treatment	n	Motility (%)	Fertilization (%)	Hatch (%)
Control (egg quality)	2	95 \pm 0	86 \pm 0	32 \pm 15
Control (no antibiotic)	1	15	23	7
Enrofloxacin 5 μ g/ml	1	10	24	21
Enrofloxacin 10	1	20	15	8
Gentamicin 2	2	10 \pm 7	19 \pm 26	11 \pm 13
Gentamicin 10	1	20	76	22
Gentamicin 20	2	15 \pm 7	26 \pm 28	11 \pm 9
Oxytetracycline 6	1	20	81	45
Oxytetracycline 30	1	20	77	36
Oxytetracycline 60	1	10	41	22

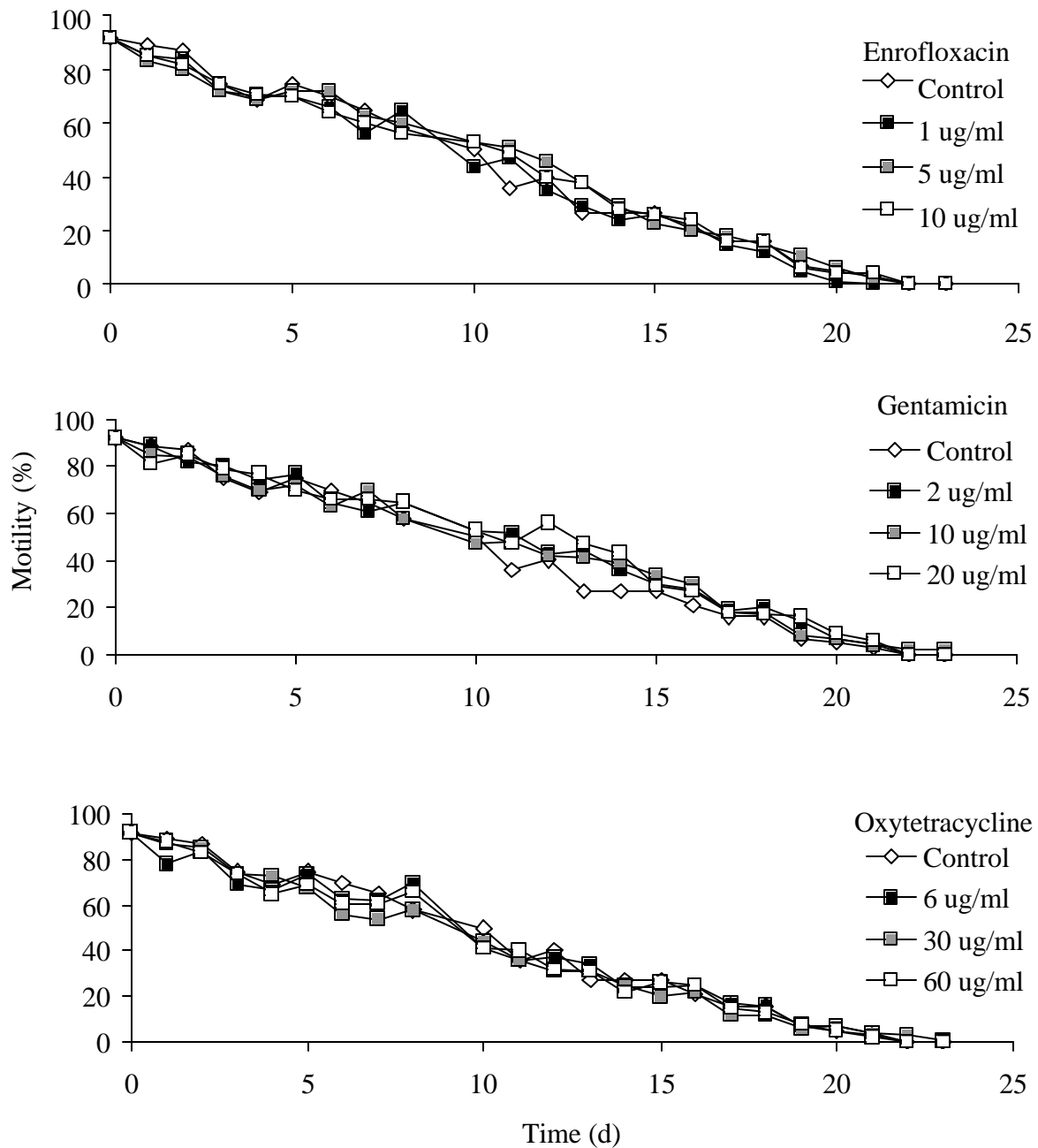


Figure 4-1. Motility of shortnose sturgeon sperm exposed to various concentrations of antibiotics for as long as 23 d. Motility was estimated daily for refrigerated samples diluted 1:4 (v:v) with a modified HBSS extender with antibiotics added. The antibiotics used were: enrofloxacin at concentrations of 1, 5, and 10 $\mu\text{g/ml}$; gentamicin at concentrations of 2, 10, and 20 $\mu\text{g/ml}$, and oxytetracycline at concentrations of 6, 30, and 60 $\mu\text{g/ml}$. Each point represents the mean of five fish. Overall motility decreased significantly throughout the study ($P < 0.0001$). There were no differences ($P = 0.0655$) for percent motility values among treatments at any time period throughout the study.

Table 4-2. Identification of bacterial isolates collected from sperm samples from three sturgeon species. Sperm were collected using methods that are commonly practiced in the field. Routine sperm sampling procedures were used, and no additional effort was made to collect the samples aseptically.

Lake Sturgeon	Pallid Sturgeon	Shovelnose Sturgeon
<i>Aeromonas caviae</i> *	<i>Acinetobacter lwoffii</i>	<i>Actinomyces</i> sp.
<i>Aeromonas hydrophila</i> *	<i>Aeromonas caviae</i>	<i>Flavobacterium</i> sp.*
<i>Aeromonas sobria</i> *	<i>Alkaligenes faecalis</i>	<i>Kocuria rosea</i>
<i>Bacillus</i> sp.	<i>Brevundimonas vesicularis</i>	<i>Listeria</i> sp.
<i>Micrococcus luteus</i> *	<i>Enterococcus</i> sp.	<i>Micrococcus luteus</i> *
<i>Micrococcus lylae</i>	<i>Micrococcus lylae</i>	<i>Micrococcus lylae</i>
<i>Staphylococcus cohnii</i>	<i>Pseudomonas putida</i> *	<i>Micrococcus sedentarius</i>
<i>Staphylococcus</i> sp.	<i>Stenotrophomonas maltophilia</i> *	<i>Pseudomonas aeruginosa</i>
<i>Shewanella putrefaciens</i> *		<i>Pseudomonas fluorescens</i> *
		<i>Pseudomonas</i> sp.*
		<i>Staphylococcus epidermidis</i>
		<i>Staphylococcus hominis</i>
		<i>Staphylococcus xylosus</i>
		<i>Vibrio hollisae</i>

* Species of bacteria that are pathogenic to fish.

Storage Study

There was a significant decline in motility over time for all samples ($P < 0.0001$). Motility was retained for a maximum of 20 d (Figure 4-2). At this time motility had decreased to $\leq 1\%$ for all samples that retained motility. There were no effects due to antibiotic or antibiotic by time interactions ($P = 0.2953$). All treated samples had motilities similar to that of the control samples throughout the study.

Shovelnose Sturgeon

Bacterial Identifications

From the 40 isolates obtained from the sperm samples, 17 bacterial species representing eight genera were identified (Table 4-2). Identifications could not be determined for 11 of the isolates. These unidentified isolates were all Gram-positive rod-shaped non-motile bacteria. The identified bacteria included pathogenic and non-pathogenic species.

Bacterial Survival

There was no significant difference ($P = 0.8126$) between the number of CFU/ml prior to freezing and after thawing for bacterial samples collected from shovelnose sperm. The number of CFU/ml prior to freezing was $1.1 \times 10^8 \pm 1.1 \times 10^8$ (mean \pm SD). The number of CFU/ml after thawing was $1.0 \times 10^8 \pm 1.2 \times 10^8$ (mean \pm SD) (Table 4-3).

Discussion

Bacterial contamination of sperm samples can cause degradation of samples, and the complete loss of motility (Jenkins and Tiersch, 1997). The addition of antibiotics has been used

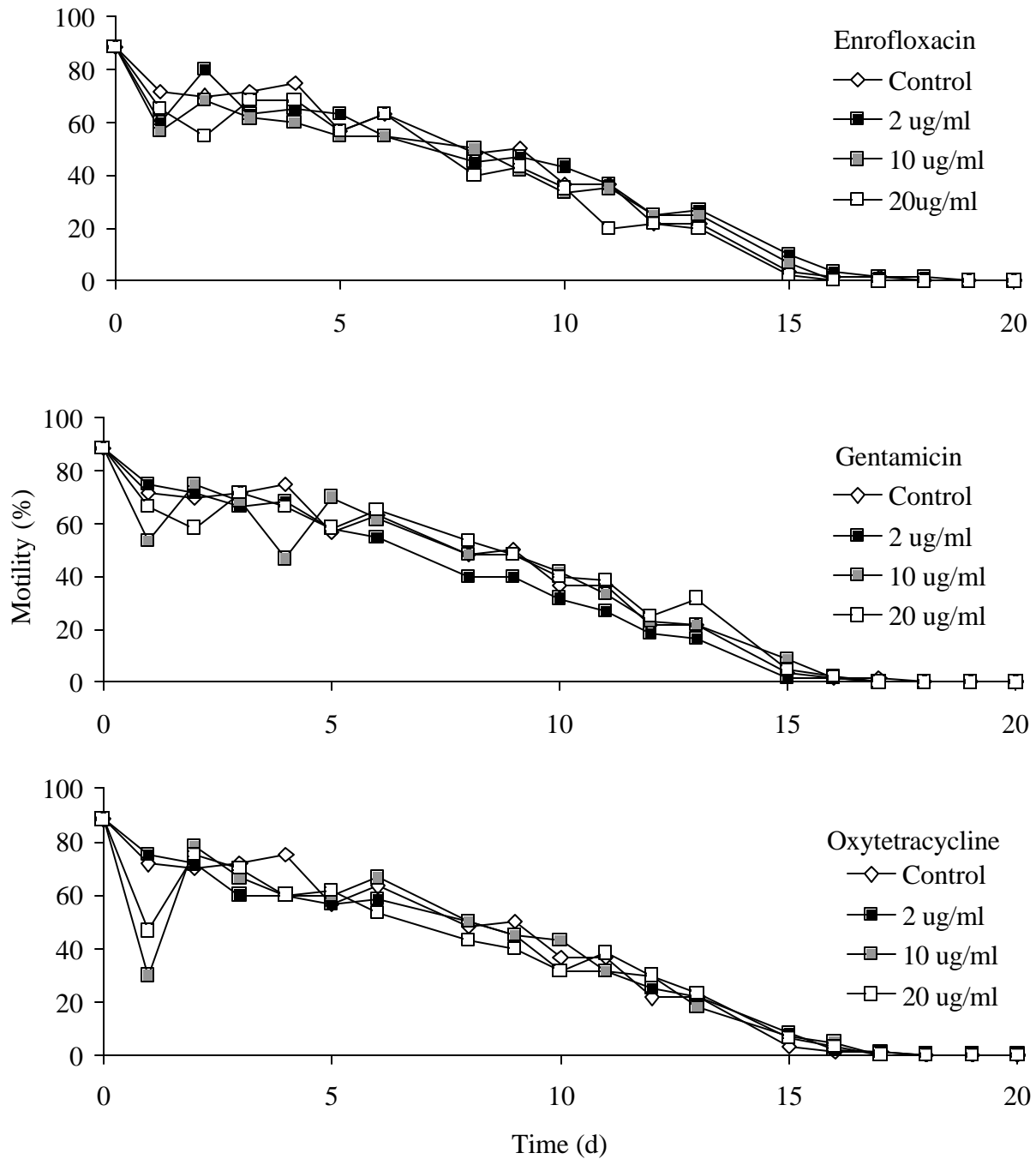


Figure 4-2. Motility of pallid sturgeon sperm after exposure to antibiotics for as long as 20 d. Motility was estimated daily for refrigerated sperm in modified Hanks' balanced salt solution as an extender with varied concentrations of antibiotics. The antibiotics used were enrofloxacin, gentamicin, and oxytetracycline, each at a concentration of 2, 10, and 20 µg/ml of extender. Each sperm sample was diluted 1:4 (v:v) with an extender/antibiotic solution. Each point represents the mean of three fish.

Table 4-3. Colony forming units (CFU/ml) for bacterial isolates from shovelnose sturgeon sperm prior to and after cryopreservation. The samples were frozen in 0.5-ml straws, which were packed in goblets at the lower position on aluminum canes. The samples were cooled in a nitrogen-vapor shipping dewar at a rate of 31 °C/min. The samples were stored for 5 d in liquid nitrogen and thawed at 40 °C for 9 sec. Thawed samples were plated on BHIA plates, incubated at 22 °C for 24 hr, and the CFU/ml counted. There was no significant difference ($P = 0.8126$) between CFU/ml counts prior to freezing or after thawing for each culture.

Bacterial sample	CFU/ml	
	Prior to freezing	After thawing
<i>Flavobacterium sp.</i>	1.16×10^8	1.07×10^8
<i>Vibrio hollisae</i>	2.55×10^8	1.60×10^8
<i>Psuedomonas sp.</i>	1.60×10^8	1.50×10^8
Unidentified*	2.71×10^7	2.18×10^7
<i>Flavobacterium sp.</i>	2.65×10^8	3.59×10^8
<i>Kocuria rosea</i>	3.59×10^7	1.27×10^7
<i>Staphylococcus epidermidis</i>	4.06×10^5	4.09×10^4
<i>Micrococcus lylae</i>	2.07×10^7	1.34×10^7

*The identification of this culture to species was not possible, although the culture was classified as a Gram positive rod-shaped non-motile bacteria.

to increase storage time of refrigerated sperm samples of channel catfish *Ictalurus punctatus* (Christensen and Tiersch, 1996), paddlefish *Polyodon spathula* (Mims, 1991), and Atlantic salmon *Salmo salar* (Stoss and Refstie, 1983). The addition of antibiotics to sperm of shortnose sturgeon and pallid sturgeon in this study did not provide storage times longer than those of sperm samples stored without antibiotics. The addition of antibiotics to refrigerated sperm samples of walleye *Stizostedion vitreum* also did not increase storage times compared to untreated control samples (Satterfield and Flickinger, 1995), but samples contaminated with feces did have reduced storage times. The 1995 study did not incorporate a treatment combining antibiotic addition and fecal contamination, which may have shown the benefits of antibiotics to prolonged storage time. The samples in the present study were collected by inserting a tube into the urogenital opening, which would reduce the possibility of fecal contamination of the samples. The addition of antibiotics to sperm of Nile tilapia *Oreochromis niloticus* did not have an effect on percent motility of stored sperm samples, but did retain higher levels of viability and mitochondrial function (Segovia et al., 2000). Other measures of sperm quality, such as membrane integrity or mitochondrial function, may have revealed benefits of the addition of antibiotics in the current study that were not evident using motility retention.

Antibiotics have also been shown to be toxic to sperm samples. The addition of high concentrations of penicillin or streptomycin (> 9,000 I. U.) to sperm of rainbow trout *Oncorhynchus mykiss* reduced storage times (Stoss et al., 1978). The addition of gentamicin, enrofloxacin, or oxytetracycline at the concentrations used in this study did not reduce sperm storage time as compared to untreated controls. However, it is possible that the concentrations of the antibiotics used in this study were not sufficient to control bacterial growth. Bacterial counts

were not conducted during the present study to determine if the antibiotics reduced bacterial numbers throughout the study.

Incorporation of antibiotics into human sperm samples has reduced motility and fertilization abilities (King et al., 1997). In this study, at the time eggs were available (21 d into the refrigerated storage study), the remaining shortnose sturgeon sperm samples from this study retained fertilizing ability. Although some of the samples had fertilization and hatch rates equal to or greater than the controls, the lack of replication made statistical comparisons impossible.

The bacterial species identified in the samples in the current study contained typical ubiquitous non-pathogenic and pathogenic flora. Three of the isolates identified came from genera (*Acinetobacter*, *Micrococcus*, and *Alkaligenes*) that are known to be common environmental bacteria (Krieg and Holt, 1984), and considered to be non-pathogenic. *Aeromonas* species (motile aeromonas septicemia) (Plumb, 1999) and *Pseudomonas* species (fin rot) (Noga, 1996) are known to be pathogenic bacteria for aquatic species, and *Stenotrophomonas maltophilia* is known to have antibiotic resistance (Zhang et al., 2000). Few bacterial identification studies have been done with sperm samples from aquatic species. Bacteria of the genus *Pseudomonas* were the most frequently encountered genus from refrigerated sperm of channel catfish, and showed a direct relationship between increasing bacterial numbers and decreasing sperm motility (Jenkins and Tiersch, 1997). In the current study, the effect of bacteria contamination on sperm motility is unclear, because bacterial concentrations were not determined for stored samples.

Sperm samples for the present study were collected as typically done during normal sturgeon spawning. No measures were taken to reduce the levels of bacterial contamination. To improve the quality of sperm samples and reduce the chance of bacterial contamination certain steps

should be taken. Use of sterile sampling equipment and sterile extenders could help to reduce bacterial contamination. Disinfecting the area around the urogenital opening prior to sample collection could also reduce the possibility of contaminating sperm samples with bacteria. These steps would help to minimize bacterial contamination and reduce the risks associated with the transport of sperm samples.

Bacterial survival through cryopreservation of mammalian and avian sperm has been shown (Ferrier et al., 1982). The bovine artificial insemination industry adds antibiotics to sperm samples to reduce the transfer of pathogens (Jones, 1958; Sullivan et al., 1966). No studies have been done to determine the survival of bacterial pathogens through the cryopreservation process for aquatic species. In this study there was no effect on the number of CFU/ml for the eight bacterial samples examined.

Bacterial contamination of sperm samples from sturgeon species could be a problem when using the current practices. As many as 14 species of bacteria were found in the samples studied, and these bacteria were not eliminated or reduced by the cryopreservation procedure for sturgeon sperm. The use of antibiotics at the concentrations used in this study did not have an adverse effect on sperm motility and fertility during refrigerated storage. Further research into the effects of antibiotics on bacterial concentrations during sperm storage could provide a method to reduce or eliminate the bacterial contamination of sperm samples.

Other agricultural industries that routinely use cryopreserved sperm, such as cattle and swine, have addressed the issue of bacterial contamination of cryopreserved samples (Althouse et al., 2000; Shin et al., 1988). However, cryopreservation of sperm from aquatic species is a recent development, and has not yet been used on a commercial scale. The identification of contamination issues regarding cryopreserved sperm from aquatic species has been addressed

(Tiersch and Jenkins, 2003). The report identified several procedures to limit the risk of disease transmission through cryopreserved samples, including adequate training of personnel about techniques to minimize contamination of samples, maintenance of disease-free broodstocks, maintenance of sterile extenders and cryoprotectants, use of antimicrobial compounds, and proper labeling and storage of cryopreserved samples to reduce the risk of unintentional use of samples. Also the incorporation of existing regulations for the transfer of fish and gametes (primarily fertilized eggs) and formulation of new regulations for the transfer of cryopreserved sperm samples would help to ensure the biosecurity of cryopreserved sperm (Jenkins, 2000).

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Chapter 5

Development of Refrigerated Storage Methods

Introduction

The development of refrigerated storage techniques can provide management tools for the genetic improvement of aquaculture species and the genetic conservation of threatened and endangered species. Stored sperm can be used to transfer genes from wild stocks into hatchery stocks to maintain genetic diversity (Cloud et al., 1990), aid in induced spawning protocols, and aid in the propagation of populations where reproductive asynchrony occurs between males and females (Bromage and Cumararatunga, 1988). Refrigerated storage techniques are also necessary for the transport of sperm to a cryopreservation facility, which assures repeatability and quality control of cryopreserved samples.

Typically, refrigerated storage involves dilution of collected sperm in an extender (solutions designed to maintain sperm in a quiescent state), and storage of the sperm at cool temperatures for days to weeks while maintaining fertilizing ability. Methods for refrigerated storage of sperm have been developed for numerous freshwater species (e.g. reviews by Scott and Baynes, 1980 and Rana, 1995) and marine species (Chereguini et al., 1997; Wayman et al., 1997; 1998; Yao et al., 1999; Ritar and Campet, 2000; and Riley, 2002). The majority of research on freshwater species has focused on salmonids, while few studies have addressed refrigerated storage of sturgeon sperm. White sturgeon sperm (Conte et al., 1988) and Atlantic sturgeon sperm (DiLauro et al., 1994) have been stored undiluted for as long as 14-17 d, but few studies on the use of extenders for refrigerated storage have been reported.

The use of extenders can increase storage time by minimizing the effects of urine contamination. Urine, which is hypotonic to bodily fluids of freshwater fishes, can cause sperm

activation due to a dilution of ionic concentrations or osmotic pressure (Poupard et al., 1998).

Use of an extender can help retain ion concentrations and osmotic pressure at levels close to that of the seminal plasma, preventing sperm activation.

The goal of this study was to develop refrigerated storage techniques for four species of sturgeon representing the genera *Acipenser* and *Scaphirhynchus* by determining the osmolality and chemical constituents of seminal plasma and their effects on sperm motility. The specific objectives of this study are to: 1) determine the osmotic pressure and ionic composition of blood and seminal plasma; 2) characterize the duration of sperm motility; 3) determine the effects of osmotic pressure on sperm activation; 4) determine the effects of specific ions on sperm activation; and 5) determine the effects of ionic and osmotic solutions on sperm storage time.

Materials and Methods

Plasma Osmolality

Blood and sperm samples were collected from lake sturgeon *Acipenser fulvescens* (n = 8), shortnose sturgeon *A. brevirostrum* (n = 18), pallid sturgeon *Scaphirhynchus albus* (n = 7), and shovelnose sturgeon *S. platyrhynchus* (n = 9). Blood plasma samples were collected according to SOP 1 (Appendix A), except as follows. Pallid sturgeon blood samples (600 µl) were transferred to plasma separator tubes containing lithium heparin (Catalog # 0266939, Fisher Scientific, Atlanta, Georgia), and were centrifuged for 1.5 min at 6,000- \times g (Micro 7 Centrifuge, Fisher Scientific). Shortnose sturgeon blood samples were collected without anticoagulant, transferred to 3-ml plain vacutainers (#6434, Becton Dickinson and Company, Rutherford, New Jersey), and allowed to clot. Blood (1 ml) from lake sturgeon and shovelnose sturgeon was collected and transferred to 1.5-ml tubes and centrifuged at 5,000- \times g for 10 min. Ten microliters of blood

plasma were removed from each sample and used for the determination of osmolality. Sperm samples were collected (SOP 5, Appendix A) and 1 ml of sperm was transferred into 1.5-ml tubes. The tubes were centrifuged at 5,000- \times g for 10 min to pack the sperm cells at the bottom of the tubes. Ten microliters of seminal plasma were removed and used for the determination of osmolality. The osmotic pressure of the blood and seminal plasma was determined using a vapor pressure osmometer (Vapro 5520, Wescor, Logan, Utah). The remaining plasma was frozen for plasma composition analysis.

Seminal and Blood Plasma Composition Analysis

The seminal and blood plasma samples collected above were sent frozen in nitrogen-vapor shipping dewars to the Olympia Fish Health Center (United States Fish and Wildlife Service, Olympia, Washington) for composition analysis. Glucose, magnesium, phosphorus, and calcium concentrations were determined by standard spectrophotometric methods (Model HCL 2000; Hycel Groupe Lisabio Company, Morangis, France). Sodium, potassium, and chloride concentrations were determined by standard isoelectric methods (EasyVet; Medica Corporation, Bedford, Massachusetts).

Duration of Motility

Sperm (2 μ l) were activated with 20 μ l of deionized water and the level and duration of motility were estimated as described in SOP 3 (Appendix A). For lake sturgeon sperm ($n = 2$), motility was estimated every 15 sec for the first minute, every 30 sec until 5 min, and every 1 min after 5 min until forward motility for each sample ceased. For sperm of shovelnose sturgeon ($n = 10$), motility was estimated every 15 sec until all forward motility for each sample ceased.

Osmotic Activation

Sperm were activated with 20 μ l of specific solutions (described below). Motility was estimated immediately after activation, and after motility was estimated, 10 μ l of the activated sperm were removed directly from the slide. The osmotic pressure of the activated sample was determined using a vapor pressure osmometer. Threshold activation was defined as the highest osmotic pressure that elicited 10% motility (Bates et al., 1996). Complete activation was defined as the highest osmotic pressure that elicited the highest motility for a sample.

For sperm of lake sturgeon (n = 5), shortnose sturgeon (n = 5), and shovelnose sturgeon (n = 5), the activating solutions consisted of serial dilutions (range 61 – 316 mOsm/kg) of a modified Hanks' balanced salt solution (mHBSS) (H-4385, Sigma Chemical Corporation, St. Louis, Missouri) (SOP 6, Appendix A), 5.75% sucrose solution, and a 1.25% sodium chloride solution. Each activating solution was initially made at an osmolality of ~300 mOsm/kg, and serially diluted with deionized water.

For pallid sturgeon (n = 4), the activating solutions consisted of 10 serial dilutions (range 6-300 mOsm/kg) of mHBSS, Hanks' balanced salt solution (HBSS) (Tiersch et al., 1994) (SOP 7, Appendix A), a 1.25% sodium chloride (Sigma Chemical Corporation) solution, or a 5.75% sucrose (Sigma Chemical Corporation) solution.

Ionic Activation

Sperm (2 μ l) from lake sturgeon (n = 2), pallid sturgeon (n = 3), shortnose sturgeon (n = 5), and shovelnose sturgeon (n = 5) were activated with 20 μ l of various activating solutions. There were five activating solutions for lake sturgeon and shovelnose sturgeon consisting of 0.4 mM to

2.0 mM sodium bicarbonate, potassium phosphate, calcium chloride, potassium chloride, glucose, sodium phosphate, magnesium sulfate, or sodium chloride. The twelve activating solutions for pallid sturgeon sperm consisted of 0.2 mM to 2.0 mM magnesium chloride, potassium chloride, calcium chloride, or sucrose. The seven activating solutions for shortnose sturgeon sperm consisted of 0.1 mM to 1 mM calcium chloride, sodium chloride, or potassium chloride. Motility was estimated immediately after activation.

Refrigerated Storage

Sperm of pallid sturgeon (n = 4), shortnose sturgeon (n = 4), or shovelnose sturgeon (n = 5) were diluted with each of four extenders (mHBSS, HBSS, sodium chloride, and sucrose) each at three concentrations (100, 200, and 300 mOsmol/kg) at a ratio of 1:4 (v:v; sperm:extender). To determine the effects of each extender combination on sperm motility prior to activation, motility was determined immediately after mixing the sperm with the extender (no deionized water was used to activate the sperm). The extended sperm were stored in upright 50-ml tubes in the refrigerator. Motility was estimated daily according to SOP 3 (Appendix A). Compressed oxygen gas was added to the tubes after motility estimation and the tubes were re-capped.

After 6 and 14 d of storage, the shortnose sturgeon sperm samples were used in a fertilization trial. In an effort to equalize sperm quantities per egg, 1 ml from extended sperm samples or 0.2 ml from undiluted controls were added to ~200 eggs. Fresh sperm (0.2 ml) were used as a control for egg quality. Water was added to activate the gametes. After 5 min, the water was poured off and a Fuller's earth solution (Humco, Texarkana, Texas) was added and mixed to reduce egg adhesiveness. After 30 min, the eggs were transferred to 5-cm diameter egg hatching jars and incubated at 16 °C. After 6 hr, approximately half of the eggs were removed and

preserved in 10% buffered formalin (SOP 8, Appendix A) to determine percent fertilization at the 4-cell stage. The remaining eggs were incubated to determine hatch rates.

Statistical Analysis

Percent motility data were arc-sine square root transformed prior to analysis. Differences in seminal and blood plasma osmolality and chemical composition among species were determined using a one-way analysis of variance (ANOVA) (NCSS 2000, Number Cruncher Statistical Systems, Kaysville, Utah). Means were separated at the $P = 0.05$ level using a Tukey-Kramer multiple comparison test. When data were not normally distributed (determined by testing the normality of the residuals) (NCSS 2000), values were compared using a Kruskal-Wallis ANOVA, and means were separated using a Kruskal-Wallis multiple comparison Z-test (NCSS 2000). Comparisons of seminal and blood plasma osmolality within species were performed using a T-test (Microsoft Excel 2000, Microsoft Corporation, Redmond, Washington).

Differences in osmotic pressures that elicited threshold and complete activation for a particular solution within a species were determined using a T-test. Differences in threshold activation and osmotic activation among species were determined using a one-way ANOVA. Differences in solution concentrations that elicited threshold and complete activation were determined among species using either a one-way ANOVA or a Kruskal Wallis one-way ANOVA depending on normality.

Storage study data were analyzed using a 4 (extender) x 3 (osmolality) factorial repeated measures (time) ANOVA (SAS, SAS Institute, Cary, North Carolina) for main effects of extender and osmolality and their interactions over time. Means were separated using a Tukey-Kramer multiple comparison test at the $P = 0.05$ level.

Results

Plasma Osmolality

Lake sturgeon seminal plasma osmolality was significantly higher ($P = 0.0002$) than the seminal plasma osmolality of the other species tested (Table 5-1). The seminal plasma osmolality of pallid sturgeon, shortnose sturgeon, and shovelnose sturgeon was significantly lower ($P < 0.0001$) than their respective blood plasma osmolalities (Table 5-2). The blood plasma osmolality for each species was significantly different ($P < 0.0001$) from each other.

Seminal Plasma Composition

Glucose concentration was more than 8 times higher ($P = 0.0005$) in shortnose sturgeon seminal plasma than in seminal plasma of lake sturgeon, pallid sturgeon, or white sturgeon (Table 5-1). The magnesium concentration was significantly lower ($P = 0.0007$) in shortnose sturgeon (0.3 ± 0.1 mM) seminal plasma than in seminal plasma of pallid sturgeon (0.4 ± 0.1 mM) or white sturgeon (0.6 ± 0.1 mM). Lake sturgeon (5.0 ± 1.1 mM) and white sturgeon (5.4 ± 2.4 mM) seminal plasma had a significantly higher ($P = 0.0002$) potassium concentration than did seminal plasma of pallid sturgeon (2.1 ± 0.9 mM) or shortnose sturgeon (1.9 ± 1.2 mM). Shortnose sturgeon (25.4 ± 5.9 mM) seminal plasma had a significantly lower ($P = 0.0162$) concentration of chloride ions than did lake sturgeon (38.7 ± 6.6 mM) and white sturgeon (35.7 ± 4.2 mM). There were no differences among the ion concentrations for phosphorus ($P = 0.1739$), calcium ($P = 0.5551$), and sodium ($P = 0.4933$) among seminal plasma of lake sturgeon, pallid sturgeon, shortnose sturgeon, and white sturgeon.

Table 5-1. Mean (\pm SD) osmolality and chemical composition for seminal plasma of lake sturgeon (n = 5), shortnose sturgeon (n = 8), pallid sturgeon (n = 7), shovelnose sturgeon (n = 9), and white sturgeon (n = 3). Seminal plasma was collected by centrifugation at 5,000-X g for 10 min. Values sharing superscripts within a column were not significantly different ($P > 0.05$).

Sturgeon species	Osmolality (mOsmol/kg)	Glucose (mM)	Magnesium (mM)	Phosphorus (mM)	Calcium (mM)	Sodium (mM)	Potassium (mM)	Chloride (mM)
Lake	112 \pm 20 ^a	0.6 \pm 0.1 ^a	0.4 \pm 0.0 ^{ab}	3.1 \pm 0.6 ^a	0.1 \pm 0.0 ^a	34.0 \pm 5.8 ^a	5.0 \pm 1.1 ^a	38.7 \pm 6.6 ^a
Shortnose	67 \pm 33 ^b	5.4 \pm 0.0 ^b	0.3 \pm 0.1 ^a	2.4 \pm 1.9 ^a	0.1 \pm 0.1 ^a	30.4 \pm 12.3 ^a	1.9 \pm 1.2 ^b	25.4 \pm 5.9 ^b
Pallid	52 \pm 31 ^b	0.2 \pm 0.4 ^a	0.4 \pm 0.1 ^b	1.4 \pm 0.4 ^a	0.1 \pm 0.0 ^a	29.8 \pm 14.7 ^a	2.1 \pm 0.9 ^b	30.4 \pm 9.9 ^{ab}
Shovelnose	79 \pm 8 ^b	--	--	--	--	--	--	--
White	--	0.5 \pm 0.1 ^a	0.6 \pm 0.1 ^b	2.3 \pm 1.2 ^a	0.1 \pm 0.1 ^a	32.2 \pm 9.1 ^a	5.4 \pm 2.4 ^a	35.7 \pm 4.2 ^{ab}

Table 5-2. Mean (\pm SD) osmolality and chemical composition for blood plasma of lake sturgeon (n= 5), shortnose sturgeon (n = 8), pallid sturgeon (n = 7), and shovelnose sturgeon (n = 9). Blood plasma was collected by centrifugation at 5,000-X g for 10 min. Values sharing superscripts within a column were not significantly different ($P > 0.05$).

Sturgeon species	Osmolality (mOsmol/kg)	Glucose (mM)	Magnesium (mM)	Phosphorus (mM)	Calcium (mM)	Sodium (mM)	Potassium (mM)	Chloride (mM)
Lake	---	12.1 ± 1.2^{bc}	1.0 ± 0.2^{ab}	2.1 ± 0.4^{ab}	0.9 ± 0.2^b	108.4 ± 26.1^{bc}	4.3 ± 1.0^a	84.8 ± 13.0^{bc}
Shortnose	268 ± 6^a	8.3 ± 0.2^c	0.8 ± 0.2^b	2.0 ± 0.3^b	0.9 ± 0.6^b	136.0 ± 5.3^a	2.6 ± 0.4^b	109.9 ± 1.6^a
Pallid	258 ± 6^b	22.0 ± 0.6^{ab}	1.2 ± 0.1^a	1.7 ± 0.3^b	1.1 ± 0.1^{ab}	123.9 ± 2.6^b	2.4 ± 0.2^b	88.3 ± 0.8^c
Shovelnose	280 ± 4^c	23.7 ± 1.5^a	1.2 ± 0.0^a	2.7 ± 0.3^a	1.2 ± 0.1^a	133.6 ± 3.9^{ac}	2.7 ± 0.4^b	93.3 ± 2.0^b

Blood Plasma Composition

The glucose concentration in shovelnose sturgeon blood plasma (23.7 ± 1.5 mM) was significantly higher ($P < 0.0001$) than in shortnose sturgeon (8.3 ± 0.2 mM) or lake sturgeon (12.1 ± 1.2 mM) (Table 5-2). Magnesium ion concentration in pallid sturgeon (1.2 ± 0.1 mM) or shovelnose sturgeon (1.2 ± 0.0 mM) was significantly higher ($P = 0.0002$) than in shortnose sturgeon blood plasma (0.8 ± 0.2 mM). The concentration of phosphorus ions in shovelnose sturgeon blood plasma (2.7 ± 0.3 mM) was significantly higher ($P = 0.0001$) than in shortnose sturgeon (2.0 ± 0.3 mM) or pallid sturgeon (1.7 ± 0.3 mM). Calcium ion concentration in shovelnose sturgeon blood plasma (1.2 ± 0.1 mM) was significantly higher ($P = 0.0130$) than in lake sturgeon (0.9 ± 0.2 mM) and shortnose sturgeon (0.9 ± 0.6 mM). Sodium ion concentration in shortnose sturgeon blood plasma (136.0 ± 5.3 mM) was significantly higher ($P = 0.0038$) than in lake sturgeon (108.4 ± 26.1 mM) or pallid sturgeon (123.9 ± 2.6 mM). Potassium ion concentration in lake sturgeon blood plasma (4.3 ± 1.0 mM) was significantly higher ($P = 0.0274$) than in the other sturgeon species (<3.7 mM). Chloride ion concentration in shortnose sturgeon blood plasma (109.9 ± 1.6 mM) was significantly higher ($P < 0.0001$) than in the other sturgeon species (<93.3 mM).

Duration of Motility

Duration of sperm motility was characterized for lake sturgeon and shortnose sturgeon sperm. For each species, sperm were at maximal motility as soon as they could be visualized using the microscope, approximately 3 sec after activation (Figure 5-1). Sperm maintained maximal motility for 15 sec before motility began to slowly decrease. The mean time for lake

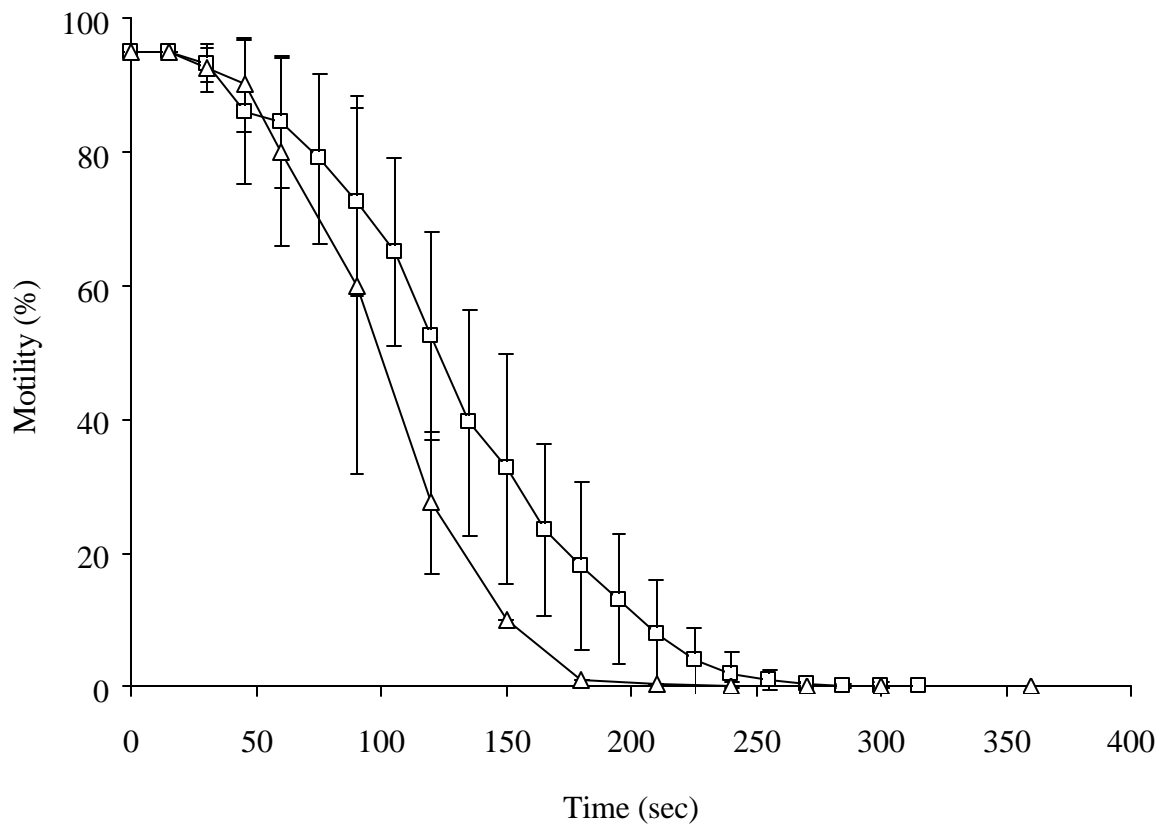


Figure 5-1. Motility characterization of sperm of lake sturgeon (triangles) and shovelnose sturgeon (squares). Sperm were activated with deionized water, and motility was estimated until all sperm had ceased progressive motility. Each point represents the mean of 2 fish for lake sturgeon and the mean of 10 fish for shovelnose sturgeon. Error bars represent ± 1 standard deviation.

sturgeon sperm cells to cease all forward movement was 285 ± 106 sec. The mean time for shovelnose sturgeon sperm cells to cease all forward movement was 265 ± 39 sec.

Osmotic Activation

For lake sturgeon, the osmotic pressure that elicited threshold activation was not different ($P = 0.2355$) among HBSS (173 mOsmol/kg), sodium chloride (202 mOsmol/kg), or sucrose (210 mOsmol/kg) (Figure 5-2). The osmotic pressure that elicited complete activation was significantly lower ($P = 0.0083$) for sperm activated with HBSS (79 ± 11 mOsmol/kg) than for sperm activated with sodium chloride (118 ± 33 mOsmol/kg) or sucrose (130 ± 16 mOsmol/kg).

For pallid sturgeon, the osmotic pressure that elicited threshold activation was significantly lower ($P < 0.0001$) for sperm activated with mHBSS (52 ± 10 mOsmol/kg) than for sperm activated with HBSS (116 ± 23 mOsmol/kg), sodium chloride (265 ± 20 mOsmol/kg), or sucrose (246 ± 1 mOsmol/kg) (Figure 5-3). The osmotic pressure that elicited complete activation was significantly lower ($P < 0.0001$) for mHBSS (8 ± 3 mOsmol/kg) and HBSS (32 ± 1 mOsmol/kg) than for sperm activated with sodium chloride (123 ± 29 mOsmol/kg) or sucrose (144 ± 18 mOsmol/kg).

For shortnose sturgeon, the osmotic pressure that elicited threshold activation was significantly lower ($P < 0.0001$) for sperm activated with mHBSS (52 ± 10 mOsmol/kg) than for sperm activated with sodium chloride (268 ± 19 mOsmol/kg) or sucrose (273 ± 25 mOsmol/kg) (Figure 5-4). The osmotic pressure that elicited complete activation was significantly lower ($P < 0.0001$) for sperm activated with mHBSS (10 ± 8 mOsmol/kg) than for sperm activated with sodium chloride (153 ± 19 mOsmol/kg) or sucrose (143 ± 22 mOsmol/kg).

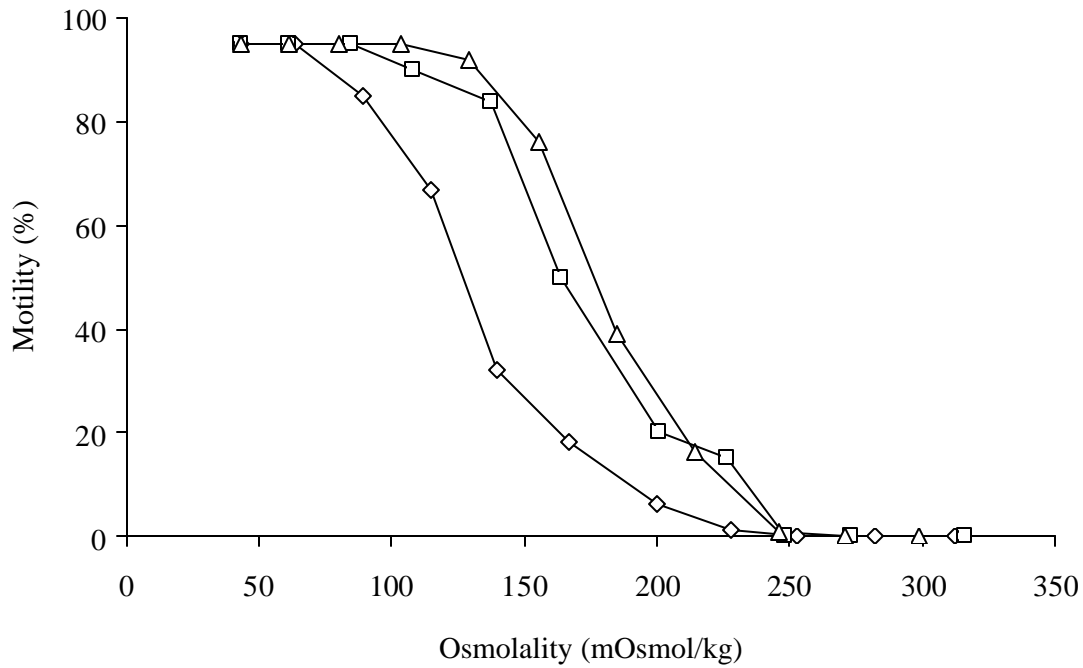


Figure 5-2. Mean percent sperm motility of lake sturgeon ($n = 5$) activated with eleven concentrations of three solutions. Sperm ($2 \mu\text{l}$) were activated with $20 \mu\text{l}$ of activating solution and motility was estimated using $100\times$ dark-field microscopy. The activating solutions consisted of serial dilutions of 300 mOsmol/kg Hanks' balanced salt solution (diamonds), sodium chloride solution (squares), and sucrose solution (triangles).

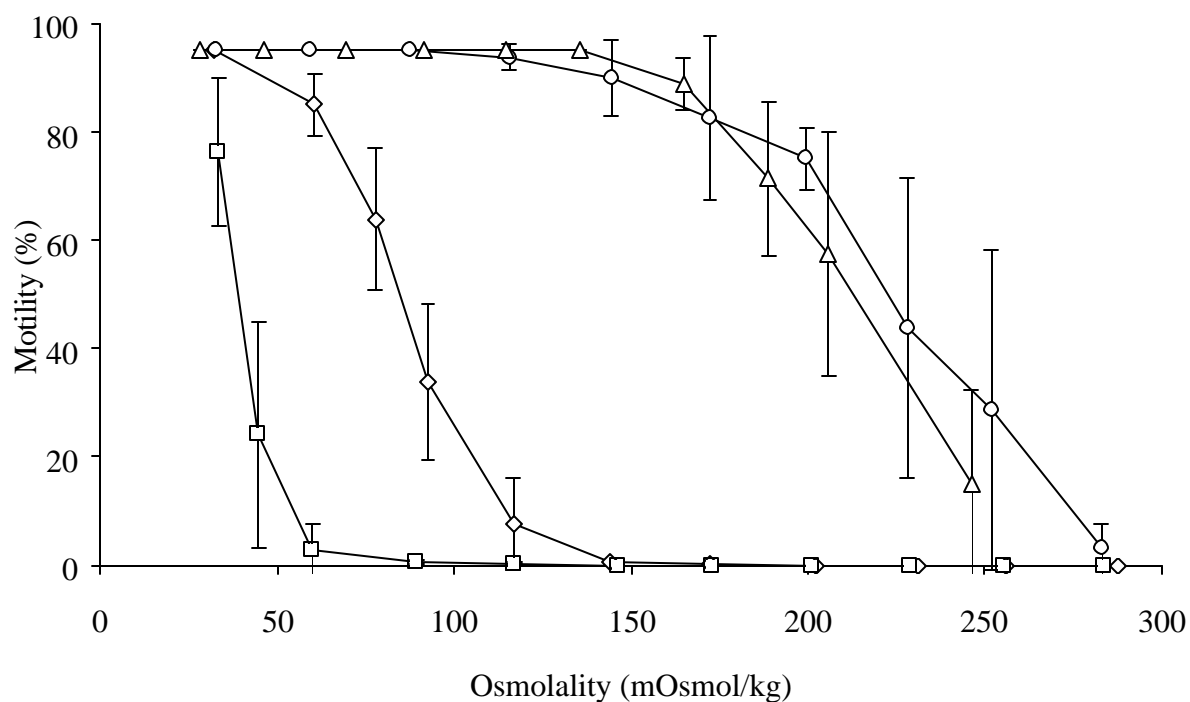


Figure 5-3. Mean percent sperm motility of pallid sturgeon ($n = 4$) activated with serial dilutions of modified Hanks' balanced salt solution (squares), Hanks' balanced salt solution (diamonds), sodium chloride solution (circles), or sucrose solution (triangles). Sperm ($2 \mu\text{l}$) were activated with $20 \mu\text{l}$ of each solution, and motility was estimated using $100\times$ dark-field microscopy. Ten μl of solution were removed directly from the slide and analyzed using a vapor-pressure osmometer to determine the osmotic pressure. Error bars represent ± 1 standard deviation.

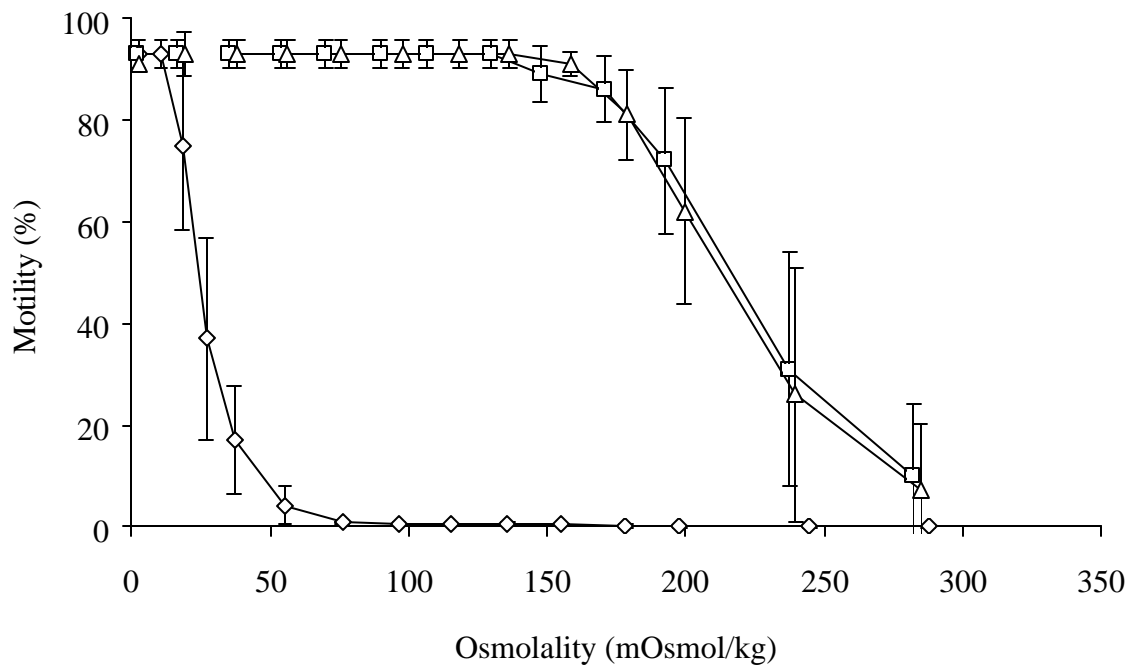


Figure 5-4. Percent sperm motility of shortnose sturgeon ($n = 5$) activated with 14 dilutions of Hanks' balanced salt solution (diamonds), sodium chloride solution (squares), and sucrose solution (triangles). Sperm ($2 \mu\text{l}$) were activated with $20 \mu\text{l}$ of solution, and motility was estimated using 100- \times dark-field microscopy. Error bars represent ± 1 standard deviation.

For shovelnose sturgeon, the osmolality that elicited threshold activation was significantly lower ($P = 0.0411$) for sperm activated with mHBSS (84 ± 37 mOsmol/kg) than for sperm activated with sodium chloride (201 ± 43 mOsmol/kg) or sucrose (182 ± 46 mOsmol/kg) (Figure 5-5). The osmolality that elicited complete activation was significantly ($P = 0.0019$) lower for sperm activated with mHBSS (33 ± 27 mOsmol/kg) than for sperm activated with sodium chloride (110 ± 38 mOsmol/kg) or sucrose (104 ± 66 mOsmol/kg).

Ionic Activation

All activating solutions used in this study had an osmolality of less than 20 mOsmol/kg. For lake sturgeon, sperm samples activated by 0.4 mM potassium chloride ($25 \pm 21\%$) had significantly lower ($P = 0.0248$) motility than did sperm activated with glucose ($95 \pm 0\%$), sodium chloride ($95 \pm 0\%$), sodium phosphate ($95 \pm 0\%$), sodium bicarbonate ($95 \pm 0\%$), or magnesium sulfate ($95 \pm 0\%$) (Figure 5-6). Motility of sperm samples activated with potassium chloride or potassium phosphate solutions at concentrations greater than 0.4 mM was significantly lower ($P < 0.0001$) than for sperm activated with other solutions at equal concentrations. At 2.0 mM, calcium chloride solution ($75 \pm 0\%$) activated significantly fewer sperm ($P < 0.0001$) than did glucose ($95 \pm 0\%$), sodium chloride ($95 \pm 0\%$), sodium phosphate ($88 \pm 4\%$), sodium bicarbonate ($95 \pm 0\%$), or magnesium sulfate ($92 \pm 4\%$) solutions.

For pallid sturgeon, sperm activated with potassium chloride at each concentration had significantly lower ($P < 0.0182$) motility than did sperm activated with other solutions (Figure 5-7). Sperm activated with 2.0 mM calcium chloride ($75 \pm 9\%$) had significantly lower ($P = 0.0079$) motility than did sperm activated with 2.0 mM sodium chloride ($90 \pm 0\%$), magnesium chloride ($90 \pm 0\%$), or sucrose ($90 \pm 0\%$).

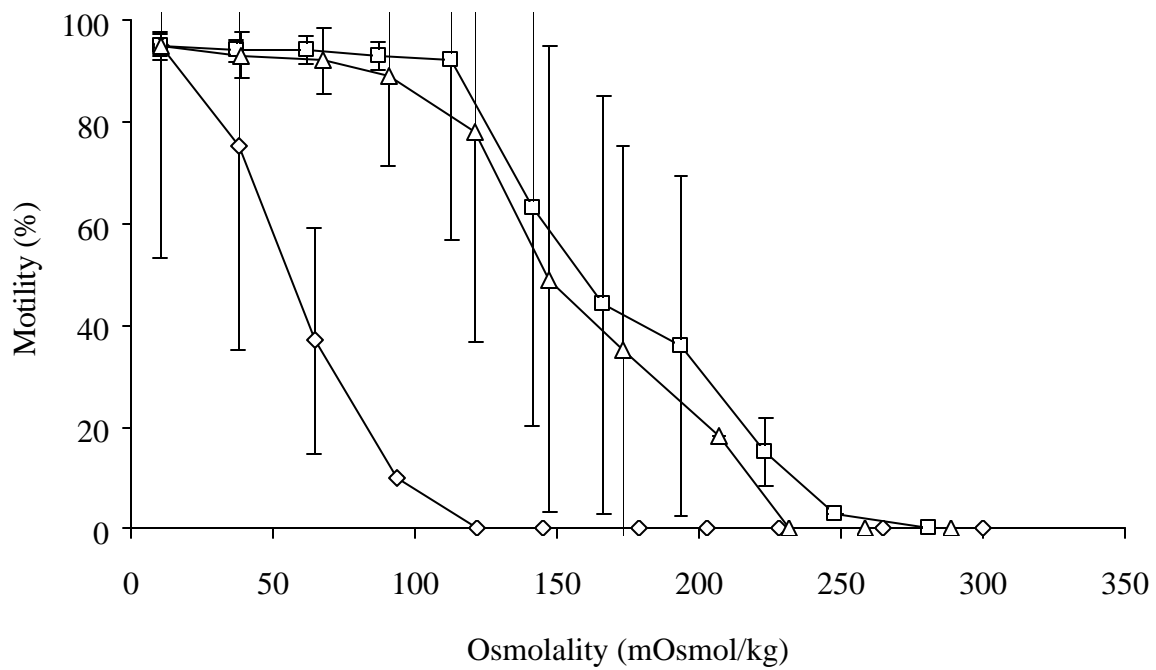


Figure 5-5. Percent sperm motility of shovelnose sturgeon ($n = 5$) activated with eleven dilutions of modified Hanks' balanced salt solution (diamonds), sodium chloride solution (squares), or sucrose solution (triangles). Sperm ($2 \mu\text{l}$) were activated with $20 \mu\text{l}$ of solution, and motility was estimated using 100- \times dark-field microscopy. Error bars represent ± 1 standard deviation.

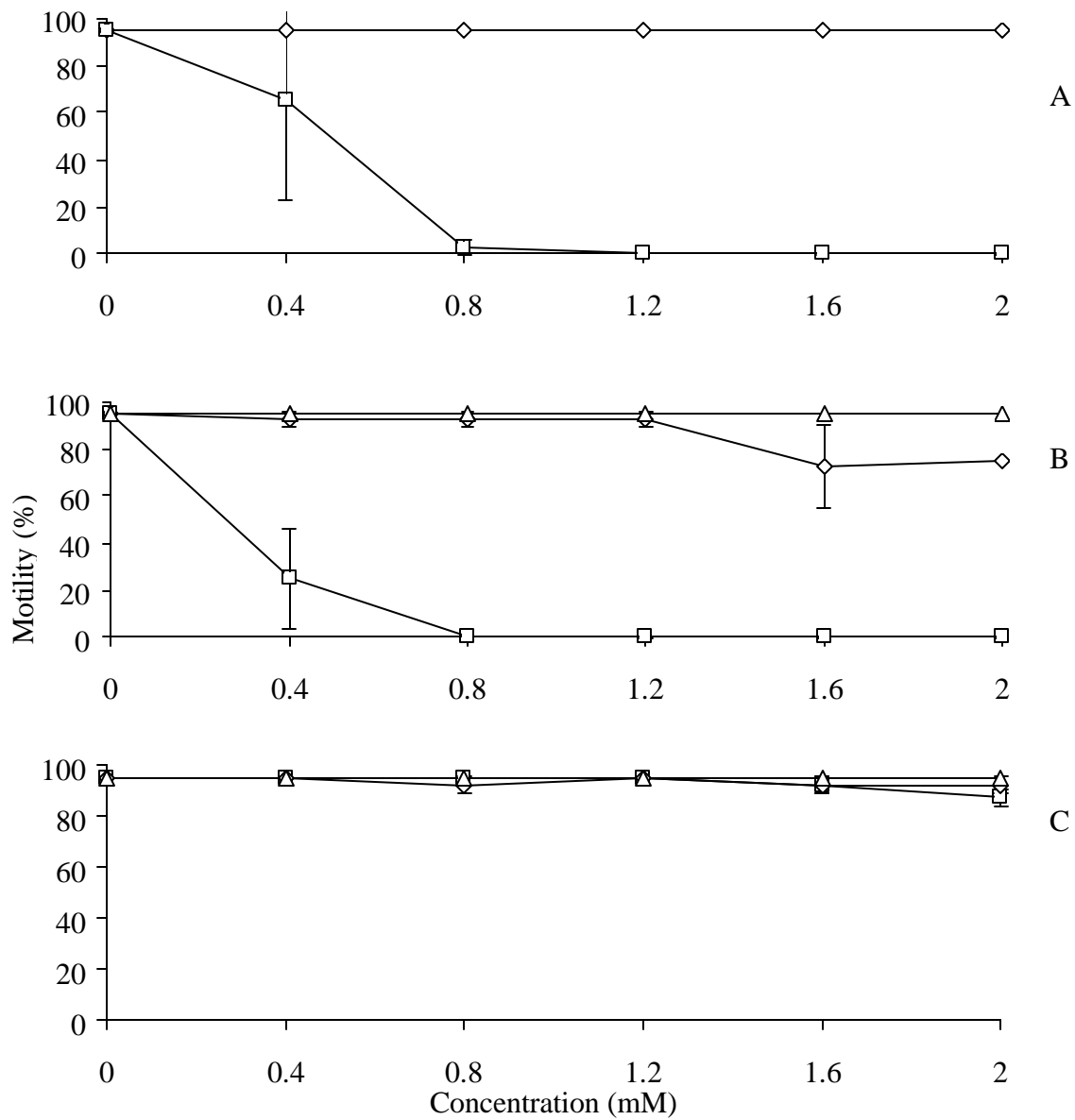


Figure 5-6. Percent sperm motility of lake sturgeon (n = 2) activated with dilutions of 2 mM concentrations of 8 solutions: sodium bicarbonate (A, diamonds), potassium phosphate (A, squares), glucose (B, triangles), calcium chloride (B, diamonds), potassium chloride (B, squares), sodium phosphate (C, squares), magnesium sulfate (C, diamonds), and sodium chloride (C, triangles). Sperm (2 μ l) were activated with 20 μ l of solution, and motility was estimated using 100-X dark-field microscopy. Error bars represent ± 1 standard deviation.

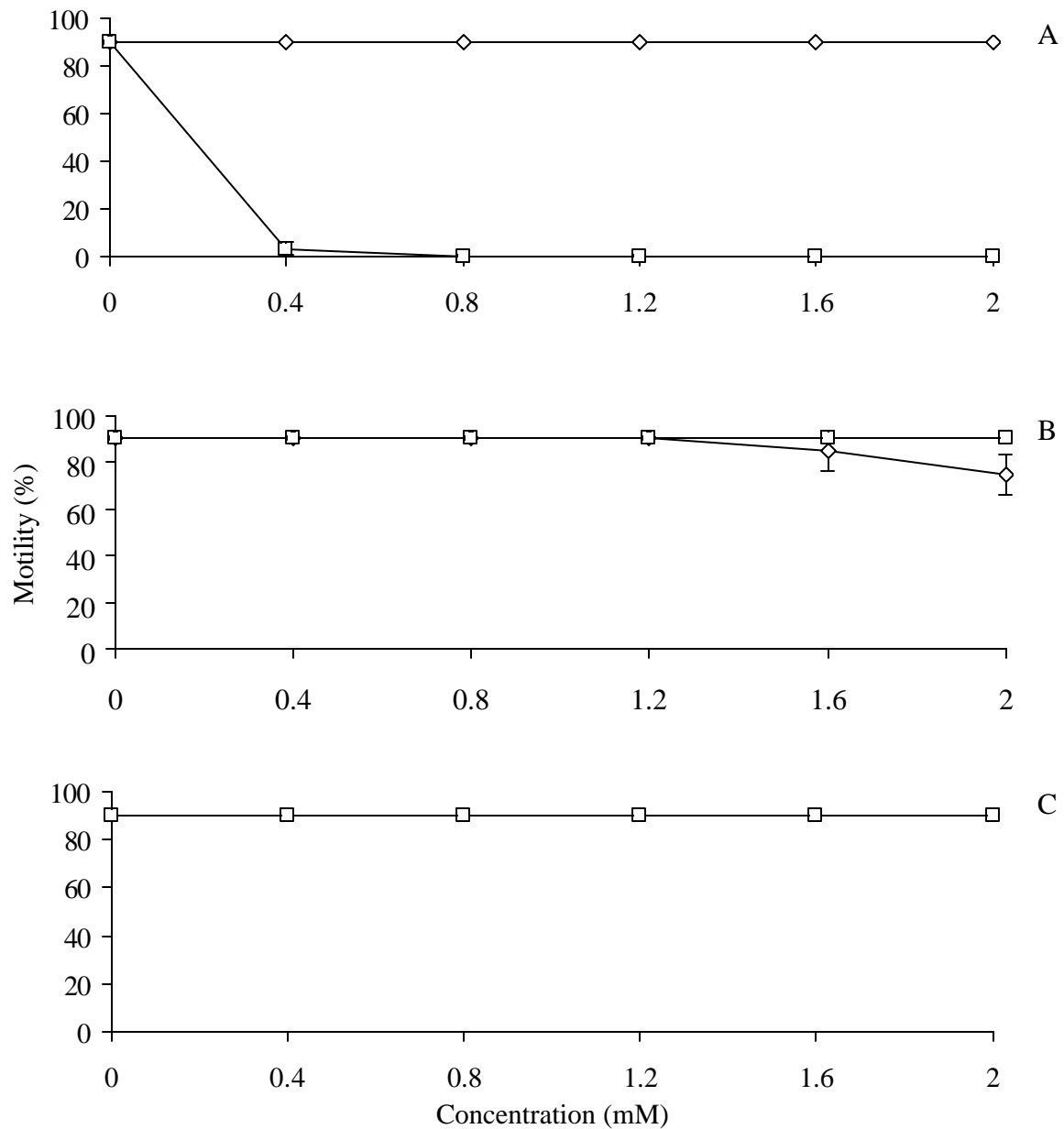


Figure 5-7. Mean percent sperm motility of pallid sturgeon ($n = 3$) activated with dilutions of 2 mM concentrations of solutions: magnesium chloride (A, diamonds), potassium chloride (A, squares), calcium chloride (B, diamonds), sucrose (B, squares), and sodium chloride (C, squares). Sperm ($2 \mu\text{l}$) were activated with $20 \mu\text{l}$ of solution, and motility was estimated using 100-X dark-field microscopy. Error bars represent ± 1 standard deviation.

For shortnose sturgeon, sperm activated with any solution at 0.1 mM and 0.2 mM concentrations had similar ($P > 0.4926$) motility (90 – 93%) (Figure 5-8). Sperm activated with potassium chloride at concentrations greater than 0.2 mM had significantly lower ($P < 0.0002$) motility than did sperm activated with calcium chloride or sodium chloride at the same concentrations.

For shovelnose sturgeon, motility of sperm activated with potassium chloride or potassium phosphate was significantly lower ($P < 0.0001$) than for sperm activated with any other solution at equal concentration (Figure 5-9). Sperm activated with calcium chloride at concentrations greater than 0.8 mM had motility that was significantly lower ($P < 0.0001$) than for sperm activated with other solutions except potassium chloride or potassium phosphate.

Refrigerated Storage

Pallid Sturgeon

Overall, sperm motility declined in all treatments over time ($P < 0.0001$). Upon initial dilution, sperm extended with sodium chloride or sucrose at 100 mOsmol/kg had motility equal to their initial motility (95%) when activated with de-ionized water (3 mOsmol/kg). More than 40% of sperm were activated when extended with sodium chloride or sucrose at 200 mOsmol/kg, or HBSS at 100 mOsmol/kg. Sperm extended with mHBSS at 200 or 300 mOsmol/kg, or HBSS at 300 mOsmol/kg had significantly lower ($P < 0.0001$) motility than did sperm diluted with the other extender combinations tested (Figure 5-10).

During storage there were significant main effects of extender ($P < 0.0001$) and osmolality ($P < 0.0001$) on percent motility over time (Figure 5-11). After 1 d of storage, sperm in sodium chloride solution had lower motility (18%) than sperm stored in the other extenders (>38%).

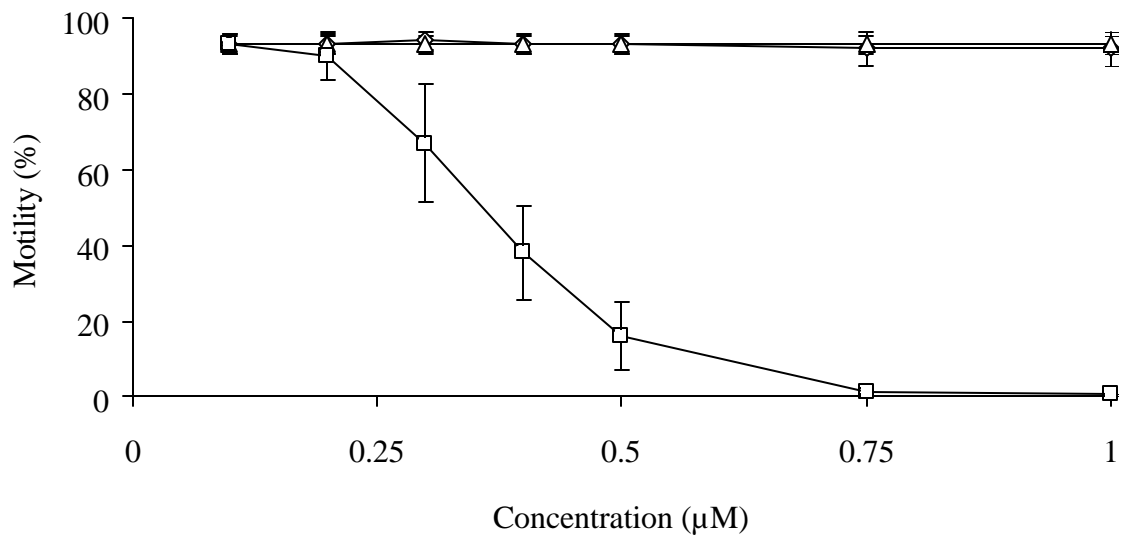


Figure 5-8. Percent sperm motility of shortnose sturgeon ($n = 5$) activated with seven dilutions of ionic solutions: potassium chloride (squares), calcium chloride (diamonds), and sodium chloride (triangles). Sperm ($2 \mu\text{l}$) were activated with $20 \mu\text{l}$ of solution, and motility was estimated using 100- \times dark-field microscopy. Error bars represent ± 1 standard deviation.

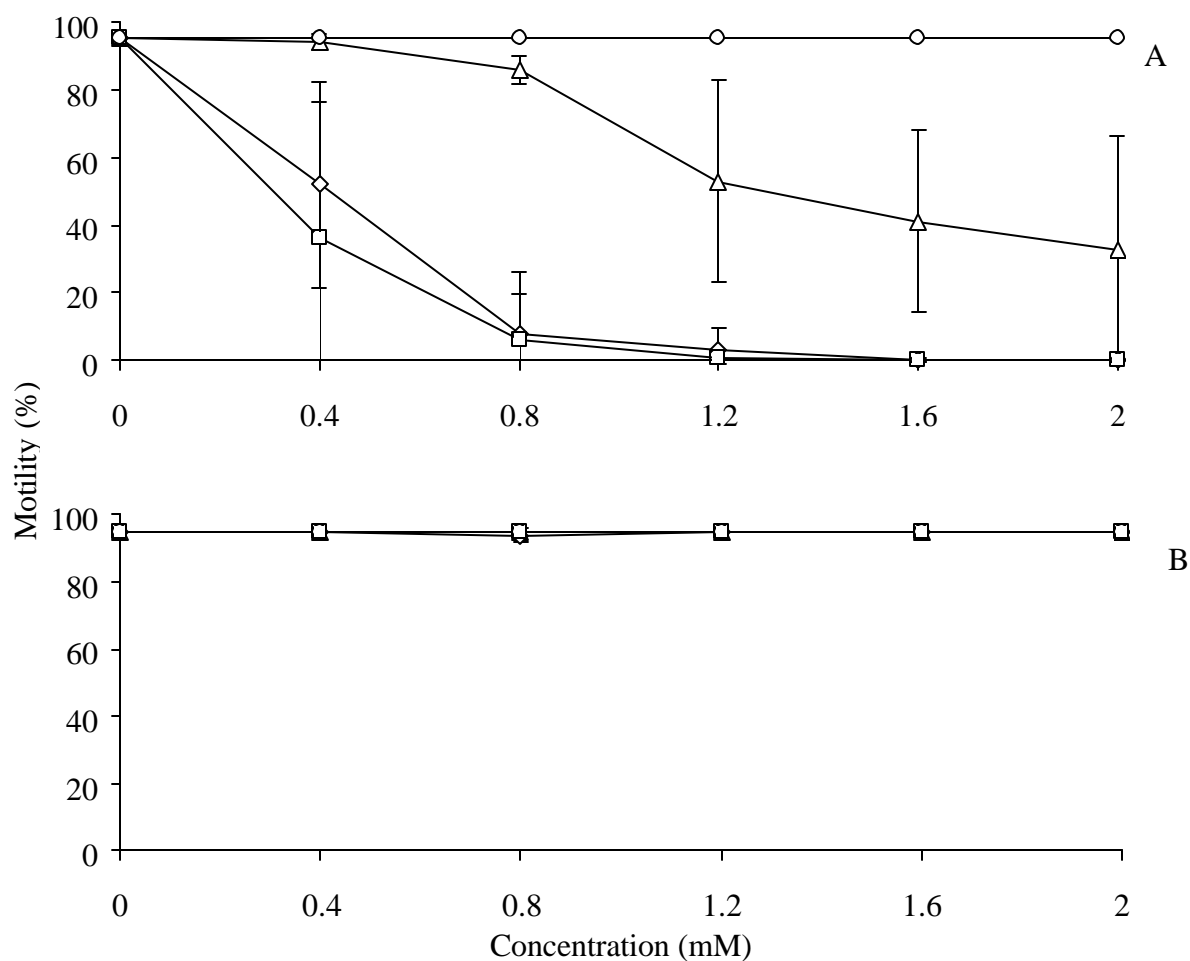


Figure 5-9. Percent sperm motility of shovelnose sturgeon (n = 5) activated with five dilutions of eight solutions: potassium chloride (A, squares), potassium phosphate (A, diamonds), calcium chloride (A, triangles), sodium phosphate (A, circles), magnesium sulfate (B, diamonds), sodium bicarbonate (B, squares), glucose (B, triangles), and sodium chloride (B, circles). Sperm (2 μ l) were activated with 20 μ l of solution, and motility was estimated using 100-X dark-field microscopy. Error bars represent ± 1 standard deviation.

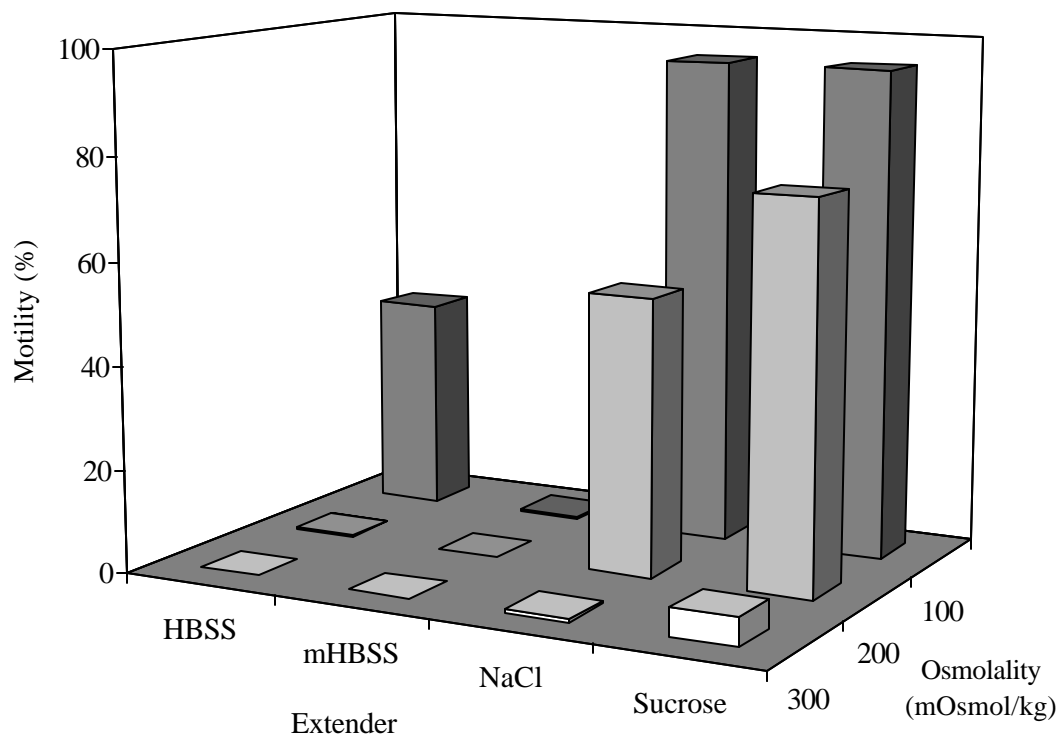


Figure 5-10. Mean percent sperm motility of pallid sturgeon ($n = 4$) upon dilution with various extenders. The extenders were Hanks' balanced salt solution (HBSS), modified HBSS (mHBSS), sodium chloride solution (NaCl), and sucrose solution. Each extender was used at three concentrations (100, 200, and 300 mOsmol/kg). Sperm were diluted 1:4 (sperm:extender) with each extender and motility was estimated.

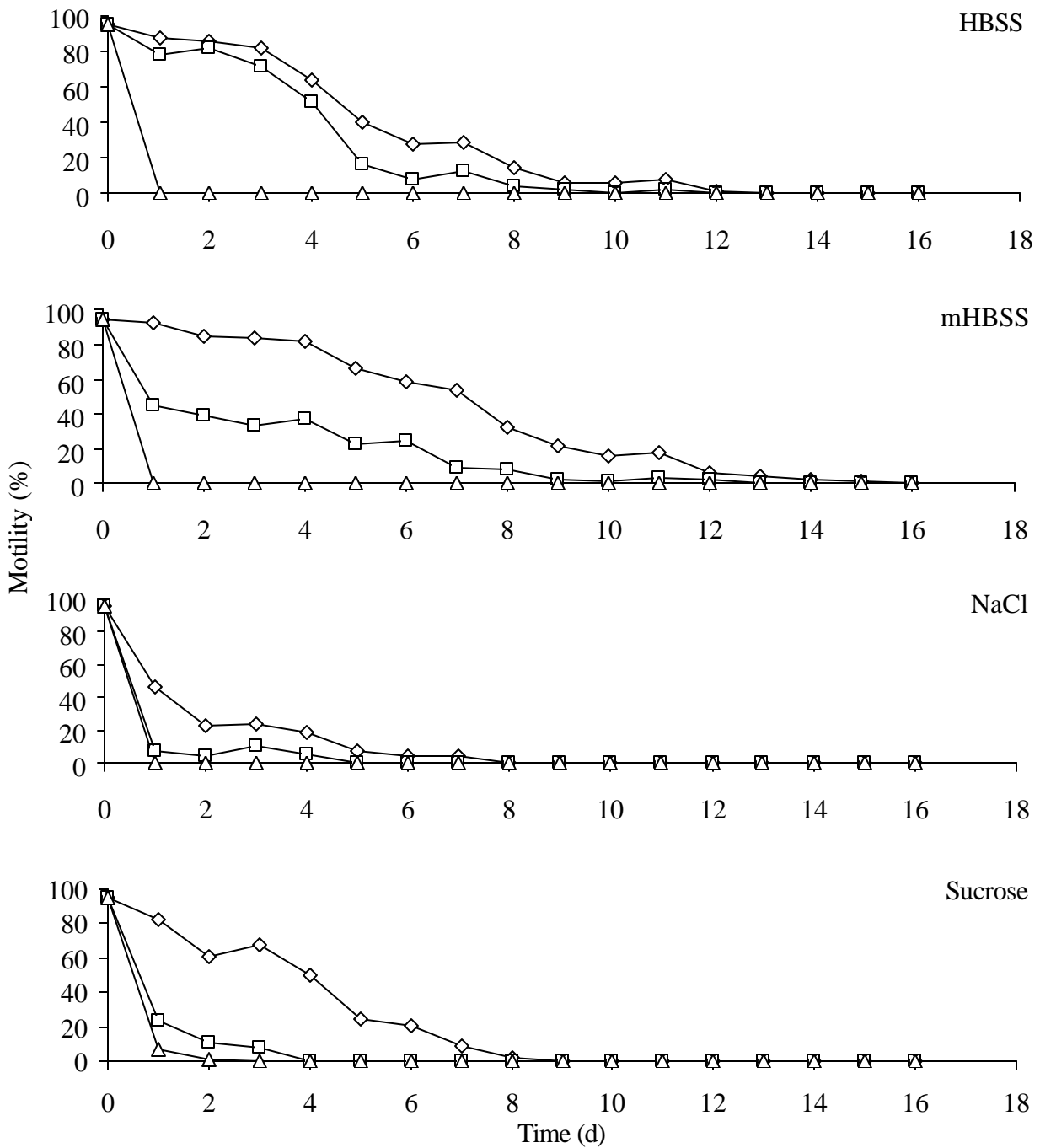


Figure 5-11. Percent sperm motility of pallid sturgeon ($n = 4$) stored in four different extenders at 100 (diamonds), 200 (squares), and 300 (triangles) mOsm/kg. Sperm ($2 \mu\text{l}$) were activated with $20 \mu\text{l}$ of deionized water, and motility was estimated using 100-X dark-field microscopy.

Sperm stored in HBSS or mHBSS for as long as 7 d retained significantly higher motility ($P < 0.05$) than did sperm stored in sucrose or sodium chloride solution. From day 8 to day 10 of storage, sperm stored in mHBSS retained significantly higher motility than sperm stored in sucrose or sodium chloride. After day 10, there were no significant differences ($P > 0.05$) among the extenders (mean motility was $<10\%$).

Sperm retained higher motility (77%) stored in extenders at 100 mOsmol/kg than did sperm stored in 200 mOsmol/kg (38%) or 300 mOsmol/kg ($<1\%$) after 1 d of storage. This relationship continued through 10 d of storage, when mean motility dropped below 6% for all treatments. Sperm stored at 300 mOsmol/kg lost all motility after 3 d of storage. Whereas sperm stored in 200 mOsmol/kg retained motility greater than 10% as long as 5 d, and sperm stored at 100 mOsmol/kg retained motility greater than 10% as long as 8 d. After day 12 there were no differences among the treatments (mean motility was $<2\%$).

There were also significant interaction effects between extender and osmolality over time ($P < 0.0001$). After 1 d, sperm retained higher motility stored in HBSS at 100 mOsmol/kg (88%), 200 mOsmol/kg (78%), mHBSS at 100 mOsmol/kg (92%), and sucrose at 100 mOsmol/kg (82%) than did sperm stored in other extender and osmolality combinations ($<46\%$). After 4 d of storage, sperm stored in mHBSS at 100 mOsmol/kg (82%) retained higher motility than did sperm stored in any other combination ($<64\%$). After 12 d of storage, motility in all samples was $<10\%$.

Shortnose Sturgeon

Overall, sperm motility declined in all treatments over time ($P < 0.0001$). Sperm stored undiluted retained motility equal to or greater than the motility of sperm stored in any of the

extender and osmolality combinations throughout the study. Within the extended samples, there were significant main effects of extender ($P < 0.0001$) and osmolality ($P < 0.0001$) on sperm motility over time (Figure 5-12). After 1 d of storage, sperm stored in sucrose solution (37%), mHBSS (25%), or HBSS (30%) had significantly higher motility ($P < 0.05$) than did sperm stored in Mounib's solution (1%). Sperm stored in Mounib's solution lost all motility after 6 d of storage, while the other extenders allowed storage for at least 13 d (motility $\geq 1\%$). After 8 d of storage, there were no differences among the extenders (motility $\leq 5\%$).

After 1 d of storage, sperm stored at 100 mOsmol/kg solutions (50%) had higher motility than did sperm stored at 200 mOsmol/kg (16%) or 300 mOsmol/kg (5%). This relationship continued until day 10. Sperm stored at 300 mOsmol/kg lost all motility after 3 d of storage. However sperm retained motility ($>1\%$) at 100 mOsmol/kg or 200 mOsmol/kg for at least 8 d. After 11 d of storage, sperm motility in all samples declined to $<5\%$.

There was also a significant ($P < 0.0001$) interaction effect between extender and osmolality over time. After 1 d of storage, sperm retained higher motility stored in HBSS (71%) or mHBSS (71%) at 100 mOsmol/kg than did sperm stored in all other extender and osmolality combinations ($< 39\%$), except sucrose solution at 100 mOsmol/kg (54%). At 10 d of storage, sperm stored in mHBSS at 100 mOsmol/kg had significantly higher motility ($P < 0.05$) (11%) than all other combinations except HBSS at 100 mOsmol/kg (6%). After day 13, sperm motility had declined in all samples to $<5\%$.

Shovelnose Sturgeon

Overall, sperm motility declined in all treatments over time ($P < 0.0001$). Sperm stored undiluted retained motility equal to or greater than that retained by sperm stored in any other

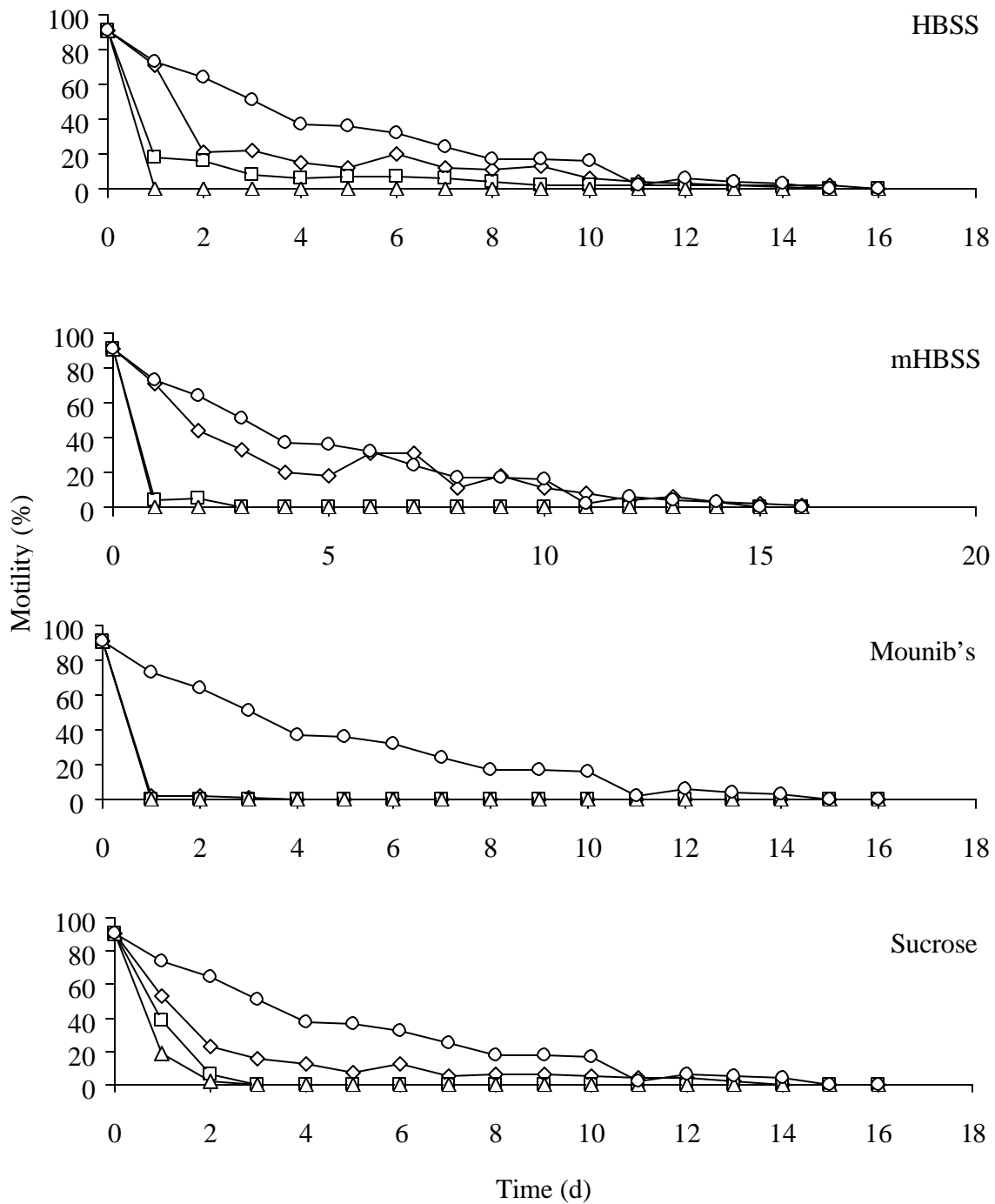


Figure 5-12. Percent sperm motility of shortnose sturgeon ($n = 4$) stored at concentrations of 100 mOsmol/kg (diamonds), 200 mOsmol/kg (squares), or 300 mOsmol/kg (triangles) in four extenders and undiluted (circles). Sperm ($2 \mu\text{l}$) were activated with $20 \mu\text{l}$ of deionized water, and motility was estimated using 100-X dark-field microscopy.

extender and osmolality combination throughout the study, except on day 4 and near the end of the study, when motilities were <10% (days 17, 18, 20, and 21). Among the extended samples, there were significant main effects of extender ($P < 0.0001$) and osmolality ($P < 0.0001$) over time (Figure 5-13).

Sperm stored in sodium chloride solution consistently had lower motility than did sperm stored in the other extenders. After 1 d of storage, sperm retained equal or higher motility stored in sucrose solution (64%) than did sperm stored in the other extenders, but from d 7 to the end of the study, sperm stored in sucrose had the lowest motility (<17%) of the extenders tested. Sperm stored in extenders at 100 mOsmol/kg retained higher motility than did sperm stored at 200 or 300 mOsmol throughout the study. Sperm stored at 100 mOsmol/kg or 200 mOsmol/kg retained motility (>1%) for at least 18 d, but sperm stored at 300 mOsmol/kg lost all motility within 4 d of storage.

There were also significant interaction effects between extender and osmolality ($P < 0.0001$) over time. Sperm stored in mHBSS at 100 mOsmol/kg retained equal or higher motility than did the other extender and osmolality combinations throughout the study.

Discussion

The development of extenders for refrigerated storage of fish sperm is often based on the osmolality of the seminal or blood plasma osmolality (Wayman et al., 1996). The blood plasma osmolalities of shortnose sturgeon, pallid sturgeon, and shovelnose sturgeon in this study were similar to blood plasma osmolalities reported for other sturgeon species captured or held in freshwater. For example, a blood plasma osmolality of 247 mOsmol/kg has been reported for lake sturgeon (LeBreton and Beamish, 1998), 261 mOsmol/kg for Gulf sturgeon *Acipenser*

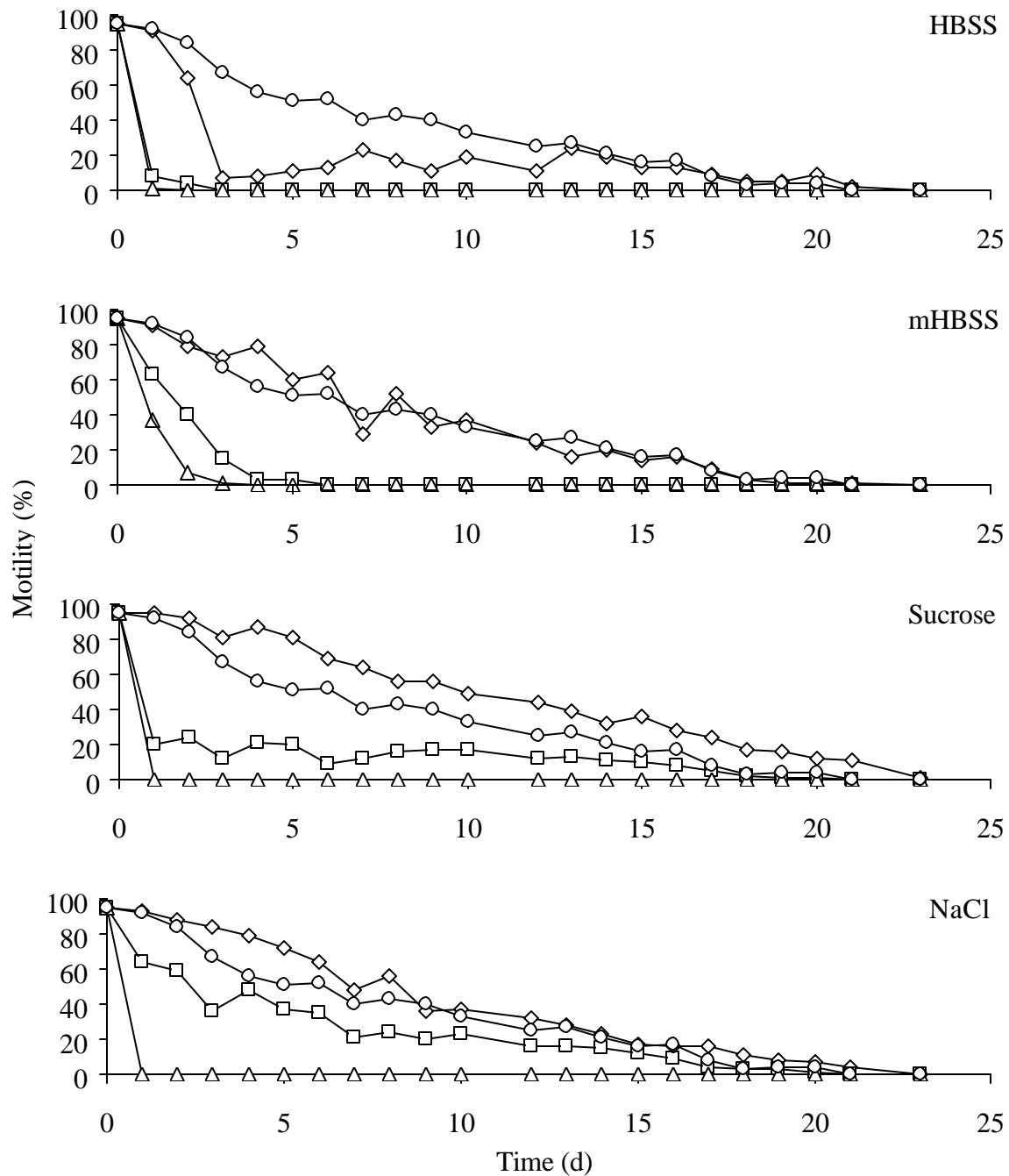


Figure 5-13. Percent sperm motility of shovelnose sturgeon ($n = 5$) stored in four extenders at 100 mOsmol/kg (diamonds), 200 mOsmol/kg (squares), and 300 mOsmol/kg (triangles) or undiluted (circles). Sperm ($2 \mu\text{l}$) were activated with $20 \mu\text{l}$ of solution, and motility was estimated using 100-X dark-field microscopy.

oxyrinchus desotoi (Altinok et al., 1998), 248 mOsmol/kg for Russian sturgeon *Acipenser gueldenstaedti* (Natochin et al., 1995), and 236 mOsmol/kg for white sturgeon (McEnroe and Cech, Jr., 1985). These values are similar to those reported for freshwater fishes such as channel catfish *Ictalurus punctatus* (273 mOsmol/kg) (Bates et al., 1996) and razorback sucker *Xyrauchen texanus* (282 mOsmol/kg) (Tiersch et al., 1997), but were lower than other species such as white bass *Morone chrysops* (330 mOsmol/kg) (Allyn et al., 2001).

Typically in fishes the osmotic pressure of blood plasma and seminal plasma osmotic pressure are similar. Accordingly, when seminal plasma is not available the osmotic pressure of blood plasma can be used as a substitute value for preliminary development of sperm extenders (Wayman et al., 1996). However, the osmotic pressures of seminal plasma (52-112 mOsmol/kg) from all four sturgeon species in this study were significantly lower than the osmotic pressures of their respective blood plasma (258-280 mOsmol/kg). These seminal plasma osmolalities were similar to values reported for other sturgeon species (Gallis et al., 1991), but were significantly lower than values reported for other freshwater fishes. For example, a seminal plasma value of 322 mOsmol/kg was reported for rainbow trout *Oncorhynchus mykiss* (Glogowski et al., 2000), 261 mOsmol/kg for bleak *Alburnus alburnus* (Lahnsteiner et al., 1996), 249 mOsmol/kg for sea lamprey *Petromyzon marinus* (Cierszko et al., 2002), 302 mOsmol/kg for common carp *Cyprinus carpio*, and 317 mOsmol/kg for goldfish *Carassius auratus* (Morisawa et al., 1983). This difference indicates that blood plasma osmolality of sturgeon species cannot be used directly as a basis for extender development, however an approximation of seminal plasma osmolality could be made by subtracting 200 mOsmol/kg from the blood plasma osmolality (data not shown).

The differences in the seminal and blood plasma osmolalities encountered in sturgeon can be attributed to differences in ionic composition. The concentrations of glucose, sodium and chloride ions in the blood plasma of sturgeon in this study were higher than their respective concentrations within seminal plasma. The blood and seminal plasma ion concentrations found in this study were similar to values previously reported for sturgeon (Natochin et al., 1975; Gallis et al., 1991; Natochin et al., 1995; Toth et al., 1997; LeBreton and Beamish, 1998; and Altinok et al., 1998). However, the seminal plasma ion concentrations reported here and in other sturgeon species are lower than the concentrations reported for other freshwater species (Morisawa et al., 1983; Lahnsteiner et al., 1996; Glogowski et al., 2000). This difference also suggests a mechanism for sperm activation in sturgeon different from the reduction of osmotic pressure that is commonly reported for freshwater fishes (Morisawa et al., 1983; Bates et al., 1996; Mansour et al., 2002), with the exception of the salmonids (Billard, 1992).

Sperm from fishes that spawn in freshwater are typically activated by a decrease in osmotic pressure to below that of the seminal plasma. For example, common carp *Cyprinus carpio* sperm, with a seminal plasma osmolality of 266 mOsmol/kg, were reported at threshold activation (10% motility) at 252 mOsmol/kg (Glenn, 1998). In the present study, sturgeon sperm were completely activated by sucrose and sodium chloride solutions at osmolalities greater than the seminal plasma osmolality. This is in contrast to some reports on sturgeon sperm activation. Sperm motility of Siberian sturgeon was inhibited by sucrose solutions of greater than 100 mOsmol/kg (Gallis et al., 1991). Sperm of paddlefish *Polyodon spathula* were inhibited in sodium chloride or Tris solutions at osmolalities of greater than 120 mOsmol/kg (Linhart et al., 1995). The addition of 50 mM sodium chloride caused a complete cessation of motility for lake sturgeon sperm (Toth et al., 1997). Motility of sperm in the present study at osmolalities greater

than that of the seminal plasma would indicate that osmolality is not the primary cause of sperm activation.

In this study, sperm activated with mHBSS or HBSS reached threshold and complete activation at osmolalities well below those for sperm activated with sodium chloride or sucrose solutions (for all 4 sturgeon species examined). Modified HBSS and HBSS contain a variety of ions, which would suggest that sperm motility was inhibited by a particular ion concentration.

In this study, sperm motility was reduced by potassium chloride or potassium phosphate solutions, and was completely inhibited at concentrations greater than or equal to 0.8 mM. High concentrations (≥ 1.2 mM) of calcium chloride also reduced sperm motility. In other reports, the addition of potassium produced a dose-dependent reduction of lake sturgeon sperm motility with motility reduced to ~10% with a 1.0 mM potassium chloride solution (Toth et al., 1997).

Siberian sturgeon *Acipenser baerii* sperm motility was also completely inhibited by potassium ion concentrations above 0.1 mM (Gallis et al., 1991). Motility of salmonid sperm was inhibited by potassium ions at concentration greater than 1 mM, and when extended in a 140 mM sodium chloride and 20 mM potassium chloride solution sperm could be stored indefinitely (Scott and Baynes, 1980). The inclusion of potassium in extenders for refrigerated storage of sturgeon sperm could maintain sperm in a quiescent state and enable longer storage times. Sperm stored in solutions containing potassium retained higher motility than did sperm stored in other ionic or non-ionic solutions.

Regarding duration of motility, sperm of lake sturgeon and shovelnose sturgeon maintained motility for longer (>240 sec) than sperm of most freshwater fishes (< 120 sec) (Scott and Baynes, 1980; Billard et al., 1995; Jayaprakas and Bimal Lal, 1996, and Tiersch et al., 1997). The duration of sperm motilities in the present study were similar to values reported for other

fishes of the family Acipenseridae. Shovelnose sturgeon sperm maintained >50% motility for as long as 80 sec and maintained forward motility for 2-3 min when activated with distilled water (Cosson et al., 2000). White sturgeon sperm maintained motility for as long as 5 min when diluted in fresh water (Cherr and Clark, 1985). Siberian sturgeon sperm remained motile for 2-3 min when activated with a 30 mM Tris-HCl solution (Billard et al., 1999). Paddlefish sperm remained motile for as long as 4 min when activated with dechlorinated water (Mims, 1991).

Sturgeon sperm, in the present study, stored undiluted retained equal or greater motility than sperm stored in any of the extender and osmolality combinations. Unlike undiluted sperm from some species in which quality decreases rapidly (Jenkins-Keeran and Woods, 2001), undiluted sturgeon sperm can be stored for as long as 14 d before complete loss of motility. Atlantic sturgeon sperm retained at least 80% motility and 99% viability (as determined by trypan blue vital staining) after 5 d of storage on ice (DiLauro et al., 1994). White sturgeon sperm were maintained for 14 d refrigerated in syringes supplemented with oxygen (Conte et al., 1988).

Sperm for the present study were collected by inserting a tube into the urogenital opening and aspirating to withdraw the sperm. This method minimized the possibility of urine contamination within the samples. Urine can cause activation of sperm and reduced storage time (Poupard et al., 1998). A benefit of extenders such as HBSS or mHBSS is that they can help maintain osmolality and ionic concentrations when samples are contaminated with small amounts of urine, and thus can extend storage times.

The composition of the extender and its osmolality had significant influences on the duration of refrigerated storage. Sperm stored in solutions with osmolalities greater than the seminal plasma had reduced motility retention as compared to sperm stored at or near seminal plasma osmolality or left undiluted. This is in contrast to the results of other storage studies with

sturgeon or paddlefish sperm. In these reports, shovelnose sturgeon sperm were stored in 150 mM glucose plus 20 mM Tris (pH 8.5, ~330 mOsmol/kg) for as long as 3 d without loss of motility (Linhart et al., 1995). Paddlefish sperm retained <80% motility for as long as 48 hr in 100 mM glucose plus 20 mM Tris (~220 mOsmol/kg) at a pH of 8.5 (Linhart et al., 1995), and retained motility (5-25%) for as long as 56 d when stored in 310 mOsmol/kg sodium chloride and antibiotics (Brown and Mims, 1995). The loss of motility in samples with higher osmolality in the present study could have been decreased by the addition of antibiotics, but this is unlikely given that undiluted samples and samples stored at 100 mOsmol/kg retained motility for as long as 21 d.

Sperm that were completely activated by the sucrose solution at 100 mOsmol/kg also retained motile sperm (>1%) when stored in sucrose solution at 100 mOsmol/kg for as long as 8 d. It has been reported that activated sperm remained motile until their ATP supply was exhausted, and then die (Ginsburg, 1963; Truscott and Idler, 1969). However it has been shown that activated sperm of common carp *Cyprinus carpio* can be placed in an immobilizing solution (200 mM potassium chloride and 30 mM Tris-HCl, pH 8.0), become quiescent, and then be reactivated (Poupard et al., 1998). Glucose, galactose, and fructose are commonly found as constituents of chub *Leuciscus cephalus* (Lahnsteiner et al., 1992) and perch *Perca fluviatilis* (Lahnsteiner et al., 1995) seminal plasma and can be used by sperm cells for the production of ATP through glycolysis. Sperm stored in sucrose may be able to transport sucrose across the cellular membrane and use it as an energy source. Further study of the sugar metabolism of sperm during storage could determine if extracellular sucrose can be used as an energy source.

The development of refrigerated storage techniques for sturgeon in this study was based on the seminal plasma osmolality, chemical composition, and sperm activation. To attain the

longest period of storage with the highest motility, sperm should be stored in a solution that contains potassium at a concentration of at least 1 mM and adjusted to an osmotic pressure similar to that of the seminal plasma osmolality (~100 mOsmol/kg). There is also some evidence that the use of external sugars as an energy source could prolong the motility of sperm samples, but further investigation is necessary. The development of extenders containing potassium ions (mHBSS or HBSS) and an osmolality of 100 mOsmol/kg allowed storage of sperm for up to 21 d. These extenders could be used to aid managers in their efforts at induced spawning of sturgeon species, and allow sperm to be shipped to a cryopreservation facility for incorporation into a repository system. The use of an extender can also allow the addition of antibiotics to sperm samples to minimize the effects of bacteria on the sperm and to reduce the spread of diseases.

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Chapter 6

Development of Cryopreservation Techniques

Introduction

The development of cryopreservation techniques for endangered fishes could help to conserve remaining genetic diversity (Harvey, 2000; Gwo et al., 1999; Tiersch et al., 1998), and enhance genetic research by providing a non-invasive source of DNA suitable for the development of genetic markers and the study of specific DNA sequences (Pittman-Cooley and Tiersch, 2000). Development of a repository of cryopreserved sturgeon sperm would therefore be of benefit to the study and recovery of threatened and endangered sturgeon species.

Commercial aquaculture of sturgeon species has historically been a small industry. However the decline of commercial fisheries has allowed aquaculture production to become more profitable as the price for sturgeon products has increased. Currently the only species in the United States that is being raised commercially is the white sturgeon *Acipenser transmontanus*. Cryopreservation of sturgeon sperm offers many advantages to the artificial spawning of these fishes. Mature sturgeon can weigh from 4 kg to more than 800 kg and require large holding facilities. Sperm cryopreservation can be used to reduce the number of males maintained in hatcheries, allowing more resources to be devoted to females for caviar development. A major problem in the artificial spawning of sturgeon is asynchronous maturation of broodstock (DiLauro and Krise, 1994). Cryopreserved sperm would allow artificial spawning whenever eggs were available. Development of a repository of cryopreserved sturgeon sperm could thus also benefit the development of commercial sturgeon aquaculture.

Techniques were developed for the cryopreservation of sturgeon sperm (reviewed by Mims et al., 2000), but the majority of these techniques were for European species. These techniques

primarily used dimethyl sulfoxide (DMSO), ethylene glycol, or glycerol as the cryoprotectant, although a recent study investigated the use of methanol as a cryoprotectant for Siberian sturgeon sperm (Glogowski et al., 2002). Sperm were cryopreserved from only one North American species, the lake sturgeon *Acipenser fulvescens* (Cierszko et al., 1996a,b), but these studies did not investigate the use of methanol as a cryoprotectant.

The goal of this research is to develop cryopreservation techniques for four species of North American sturgeon representing the genera *Acipenser* and *Scaphirhynchus*. The specific objectives are to: 1) determine the toxicity of varied concentrations of cryoprotectants to sturgeon sperm; 2) evaluate the effects of two different extenders for the cryopreservation of sturgeon sperm; and 3) evaluate various freezing methods and rates on post-thaw motility, percent fertilization, and percent hatch.

Materials and Methods

Shortnose Sturgeon

Sperm were collected from 3 males at the U. S. Fish and Wildlife Service Bears Bluff National Fish Hatchery (BBNFH) (Wadmalaw Island, South Carolina, 32° 39' N, 80° 15' W), and were held undiluted in 50-ml tubes on ice (~2 hr). Initial motility of all samples was >75%. Sperm were diluted 1:1 (v:v) with modified Hanks' balanced salt solution (mHBSS) (SOP 6, Appendix A). Methanol and DMSO were used as cryoprotectants at concentrations of 5% and 10%. Cryoprotectants were mixed 1:1 (v:v) with extender prior to addition to sperm to minimize toxic effects. Sperm were allowed to equilibrate in the cryoprotectants for 20 min, and motility was estimated (SOP 3, Appendix A) to determine cryoprotectant toxicity. Sperm were loaded into 0.5-ml straws (bovine medium straws; IMV International Corporation, Minneapolis,

Minnesota), placed in 1-cm plastic goblets (IMV International Corporation) (4 straws per goblet), attached to the upper or lower position on 10-cm aluminum canes (IMV International Corporation), and placed in a computer-controlled freezer (Kryo-10, Planer Products Ltd., Sunbury on Thames, United Kingdom). Sperm were frozen at cooling rates of 5, 10, 20, 30, and 40 °C/min. The frozen straws were transferred into liquid nitrogen. After 7 d, the straws were thawed and used in hatching trials (SOP 10, Appendix A).

In a second experiment, sperm were collected from 3 males held at the U. S. Fish and Wildlife Service Warm Springs Fish Technology Center (WSFTC) (Warm Springs, Georgia), and were held undiluted in 50-ml tubes on ice (~2 hr). All sperm samples had motility of 95% prior to initiation of the study. The sperm were diluted with mHBSS (SOP 6, Appendix A) or mTsvetkova (SOP 11, Appendix A) at a ratio of 1:4 (v:v, sperm:extender). Methanol and DMSO were used as cryoprotectants at concentrations of 5% and 10%. Cryoprotectants were mixed 1:1 (v:v) with extenders prior to addition to sperm to minimize toxic effects. The cryoprotectants were allowed to equilibrate with the sperm for 37 min, and motility was estimated (SOP 3, Appendix A). Sperm were loaded into 0.5-ml straws, placed in 1-cm goblets, attached to 10-cm aluminum canes, and frozen in a computer-controlled freezer (Kryo-10) using a 5-step program (Table 6-1) comparable to the rate of freezing in a nitrogen-vapor shipping dewar (CP-100, TS-Scientific, Perkasi, Pennsylvania). After the straws were frozen, they were stored in liquid nitrogen for 20 d. The straws were transferred to BBNFH and used in fertilization (SOP 9, Appendix A) and hatch trials (SOP 10, Appendix A).

In a third experiment, sperm were collected from 4 males at the BBNFH and shipped overnight to the Louisiana State University Agricultural Center Aquaculture Research Station (ARS) in Baton Rouge, Louisiana. All sperm samples had motility of 95%. The sperm were

Table 6-1. Computer-controlled freezer program used to mimic the cooling rate in a nitrogen-vapor shipping dewar. After the 10 min holding period at -80°C , the straws were plunged into liquid nitrogen.

Cooling steps	Start temperature ($^{\circ}\text{C}$)	End temperature ($^{\circ}\text{C}$)
Hold (5 min)	5	5
-18 $^{\circ}\text{C}/\text{min}$	5	-5
-6 $^{\circ}\text{C}/\text{min}$	-5	-15
-36 $^{\circ}\text{C}/\text{min}$	-15	-80
Hold (10 min)	-80	-80

diluted 1:1 (v:v) with HBSS (SOP 7, Appendix A) and frozen at Genex Cooperative, Inc. located at the LSU T. E. Patrick Dairy Improvement Center. Methanol, glycerol, or DMSO was used as a cryoprotectant at a concentration of 5%. The sperm were allowed to equilibrate with the cryoprotectants for 25 min prior to freezing, and motility was estimated (SOP 3, Appendix A). Sperm were frozen in 0.5-ml straws at a mean cooling rate of 16 $^{\circ}\text{C}/\text{min}$. After freezing the straws were transferred to liquid nitrogen and stored for 6 d. The frozen samples were shipped to the BBNFH and used in fertilization trials (SOP 9, Appendix A).

Lake Sturgeon

Sperm were collected from 5 males captured in the Sturgeon River, Michigan ($46^{\circ} 44' \text{ N}$, $88^{\circ} 39' \text{ W}$), and were held undiluted in 50-ml tubes on ice (~6 hr). All samples had 95% motility prior to the initiation of the study. Sperm were diluted 1:4 (v:v; sperm:extender) in mHBSS. Methanol and DMSO were used as cryoprotectants at concentrations of 5% and 10%. Cryoprotectants were diluted (1:1, v:v) in mHBSS prior to addition to samples. Cryoprotectants were added to the sperm and allowed to equilibrate for 2 min, and motility was estimated (SOP 3, Appendix A). Sperm were frozen in a nitrogen-vapor shipping dewar (SOP 12, Appendix A). The mean cooling rate was 22 $^{\circ}\text{C}/\text{min}$. Frozen straws were transferred to a shipping dewar filled

with liquid nitrogen. After 8 d, the frozen sperm were used in fertilization (SOP 9, Appendix A) and hatch trials (SOP 10, Appendix A).

In a second experiment, sperm were collected from 4 males captured in the Black River, Michigan (45° 24' N, 84° 24' W), and held in 50-ml tubes on ice until use (~ 6 hr). All sperm samples had motilities >90% prior to initiation of the experiment. Sperm were extended 1:4 (v:v, sperm:extender) with mTsvetkova solution (SOP 11, Appendix A). Methanol and DMSO were mixed 1:1 (v:v) with extender prior to addition to sperm, and were used as cryoprotectants at concentrations of 5% and 10%. Cryoprotectants were added to the sperm and allowed to equilibrate for 5 min, and motility was estimated (SOP 3, Appendix A). Sperm were frozen in a nitrogen-vapor shipping dewar (SOP 12, Appendix A) at a mean cooling rate of 22 °C/min. After freezing, straws were transferred to the WSFTC and stored in liquid nitrogen in a storage dewar (Model 8038, Thermo Forma, Marietta, Ohio) for 5 months. Straws were thawed in a 40 °C water bath for 9 sec, and motility was estimated (SOP 3, Appendix A).

Pallid Sturgeon

Sperm were collected from 4 males at the U. S. Fish and Wildlife Service Garrison Dam National Fish Hatchery (GDNFH) Riverdale, North Dakota (47° 29' N, 101° 25' W), and were held undiluted in 50-ml tubes on ice (~8 hr). Sperm were diluted in mHBSS at a ratio of 1:4 (v:v, sperm:extender). Methanol, glycerol and DMSO were used as cryoprotectants at concentrations of 5%, 10% and 15%. Cryoprotectants were mixed with mHBSS at a ratio of 1:1 (v:v) prior to addition to sperm. Sperm were allowed to equilibrate in cryoprotectants for 2 min, and motility was estimated (SOP 3, Appendix A). The freezing was performed in a nitrogen-vapor shipping dewar (SOP 12, Appendix A). The mean cooling rate was 22 °C/min. Frozen

samples were transferred to the WSFTC and stored in liquid nitrogen in a storage dewar for 1 year. The sperm were shipped back to GDNFH for use in fertilization (SOP 9, Appendix A) and hatch trials (SOP 10, Appendix A), with the exception that eggs were incubated at 21 °C.

Shovelnose Sturgeon

(1) Sperm were collected from 5 males collected from the Missouri River (47° 37' N, 108° 37' W) above Fort Peck Dam and held at the Charles M. Russell National Wildlife Refuge, U. S. Fish and Wildlife Service, Lewistown, Montana (47° 35' N, 108° 43' W). Sperm were held undiluted in 50-ml tubes on ice (~1 hr) and then diluted 1:4 (v:v) with mHBSS. Methanol and DMSO were used as cryoprotectants at concentrations of 5% and 10%. Cryoprotectants were mixed with mHBSS at a ratio of 1:1 (v:v) prior to addition to sperm. Sperm were allowed to equilibrate with cryoprotectants for 3 min, and motility was estimated (SOP 3, Appendix A). The sperm were frozen in a nitrogen-vapor shipping dewar (SOP 12, Appendix A). The mean cooling rate was 26 °C/min. After 48 hr, the sperm were used in fertilization (SOP 9, Appendix A) and hatch trials (SOP 10, Appendix A).

In a second experiment, sperm were collected from 5 males, held undiluted in 50-ml tubes on ice (~1 hr), and diluted 1:4 (v:v) with mTsvetkova solution. Methanol and DMSO were used as cryoprotectants at concentrations of 5% and 10%. Cryoprotectants were mixed with mTsvetkova at a ratio of 1:1 (v:v) prior to addition to sperm. Sperm were allowed to equilibrate with cryoprotectants for 3 min, and motility was estimated (SOP 3, Appendix A). The sperm were frozen in a nitrogen-vapor shipping dewar (SOP 12, Appendix A). The mean cooling rate was 24 °C/min. After 24 hr, the sperm were used in fertilization (SOP 9, Appendix A) and hatch trials (SOP 10, Appendix A).

Statistical Analysis

Percent motility, percent fertilization, and percent hatch data were arc-sine square root transformed prior to analysis. One-way analysis of variance (ANOVA) was used to determine differences among equilibration motility, post-thaw motility, percent fertilization, and percent hatch for each of the studies. Means were separated using a Tukey-Kramer multiple comparison test at the $P = 0.05$ level.

Results

Shortnose Sturgeon

In the first study, significant differences ($P < 0.0271$) were found among the equilibration motilities in the 5, 10, and 40 °C/min cooling rates. Sperm equilibrated with 10% DMSO for 20 min had significantly lower ($P < 0.0500$) motility ($\leq 65\%$) than did sperm equilibrated with 5% MeOH ($\geq 90\%$) (Table 6-2). However, there were no differences ($P > 0.0657$) in equilibration motility ($\geq 63\%$) for any cryoprotectant and combination in the 20 and 30 °C/min cooling rates.

A significant difference ($P < 0.0363$) was also found for post-thaw motility among the treatments. Post-thaw motility of sperm cryopreserved with 10% DMSO (range 8-17%) was significantly higher ($P < 0.0500$) than that of sperm cryopreserved with either concentration of MeOH at all cooling rates (range 0-5%) (Table 6-2). At the 20 and 40 °C/min cooling rates, sperm cryopreserved with 5% DMSO had post-thaw motility (7-8%) that was significantly higher ($P < 0.0500$) than that of sperm cryopreserved with methanol at either concentration ($\leq 1\%$).

The percent hatch of eggs fertilized with control sperm ($\geq 15\%$) was significantly different ($P < 0.0002$) from that of eggs fertilized with any of the cryopreserved sperm ($\leq 1\%$). The

Table 6-2. Mean (\pm SD) equilibration motility, post-thaw motility, and percent hatch for sperm of shortnose sturgeon cryopreserved using cooling rates of 5, 10, 20, 30, or 40 °C/min. Methanol and DMSO were used as cryoprotectants at concentrations of 5% and 10%, and were equilibrated with the sperm for 20 min prior to freezing. Sperm were frozen in 0.5-ml straws, and thawed in a water bath at 40 °C for 9 sec. Motility was estimated by activating 2 μ l of sperm with 20 μ l of deionized water. Within a cooling rate, values within a column that share a superscript were not significantly different ($P > 0.05$).

Cryoprotectant	Concentration	Motility		Hatch (%)
	(%)	Equilibration (%)	Post-thaw (%)	
Cooling rate 5 °C/min				
DMSO	5	87 ± 6 ^{ab}	1 ± 0 ^b	0 ± 0 ^b
	10	65 ± 15 ^b	8 ± 3 ^a	0 ± 0 ^b
MeOH	5	90 ± 5 ^a	0 ± 0 ^b	0 ± 0 ^b
	10	90 ± 5 ^a	0 ± 0 ^b	0 ± 1 ^b
Control	--	--	90 ± 0*	17 ± 12 ^a
Cooling rate 10 °C/min				
DMSO	5	85 ± 10 ^{ab}	4 ± 3 ^b	0 ± 0 ^b
	10	60 ± 10 ^b	15 ± 5 ^a	0 ± 0 ^b
MeOH	5	92 ± 3 ^a	0 ± 1 ^b	0 ± 0 ^b
	10	75 ± 15 ^{ab}	0 ± 1 ^b	0 ± 0 ^b
Control	--	--	90 ± 0*	15 ± 1 ^a
Cooling rate 20 °C/min				
DMSO	5	80 ± 10 ^a	8 ± 3 ^a	0 ± 0 ^b
	10	63 ± 15 ^a	15 ± 5 ^a	0 ± 0 ^b
MeOH	5	90 ± 5 ^a	0 ± 0 ^b	1 ± 2 ^b
	10	82 ± 8 ^a	1 ± 1 ^b	0 ± 0 ^b
Control	--	--	95 ± 0*	48 ± 18 ^a
Cooling rate 30 °C/min				
DMSO	5	82 ± 8 ^a	4 ± 2 ^{ab}	0 ± 0 ^b
	10	70 ± 15 ^a	15 ± 5 ^a	0 ± 0 ^b
MeOH	5	92 ± 3 ^a	1 ± 0 ^b	0 ± 0 ^b
	10	85 ± 5 ^a	5 ± 8 ^{ab}	0 ± 0 ^b
Control	--	--	90 ± 0*	54 ± 23 ^a
Cooling rate 40 °C/min				
DMSO	5	83 ± 8 ^a	7 ± 3 ^b	0 ± 0 ^b
	10	53 ± 14 ^b	17 ± 3 ^a	0 ± 0 ^b
MeOH	5	90 ± 5 ^a	0 ± 0 ^c	0 ± 0 ^b
	10	88 ± 8 ^a	0 ± 0 ^c	0 ± 1 ^b
Control	--	--	95 ± 0*	22 ± 5 ^a

*Motility of fresh sperm used as a control to determine egg quality.

highest mean percent hatch for any of the cryopreserved samples, at any freezing rate, was $1 \pm 2\%$ (Table 6-2).

In the second study, extender did not have a significant effect on equilibration motility ($P = 0.9046$), but cryoprotectant did ($P < 0.0001$). Sperm exposed to 10% DMSO had significantly ($P < 0.0500$) lower motility ($\leq 25\%$) than did sperm exposed to the other cryoprotectants ($\geq 87\%$) (Table 6-3).

Overall, post-thaw motility of sperm cryopreserved using mTsvetkova as an extender ($13 \pm 13\%$) was significantly different ($P = 0.0183$) than that of sperm cryopreserved using mHBSS ($5 \pm 9\%$) (Table 6-3). Also, significant differences ($P = 0.0005$) were detected due to cryoprotectant. Sperm cryopreserved using 5% DMSO had significantly higher ($P < 0.0500$) post-thaw motility than did sperm cryopreserved with MeOH at either concentration. Sperm cryopreserved using mTsvetkova and 5% DMSO had significantly higher ($P < 0.0500$) post-thaw motility ($30 \pm 5\%$) than did sperm cryopreserved using mTsvetkova and 10% MeOH or mHBSS and MeOH at either concentration ($\leq 1\%$).

In all trials using thawed sperm, percent fertilization and percent hatch were 0% (Table 6-4). With fresh sperm, 40-81% of the eggs were fertilized and 35-68% hatched. In the third study, sperm frozen at the LSU T. E. Dairy Improvement Center, significant differences in equilibration and post-thaw motility of sperm were detected ($P < 0.0001$). Sperm equilibrated in MeOH had significantly higher ($P < 0.0500$) motility than did sperm equilibrated in DMSO or glycerol. Post-thaw motility of sperm cryopreserved with 5% DMSO ($24 \pm 8\%$) was significantly higher ($P < 0.0500$) than that for 5% MeOH ($2 \pm 2\%$) or 5% glycerol ($1 \pm 0\%$) (Table 6-5).

Table 6-3. Mean (\pm SD) equilibration motility and post-thaw motility for sperm of shortnose sturgeon cryopreserved using modified Hanks' balanced salt solution (mHBSS) or modified Tsvetkova solution as an extender and a freezing rate designed to mimic that of a nitrogen-vapor shipping dewar. Methanol (MeOH) and dimethyl sulfoxide (DMSO) were used as cryoprotectants at a concentration of 5% or 10%. Sperm were allowed to equilibrate with the cryoprotectants for 37 min prior to being loaded into 0.5-ml straws. The straws were placed in goblets (5 straws per goblet), which were placed on the lower position of aluminum canes and frozen using a 5-step cooling rate (Table 6-1) in a computer-controlled freezer. Sperm were thawed in a 40 °C water bath and motility was estimated by activating 2 μ l of sperm with 20 μ l of deionized water. Values within a column that share a superscript were not significantly different ($P > 0.05$).

Extender	Cryoprotectant	Motility (%)	
		Equilibration	Post-thaw
mTsvetkova	MeOH 5%	93 \pm 3 ^a	10 \pm 13 ^{ab}
	MeOH 10%	93 \pm 3 ^a	1 \pm 0 ^b
	DMSO 5%	87 \pm 10 ^a	30 \pm 5 ^a
	DMSO 10%	25 \pm 13 ^b	12 \pm 12 ^{ab}
mHBSS	MeOH 5%	93 \pm 3 ^a	0 \pm 0 ^b
	MeOH 10%	93 \pm 3 ^a	0 \pm 0 ^b
	DMSO 5%	87 \pm 14 ^a	15 \pm 13 ^{ab}
	DMSO 10%	22 \pm 12 ^b	5 \pm 5 ^{ab}

Table 6-4. Mean (\pm SD) percent fertilization and percent hatch for eggs fertilized with sperm of shortnose sturgeon cryopreserved using modified Hanks' balanced salt solution (mHBSS) or modified Tsvetkova solution as an extender and frozen at a cooling rate designed to mimic that in a nitrogen-vapor shipping dewar. Methanol (MeOH) and dimethyl sulfoxide (DMSO) were used as cryoprotectants at a concentration of 5% or 10%. Sperm were allowed to equilibrate with the cryoprotectants for 37 min prior to being loaded into 0.5-ml straws. The straws were placed in goblets (5 straws per goblet), which were placed on the lower position of aluminum canes and frozen using a 5-step cooling rate (Table 6-1) in a computer-controlled freezer. Sperm were thawed in a 40 °C water bath for 9 sec and used in fertilization (SOP 9, Appendix A) and hatch trials (SOP 10, Appendix A). Within an extender, values within a column that share a superscript were not significantly different ($P > 0.05$).

Extender*	Cryoprotectant	Fertilization (%)	Hatch (%)
mTsvetkova	MeOH 5%	0 ± 0^b	0 ± 0^b
	MeOH 10%	0 ± 0^b	0 ± 0^b
	DMSO 5%	0 ± 0^b	0 ± 0^b
	DMSO 10%	0 ± 0^b	0 ± 0^b
	Control	40 ± 8^a	35 ± 14^a
mHBSS	MeOH 5%	0 ± 0^b	0 ± 0^b
	MeOH 10%	0 ± 0^b	0 ± 0^b
	DMSO 5%	0 ± 0^b	0 ± 0^b
	DMSO 10%	0 ± 0^b	0 ± 0^b
	Control	81 ± 12^a	68 ± 2^a

* Eggs from different females were used for each extender, so statistical comparisons of fertilization percent and hatch percent were made only within extenders.

Table 6-5. Mean (\pm SD) equilibration motility, post-thaw motility and fertilization rate for sperm of shortnose sturgeon cryopreserved at the Dairy Improvement Center. Methanol (MeOH), glycerol and dimethyl sulfoxide (DMSO) were used as cryoprotectants at a concentration of 5%, and were allowed to equilibrate with the sperm for 25 min prior to freezing. Sperm were frozen in 0.5-ml straws in plastic goblets at a cooling rate of 16 °C/min. Straws were thawed in a water bath at 40 °C for 9 sec. Motility was estimated by activating 2 μ l of sperm with 20 μ l of deionized water. Values within a column that share a superscript were not significantly different ($P > 0.05$).

Cryoprotectant	Motility		Fertilization (%)
	Equilibration (%)	Post-thaw (%)	
MeOH	85 \pm 7 ^a	2 \pm 2 ^b	3 \pm 2 ^b
Glycerol	1 \pm 0 ^b	1 \pm 0 ^b	1 \pm 1 ^b
DMSO	73 \pm 5 ^c	24 \pm 8 ^a	0 \pm 0 ^b
Control	--	90 \pm 0*	74 \pm 11 ^a

*Motility of fresh sperm used as a control to determine egg quality.

Significant differences ($P < 0.0001$) in percent fertilization were also detected. Eggs fertilized with fresh sperm had significantly higher ($P < 0.0500$) percent fertilization ($74 \pm 11\%$) than did eggs fertilized with any of the cryoprotectants ($\leq 3\%$) (Table 6-5). Sperm cryopreserved with DMSO were unable to fertilize eggs.

Lake Sturgeon

With mHBSS as an extender, significant differences ($P < 0.0001$) were found in equilibration motility of sperm exposed to cryoprotectants for 2 min. Sperm exposed to 10% DMSO had significantly lower ($P < 0.0500$) motility (72%) than did sperm exposed to the other cryoprotectant concentrations ($> 88\%$) (Table 6-6). No differences in post-thaw motility were found among the cryoprotectants tested ($P = 0.3581$). However, significant differences ($P < 0.0001$) in percent fertilization were detected among the treatments. Sperm cryopreserved with 5% MeOH ($48 \pm 14\%$) or 10% MeOH ($46 \pm 15\%$) fertilized a significantly higher ($P < 0.0500$) percentage of eggs than did sperm cryopreserved with 5% DMSO ($1 \pm 0\%$) or 10% DMSO ($1 \pm 1\%$), although fertilization with cryopreserved sperm was significantly lower than that with fresh control sperm (95%).

With mTsvetkova as an extender, significant differences ($P = 0.0160$) were detected in equilibration motility of sperm exposed to cryoprotectants for 5 min (Table 6-7). Sperm exposed to 10% DMSO had significantly lower ($P < 0.0500$) motility ($39 \pm 33\%$) than did sperm exposed to other cryoprotectants and concentrations ($\geq 92\%$). Significant differences ($P = 0.0142$) in post-thaw motility were also found. Sperm cryopreserved with 10% MeOH had significantly higher ($P < 0.0500$) post-thaw motility ($50 \pm 22\%$) than did sperm cryopreserved with 10% DMSO ($7 \pm 7\%$) (Table 6-7).

Table 6-6. Mean (\pm SD) equilibration motility, post-thaw motility, and percent fertilization for lake sturgeon sperm cryopreserved with modified Hanks' balanced salt solution (mHBSS) as an extender. Sperm were diluted at a ratio of 1:4 (v:v, sperm:mHBSS). Sperm were cryopreserved using two concentrations (5% or 10%) of MeOH or DMSO as cryoprotectants, and frozen at a cooling rate of 22 °C/min. Equilibration time was 2 min. Sperm were thawed in a water bath at 40 °C for 9 sec. Motility was estimated by activating 2 μ l of sperm with 20 μ l of deionized water. Values within a column that share a superscript were not significantly different ($P > 0.05$).

Cryoprotectant	Motility		Fertilization (%)
	Equilibration (%)	Post-thaw (%)	
Control	--	75 \pm 0*	95 \pm 2 ^a
5% DMSO	92 \pm 3 ^a	11 \pm 13 ^a	1 \pm 0 ^b
10% DMSO	72 \pm 7 ^b	17 \pm 10 ^a	1 \pm 1 ^b
5% MeOH	92 \pm 3 ^a	7 \pm 5 ^a	48 \pm 14 ^c
10% MeOH	88 \pm 7 ^a	8 \pm 7 ^a	46 \pm 15 ^c

*Motility of fresh sperm used as a control to determine egg quality.

Table 6-7. Mean (\pm SD) equilibration motility and post-thaw motility for lake sturgeon sperm cryopreserved using modified Tsvetkova solution (mTsvetkova) as an extender. Sperm were diluted at a ratio of 1:4 (v:v, sperm: mTsvetkova). Methanol (MeOH) and dimethyl sulfoxide (DMSO) were used as cryoprotectants at concentrations of 5% and 10%, and were allowed to equilibrate with sperm for 5 min prior to freezing. Sperm were frozen in 0.5-ml straws and at a cooling rate of 22 °C/min. Straws were thawed in a 40 °C water bath for 9 sec, and motility was estimated by activating 2 μ l of sperm with 20 μ l of deionized water. Values within a column that share a superscript were not significantly different ($P > 0.05$).

Cryoprotectant	Motility (%)	
	Equilibration	Post-thaw
MeOH 5%	92 \pm 5 ^a	32 \pm 22 ^{ab}
MeOH 10%	94 \pm 3 ^a	50 \pm 22 ^a
DMSO 5%	94 \pm 3 ^a	21 \pm 2 ^{ab}
DMSO 10%	39 \pm 33 ^b	7 \pm 7 ^b

Pallid Sturgeon

Significant differences ($P < 0.0001$) were detected in equilibration motility after exposure to cryoprotectants for 2 min. Sperm exposed to 15% glycerol ($0 \pm 0\%$) had significantly ($P < 0.0500$) lower motility than did sperm exposed to any concentration of MeOH ($\geq 90\%$) or DMSO ($\geq 45\%$) (Table 6-8). Significant differences ($P = 0.0002$) were also found in post-thaw motility. Sperm cryopreserved with glycerol at any concentration had significantly ($P < 0.0500$) lower post-thaw motility ($\leq 1\%$) than did sperm cryopreserved with MeOH or DMSO at concentrations as high as 10% ($\geq 34\%$). Significantly higher ($P < 0.0500$) post-thaw motilities were observed with 5% concentrations of MeOH (42%) and DMSO (54%) than with 15% concentrations (20% motility for MeOH and 5% motility for DMSO).

Due to the low post-thaw motility ($\leq 1\%$), sperm cryopreserved with glycerol were not used in the fertilization trials. Significant differences ($P < 0.0001$) in percent fertilization were observed among the cryoprotectants. Eggs fertilized using sperm cryopreserved with 5% or 10% MeOH had fertilization rates ($\geq 91\%$) that were not significantly different ($P > 0.0500$) from that of control sperm ($97 \pm 2\%$), but were significantly higher ($P < 0.0500$) than percent fertilization for eggs fertilized using sperm cryopreserved with other cryoprotectants or concentrations (1-38%) (Table 6-8). Significant differences ($P < 0.0001$) were also found in percent hatch among the cryoprotectants. Percent hatch of eggs fertilized with sperm cryopreserved using 5% or 10% methanol (77%) were not significantly different ($P > 0.0500$) from that of control sperm ($69 \pm 5\%$) but were significantly higher ($P < 0.0500$) than for eggs fertilized using sperm cryopreserved with other cryoprotectants or concentrations ($\leq 34\%$) (Table 6-8).

Table 6-8. Mean (\pm SD) equilibration motility, post-thaw motility, percent fertilization and percent hatch for pallid sturgeon sperm cryopreserved with modified Hanks' balanced salt solution (mHBSS) as an extender. Sperm were diluted at a ratio of 1:4 (v:v, sperm:mHBSS). Sperm were equilibrated in cryoprotectants for 2 min prior to being frozen in 0.5-ml straws in a nitrogen-vapor shipping dewar at a cooling rate of 22 °C/min. Sperm were thawed in a water bath at 40 °C for 9 sec, and motility was estimated by activating 2 μ l of sperm with 20 μ l of deionized water. Thawed sperm were used to fertilize ~300 eggs. The eggs were incubated at 18 °C. After 6 hrs, one-half of the eggs were removed to determine fertilization rates, and the remaining eggs were incubated until hatch. Values within a column that share a superscript were not significantly different ($P > 0.05$).

Cryoprotectant	Motility		Fertilization (%)	Hatch (%)
	Equilibration (%)	Post-thaw (%)		
MeOH 5%	94 \pm 2 ^a	42 \pm 10 ^{ab}	91 \pm 5 ^a	77 \pm 6 ^a
MeOH 10%	91 \pm 2 ^a	35 \pm 11 ^{ab}	92 \pm 5 ^a	77 \pm 8 ^a
MeOH 15%	90 \pm 0 ^a	20 \pm 14 ^{bcd}	12 \pm 12 ^c	10 \pm 12 ^c
DMSO 5%	95 \pm 0 ^a	54 \pm 24 ^a	38 \pm 8 ^b	34 \pm 3 ^b
DMSO 10%	79 \pm 26 ^{ab}	34 \pm 19 ^{abc}	2 \pm 4 ^{cd}	3 \pm 5 ^c
DMSO 15%	45 \pm 38 ^{bc}	5 \pm 4 ^{cd}	1 \pm 3 ^d	1 \pm 2 ^c
Glycerol 5%	38 \pm 12 ^c	1 \pm 0 ^d	--	--
Glycerol 10%	9 \pm 5 ^{cd}	0 \pm 0 ^d	--	--
Glycerol 15%	0 \pm 0 ^d	0 \pm 0 ^d	--	--
Control	--	95 \pm 0*	97 \pm 2 ^a	69 \pm 5 ^a

*Motility of fresh sperm used as a control to determine egg quality.

Shovelnose Sturgeon

In the first study, there were no significant differences among cryoprotectants and concentrations in equilibration motility (89-95%) ($P = 0.0571$) or post-thaw motility (22-28%) ($P = 0.0637$) for sperm cryopreserved with mHBSS as an extender (Table 6-9). Differences in percent fertilization and percent hatch could not be determined between the cryoprotectants due to the small quantities of eggs used for fertilizations. Eggs fertilized with sperm cryopreserved using MeOH at either concentration yielded percent fertilization (57-63%) and percent hatch (44-49%) that were not significantly different ($P > 0.0664$) among the 5% and 10% concentrations or fresh sperm controls (56-78%) (Table 6-10). Similarly, eggs fertilized with sperm cryopreserved using DMSO at either concentration yielded percent fertilization (36-40%) and percent hatch (30-31%) that were not significantly different ($P > 0.0735$) among the 5% and 10% concentrations or fresh sperm controls (68-97%).

In the second study, when mTsvetkova was used as an extender, significant differences were found in equilibration motility of sperm exposed to cryoprotectants for 3 min (Table 6-9). Sperm exposed to 10% DMSO had significantly ($P = 0.0027$) lower motility ($90 \pm 4\%$) than did sperm exposed to the other cryoprotectant combinations ($95 \pm 0\%$). There were no significant differences in post-thaw motility ($P = 0.6534$) for sperm cryopreserved with mTsvetkova as an extender. Eggs fertilized with sperm cryopreserved with MeOH at either concentration yielded percent fertilization (79-92%) and percent hatch (51-73%) that were not significantly different ($P > 0.1349$) from that of fresh sperm controls (75-95%) (Table 6-10). Significant differences ($P < 0.0001$) were detected in percent fertilization and percent hatch for eggs fertilized with sperm cryopreserved using DMSO and eggs fertilized with control sperm. Eggs fertilized with sperm cryopreserved using DMSO at either concentration yielded percent fertilization (8-17%)

Table 6-9. Mean (\pm SD) equilibration motility and post-thaw motility of shovelnose sturgeon sperm frozen using modified Hanks' balanced salt solution (mHBSS) or modified Tsvetkova solution (mTsvetkova) as extenders. Sperm were equilibrated in cryoprotectants for 3 min prior to freezing. Sperm were frozen in 0.5-ml straws in a nitrogen vapor shipping dewar at a cooling rate of 22-24 °C/min. Sperm were thawed for 9 sec in a 40 °C water bath, and motility was estimated by activating 2 μ l of sperm with 20 μ l of deionized water. Within an extender, values within a column that share a superscript were not significantly different ($P > 0.05$).

Cryoprotectant	Motility	
	Equilibration (%)	Post-thaw (%)
mHBSS		
MeOH 5%	95 \pm 0 ^a	22 \pm 15 ^a
MeOH 10%	95 \pm 0 ^a	28 \pm 21 ^a
DMSO 5%	94 \pm 2 ^a	24 \pm 11 ^a
DMSO 10%	89 \pm 6 ^a	26 \pm 16 ^a
mTsvetkova		
MeOH 5%	95 \pm 0 ^a	47 \pm 8 ^a
MeOH 10%	95 \pm 0 ^a	50 \pm 4 ^a
DMSO 5%	95 \pm 0 ^a	42 \pm 11 ^a
DMSO 10%	90 \pm 4 ^b	48 \pm 8 ^a

Table 6-10. Mean (\pm SD) percent fertilization and percent hatch for eggs fertilized with shovelnose sturgeon sperm cryopreserved using modified Hanks' balanced salt solution (mHBSS) or modified Tsvetkova solution (mTsvetkova) as extenders. Sperm were thawed in a water bath at 40 °C for 9 sec, and motility was estimated by activating 2 μ l of sperm with 20 μ l of deionized water. Fresh sperm (95% motility) were used as controls to determine egg quality. Sperm were used to fertilize ~300 eggs, which were then incubated at 18 °C. After 6 hrs, one-half of the eggs were removed to determine fertilization rates, and the remaining eggs were incubated until hatch. Due to insufficient sample sizes of eggs, sperm cryopreserved using each cryoprotectant within an extender were applied to separate samples of eggs and analyzed separately. Within an extender and cryoprotectant, values within a column that share a superscript were not significantly different ($P > 0.05$).

Cryoprotectant	Fertilization (%)	Hatch (%)
mHBSS		
MeOH 5%	63 \pm 32 ^a	49 \pm 24 ^a
MeOH 10%	57 \pm 34 ^a	44 \pm 26 ^a
Control*	78 \pm 6 ^a	56 \pm 7 ^a
DMSO 5%	40 \pm 22 ^a	31 \pm 20 ^a
DMSO 10%	36 \pm 26 ^a	30 \pm 20 ^a
Control*	97 \pm 3 ^a	68 \pm 17 ^a
mTsvetkova		
MeOH 5%	92 \pm 10 ^a	73 \pm 15 ^a
MeOH 10%	79 \pm 31 ^a	51 \pm 21 ^a
Control*	95 \pm 1 ^a	75 \pm 3 ^a
DMSO 5%	17 \pm 8 ^a	13 \pm 7 ^a
DMSO 10%	8 \pm 9 ^a	4 \pm 8 ^a
Control*	98 \pm 1 ^b	69 \pm 2 ^b

*Motility of fresh sperm used as a control to determine egg quality.

and percent hatch (4-13%) that were significantly lower ($P < 0.05$) than that of fresh sperm controls (69-98%), however there were no significant differences ($P > 0.05$) between the cryoprotectant concentrations.

Discussion

Although cryoprotectants are used to help protect cells during the freezing and thawing processes, these chemicals can be toxic to the cells, and care must be taken to balance the cryoprotective benefits and the toxicity. In the current study, exposure of sperm to 10% DMSO for as little as 5 min reduced motility of lake sturgeon sperm by ~50%. Similar results were obtained with Atlantic sturgeon sperm which were reduced to 1% motility when exposed to 20% DMSO for 30 min (Kopeika et al., 2000). Paddlefish sperm motility was reduced by as much as 25% by exposure to DMSO for 15 min (Brown and Mims, 1999). Methanol was shown to be non-toxic to cells when used as a cryoprotectant for sperm of spotted seatrout *Cynoscion nebulosus* (Wayman et al., 1996), channel catfish *Ictalurus punctatus* (Christensen and Tiersch, 1997), and razorback sucker *Xyrauchen texanus* (Tiersch et al., 1998). In the current study, exposure of sperm to MeOH (5 - 15%) for as long as 20 min had no effect on sperm motility. In the only other report of the use of MeOH as a cryoprotectant for sturgeon sperm (Glogowski et al., 2002), information was not reported for sperm motility of Siberian sturgeon after exposure to MeOH. Based on the toxicity of DMSO, cryopreservation procedures should be optimized to reduce the amount of time that sperm are in cryoprotectants prior to freezing.

Dimethyl sulfoxide was the most common cryoprotectant used for sturgeon sperm (reviewed by Mims, 2000). When 5% or 10% DMSO were used as a cryoprotectant in the current study, post-thaw motility was equal to or higher than that of sperm cryopreserved with MeOH at equal

concentrations. Post-thaw motility of sperm cryopreserved with DMSO was also reported for several species of sturgeon. Sperm of lake sturgeon cryopreserved with 10% DMSO had 14% post-thaw motility (Ciereszko et al., 1996b), which was similar to the post-thaw motility (17%) of lake sturgeon sperm when mHBSS was used as an extender in the current study. Sperm cryopreserved using 15% DMSO had post-thaw motilities of 30-40% for Siberian sturgeon, 15-20% for Sakhalin sturgeon *A. mikadoi*, and 30-40% for stellate sturgeon *A. stellatus* (Drokin and Kopeika, 1996). Sperm of Siberian sturgeon cryopreserved with methanol had post-thaw motility of 15-16% (Glogowski et al., 2002). Based on post-thaw motility in the current study, DMSO appears to be an equal or better cryoprotectant for sturgeon sperm than does MeOH.

Although sturgeon sperm cryopreserved using DMSO generally retained equal or higher post-thaw motility, sperm cryopreserved with MeOH yielded higher fertilization rates. In the current study, percent fertilization for pallid sturgeon sperm (91-92%) and shovelnose sturgeon sperm (57-63%) cryopreserved with MeOH were equal to that of fresh sperm (78-97%); however sperm cryopreserved with DMSO yielded lower percent fertilization (1-40%). Cryopreserved lake sturgeon sperm, in the current study, fertilized 46-48% of eggs when MeOH was used as a cryoprotectant, compared to 1% fertilized eggs when DMSO was used as cryoprotectant. Siberian sturgeon sperm cryopreserved with MeOH also yielded high percent fertilization (>80%) and percent hatch (30%) (Glogowski et al., 2002), but DMSO was not evaluated as the cryoprotectant so comparisons cannot be made.

In other reports where DMSO was used as a cryoprotectant for sturgeon sperm and percent fertilization or percent hatch were reported, other cryoprotectants were not evaluated. (Tsvetkova et al., 1996; Kopeika et al., 2000). Due to the fact that in the current study high post-thaw motility did not always equate to high percent fertilization or percent hatch, it is impossible to

predict fertilization ability from previous reports on post-thaw motility using DMSO as a cryoprotectant. Also, the lack of cryoprotectant comparisons within previous reports makes it difficult to determine if MeOH or DMSO provided greater protection for sperm of other sturgeon species during cryopreservation. However, based on the percent fertilization and percent hatch of eggs fertilized with sperm cryopreserved using MeOH as a cryoprotectant in the current study, MeOH would be recommended for the cryopreservation of sturgeon sperm, except for shortnose sturgeon.

Differences in extender composition were shown to have effects on the cryopreservation of sturgeon sperm. Siberian sturgeon sperm cryopreserved using three different extenders (Tris-Sucrose-KCl, Tris-NaCl-KCl, Tris-Sucrose) retained similar post-thaw motility (~15%), but sperm cryopreserved with Tris-Sucrose produced significantly lower fertilization percent (40%) and hatch percent (6%) than did sperm cryopreserved with other extenders (>80%, fertilization; >18%, hatch) (Glogowski et al., 2002). In the current study, lake sturgeon sperm cryopreserved using mTsvetkova (same extender as Tris-Sucrose-KCl in Glogowski et al., 2002) and MeOH had high post-thaw motility (32-50%), but comparisons could not be made to sperm cryopreserved using mHBSS due to differences in the males used in the studies. Also in the current study, shovelnose sturgeon sperm cryopreserved using mTsvetkova retained higher percent motility (>42%) than did sperm cryopreserved using mHBSS (<28%). Percent fertilization and percent hatch comparisons between the extenders could not be made due to small sample sizes of eggs from shovelnose sturgeon. Further research into the fertilizing ability of sperm cryopreserved in mTsvetkova extender is warranted for other species, but the extender can be recommended for use with sperm of lake sturgeon.

In the current study, although fresh sperm of shortnose sturgeon were capable of fertilizing eggs, cryopreserved shortnose sturgeon sperm had limited ability ($\leq 3\%$) to fertilize eggs. Seven cooling rates were attempted, but the highest percent fertilization of any treatment was 3% for sperm cryopreserved using the method of the LSU Dairy Improvement Center and 5% MeOH as a cryoprotectant. At least two possible explanations exist for the differences in the sperm cryopreservation of shortnose sturgeon and lake sturgeon, pallid sturgeon, or shovelnose sturgeon. First, it is possible that the methods used for other sturgeon species are not suitable for shortnose sturgeon sperm. Studies have shown that cryopreservation procedures can differ dramatically from species to species within a family or genus (Tiersch et al., 1994; Bart et al., 1998), and within subspecies (Gwo et al., 1999). Secondly, the shortnose sturgeon used in this study were part of a domesticated broodstock maintained at the BBNFH, and were the only domesticated sturgeon used during this study. Currently the nutritional requirements of shortnose sturgeon are unknown, and as such the sturgeon are not fed a specific diet, which may have contributed to the lack of fertilizing ability of cryopreserved sperm. Dietary ingredients were shown to influence the post-thaw quality of sperm of rainbow trout *Oncorhynchus mykiss* (Pustowka et al., 2000), significantly increasing the percentage of eyed embryos. Further study is necessary to develop a cryopreservation protocol for sperm of shortnose sturgeon.

Based on the results of the current study, MeOH should be considered as a useful cryoprotectant for sturgeon sperm. Dimethyl sulfoxide retained equal to or higher post-thaw motility than did MeOH, but produced lower percent fertilization and percent hatch than did MeOH. This indicates that post-thaw motility should not be used as an indicator of cryopreserved sperm quality. There was evidence that extender composition affected post-thaw sperm quality, based on motility of sperm of shovelnose sturgeon and lake sturgeon, but further

research comparing fertilization and hatch of eggs fertilized with sperm cryopreserved using mHBSS or mTsvetkova as extenders is necessary. Although currently a recommendation cannot be made for the cryopreservation of shortnose sturgeon sperm, it can be recommended that sperm of lake sturgeon, shovelnose sturgeon, and pallid sturgeon should be cryopreserved using 5% MeOH as a cryoprotectant, mHBSS as an extender at a ratio of 1:4 (v:v; sperm:extender), 0.5-ml straws, and freezing in a nitrogen-vapor shipping dewar at a cooling rate of 22 to 24 °C/min.

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Chapter 7

Development of Methods for the Objective Determination of Sperm Quality

Introduction

Several methods are currently available to evaluate the quality of sperm samples.

Fertilization trials, which directly evaluate the ability of the sperm cell to produce offspring, are the best method, but they are labor intensive and time consuming. For example, determination the percent of eyed eggs (commonly used as a measure of fertilization rates for salmonids) from chinook salmon *Oncorhynchus tshawytscha*, can take as long as 18 d at 14 °C (Piper et al., 1982). Also egg availability is typically seasonal in fishes, often lasting only a few weeks (McNiven et al., 1992). Visual estimation of the percentage of motile sperm is the most common form of analysis (see SOP 3, Appendix A). This method is fast and inexpensive, but is subjective and can be influenced by the person reading the samples (McNiven et al., 1992). Computer-assisted sperm analysis (CASA) is also based on motility characteristics. Unlike motility estimation, CASA is objective. The system uses a camera, computer, and software to record and analyze sperm motility. However, the system has to be fully validated and the conditions for analysis optimized for each species (Kime et al., 2001). Another drawback of motility analysis for determining sperm quality is that sperm motility does not always correlate well with fertilizing ability (Stein and Bayrle, 1978). The development of fast and accurate methods of analyzing sperm quality would benefit the development of cryopreservation programs for sturgeon species.

Flow cytometry can offer many advantages to the analysis of sperm quality. The technique provides objective and rapid (300 cells/sec) analysis of individual cells, and allows quantification of tens of thousands of cells per sample. Several flow cytometry techniques have been

developed for evaluating sperm quality (e.g. Evenson et al., 1994; Thomas et al., 1998; Honeyfield and Krise, 2000; Segovia et al., 2000). One such technique uses two dyes to evaluate the integrity of the cell membrane. A membrane permeable nucleic acid stain, SYBR-14, is added to the cells, and yields green fluorescence. A second nucleic acid stain, propidium iodide (PI), which is not membrane permeable and can only enter into damaged cells, counter-stains damaged cells yielding red fluorescence. After staining with both dyes, cells that stain red are counted as non-viable (membrane incapable of excluding PI) and cells that stain green are counted as viable (membrane capable of excluding PI). This technique has been used effectively for sperm from mammals (Garner and Johnson, 1995), birds (Donoghue et al., 1995), mollusks (Paniaqua-Chavez and Tiersch, 2001) and fish (McNiven et al., 1992).

This study sought to evaluate a dual-staining technique and flow cytometry for the analysis of sturgeon sperm quality after cryopreservation. The specific objectives of the study are to determine if: 1) a commercially available staining kit can be used for quality analysis of cryopreserved sturgeon sperm; 2) the percentage of viable sperm determined by flow cytometry analysis correlates with motility values for thawed sperm, and 3) the percentage of viable sperm correlates with percent fertilization and percent hatch rates established from previous fertilization trials. For the purpose of these studies, viable sperm were defined as sperm with intact cell membranes capable of excluding propidium iodide.

Materials and Methods

Sperm Cryopreservation

The sperm samples used in this study were cryopreserved in the experiments presented in Chapter 6 “Cryopreservation of Sturgeon Sperm.” Sperm of lake sturgeon *Acipenser fulvescens*

from 5 males were collected in modified Hanks' balanced salt solution (mHBSS; SOP 6, Appendix A) and from 4 males in another extender ("mTsvetkova"; SOP 11, Appendix A) developed for the cryopreservation of sperm of sterlet *Acipenser ruthenus* (Horvath et al., 2000). Sperm of shovelnose sturgeon *Scaphirhynchus platyrhynchus* were collected from 5 different males for each extender (mHBSS and mTsvetkova). Sperm of pallid sturgeon *Scaphirhynchus albus* were collected from 4 males in mHBSS. All samples had motilities of 95% prior to initiation of the study. Sperm samples were extended at a ratio of 1:4 (v:v, sperm:extender).

Methanol and dimethyl sulfoxide were used as cryoprotectants at concentrations of 5% and 10%. Cryoprotectants were first diluted 1:1 (v:v) with mHBSS or mTsvetkova to reduce cryoprotectant toxicity effects. Cryoprotectants were mixed with the sperm and allowed to equilibrate for 2-3 min. The sperm were loaded into 0.5-ml straws (bovine medium straws, IMV International Corporation, Minneapolis, Minnesota). The straws were sealed with PVC sealing powder (IMV International Corporation), loaded into 10-mm plastic goblets (5 straws per goblet) (IMV International Corporation) at the bottom position on 10-mm aluminum canes (IMV International Corporation), and lowered into a nitrogen-vapor shipping dewar (CP100, TS-Scientific, Perkasié, Pennsylvania). The average cooling rate (22-26 °C/min) for the straws was determined using a datalogger (OM-550; Omega Engineering Inc., Stamford, Connecticut) and a type-T thermocouple (Omega Engineering Inc.). After 30 min, the canes were placed in another nitrogen-vapor shipping dewar containing liquid nitrogen until transferred to the United States Fish and Wildlife Service's Warm Springs Fish Technology Center (Warm Springs, Georgia). The samples were stored submerged in liquid nitrogen in a storage dewar (Model 8038, Thermo Forma, Marietta, Ohio) until use. Frozen samples were thawed for 9 sec in a

40 °C water bath. The sperm were transferred to a 1.5-ml centrifuge tube, and motility estimated within 5 min. Sperm were stained as described below.

Viability Staining

A dual-staining technique (Live/Dead Sperm Viability Kit, Molecular Probes, Eugene, Oregon) was used to determine the percentages of viable cells in thawed sperm samples. To determine percent viable sperm, thawed sperm were suspended in 500 µl of mHBSS. Sperm concentrations approximated 1×10^6 , where the volume of thawed sperm added was adjusted to maintain a rate of analysis of 300 cells per second in the flow cytometer. Two µl of lake sturgeon sperm, 8 µl of pallid sturgeon sperm, and 20 µl of shovelnose sturgeon sperm were added to 500 µl of mHBSS. Five µl of SYBR-14 (20 µM) were added to the sperm suspension, and incubated at room temperature in the dark. After 10 min, 2.5 µl of propidium iodide (PI) (1 mM) were added and incubated for 10 min at room temperature in the dark. The samples were analyzed with a flow cytometer (FACSCaliber[®], Becton Dickinson Immunocytometry Systems, BDIS, San Jose, California) equipped with an air-cooled, argon laser that emits at 488 nm. The instrument was initially calibrated using FACSComp[®] software (BDIS) and labeled fluorescent beads (Calibrite Beads, BDIS). Green and red fluorescence were plotted on a density plot, and a total of 10,000 events were counted per sample. Data were analyzed using Cell Quest[®] software (BDIS).

Validation of the Assay

To verify that the staining procedure would accurately determine the percentage of viable cells within a cryopreserved sturgeon sperm sample, a standard curve was developed from viable

and non-viable sperm. Non-viable sperm were produced by incubating sperm samples in a 50 °C water bath for 5 min, after which time all sperm were immotile upon activation and fluoresced red after viability staining. To develop the standard curve, cryopreserved sperm were thawed in a water bath at 40 °C for 9 sec, and motility was estimated (SOP 3, Appendix A). Half of the sperm sample was placed in a 50 °C water bath for 5 min. The motility was again estimated to verify that the sperm were immotile. Viable and non-viable sperm were mixed in ratios of 100:0, 75:25, 50:50, 25:75, and 0:100. The mixtures were stained using the dual-staining technique described above and analyzed by flow cytometry.

Fertilization Trials

Lake sturgeon fertilization trials were conducted on the Black River, Michigan (45° 24' N, 84° 24' W) with sperm cryopreserved with mHBSS as an extender and stored for 8 d in a nitrogen-vapor shipping dewar (CP-100). Pallid sturgeon fertilization trials were conducted at the United States Fish and Wildlife Service's Garrison Dam National Fish Hatchery (Riverdale, North Dakota, 47° 29' N, 101° 25' W) with sperm cryopreserved with mHBSS as an extender and stored for 1 yr. Shovelnose sturgeon fertilization trials were conducted at the Charles M. Russell National Wildlife Refuge (United States Fish and Wildlife Service, Lewistown, Montana, 47° 35' N, 108° 43' W) with sperm cryopreserved with mHBSS and mTsvetkova as extenders and stored for 2 d in a nitrogen-vapor shipping dewar. Fertilization and hatch trials were conducted according to SOP 9 and SOP 10 (Appendix A).

Statistical Analysis

An analysis of variance (ANOVA) (NCSS 2000, Number Cruncher Statistical Systems, Hayesville, Utah) was used to determine the main effects of cryoprotectant and concentration on viable cell percentage for thawed sperm. Coefficients of determination (r^2) (Microsoft Excel 2000, Microsoft Corporation, Redmond, Washington) were used to determine the relationships between the percentage of viable cells as determined by flow cytometry and percent motility, percent fertility, or percent hatch.

Results

Validation of the Assay

Due to the use of cryopreserved sperm, upon thawing sperm cells were not 100% viable, and therefore 100% proportions of viable cells only contained 26-68% viable cells. There was a strong linear relationship between the percentage of non-viable cells and the percentage of viable cells for thawed lake sturgeon ($r^2 = 0.9468$), pallid sturgeon ($r^2 = 0.9468$), and shovelnose sturgeon sperm ($r^2 = 0.9909$) (Figure 7-1).

Viability Staining

Lake Sturgeon

When mHBSS was used as the extender, sperm cryopreserved with 10% DMSO retained a significantly higher ($P < 0.0001$) percentage of viable cells (62%) than did sperm cryopreserved with other cryoprotectants (Table 7-1). There was a strong correlation between the percentage of viable cells and percent motility for sperm samples cryopreserved with 5% methanol ($r^2 = 0.8216$), 10% methanol ($r^2 = 0.9741$) and 5% DMSO ($r^2 = 0.7797$) (Figure 7-2). All

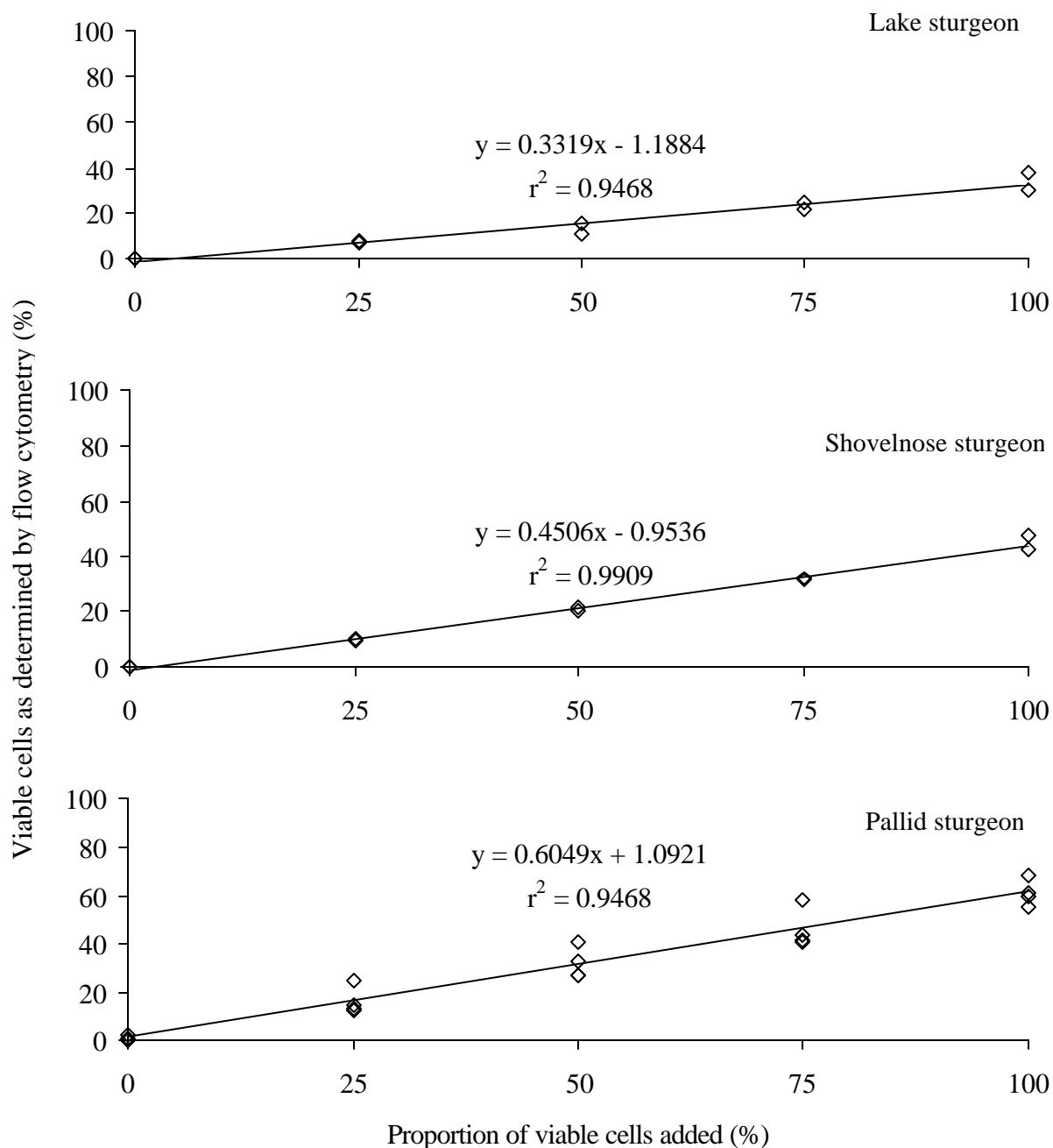


Figure 7-1. Percentage of viable cells as determined by dual-staining and flow cytometry for thawed sturgeon sperm samples containing varied proportions of heat-treated non-viable cells. Sperm samples were diluted 1:4 in modified Hanks' balanced salt solution (mHBSS), and frozen in 10% dimethyl sulfoxide. Frozen sperm samples were thawed in a 40 °C water bath for 9 sec. One-half of the thawed sample was placed in a 50 °C water bath for 5 min to produce non-viable sperm. Varied proportions of untreated and heat-treated sperm were mixed, suspended in 500 μ l of mHBSS, and stained with 5 μ l of 20 μ M SYBR-14 and 2.5 μ l of 1 mM propidium iodide.

Table 7-1. Mean (\pm SD) percentage of viable cells as determined by dual-staining and flow cytometry for thawed sturgeon sperm cryopreserved using modified Hanks' balanced salt solution (mHBSS) as an extender. Sperm were thawed for 9 sec in a 40 °C water bath, suspended in 500 μ l of mHBSS, and stained with 5 μ l of 20 μ M SYBR-14 and 2.5 μ l of 1 mM propidium iodide. The sperm cells were analyzed by flow cytometry to determine the percentage of viable cells per sample. Values in columns sharing superscript letters were not significantly different ($P < 0.05$). DMSO, dimethyl sulfoxide; MeOH, methanol.

Cryoprotectant	Viable cells (%)		
	Lake sturgeon	Pallid sturgeon	Shovelnose sturgeon
DMSO 5%	26 \pm 8 ^b	65 \pm 9 ^{ab}	58 \pm 5 ^{bc}
DMSO 10%	62 \pm 3 ^a	70 \pm 10 ^a	74 \pm 7 ^a
DMSO 15%	--	70 \pm 7 ^{ab}	--
MeOH 5%	14 \pm 8 ^b	52 \pm 3 ^{ab}	45 \pm 15 ^c
MeOH 10%	28 \pm 13 ^b	61 \pm 7 ^{ab}	60 \pm 16 ^{ab}
MeOH 15%	--	45 \pm 10 ^b	--

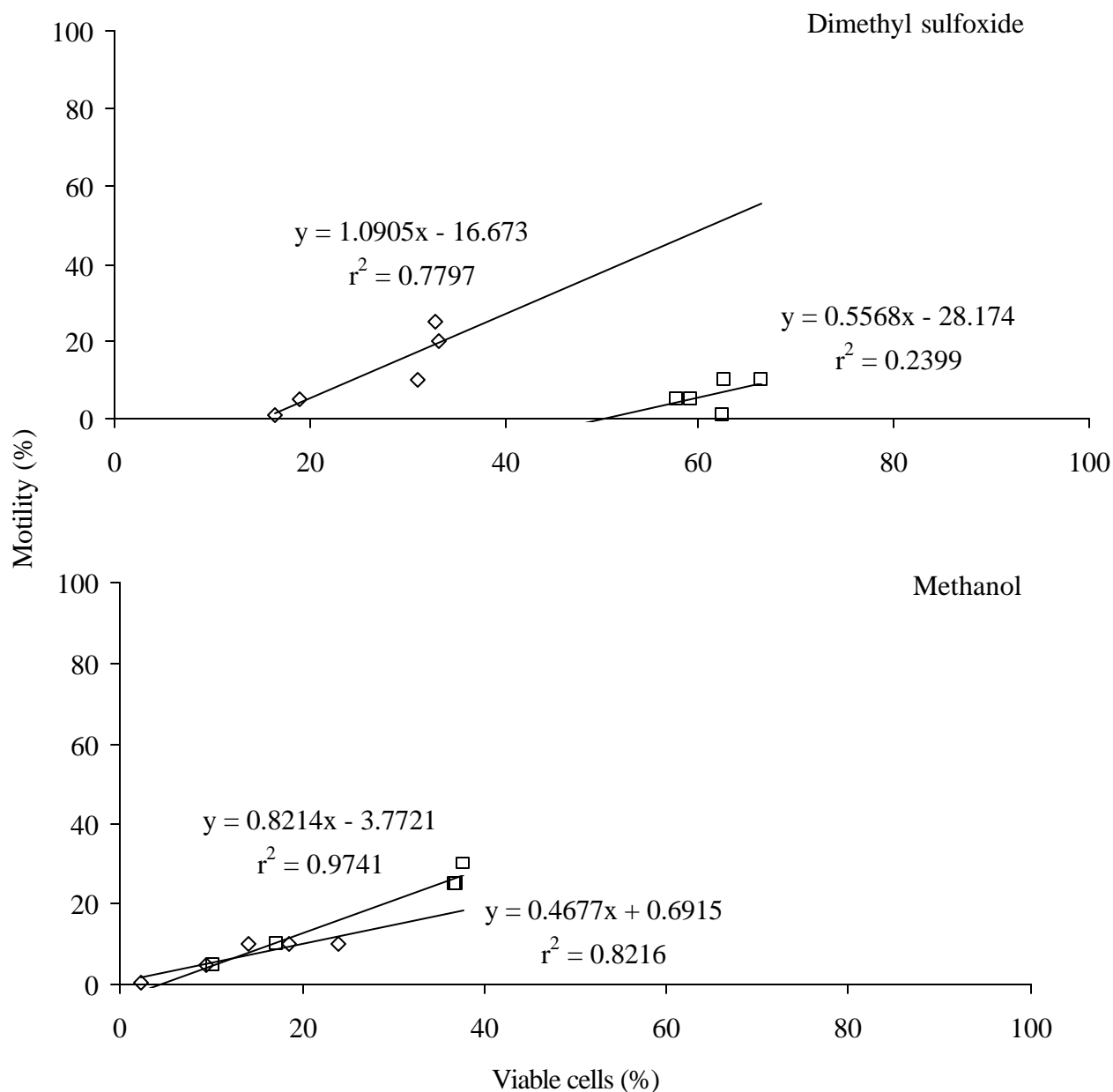


Figure 7-2. Correlations between percent motility and the percentage of viable cells for sperm of lake sturgeon cryopreserved with modified Hanks' balanced salt solution (mHBSS) as an extender. Sperm were diluted to a final dilution of 1:4 in extender with cryoprotectant prior to freezing. Frozen sperm were thawed in a 40 °C water bath for 9 sec, suspended in 500 µl of mHBSS, and stained with 5 µl of 20 µM SYBR-14 and 2.5 µl of 1 mM propidium iodide. Flow cytometry was used to determine the percentage of viable cells. (5%, diamonds; 10%, squares).

combinations of cryoprotectant and concentration yielded poor relationships between the percentage of viable cells and percent fertilization ($r^2 < 0.5$) (Figure 7-3). The strongest linear relationship ($r^2 = 0.4645$) among the combinations was for sperm cryopreserved with 10% DMSO, however the highest percentage of fertilized eggs for this treatment was 2%.

When mTsvetkova solution was used as an extender, sperm cryopreserved with 10% DMSO had significantly ($P = 0.0016$) more viable cells ($75 \pm 5\%$) than did sperm cryopreserved with 5% DMSO ($56 \pm 3\%$) or 5% MeOH ($41 \pm 15\%$) (Table 7-2). There was a strong correlation ($r^2 \geq 0.9165$) between percent motility and percent viable cells for sperm cryopreserved with MeOH at 5% or 10% (Figure 7-4). Sperm cryopreserved with DMSO had poor correlations ($r^2 \leq 0.4747$) between percent motility and percent viable cells. The percentage of viable cells was generally high ($>52\%$) for sperm cryopreserved with DMSO, but the post-thaw motility was lower ($<25\%$).

Shovelnose Sturgeon

When mHBSS was used as the extender for shovelnose sturgeon, sperm cryopreserved with 10% DMSO retained a significantly higher ($P < 0.0001$) percentage of viable cells ($74 \pm 7\%$) than did sperm cryopreserved with 5% MeOH ($45 \pm 15\%$) or 5% DMSO ($58 \pm 5\%$) (Table 7-1). Sperm cryopreserved with MeOH had strong correlations between the percentage of viable cells and motility ($r^2 \geq 0.8853$), fertilized eggs ($r^2 \geq 0.8068$), and hatched larvae ($r^2 \geq 0.8189$) (Figures 7-5, 7-6, 7-7). However, sperm cryopreserved with DMSO had weaker correlations ($r^2 \leq 0.4940$) between the percentage of viable cells and motility, fertilized eggs or hatched larvae.

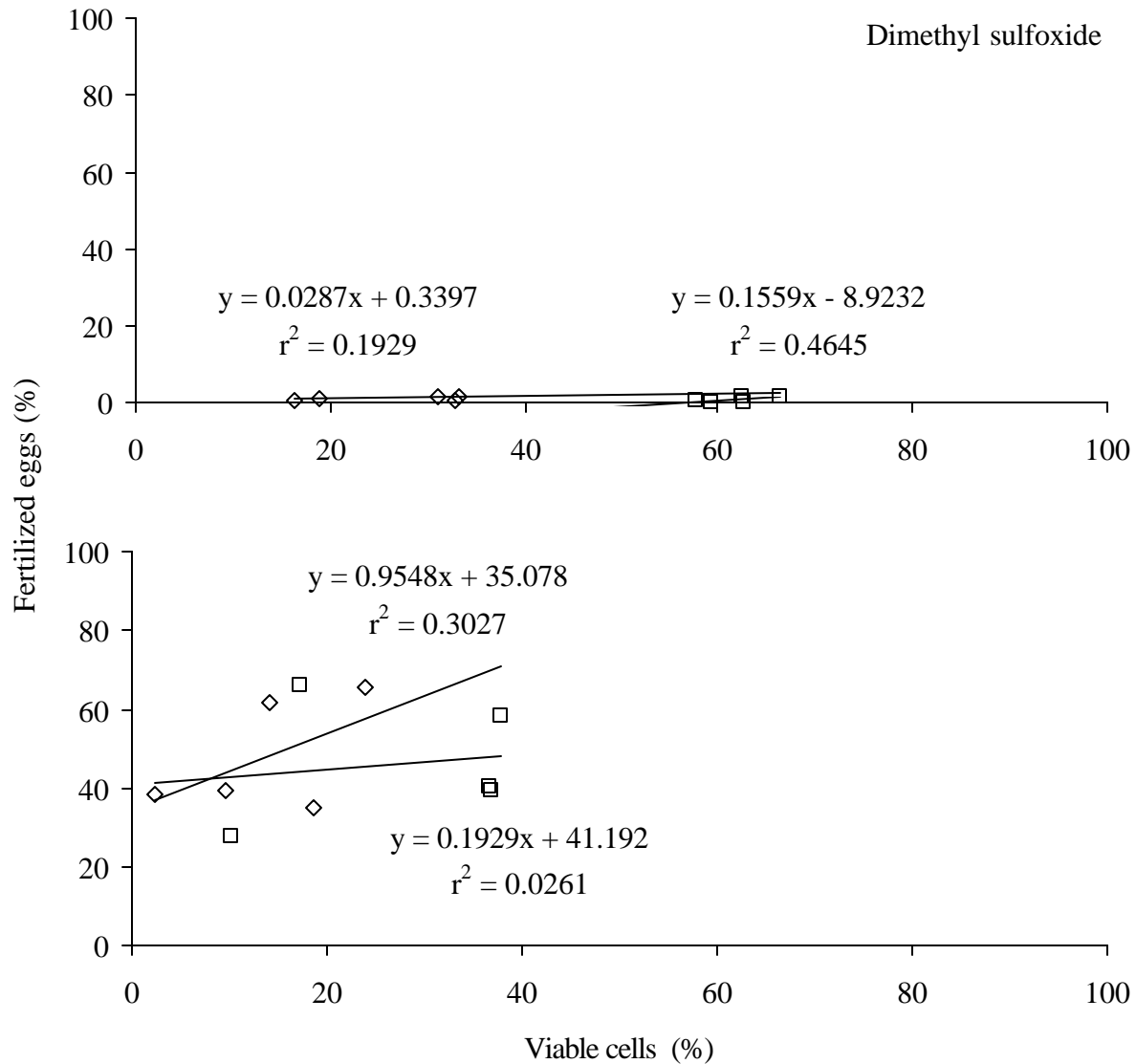


Figure 7-3. Correlations between the percentage of viable cells and the percentage of fertilized eggs as determined by dual-staining and flow cytometry for sperm of lake sturgeon cryopreserved using modified Hanks' balanced salt solution (mHBSS) as an extender. Sperm were diluted to a final dilution of 1:4 in extender with cryoprotectant prior to freezing. Frozen sperm were thawed in a 40 °C water bath for 9 sec, suspended in 500 µl of mHBSS, and stained with 5 µl of 20 µM SYBR-14 and 2.5 µl of 1 mM propidium iodide. Flow cytometry was used to determine the percentage of viable cells. (5%, diamonds; 10%, squares)

Table 7-2. Mean (\pm SD) percentage of viable cells as determined by dual-staining and flow cytometry for thawed sturgeon sperm cryopreserved using modified Tsvetkova solution as an extender. Sperm were thawed for 9 sec in a 40 °C water bath, suspended in 500 μ l of modified Hanks' balanced salt solution, and stained with 5 μ l of 20 μ M SYBR-14 and 2.5 μ l of 1 mM propidium iodide. The sperm cells were analyzed by flow cytometry to determine the percentage of viable cells per sample. Values in columns sharing superscript letters were not significantly different ($P < 0.05$). DMSO, dimethyl sulfoxide; MeOH, methanol.

Cryoprotectant	Viable Cells (%)	
	Lake sturgeon	Shovelnose sturgeon
DMSO 5%	56 \pm 3 ^b	47 \pm 7 ^a
DMSO 10%	75 \pm 5 ^a	57 \pm 7 ^a
MeOH 5%	41 \pm 15 ^b	47 \pm 11 ^a
MeOH 10%	57 \pm 16 ^{ab}	47 \pm 13 ^a

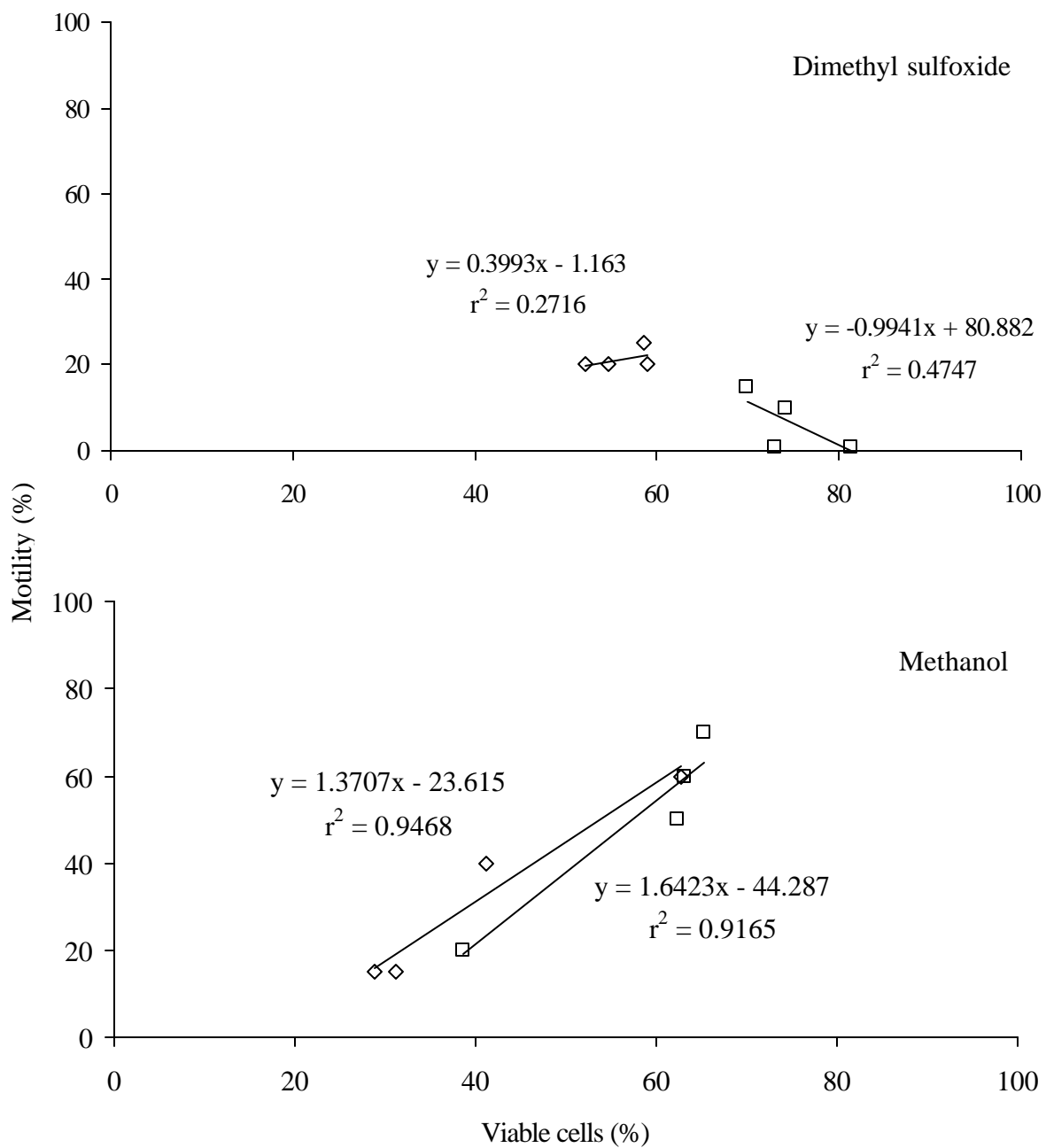


Figure 7-4. Correlations between percent motility and the percentage of viable cells for sperm of lake sturgeon cryopreserved using a modified Tsvetkova extender. Sperm were diluted to a final dilution of 1:4 in extender with cryoprotectant prior to freezing. Frozen sperm were thawed in a 40 °C water bath for 9 sec, suspended in 500 µl of modified Hanks' balanced salt solution, and stained with 5 µl of 20 µM SYBR-14 and 2.5 µl of 1 mM propidium iodide. Flow cytometry was used to determine the percentage of viable cells. (5%, diamonds; 10%, squares)

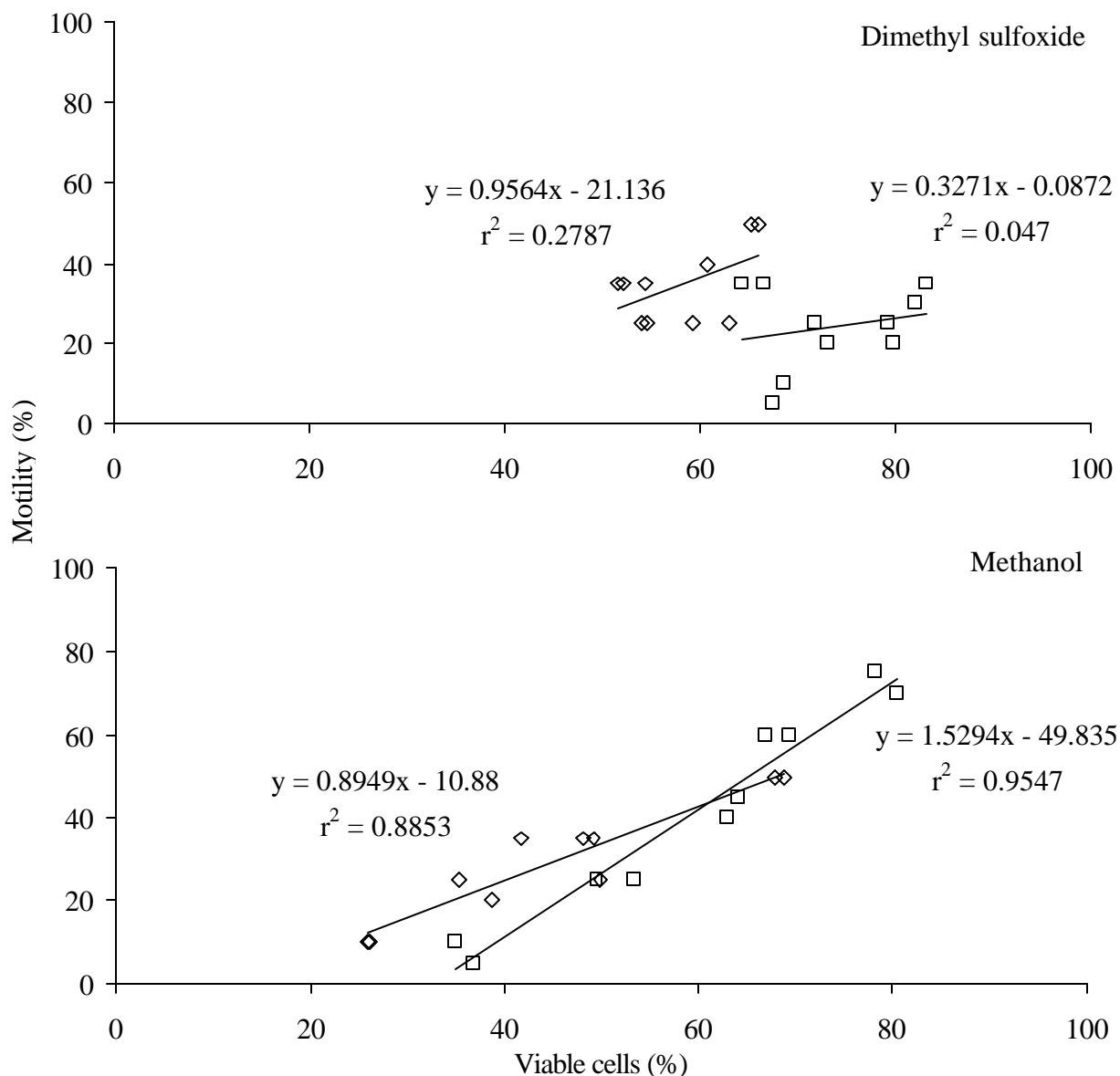


Figure 7-5. Correlations between post-thaw sperm motility and the percentage of viable cells for sperm of shovelnose sturgeon cryopreserved using modified Hanks' balanced salt solution (mHBSS) as an extender. Sperm were diluted to a final dilution of 1:4 in extender with cryoprotectant prior to freezing. Frozen sperm were thawed in a 40 °C water bath for 9 sec, suspended in 500 µl of mHBSS, and stained with 5 µl of 20 µM SYBR-14 and 2.5 µl of 1 mM propidium iodide. Flow cytometry was used to determine the percentage of viable cells. (5%, diamonds; 10%, squares).

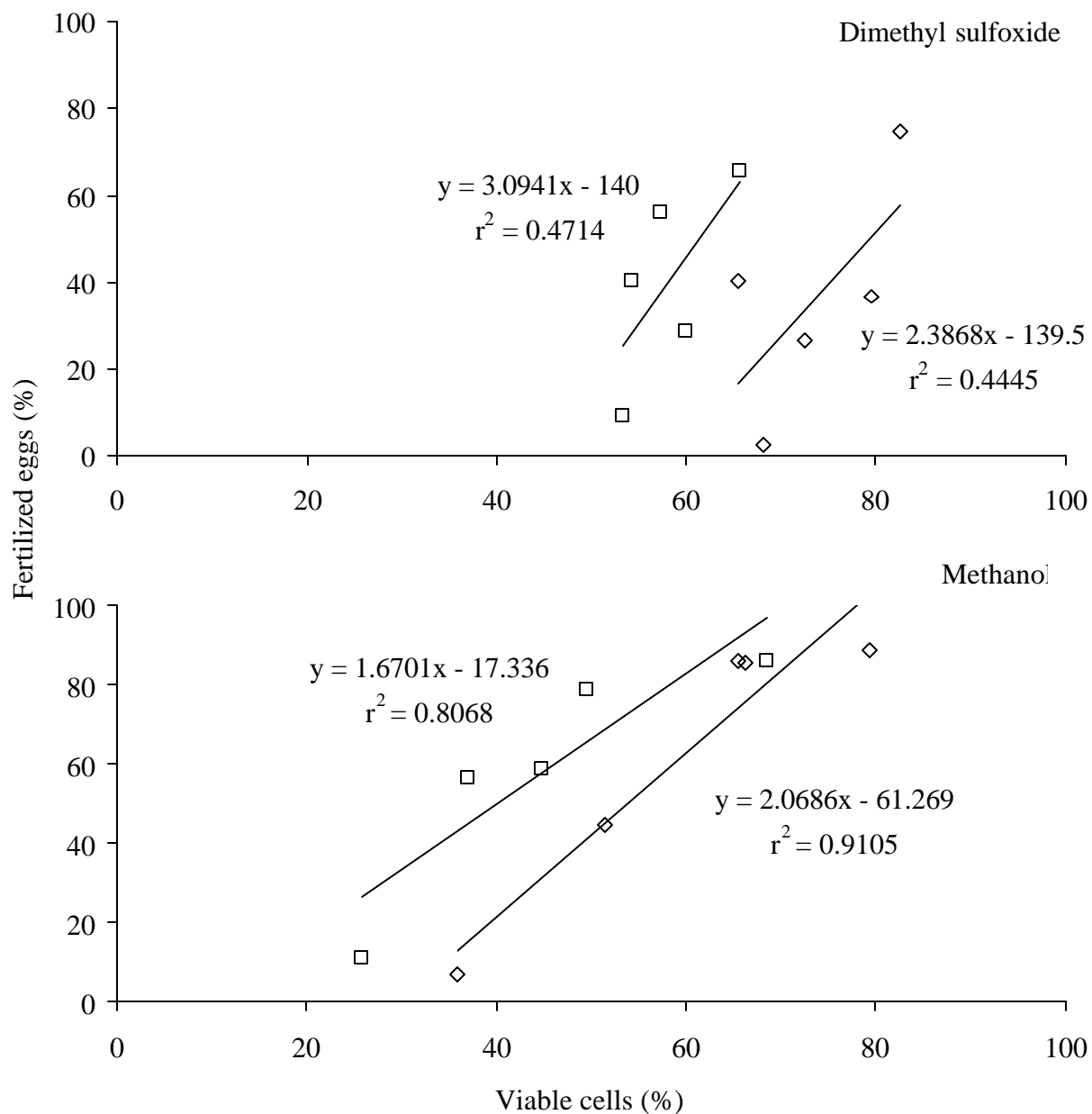


Figure 7-6. Correlations between the percentage of fertilized eggs and the percentage of viable cells for sperm of shovelnose sturgeon cryopreserved using modified Hanks' balanced salt solution (mHBSS) as an extender. Sperm were diluted to a final dilution of 1:4 in extender with cryoprotectant prior to freezing. Frozen sperm were thawed in a 40 °C water bath for 9 sec, suspended in 500 µl of mHBSS, and stained with 5 µl of 20 µM SYBR-14 and 2.5 µl of 1 mM propidium iodide. Flow cytometry was used to determine the percentage of viable cells. (5%, diamonds; 10%, squares)

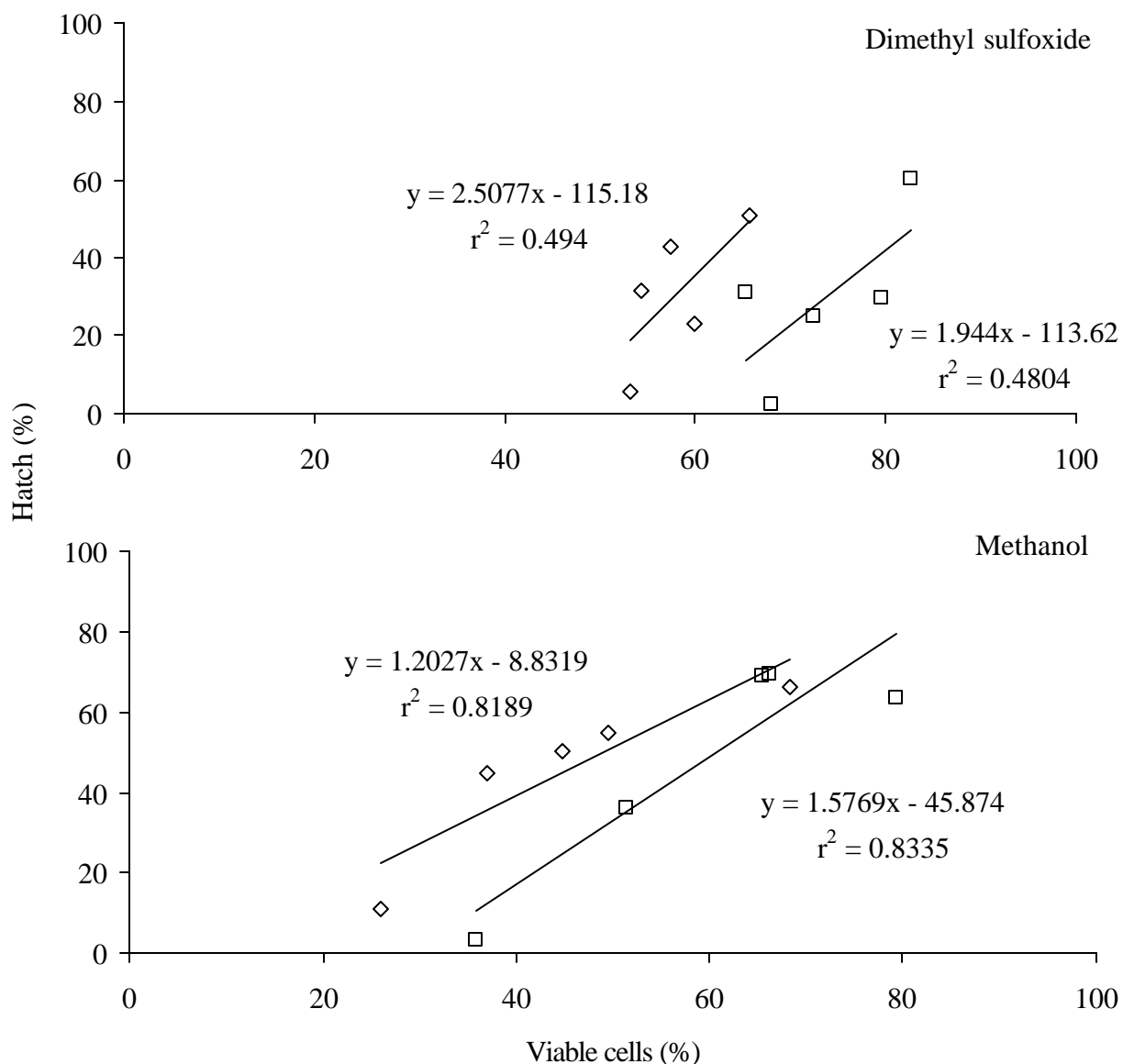


Figure 7-7. Correlations between the percentage of live cells and the percentage of hatched larvae for sperm of shovelnose sturgeon cryopreserved using modified Hanks' balanced salt solution (mHBSS) as an extender. Sperm were diluted to a final dilution of 1:4 in extender with cryoprotectant prior to freezing. Frozen sperm were thawed in a 40 °C water bath for 9 sec, suspended in 500 µl of mHBSS, and stained with 5 µl of 20 µM SYBR-14 and 2.5 µl of 1 mM propidium iodide. Flow cytometry was used to determine the percentage of viable cells. (5%, diamonds; 10%, squares)

When mTsvetkova solution was used as an extender, sperm cryopreserved with MeOH or DMSO had similar ($P = 0.4812$) percentages of viable cells (Table 7-2). There was a strong correlation ($r^2 \geq 0.7950$) between percent motility and the percentage of viable cells for sperm cryopreserved with MeOH at either concentration (Figure 7-8). However this strong correlation did not exist for fertilized eggs ($r^2 \leq 0.5985$) (Figure 7-9) or hatched larvae ($r^2 \leq 0.3417$) (Figure 7-10). There was a weak correlation ($r^2 \leq 0.0680$) between percent motility and the percentage of viable cells for sperm cryopreserved with DMSO (Figure 7-8). However, there was a strong correlation between the percentage of viable cells and fertilized eggs ($r^2 = 0.7418$) and hatched larvae ($r^2 = 0.7150$) for sperm cryopreserved with 10% DMSO (Figures 7-9, 7-10). It should be noted that sperm cryopreserved with DMSO had a mean fertilization percentage of $8 \pm 9\%$ and a hatch percentage of $4 \pm 7\%$.

Pallid Sturgeon

When mHBSS was used as the extender, sperm cryopreserved with 10% DMSO had a significantly higher ($P = 0.0219$) percent viable cells ($70 \pm 10\%$) than did sperm cryopreserved with 15% MeOH ($45 \pm 10\%$) (Table 7-1). Sperm cryopreserved with 5% MeOH or 5% DMSO had strong correlations between the percentage of viable cells and percent motility ($r^2 \geq 0.765$) and between the percentage of viable cells and fertilized eggs ($r^2 \geq 0.8624$) (Figures 7-11, 7-12), however there was a weaker correlation between the percentage of viable cells and the percentage of hatched larvae ($r^2 \leq 0.4262$) (Figure 7-13). The percentage of viable cells for sperm cryopreserved with 10% MeOH was strongly correlated with percent motility ($r^2 = 0.7362$) and percent hatched larvae ($r^2 = 0.9909$), but not with percent fertilized eggs ($r^2 = 0.1027$). The percentage of viable cells for sperm cryopreserved with 10% DMSO showed

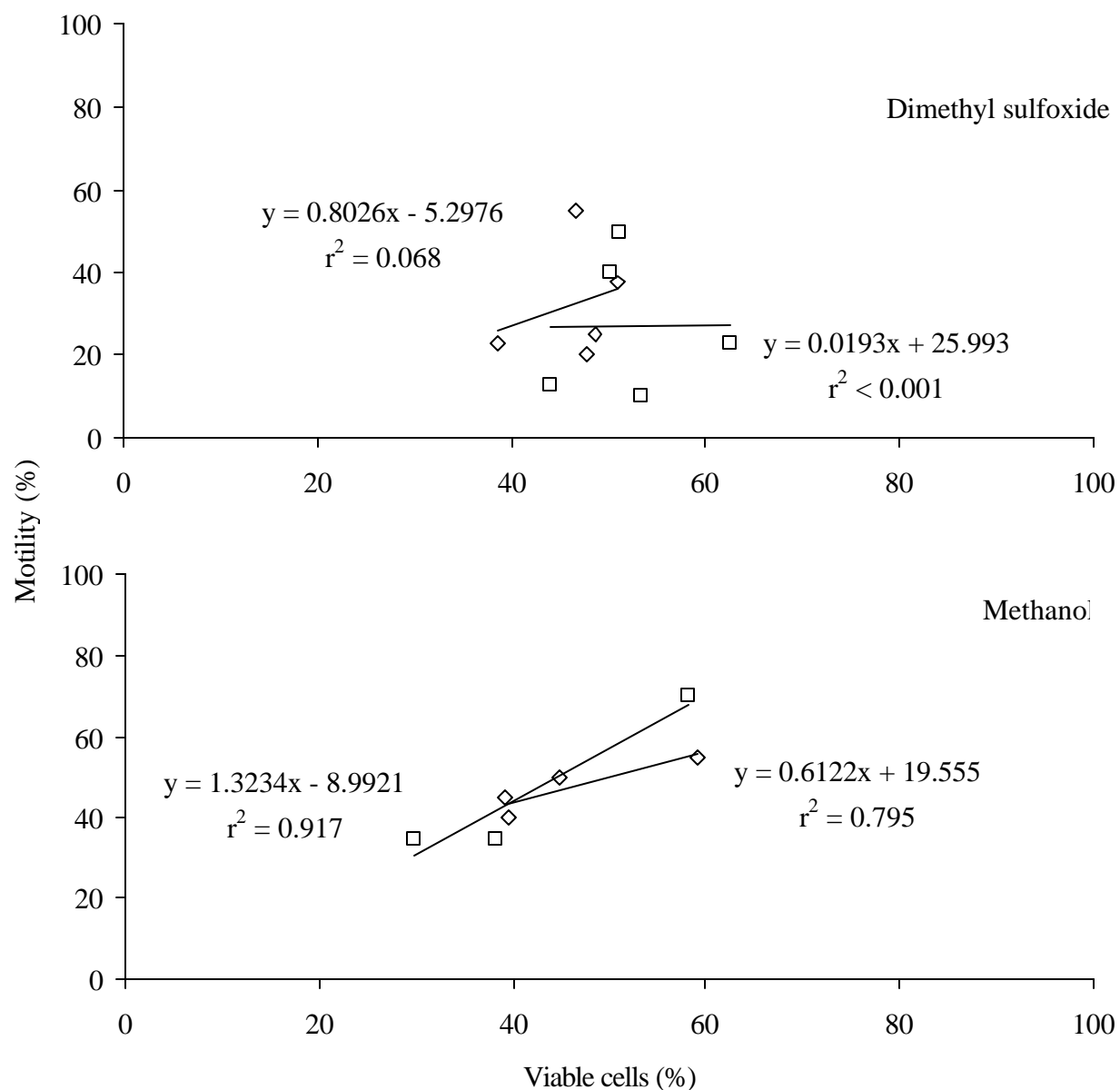


Figure 7-8. Correlations of post-thaw percent motility and the percentage of viable cells for sperm of shovelnose sturgeon cryopreserved using a modified Tsvetkova solution as an extender. Sperm were diluted to a final dilution of 1:4 in extender with cryoprotectant prior to freezing. Frozen sperm were thawed in a 40 °C water bath for 9 sec, suspended in 500 μ l of modified Hanks' balanced salt solution, and stained with 5 μ l of 20 μ M SYBR-14 and 2.5 μ l of 1 mM propidium iodide. Flow cytometry was used to determine the percentage of viable cells. (5%, diamonds; 10%, squares)

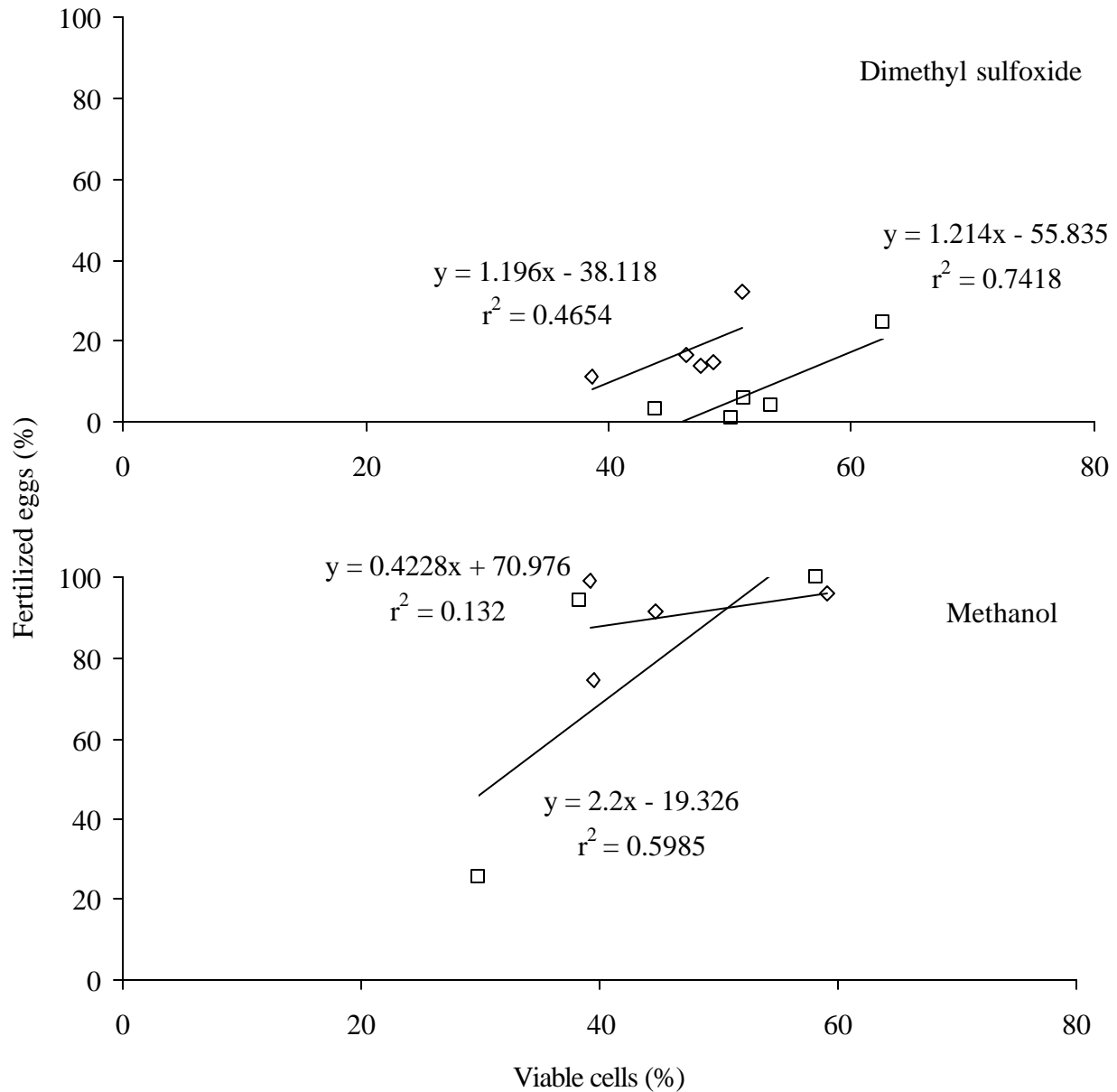


Figure 7-9. Correlations of the percentage of viable cells and the percentage of fertilized eggs for sperm of shovelnose sturgeon cryopreserved using a modified Tsvetkova solution as an extender. Sperm were diluted to a final dilution of 1:4 in extender with cryoprotectant prior to freezing. Frozen sperm were thawed in a 40 °C water bath for 9 sec, suspended in 500 µl of modified Hanks' balanced salt solution, and stained with 5 µl of 20 µM SYBR-14 and 2.5 µl of 1 mM propidium iodide. Flow cytometry was used to determine the percentage of viable cells. (5%, diamonds; 10%, squares)

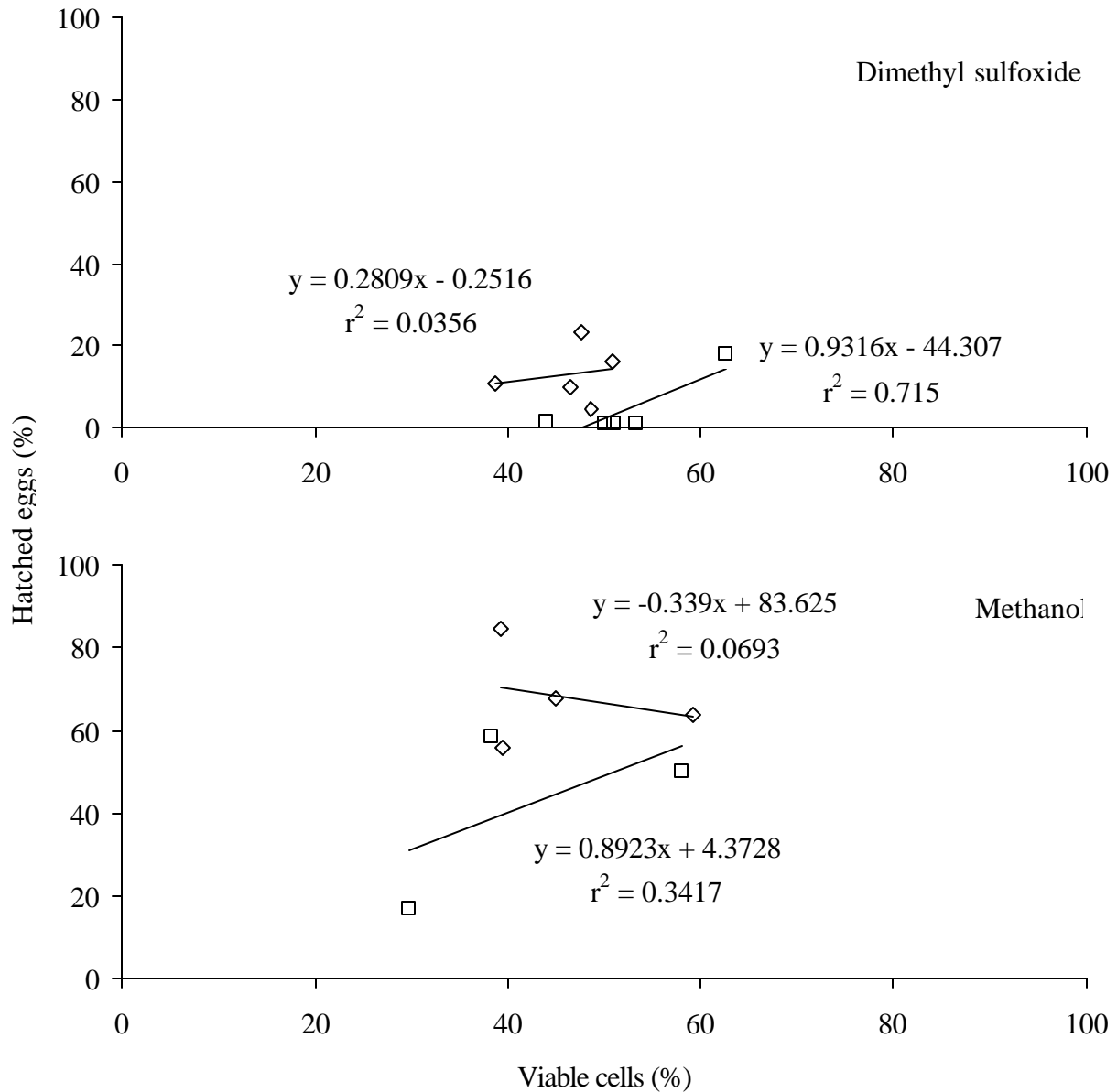


Figure 7-10. Correlations of the percentage of viable cells and the percentage of hatched eggs for sperm of shovelnose sturgeon cryopreserved using a modified Tsvetkova solution as an extender. Sperm were diluted to a final dilution of 1:4 in extender with cryoprotectant prior to freezing. Frozen sperm were thawed in a 40 °C water bath for 9 sec, suspended in 500 µl of modified Hanks' balanced salt solution, and stained with 5 µl of 20 µM SYBR-14 and 2.5 µl of 1 mM propidium iodide. Flow cytometry was used to determine the percentage of viable cells. (5%, diamonds; 10%, squares)

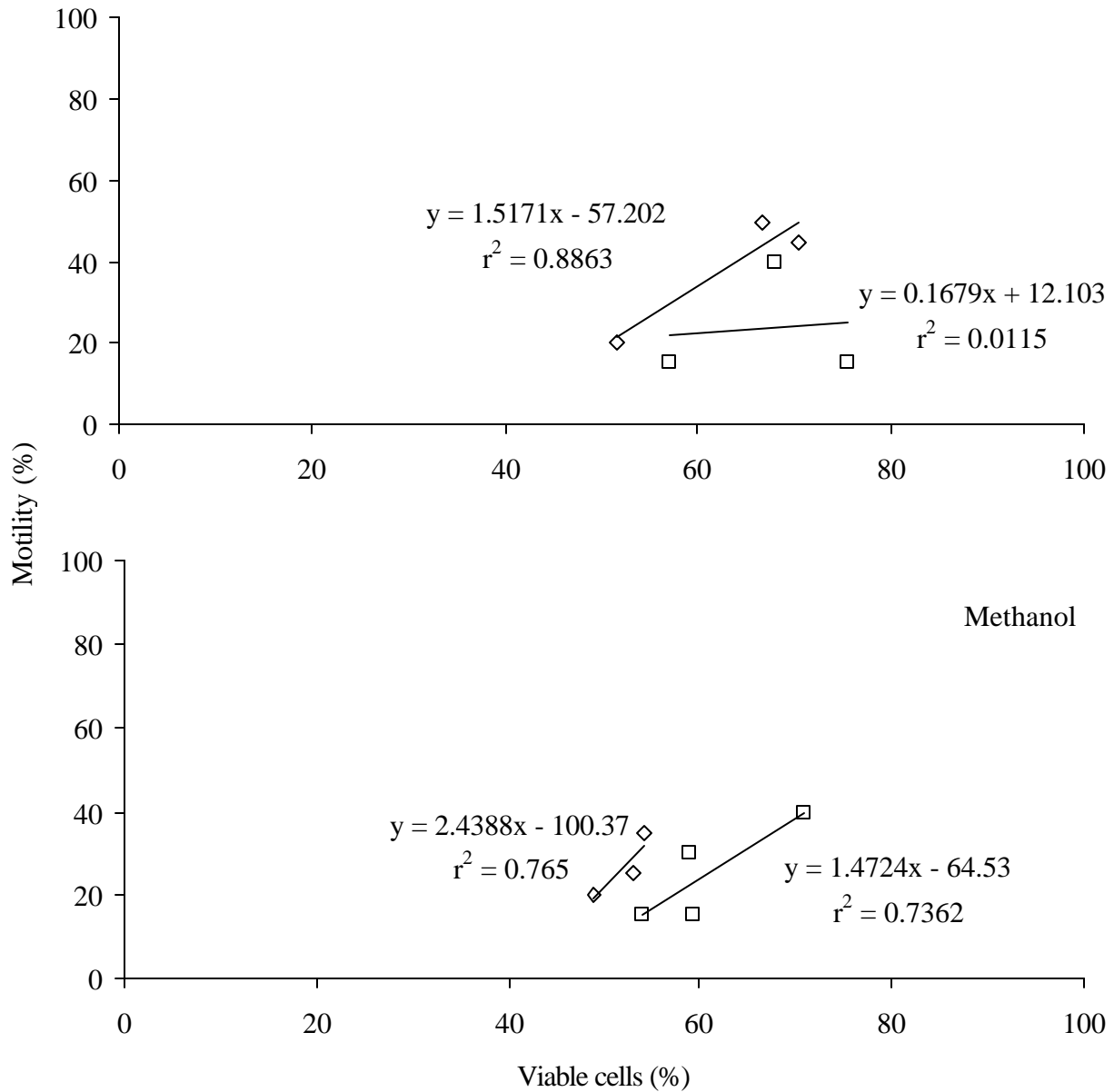


Figure 7-11. Correlations between post-thaw percent motility and the percentage of viable cells for sperm of pallid sturgeon cryopreserved using modified Hanks' balanced salt solution (mHBSS) as an extender. Sperm were diluted to a final dilution of 1:4 in extender with cryoprotectant prior to freezing. Frozen sperm were thawed in a 40 °C water bath for 9 sec, suspended in 500 µl of mHBSS, and stained with 5 µl of 20 µM SYBR-14 and 2.5 µl of 1 mM propidium iodide. Flow cytometry was used to determine the percentage of viable cells. (5%, diamonds; 10%, squares)

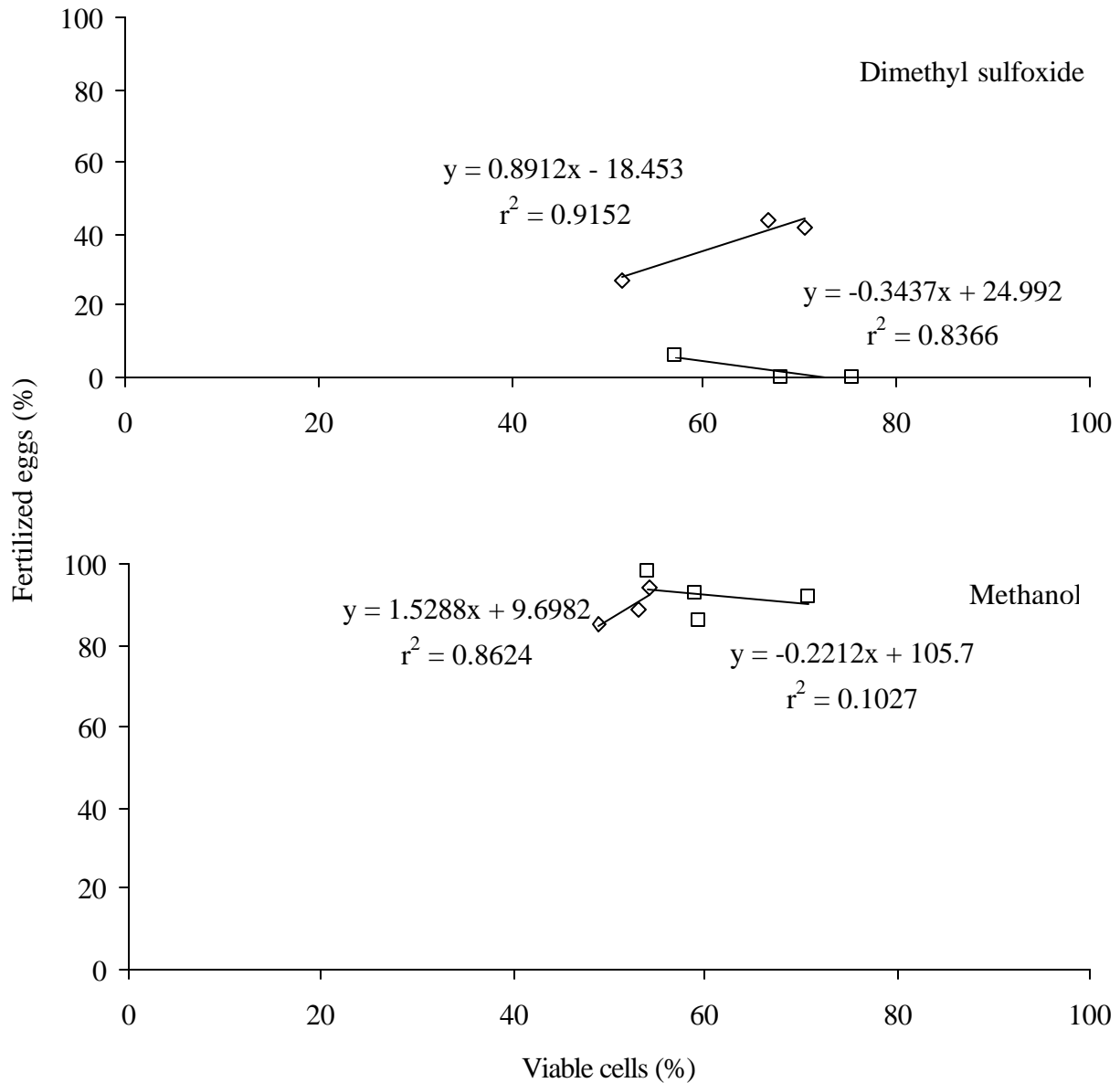


Figure 7-12. Correlations between the percentage of viable cells and the percentage of fertilized eggs for sperm of pallid sturgeon cryopreserved using modified Hanks' balanced salt solution (mHBSS) as an extender. Sperm were diluted to a final dilution of 1:4 in extender with cryoprotectant prior to freezing. Frozen sperm were thawed in a 40 °C water bath for 9 sec, suspended in 500 μ l of mHBSS, and stained with 5 μ l of 20 μ M SYBR-14 and 2.5 μ l of 1 mM propidium iodide. Flow cytometry to determine the percentage of viable cells. (5%, diamonds; 10%, squares)

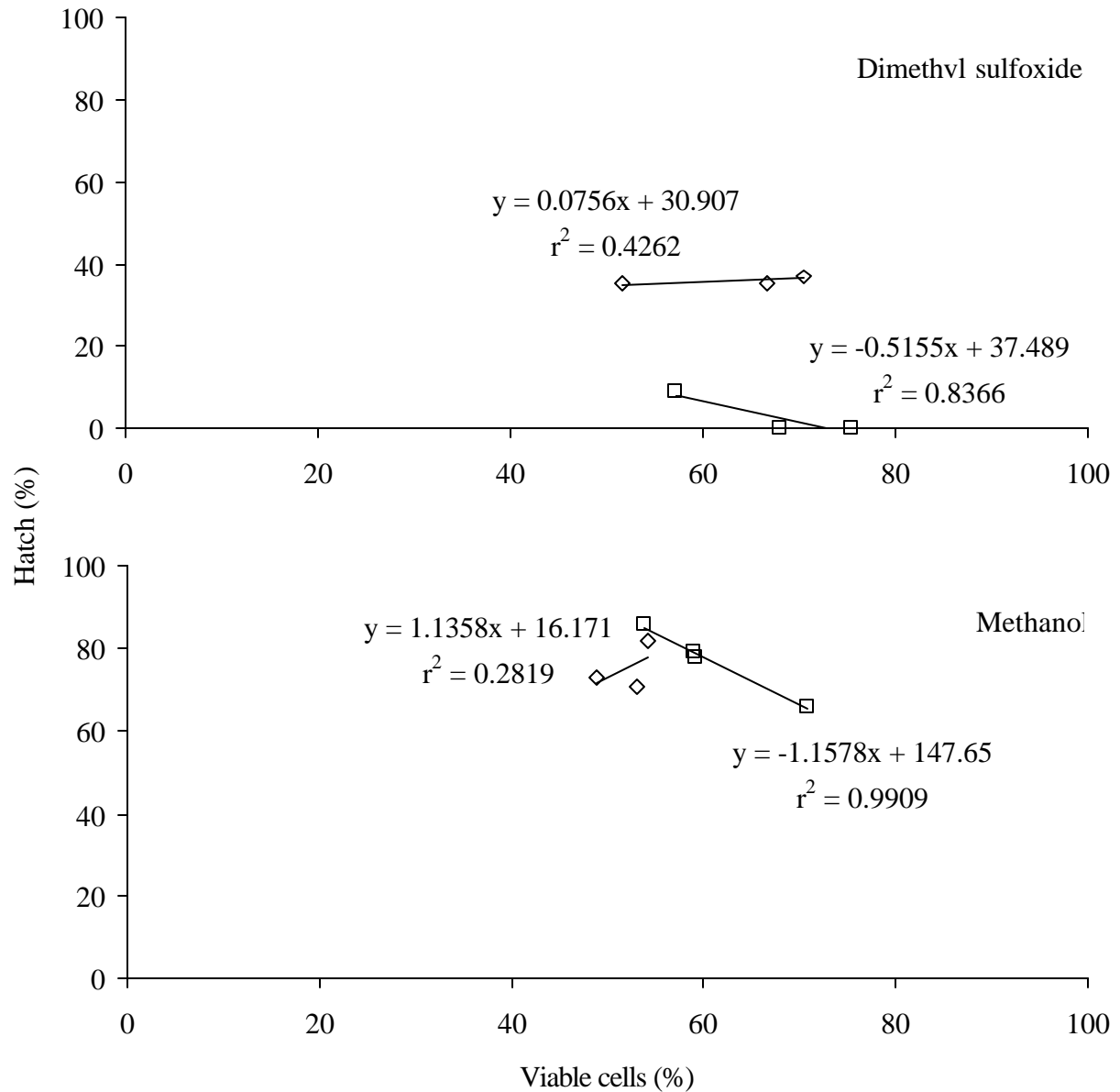


Figure 7-13. Correlations between the percentage of viable cells and the percentage of hatched larvae for sperm of pallid sturgeon cryopreserved using modified Hanks' balanced salt solution (mHBSS) as an extender. Sperm were diluted to a final dilution of 1:4 in extender with cryoprotectant prior to freezing. Frozen sperm were thawed in a 40 °C water bath for 9 sec, suspended in 500 µl of mHBSS, and stained with 5 µl of 20 µM SYBR-14 and 2.5 µl of 1 mM propidium iodide. Flow cytometry was used to determine the percentage of viable cells. (5%, diamonds; 10%, squares)

a weak correlation with motility ($r^2 = 0.0115$), and a strong negative correlation with the percent fertilized eggs ($r^2 = 0.8366$) and percent hatched larvae ($r^2 = 0.8366$).

Discussion

Due to a lack of correlations between post-thaw motility and fertilization or hatch rates (Chapter 5), the present study was designed to investigate the ability of a dual-staining procedure to predict fertilization and hatch rates of eggs fertilized with cryopreserved sperm based on the percentage of viable sperm. As such, cryopreserved sperm were used throughout the study, and therefore in the validation of the assay, the percentage of viable cells in the nominal 100% viable treatment was 26-68% viable. The strong correlation between the treatment proportion of membrane intact cells and the percentage of viable cells as determined by flow cytometry verified that the staining technique was accurate with sperm of 3 species of sturgeon, and demonstrated the effectiveness of this procedure for discriminating viable from non-viable sperm cells. This procedure has also been reported for sperm of Nile tilapia *Oreochromis niloticus* (Segovia et al., 2000), red snapper *Lutjanus campechanus* and grey snapper *Lutjanus griseus* (Riley, 2002), and rainbow trout *Oncorhynchus mykiss* (Cloud and Kersten, 1996).

In this study, sperm cryopreserved with 10% DMSO produced the highest percentages of viable cells after thawing, regardless of species or extender used. Dimethyl sulfoxide has also been shown to be a beneficial additive for the preservation of membrane integrity during cryopreservation of sperm of rainbow trout (Ogier de Baulny, 1997) and African catfish *Clarias gariepinus* (Rurangwa et al., 2001). Dimethyl sulfoxide has been commonly used as a cryoprotectant for fish sperm (see reviews by Scott and Baynes, 1980; Leung and Jamieson, 1991; and Suquet et al., 2000), and also for the cryopreservation of sturgeon sperm (Ciereszko et

al., 1996a, b; Drokin and Kopeika, 1996; Kopeika et al., 2000; Tsvetkova et al., 1996).

However, the values for viable cells from sperm cryopreserved with 10% DMSO in our study did not correlate strongly with motility, fertility, or hatch, except for sperm of pallid sturgeon cryopreserved using mHBSS or sperm of shovelnose sturgeon cryopreserved using modified Tsvetkova solution. However, it is important to note that the correlations for pallid sturgeon sperm were negatively sloped (increasing viability resulted in decreased fertilization and hatch rates), and the fertilization and hatch rates for shovelnose sturgeon sperm were less than 10%. In fact, 10% DMSO produced the lowest fertilization rates of any of the cryoprotectant combinations, regardless of extender or cryoprotectant. This suggests that DMSO was effective at preserving the cellular membrane, but was ineffective at preserving other attributes of sturgeon sperm required for fertilization.

Rainbow trout sperm cryopreserved with DMSO did not allow PI to enter sperm cells when incubated in extender that was isosmotic to the seminal plasma, but the damage caused the membrane to rupture when exposed to hyposmotic conditions, which would be encountered during activation and fertilization (Cabrita et al., 2001). Osmotic stress has been suggested as a factor in damage caused by DMSO (Stoss and Holtz, 1983), which at a concentration of 10% can raise the osmotic pressure of the freezing medium by ~1500 mOsmol/kg (Ogier De Baulny et al., 1997). Sturgeon sperm, which has a low plasma osmolality (~100 mOsmol/kg) and is damaged by storage at high osmotic pressures (<200 mOsmol/kg) (Chapter 4), may be especially sensitive to osmotic stress. In the present study, sperm cryopreserved using 5% DMSO had higher post-thaw motility than did sperm cryopreserved with 10% DMSO, possibly due to the lower osmotic stress. Similarly, post-thaw motility of Atlantic sturgeon *Acipenser sturio* sperm was increased by decreasing the percentage of DMSO used as a cryoprotectant (Kopeika et al., 2000).

However, in the present study, sperm cryopreserved with 5% or 10% DMSO had similar fertilization and hatch rates, except for sperm of pallid sturgeon sperm. Further research investigating other aspects of post-thaw sperm quality, such as mitochondrial activity, acrosome integrity, and DNA stability, would help to clarify the cryoprotective effects of DMSO on sturgeon sperm during cryopreservation.

In the present study, sperm cryopreserved with 5% methanol had the lowest percentage of viable cells after thawing regardless of species or extender. Methanol (5%, 10% or 15%) reduced membrane integrity of cryopreserved rainbow trout sperm as compared to sperm frozen without cryoprotectant (Ogier de Baulny et al., 1997). Methanol has been commonly used as a cryoprotectant for fish sperm (Tiersch et al., 1994; 1998; Lahnsteiner et al., 1997; Lahnstein et al., 1998; Viveiros et al., 2001), but has rarely been used with sturgeon sperm (Horvath et al., 2000; Glogowski et al., 2002). Unlike DMSO, methanol has no impact on the osmotic pressure (Ogier De Baulny et al., 1997), and typically was less toxic to sperm cells than DMSO (Lahnsteiner, 2000). In fact, exposure to methanol does not appear to have any deleterious effects on motility of spotted seatrout *Cynoscion nebulosus* (Wayman et al., 1996) or razorback sucker *Xyrauchen texanus* sperm (Tiersch et al., 1998) prior to freezing and has been shown to increase refrigerated storage time of sperm of channel catfish *Ictalurus punctatus* (Christensen and Tiersch, 1996). Although methanol may generally be less toxic than DMSO, when used as a cryoprotectant methanol did not maintain membrane integrity or retain higher post-thaw motility than did DMSO.

In the present study, sperm cryopreserved with methanol yielded viability percentages after thawing that were highly correlated with motility, irrespective of species or extender used. However, viability was only correlated to fertilization rates and hatch rates for shovelnose

sturgeon sperm cryopreserved using mHBSS and MeOH. The viability of sperm of pallid sturgeon cryopreserved with mHBSS and 5% MeOH was also strongly correlated to fertility rates, however it was not correlated to hatch rates. Post-thaw motility of Siberian sturgeon *Acipenser baeri* sperm cryopreserved with 10% methanol was not correlated with fertilizing ability (Glogowski et al., 2002). In the instances in the present study where post-thaw viability was correlated with percent fertilization and percent hatch, viability was also highly correlated to motility, indicating that motility estimation would work equally well at predicting sperm quality as the more complicated viability assay.

Although the procedure for dual-staining outlined here was accurate at determining the percentage of viable cells in post-thaw sturgeon sperm samples, the procedure was not able to forecast the fertilizing ability of cryopreserved sperm. The staining procedure used in this study identified viable cells by membrane integrity, but did not produce information regarding other intracellular components that may be necessary for fertilization to occur. It is likely that mitochondrial function, acrosome integrity, and nuclear stability are necessary for sturgeon sperm to successfully fertilize eggs. In this regard, development of assays that measure multiple sperm attributes simultaneously may be necessary to allow accurate correlations with fertility (Graham, 2001), such as the simultaneous use of rhodamine and PI, which has been used to evaluate mitochondrial function and membrane integrity of Nile tilapia *Oreochromis niloticus* sperm (Segovia et al., 2000).

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Chapter 8

Development of a Model Cryopreserved Sperm Repository Database for Fishes

Introduction

The development of cryopreservation techniques allows the conservation of genetic material from declining populations of endangered species by creating a repository of cryopreserved samples. The necessary information regarding these cryopreserved samples is extensive. To make this vast information available to various entities and to maintain control of the inventory, a well-designed computerized database is necessary.

Various commercial database systems exist for the inventory control of cryopreserved samples, but these systems are typically not user modifiable, and so they cannot be adjusted to fit particular situations. Typically, these commercial databases are developed for use in the fields of human reproduction and medical research, and as such, do not allow entry of information that is critical for a fish sperm repository. Commercially available inventory control systems that are fully user modifiable are often expensive (for example, \$11,000 for Freezerworks Unlimited[®]), making the use of these systems for small-scale repositories problematic.

Various categories of information have been identified as necessary for the operation of a cryopreserved sperm repository database (Kincaid, 2000). These categories include information on baseline characterization of the fish, sperm collection, sperm processing, sperm usage, and quality control.

The main objective of this work was to develop a low-cost relational database system (a database system based on inter-related tables of information) that would be able to maintain inventory control over the cryopreserved samples at the Warm Springs Fish Technology Center, and retain the information necessary for the use of those samples by the Center's partners. In

particular the database was designed to be able to track a single straw from when it is frozen to when it is thawed and used for the production of fish. This database system is flexible and should be able to serve as a model for other sperm storage applications. Information on development and functioning of the database are presented below.

Database Development

The database was developed in Microsoft Access 2000 (Version 9.0.3821 SR-1, Microsoft Corporation, Redmond, Washington). Design criteria included the ability to enter data from a wide range of categories, maintain inventory control, track individual straws, and allow the user to easily search the database contents. Throughout the development of the database, methods were employed to help make the database easy to use. These methods included warning screens when a user is about to change existing data, message boxes that warn the user of missing information, and error messages if the user enters data (such as straw identification numbers) that are already in the system. As a way of illustrating the functioning of the database, a hypothetical 2-ml sample diluted 1:4 with extender and frozen in 0.5-ml straws will be shown as it progresses through the different features of the database.

This hypothetical sperm sample was collected from a red drum *Sciaenops ocellatus* caught along the coast of Louisiana. The sample was sent to the Warm Springs Fish Technology Center, where 11 straws were frozen, and stored in the repository. After 3 months, 1 straw (# 152684) was shipped as part of a larger shipment to the Louisiana State Agricultural Center Aquaculture Research Station. The straw was thawed and motility was 80%. The thawed sperm were used to fertilize eggs from a single female red drum, yielding a 75% fertilization rate and

50% hatch rate. The offspring were used in a recovery effort in Lake Ponchartrain. The remaining straws were stored in the repository awaiting future needs.

Database Function

Main Server Screen

The initial server screen allows selection from several options (Figure 8-1). These options include: viewing the current inventory of a particular dewar or species, entering new samples into the repository, viewing or editing sample data already in the repository, tracking the shipping of samples, and submitting field reports. Clicking on a certain action button leads through a series of screens that allow accomplishment of that action.



Figure 8-1. Main server screen.

Entering New Samples

To enter a new sample into the cryopreserved sperm repository, click the “Enter New Sample” button. This button opens a screen that contains drop-down menus to allow the selection of the dewar and canister within the dewar in which the samples will be stored (Figure 8-2). Once a dewar and canister have been selected, clicking the “Enter Data” button allows addition of information regarding the sample.

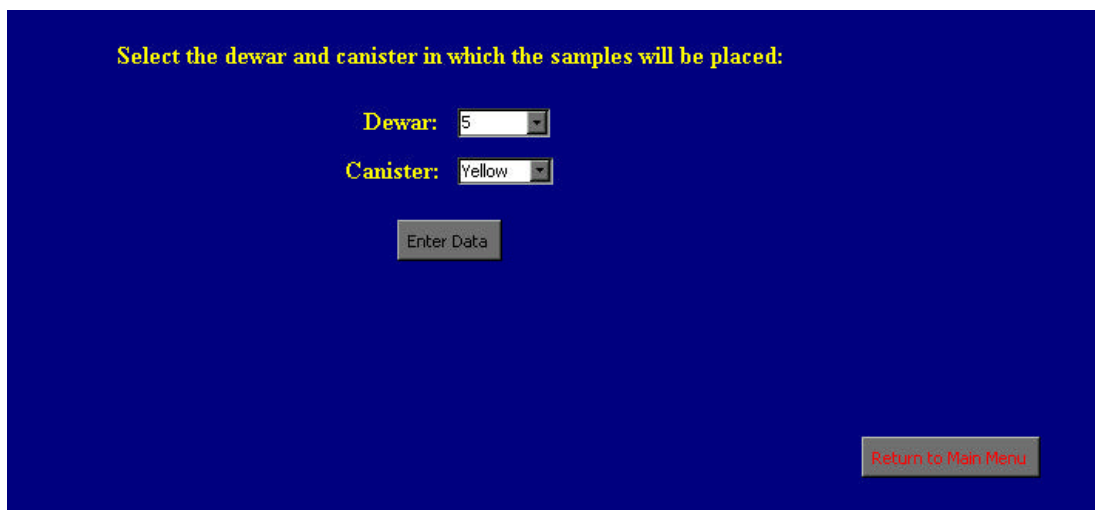


Figure 8-2. Dewar and canister selection screen.

Once the “Enter Data” button has been clicked, a new window opens allowing the entry of information regarding cane identification, straw type, and number of straws (Figure 8-3). A sample identification number is automatically generated by the computer, and is used to track the sample throughout the database. The number of straws entered into the database is determined automatically by entering the starting number and ending number of the sequentially numbered straws. At the bottom of the window are two buttons that allow the entry of information regarding the laboratory processing of samples (“Enter Lab Notes”) or collection information regarding the sperm sample and the fish that it came from (“Enter Collection Data”).

frmCane2

Cane and Straw Information

SampleID: 192

Cane Label: Red Drum

Straw Size (ml): 0.5

Straw Color: Clear

Straw Numbers

Start Number: 152684

End Number: 152694

Enter Lab Notes Enter Collection Data

Save and Return to Main Menu

Figure 8-3. Cane and straw information window

The “Enter Lab Notes” button opens a new window (Figure 8-4). The technician who is processing the sample is selected from a drop-down list of names that are currently part of the database. New technician information can be added by selecting the “Add New Tech” button, which will open a window (Figure 8-5) so that a new technician’s name can be added. The remainder of the window is organized into three sections: arrival information, cryopreservation information, and thawing information. The arrival information includes the person who shipped the sample, the method of shipping (such as frozen or on ice), the date the sample arrived, and the motility of the sample upon arrival. The cryopreservation information includes the initial motility at the time of cryopreservation, the extender used (name, osmolality, and pH), the sperm to extender ratio, the freezing protocol, the cryoprotectant and concentration, the equilibration time and the equilibration motility. The thawing information includes the protocol used for thawing the sample and the post-thaw motility. After the data has been entered a button “Submit Data” can be clicked to return to the “Cane and Straw Information Window” so that other information can be added.

The screenshot shows a software window titled "frmLabNotes". At the top, there is a "Technician" dropdown menu with "Greg Looney" selected and an "Add New Tech." button. Below this, the "Arrival Information:" section includes fields for "Shipped by:" (William Wayman), "Shipping Method:" (Ice), "Date Sample Arrived:" (2/12/2002), and "Motility at Arrival (%):" (95). The "SampleID:" field is set to 192. The "Cryopreservation Information:" section contains fields for "Initial Motility (%):" (90), "Extender:" (HBSS), "Sperm to Extender Ratio (v:v):" (1:4), "Extender Osmolality (mOsm/kg):" (300), "Extender pH:" (empty), "Freezing Rate Protocol:" (Hold at 5 C for 5 min; -40 C/min to -80C; Hold -80), "Cryoprotectant:" (DMSO), "Concentration (%):" (10), "Equilibration Time (min):" (15), and "Equilibration Motility (%):" (90). The "Thawing Information:" section has "Thawing Protocol:" (40 C for 7 sec) and "Thawed Motility (%):" (80). A "Submit Data" button is located at the bottom right.

Figure 8-4. Laboratory data screen.

The screenshot shows a software window titled "frmAddTech". It contains a single text input field labeled "Add New Technician:" and a "Return" button below it.

Figure 8-5. Enter new technician window.

Clicking the “Enter Collection Data” button opens a window (Figure 8-6) that allows entry of data from the collection of the fish and how the sperm were collected. The first drop-down menu allows entry of the name of the person who collected the sample by selecting from a list of previous collectors. If the name is not in the list, clicking a button “Enter New Collector Information” opens a window (Figure 8-7) to allow new collectors to be added to the database.

The screenshot shows the 'frmCollectionInfo' window with the following data:

- SampleID: 192
- Collected by: William Wayman
- Date Fish was Collected: 2/11/2002
- Capture Method: Hook and Line
- Body of Water Fish Collected From: Grand Isle Pass
- State: Louisiana
- County: Jefferson
- Scientific Name: Sciaenops ocellatus
- Common Name: Red drum
- Fish ID #: 7F7D32458B
- Captive: ☐
- Broodstock ID #: Grand Isle
- DiseaseHistory: N/A
- Spawning Method: LHRH-a
- Anesthetic Used: MS-222 100 ppm
- Date Sperm Collected: 2/12/2002
- Time Sperm Collected: 10:00:00 AM
- Extender: HBSS
- Osmolality (mOsm/kg): 300
- pH:
- Sample Shipping Method: Ice
- Carrier: Fed Ex
- TransitTime: 20 hrs

Buttons at the bottom include: Morphometric Data, Genetics Data, Save and Close, and an 'Enter New Collector Information' button at the top right.

Figure 8-6. Collection information screen.

The screenshot shows the 'frmCollector' window with the title 'Data Input for Collector Identification'. The data entered is as follows:

- Name: William Wayman
- Organization: LSU Aquaculture Research Sta
- Title: Graduate Student
- Address: 2410 Ben Hur Rd.
- City: Baton Rouge
- State: LA
- Zip Code: 70820
- Phone: (225) 765-2848
- Fax: (225) 765-2877

A 'Save Record' button is located at the bottom right.

Figure 8-7. New collector information screen.

The next section of the window is for the entry of when, where and how the fish was collected. Description of the collection location includes the name of the body of water, the state and county of collection, and the global positioning system (GPS) coordinates (latitude and longitude). The GPS coordinates are entered in decimal form, and a button “To enter degree GPS readings” opens a window to automatically convert and enter GPS coordinates that are in degree:minute:second format (Figure 8-8). To double check the GPS coordinates, a button “Use Geographical Locator” is available that, when connected to the internet, automatically opens the geographical locator web page (hosted by the Montana State University Environmental Statistics Group) to show the position of the GPS coordinates that were entered.

The screenshot shows a Windows-style application window titled "frmLatLongCal : Form". The window has a teal header bar with a standard Windows icon on the left and a close button (X) on the right. The main content area is gray and contains the text "Enter Latitude and Longitude:" centered at the top. Below this text, there are two rows of input fields. The first row is for Latitude, with labels "Latitude", "Degrees", "Minutes", and "Seconds" followed by four empty text boxes. The second row is for Longitude, with labels "Longitude", "Degrees", "Minutes", and "Seconds" followed by four empty text boxes. At the bottom center of the window is a button labeled "Calculate".

Figure 8-8. Decimal latitude and longitude calculator window

The next section of the “Collection Information” window is for entering information regarding the fish from which the sample was collected. The scientific or common name of the species can be selected by clicking on either of the “Scientific Name” or “Common Name” buttons. Drop-down menus list the names of 2799 species of fish. When a name is selected in

either of the drop-down menus, the scientific and common name are entered into the respective text boxes in the “Collection Information” window. The individual fish can be further identified by a fish identification number (typically a passive integrated transponder alpha-numeric identification) (PIT tag), a check-box to identify whether the fish was from a captive or wild population, a broodstock identification number (if available), and the disease history of the fish (if known).

The next section of the “Collection Information” window is for entering information on how the sperm sample was collected. Information includes how the fish was spawned, the anesthetic used, the date and time the sample was collected, and how the sample was shipped to the repository. Buttons at the end of this section allow entry of information regarding the morphometry and genetics of the fish that the sample was collected from.

Clicking the “Morphometric Data” button opens a window (Figure 8-9) that allows measurements from the fish to be entered. The information currently requested for morphometrics include the indexes that are recorded for the identification of pallid sturgeon or shovelnose sturgeon. In the future, other morphometric measurements will be added as the information is identified for a particular species, and a digital picture of the fish will be able to be added to the database. A button “Convert pounds to Kg” can be clicked to aid the user in entering weights that were collected in pounds. The “Save and Close Form” button returns the user to the “Collection Information” window.

Clicking the “Genetics Data” button opens a window (Figure 8-10) that allows the entry of genetic data regarding the fish. The first section is for entering genetic information regarding the population that the fish was collected from. This information includes: population name, strain name, status of the population, genetic analysis used, allele frequency, effective population size,

The screenshot shows a software window titled "frmMorpho". It contains several text input fields for morphometric data:

- FishID: 7F7D32458B
- Inter Rostrum Length (mm):
- Max Head Width (mm):
- Snout to Mouth Length (mm):
- Mouth to Inner Barbel Length (mm):
- Inner Barbel Length (mm):
- Outer Barbel Length (mm):
- Head Length (cm):
- Fork Length (cm): 70
- Weight (kg): 13

At the bottom right, there are two buttons: "Convert pounds to Kg" and "Save and Close Form".

Figure 8-9. Morphometric data screen.

The screenshot shows a software window titled "frmGenetics". It contains several text input fields and a dropdown menu for genetic data:

- Sample ID #: 192
- Species: Sciaenops ocellatus
- Population Level Genetic Information:**
 - Population Name: Grand Isle
 - Strain Name:
 - Status: State Special Concern (dropdown menu)
 - Genetic Analysis: N/A
 - Allele Frequency: N/A
 - Effective Population Size: N/A
 - Heterozygosity: N/A
- Sample Level Genetic Information:**
 - Genetic Analysis: N/A

At the bottom right, there is a button labeled "Save and Close".

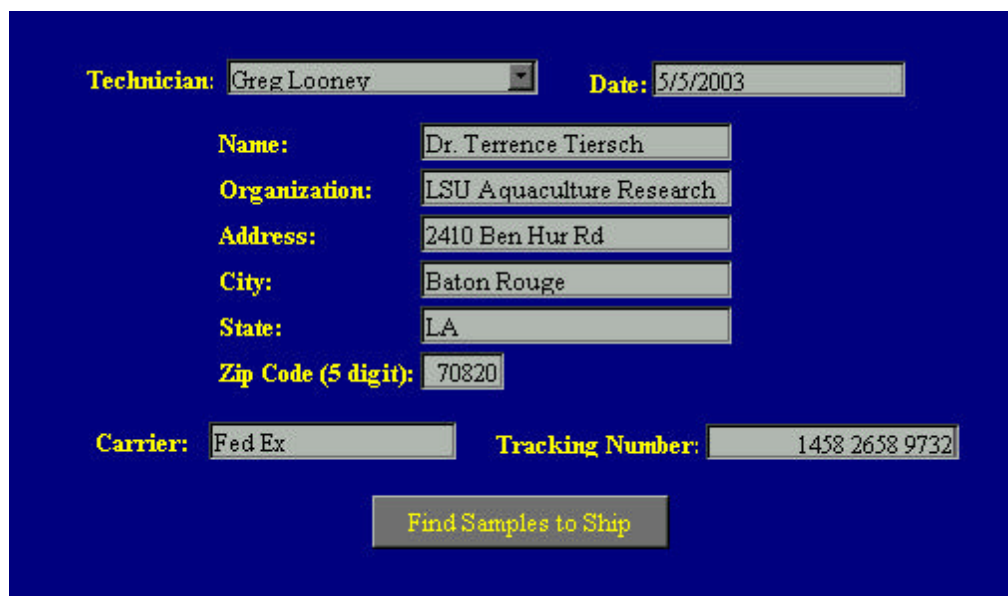
Figure 8-10. Genetics data screen.

and heterozygosity of the population. The second section is for entering genetics information regarding the individual fish. Currently only a text-box for the genetic analysis used is available, but more information will be included as specific analyses become available. A button, “Save and Close,” returns the user to the “Collection Information” window. After all the information for a particular sample has been entered, clicking the “Save and Close” button at the bottom of the “Collection Information” window returns the user to the main server window.

Shipment of Samples

To track shipment of samples, click on the “Ship Samples” button from the main server menu. This button opens a window allowing the entry of information regarding the destination of the samples (Figure 8-11). The user can select the name of the technician who shipped the samples from a drop-down menu or enter a new name in the box. The computer automatically enters the date of the shipment. The user then enters information for the name, organization, and address of whom the samples were shipped to. The name of the carrier and tracking number of the shipment are also recorded. A button “Find Samples To Ship,” allows the selection of samples from the repository that are to be shipped to the destination listed earlier.

Clicking the “Find Samples To Ship” button opens a new screen which allows the selection of the species and PIT tag number of the samples to be shipped (Figure 8-12). Once the species and PIT tag information has been selected, clicking on the “Show Straws” button opens a new screen that lists all of the straws for the selected species and PIT tag number that are currently contained within the repository (Figure 8-13). The user can click the check box to designate the straws that will be shipped. Once the straws have been selected, clicking on the “Done” button returns the user to the main server screen.



This screenshot shows a software interface for entering shipment destination information. The background is dark blue. At the top, there are two fields: 'Technician:' with a dropdown menu showing 'Greg Looney' and 'Date:' with a text box containing '5/5/2003'. Below these, a series of labels are aligned to the left, each followed by a text box: 'Name:' (Dr. Terrence Tiersch), 'Organization:' (LSU Aquaculture Research), 'Address:' (2410 Ben Hur Rd), 'City:' (Baton Rouge), 'State:' (LA), and 'Zip Code (5 digit):' (70820). Further down, 'Carrier:' is set to 'Fed Ex' and 'Tracking Number:' is '1458 2658 9732'. At the bottom center is a button labeled 'Find Samples to Ship'.

Technician: Greg Looney Date: 5/5/2003

Name: Dr. Terrence Tiersch

Organization: LSU Aquaculture Research

Address: 2410 Ben Hur Rd

City: Baton Rouge

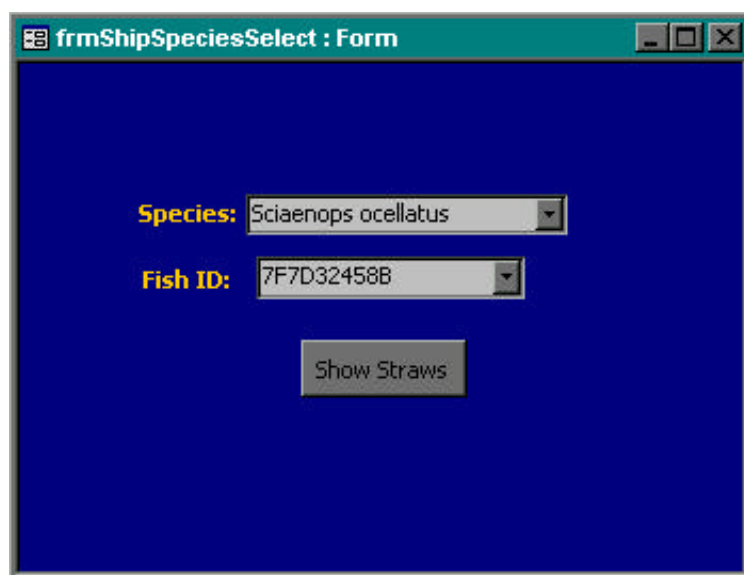
State: LA

Zip Code (5 digit): 70820

Carrier: Fed Ex Tracking Number: 1458 2658 9732

Find Samples to Ship

Figure 8-11. Sample shipment - destination screen.



This screenshot shows a software window titled 'frmShipSpeciesSelect : Form'. The background is dark blue. It contains two dropdown menus: 'Species:' with 'Sciaenops ocellatus' selected and 'Fish ID:' with '7F7D32458B' selected. Below these is a button labeled 'Show Straws'.

frmShipSpeciesSelect : Form

Species: Sciaenops ocellatus

Fish ID: 7F7D32458B

Show Straws

Figure 8-12. Sample shipment - species selection screen.

frmShipSample1

Select straws to ship: SampleID: 192 Done

Species	Fish ID #	Straw ID #	Shipped
Sciaenops ocellatus	7F7D32458B	152684	<input checked="" type="checkbox"/>
Sciaenops ocellatus	7F7D32458B	152685	<input type="checkbox"/>
Sciaenops ocellatus	7F7D32458B	152686	<input type="checkbox"/>
Sciaenops ocellatus	7F7D32458B	152687	<input type="checkbox"/>
Sciaenops ocellatus	7F7D32458B	152688	<input type="checkbox"/>
Sciaenops ocellatus	7F7D32458B	152689	<input type="checkbox"/>
Sciaenops ocellatus	7F7D32458B	152690	<input type="checkbox"/>
Sciaenops ocellatus	7F7D32458B	152691	<input type="checkbox"/>

Figure 8-13. Sample shipment - straw selection screen.

Field Reports

To enter field reports for a particular straw, click the “Submit Field Report” button. This opens a new window (Figure 8-14) allowing selection of the straw for which the report pertains from a drop-down list of all the straws that have been shipped. To verify that the correct straw was selected, the screen shows the species of fish, the technician who shipped the straws, and the date that the straws were shipped. If the correct straw has been selected, click the “Enter Field Report” button.

Select Sample Straw ID # for field report:

SampleStrawID: 152684 Enter Field Report View Field Reports

Species	Samples Shipped To :	Date shipped :
Sciaenops ocellatus	Dr. Terrence Tiersch	5/5/2003

Figure 8-14. Field report - straw selection screen.

The “Enter Field Report” button opens a new screen (Figure 8-15) for entering information regarding the program that the samples were used for, fertilization information, and stocking information. The programmatic information consists of the name and type of program. The fertilization information includes the technique used to thaw the sample, post-thaw motility, PIT tag number of the female from which the cross was made, technique used to fertilize the eggs, percent fertilization, and percent hatch. The stocking information consists of date of stocking, body of water stocked, location of stocking, GPS coordinates of the stocking location, number of fish stocked, and size of the fish at stocking. After all the information has been recorded, clicking the “Close and Save” button returns the user to the main server screen. If the field report screen was entered by mistake, clicking the “Cancel” button will return the user to the previous screen.

Dewar Inventory

To view the current inventory of a particular dewar, select the “Dewar Inventory Button” from the server screen. This button opens a new window containing a drop-down menu for dewar selection, and buttons, which once a dewar has been selected, allow the viewing or printing of the dewar inventory report (Figure 8-16). The dewar inventory report lists the canister color, sample identification number, scientific name, common name, fish identification number, the number of straws originally placed in the repository, and the number of those straws that have been shipped from the repository (Figure 8-17). If all of the straws for a particular sample have been shipped they no longer appear in the inventory report.

frmFieldReport

SampleStrawID: 152684 Species: Sciaenops ocellatus Cancel

Programatic Information:

Name of Program Sperm Used For: State Restocking Type of Program: Supplementation

Fertilization:

Technique used to thaw straws: 40 C for 7 sec Post-thaw Motility (%): 80

Cross Made: 7C7F485723

Technique used to fertilize eggs: Wet Fertilization Rate (%): 75

Hatch Rate (%): 50

Stocking:

Date: 5/7/2003 Body of Water: Lake Ponchartrain Location: Kenner Boat Ramp

GPS Coordinates of Stocking Location:

Latitude: 30.2041 Longitude: 90.2625 Convert GPS to decimals

Number of Fish Stocked: 75000 Size of Fish Stocked: 2"

Figure 8-15. Field report screen

Select dewar:

View Dewar Inventory

Preview Inventory Report

Print Inventory Report

Figure 8-16. Dewar inventory selection screen.

Inventory Report for Dewar: 5

Canister	SampleID	Species	CommonName	FishID	Straws	Shipped
Yellow	148	Scaphirhynchus albus	Pallid sturgeon	7F7D434B54	30	0
Yellow	149	Scaphirhynchus albus	Pallid sturgeon	7F7F065834	30	0
Yellow	150	Scaphirhynchus albus	Pallid sturgeon	115556461A	30	0
Yellow	151	Scaphirhynchus albus	Pallid sturgeon	1F4A3E1445	30	0
Yellow	152	Scaphirhynchus albus	Pallid sturgeon	1F477B3A65	30	0
Yellow	153	Scaphirhynchus albus	Pallid sturgeon	115544332A	30	3
Yellow	154	Scaphirhynchus albus	Pallid sturgeon	116167123A	30	0
Yellow	192	Sciaenops ocellatus	Red drum	7F7D32458B	11	1

Figure 8-17. Dewar inventory report.

Species Inventory

To view the current inventory for a particular species, click the appropriate button on the server window. The button opens a new window containing a drop-down menu listing all the species currently contained within the repository (Figure 8-18). After selecting the species of interest, clicking on a button allows the inventory to be viewed on screen or printed. The report is subcategorized by dewar, and lists the fish identification number, the canister color within the dewar, the label information for the top of the cane, the number of straws received, and the number of straws shipped (Figure 8-19). If the all of the straws that were received for a particular sample have been shipped, the sample information will not be contained within the species inventory report.



Figure 8-18. Species inventory selection screen.

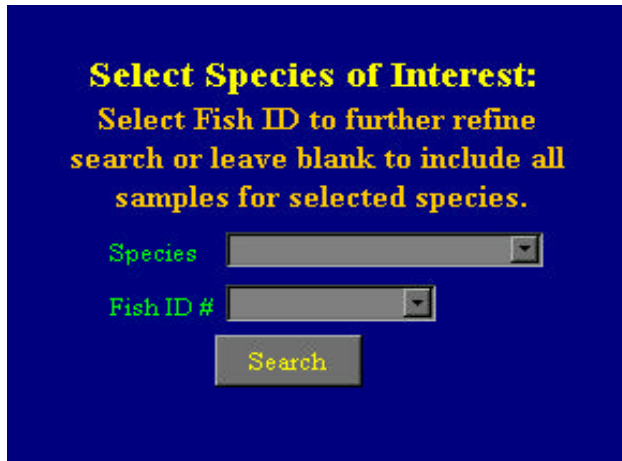
Species Inventory				
<i>Sciaenops ocellatus</i>				
Dewar: 5				
Fish ID #	Canister	CaneLabel	Straws Received	Straws Shipped
7F7D32458B	Yellow	Red Drum	11	1

Figure 8-19. Species inventory report.

View Current Sample Information

To view information for samples that are currently contained within the repository, click the “View Sample Data” from the main server screen. A new screen opens which allows the selection of the species and PIT tag number of the samples from drop-down menus (Figure 8-20). A species must be selected, but the PIT tag number can be left blank to display data on all

of the samples for the select species. After making selections, clicking the “Search” button displays the data.



Select Species of Interest:
Select Fish ID to further refine search or leave blank to include all samples for selected species.

Species

Fish ID #

Figure 8-20. View samples - species selection screen.

Sample information is displayed in a new screen (Figure 8-21). At the top of the screen, the scientific and common names of the species and the PIT tag number for that sample are displayed. If a particular PIT tag number was not selected when selecting the information to view and multiple sample records were found, the user can cycle through each record by clicking on the record selection bar at the bottom of the screen. The sample information for each fish is partitioned into six categories, which can be displayed by clicking on the appropriate tab. If more than one straw has been shipped, clicking on the record selector within the “Straw Shipped To” tab cycles through the different shipment information. Similarly, if more than one field report is available for a sample, clicking on the record selector within the “Field Reports” tab cycles through the different field reports. Clicking on the appropriate button will return the user to the species selection screen or the main server screen.

Species: Fish ID:

Common Name:

Physical Location | **Sample Info** | Genetics | Morphometrics | Straws Shipped To | Field Reports

Dewar Location

Dewar: Canister:

Straw Information

Cane Label:

Straw Size:

Straw Color:

Number of Straws Frozen:

Record: of 1

Figure 8-21. View samples - information screen. Various levels of information can be viewed by clicking on the appropriate tab.

Edit Current Sample Information

Clicking the “Edit Sample Data” button from the main server screen allows sample information currently contained within the database to be edited. When clicked, this button opens a warning message box (Figure 8-22) to make sure that the user is aware that this will change information. The user can only continue by clicking the “OK” button, or can return to the main server screen by clicking the “Cancel” button. When the “OK” button is clicked, a window opens to allow selection of the species and PIT tag number of the samples that are to be edited (Figure 8-23). A species must be selected, but the PIT tag number can be left blank to display data on all of the samples for the selected species. After making selections, clicking the “Search” button displays the data. The data for each sample is displayed identically as when sample information is viewed. To edit information, other than dewar location and straw numbers, simply type over the previous information located within the appropriate text box. To



Figure 8-22. Edit samples - warning screen.



Figure 8-23. Edit samples - species selection screen.

change the dewar location of the samples, click on the “Change Location” button. When clicked, the button opens a new window (Figure 8-24), which contains drop-down menus for selection of the new sample location. To change the number of straws for a sample, click on the “Change Straw Number” button. When clicked, the button opens a new window (Figure 8-25), which contains two text boxes to allow new straw identification numbers to be entered. Clicking the

Figure 8-24. Edit samples - dewar location edit screen.

Figure 8-25. Edit samples - number of straws screen.

“Save Changes” button saves the changed information and returns the user to the main server screen. Clicking the “Return to Main Menu – No Save” button returns the user to the main server screen without saving the edited information.

Warm Springs Cryopreserved Sperm Repository Database

Currently the cryopreserved sperm repository at the Warm Springs Fish Technology Center contains sperm from 15 species of fishes (Table 8-1). These species include recreational species (rainbow trout *Oncorhynchus mykiss*, brown trout *Salmo trutta*, striped bass *Morone saxatilis*, shovelnose sturgeon *Scaphirhynchus platyrhynchus*), species of concern (Atlantic salmon *Salmo*

Table 8-1. Contents of the Warm Springs Fish Technology Center cryopreserved sperm repository as of July 2003. The repository contains 3,952 straws of sperm from 15 species, and are divided into two purposes, archival or research. Archival straws are used for the conservation of genetic material from endangered species or species of concern.

Common name	Scientific name	# of males	# of straws	Purpose
Alabama sturgeon	<i>Scaphirhynchus suttkusi</i>	1	9	Archival
Atlantic salmon	<i>Salmo salar</i>	10	838	Archival
Atlantic sturgeon	<i>Acipenser oxyrinchus oxyrinchus</i>	3	84	Archival
Lake sturgeon	<i>Acipenser fulvescens</i>	22	240	Archival
Pallid sturgeon	<i>Scaphirhynchus albus</i>	27	450	Archival
Robust redhorse	<i>Moxastoma robustum</i>	10	78	Archival
Striped bass	<i>Morone chrysops</i>	23	564	Archival
Atlantic sturgeon	<i>Acipenser oxyrinchus oxyrinchus</i>	3	120	Research
Brook trout	<i>Salvelinus fontinalis</i>	13	90	Research
Brown trout	<i>Salmo trutta</i>	15	57	Research
Lake sturgeon	<i>Acipenser fulvescens</i>	10	54	Research
Pallid sturgeon	<i>Scaphirhynchus albus</i>	21	238	Research
Rainbow trout	<i>Oncorhynchus mykiss</i>	16	83	Research
Red drum	<i>Sciaenops ocellatus</i>	2	8	Research
Robust redhorse	<i>Moxastoma robustum</i>	15	158	Research
Shortnose sturgeon	<i>Acipenser brevirostrum</i>	16	252	Research
Shovelnose sturgeon	<i>Scaphirhynchus platorynchus</i>	7	28	Research
Striped bass	<i>Morone chrysops</i>	7	385	Research
Walleye	<i>Stizostedion vitreum</i>	12	108	Research
White sturgeon	<i>Acipenser transmontanus</i>	10	108	Research

salar, lake sturgeon *Acipenser fulvescens*, robust redhorse sucker *Moxostoma robustum*) and endangered species (Alabama sturgeon *Scaphirhynchus suttkusi*, pallid sturgeon *Scaphirhynchus albus*, shortnose sturgeon *Acipenser brevirostrum*, and white sturgeon *Acipenser transmontanus*).

The Warm Springs Cryopreserved Sperm Repository Database was developed specifically for the needs of the Warm Springs Fish Technology Center. As such, the database was organized based on the storage dewars at the facility and the information necessary for the types of sperm contained in the repository, and therefore is site specific. However, the database was developed in Microsoft Access 2000, commercially available database development software, and can be easily and rapidly modified to work at other cryopreservation facilities. For example, to change the storage design to a different dewar system, the information contained in the two tables that designate the storage location can be changed to represent the new system, or an additional table can be added and inter-related to the previous tables if necessary. Additional sample information fields can be added by including them in the appropriate table and adjusting the forms. In fact, the current database at Warm Springs will likely be changed to adjust to new facilities and programs as needed.

Literature Cited

Kincaid, H. L. 2000. Development of databases for germplasm repositories. In: *Cryopreservation in Aquatic Species*. Tiersch, T. R. and P. M. Mazik, Editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 323-331.

Chapter 9

Summary

This dissertation covers the development of methods for the creation of a cryopreserved sperm repository for North American sturgeon, and can be used as a model for a repository for any aquatic species (Figure 9-1). Techniques used for the identification of species from caviar samples (Wolf et al., 1999) were adapted for use in the identification of sperm samples. A survey was done to determine the types of bacteria that are commonly encountered during sperm collection and to determine the effects of the addition of antibiotics during storage. Methods were developed for the refrigerated storage and cryopreservation of sperm samples. An alternative method of gamete quality determination was evaluated to determine if the analysis would be correlated to fertilization and hatch rates. Finally, a database was created to organize and maintain the inventory of cryopreserved samples, and to provide the proper information needed by partners when using cryopreserved sperm.

The dissertation was outlined to follow the steps that a sample would undergo when being cryopreserved. The first of which is the verification of the proper identification of samples. The development of a cryopreserved sperm facility allows fresh sperm to be collected from distant locations and transferred to the facility for cryopreservation. Using this method, the technician at the laboratory has no way of verifying the field identification of species, many of which are similar in appearance. The adaptation of a method for species identification from caviar (Wolf et al., 1999) for identification using sperm samples was illustrated in Chapter 3. The technique used the polymerase chain reaction, three restriction enzymes, and electrophoresis to develop a banding pattern that could be used for species identification. The technique was not able to separate closely related species or subspecies, such as Alabama sturgeon, pallid sturgeon, and

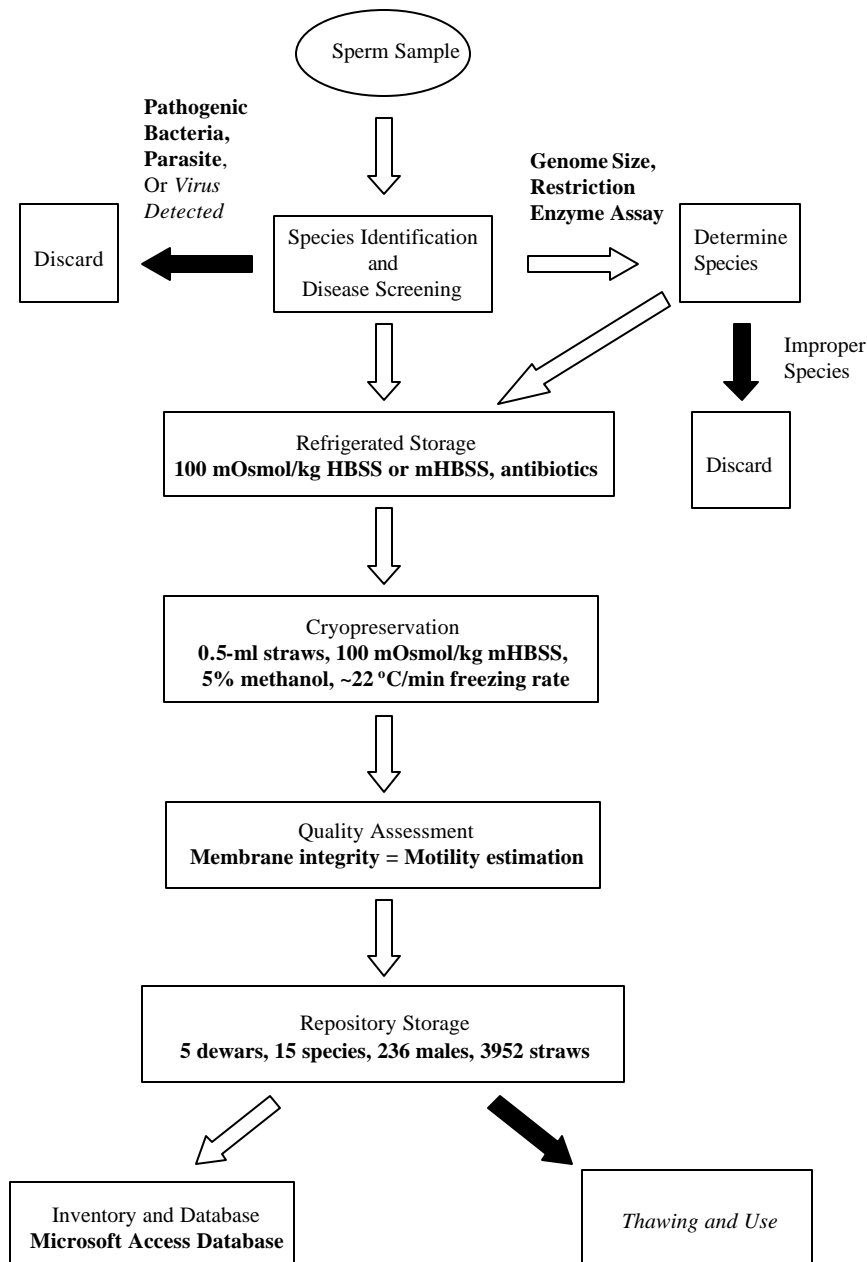


Figure 9-1. Components and methods necessary for the development of a germplasm repository. Items in bold were developed during this dissertation. Sperm samples enter the process at the top and proceed through a series of steps designed for quality assurance and quality control of the samples before they enter the repository. Black arrows represent processes in which sperm samples leave the repository. Items in italics were not specifically investigated in this dissertation. The methods identified in this dissertation have allowed the creation of an archival repository for sperm of the endangered Alabama sturgeon and pallid sturgeon.

shovelnose sturgeon or Atlantic sturgeon and Gulf sturgeon, but was able to separate many of the species. Further development of primers and restriction enzyme combinations should allow the differentiation of all the species, allowing a cryopreservation facility to verify the field identification of sperm samples.

Bacterial contamination of sperm samples is a commonly encountered problem (Jenkins 2000), and can lead to the degradation of samples, transfer of pathogens, and even the inaccurate estimation of motility (Jenkins and Tiersch, 1997). Chapter 4 illustrated the bacterial contaminants commonly encountered during the collection of sturgeon sperm samples. Collection techniques were those commonly used at production facilities, and no effort was made to reduce the levels of contamination. Commonly encountered bacterial contaminants included typical ubiquitous non-pathogenic and pathogenic flora. The use of antibiotics at the concentrations used in this study did not have an adverse effect on sperm motility and fertility during refrigerated storage; however, they also did not increase motility retention over time as compared to untreated samples. Also a cryopreservation procedure developed for sturgeon sperm did not reduce the post-thaw number of bacteria present in a sample. The incorporation of antibiotics into sampling protocols could help to reduce contamination and help to avoid the spread of bacterial pathogens.

The development of refrigerated storage techniques based on seminal plasma osmolality, seminal plasma composition, and sperm activation were reported in Chapter 5. The blood plasma osmolalities of shortnose sturgeon, pallid sturgeon, and shovelnose sturgeon were similar to blood plasma osmolalities reported for other sturgeon species captured or held in freshwater. However, the osmotic pressures of seminal plasma from all four sturgeon species in this study were significantly lower than the osmotic pressures of their respective blood plasma. This

difference indicated that blood plasma osmolality of sturgeon species cannot be used directly as a basis for extender development.

The seminal plasma ion concentrations reported in Chapter 5 and in other sturgeon species are lower than the concentrations reported for other freshwater species (Morisawa et al., 1983; Lahnsteiner et al., 1996; Glogowski et al., 2000), suggesting a mechanism for sperm activation in sturgeon which is different from the reduction of osmotic pressure, commonly reported for freshwater fishes (Morisawa et al., 1983; Bates et al., 1996; Mansour et al., 2002), with the exception of the salmonids (Billard, 1992). Motility of sturgeon sperm at osmolalities greater than that of the seminal plasma also indicated that osmolality was not the primary cause of sperm activation. Sperm motility was reduced by potassium chloride or potassium phosphate solutions, and was completely inhibited at concentrations greater than or equal to 0.8 mM. High concentrations (≥ 1.2 mM) of calcium chloride also reduced sperm motility. In this regard, sturgeon sperm activation is similar to salmonid sperm activation.

Sturgeon sperm, in the present study, stored undiluted retained equal or greater motility than sperm stored in any of the extender and osmolality combinations, however, composition of the extender and its osmolality had significant influences on the duration of refrigerated storage. The combination of 100 mOsmol/kg and Hanks' balanced salt solution or modified Hanks' balanced salt solution typically retained higher motility percentages for longer periods than did the other extender and osmolality combinations. To attain the longest period of storage with the highest motility, sperm should be stored in a solution that contains potassium at a concentration of at least 1 mM and adjusted to an osmotic pressure similar to that of the seminal plasma osmolality (~ 100 mOsmol/kg). There was some evidence that the use of external sugars as an

energy source by sperm could prolong the motility of sperm samples, but further investigation is necessary.

Although cryoprotectants are used to help protect cells during the freezing and thawing processes, these chemicals can be toxic to the cells, and care must be taken to balance the cryoprotective benefits and toxicity. Chapter 6 evaluated the use of different cryoprotectants, concentrations, and freezing rates to develop cryopreservation procedures for sturgeon sperm. Although sturgeon sperm cryopreserved using DMSO generally retained equal or higher post-thaw motility than did sperm cryopreserved with MeOH, sperm cryopreserved with DMSO had limited fertilizing ability compared to that of sperm cryopreserved with MeOH, which indicates that post-thaw motility should not always be used as an indicator of cryopreserved sperm quality. Differences in extender composition have been shown to have effects on the cryopreservation of sturgeon sperm, however in this study, extender comparisons could not be made due to inadequate quantities of eggs to allow fertilization trials with both extenders.

Cryopreserved lake sturgeon, pallid sturgeon and shovelnose sturgeon sperm were capable of fertilizing eggs at rates from 50-100% of that of fresh sperm, however, cryopreserved shortnose sturgeon sperm had only limited ability to fertilize eggs. Although currently a recommendation cannot be made for the cryopreservation of shortnose sturgeon sperm, it can be recommended that lake sturgeon, shovelnose sturgeon, or pallid sturgeon sperm should be cryopreserved using 5% MeOH as a cryoprotectant, mHBSS as an extender at a ratio of 1:4 (v:v; sperm:extender), 0.5-ml straws, and freezing in a nitrogen-vapor shipping dewar at a cooling rate of 22 to 24 °C/min. Further research into the development of a cryopreservation protocol for shortnose sturgeon sperm and into the fertilizing ability of sperm cryopreserved in mTsvetkova extender is warranted.

Several methods are currently available to evaluate the quality of sperm samples. Visual estimation of the percentage of motile sperm is the most common form of analysis, but is subjective and can be greatly influenced by the person reading the samples (McNiven et al., 1992). Chapter 7 evaluated a dual staining technique and flow cytometry for the analysis of post-thaw sperm quality. The strong correlation between the treatment proportion of viable cells and the percentage of viable cells as determined by flow cytometry verified that the staining technique was accurate, and demonstrated the effectiveness of this procedure for discriminating viable from non-viable sperm cells.

Sperm cryopreserved with 10% DMSO produced the highest percentages of viable cells after thawing, regardless of species or extender used, however, these values did not correlate strongly with motility, fertility, or hatch. Sperm cryopreserved with 5% methanol had the lowest percentage of viable cells after thawing regardless of species or extender. Sperm cryopreserved with methanol yielded viability percentages after thawing that were highly correlated with motility, irrespective of species or extender used. However, viability was only correlated to fertilization rates and hatch rates for shovelnose sturgeon sperm cryopreserved using mHBSS and MeOH. The viability of sperm of pallid sturgeon cryopreserved with mHBSS and 5% MeOH was also strongly correlated to fertility rates, however it was not correlated to hatch rates. In the instances where post-thaw viability was correlated with percent fertilization and percent hatch, viability was also highly correlated to motility, indicating that motility estimation would work equally well at predicting sperm quality as the more complicated viability assay. Although the procedure for dual-staining outlined here was accurate at determining the percentage of viable cells in post-thaw sturgeon sperm samples, the procedure showed only limited ability to forecast the fertilizing ability of cryopreserved sperm.

The objective of Chapter 8 was to develop a relational database system that would be able to maintain inventory control over the cryopreserved samples at the Warm Springs Fish Technology Center (WSFTC), and be able to track and monitor the quality of the samples. Various categories of information, including baseline characterization of the fish, sperm collection, sperm processing, sperm usage, and quality control, have been identified as necessary for the operation of a cryopreserved sperm repository database (Kincaid, 2000). The database was developed in Microsoft Access 2000 (Version 9.0.3821 SR-1, Microsoft Corporation, Redmond, Washington) and has incorporated warning screens, message boxes, and error messages to aid the user in correctly entering data. The database is currently in use by the WSFTC and will be made available to partners through the internet.

Although the techniques outlined in this dissertation have made cryopreservation of sturgeon sperm possible, there are many improvements to the techniques that can be made. The development of new PCR primer sets and restriction enzyme combinations should enable sperm samples from all of the sturgeon species in North America to be differentiated, allowing the verification of field identifications for samples sent to cryopreservation facilities. Incorporation of sterilized instruments and aseptic techniques for collection of sperm, and antibiotics within extenders could help to reduce the possibilities of bacterial contamination of sperm samples, reducing the chances of disease transmission through cryopreserved samples. Improvements in cryoprotectant and concentration combinations and freezing rates could help to improve the post-thaw motility of samples, theoretically improving fertilization and hatch rates, and creating a procedure for shortnose sturgeon sperm. Development of cryopreservation procedures for larger volumes of sperm could aid in the transition of this program from a research based genetic conservation program to a production scale. Incorporation of more stains to look at various

cellular structures, such as mitochondrial function, acrosome integrity, and DNA stability, should provide a gamete quality analysis that better correlates with percent fertilization and percent hatch. Finally, as the repository at WSFTC continues to go, the inclusion of additional types of information requested by partners will be essential.

The development of cryopreservation procedures for sperm of sturgeon species will help to aid in the recovery of threatened and endangered species and in the commercial aquaculture field. Cryopreserved sperm can be used to conserve the remaining genetic resources from declining species, help to increase genetic diversity in spawning operations, and allow spawning of females whenever they are in reproductive condition. Cryopreserved sperm can also benefit the commercial aquaculture industry by allowing females to be spawned when males are unavailable, decreasing the need to hold captive males as broodstock, aiding in genetic selection, and helping to maintain lines of selected stocks.

The methods developed within this dissertation have allowed the creation of a working repository of pallid sturgeon sperm from the upper Missouri River by the U. S. Fish and Wildlife Service. Samples are stored at the Warm Springs Fish Technology Center (Figure 9-2), the Garrison Dam National Fish Hatchery, and the Gavins Point National Fish Hatchery. It is important to caution that cryopreservation is only a tool, and does not ensure the preservation of a species. Continued efforts at habitat restoration, water quality improvement, and reductions in commercial fishing are still necessary to ensure the survival of many of these threatened or endangered species. Accurate sample identification and inventory control of samples is imperative to assure that hybrid sturgeon are not created during restocking efforts. Screening of samples for bacterial or other pathogens should be done to reduce the spread of diseases to new areas, and to protect the stocks at aquaculture facilities. Careful monitoring of the use of genetic

material is necessary to ensure that certain males are not overly used in recovery efforts, and that selection based on post-thaw viability, which could be selecting against more desirable traits, does not occur in aquaculture situations. With these cautions in mind, cryopreserved sperm can be used for the recovery and restoration of threatened and endangered species as well as in support of commercial aquaculture operations.



Figure 9-2. Warm Springs Fish Technology Center cryopreserved sperm repository. Currently the repository consists of 5 storage dewars and contains sperm from 15 species of fish.

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Appendix A

Standard Operating Procedures

SOP 1 - Blood collection

- 1) Use a 3-ml syringe with a 22-gauge 1 1/2 inch needle.
- 2) Prepare syringe prior to blood collection by drawing 0.1 ml of acid citrate dextrose anticoagulant (Catalog # 364606, Becton Dickinson Vacutainer Systems, Franklin Lakes, New Jersey) per ml of blood collected into the syringe.
- 3) Collect blood from caudal vein.
- 4) Insert the needle behind the anal fin in an area of soft tissue in front of the scutes.
- 5) Insert the needle to a depth of approximately 1/2" (depending on size of fish).
- 6) Withdraw the plunger and blood will be slowly drawn into the syringe.
- 7) After collection transfer the blood to a plain vacutainer (Catalog # 6434, Becton Dickinson Vacutainer Systems) for storage.
- 8) Remove the plunger from the syringe and re-insert the needle into the vacutainer to release the remaining vacuum.

SOP 2 – Propidium iodide solution

10-X concentrate of propidium iodide stain

100 ml of Ca^{+2} and Mg^{+2} free phosphate buffered saline

5 mg PI

100 mg sodium citrate

100 μl of Triton X-100

PI staining solution

18 ml deionized water

2 ml 10-X PI concentrate

20 μl RNase (1 mg/ml solution, R5125, Sigma Chemical Corporation)

Prepared according to:

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a lab manual. Volume 3. 2nd edition. Editor, C. Nolan. Cold Spring Harbor Laboratory Press. Plainview, New York. Page B17.

SOP 3 - Motility estimation using microscopy

- 1) Use 100-X magnification and dark-field illumination..
- 2) Place 2 μ l of sperm on a microscope slide
- 3) Activate the sperm by adding 20 μ l of deionized water.
- 4) Estimate percent motile.

Only sperm that exhibited progressive motility are considered to be motile (sperm that vibrated in place and were not progressively motile are not counted)

SOP 4 - Collection of bacteria

- 1) Dip a sterile swab into sperm sample
- 2) Using the swab coat the surface of a brain heart infusion agar (BHIA) (Product # 241830, Difco Laboratories, Detroit, MI) plate.
- 3) Incubate the plates aerobically for 48 hr at room temperature (22-24 °C)
- 4) Select individual colonies and streak on tryptic soy agar (TSA) (Product # 0369-17-6, Difco Laboratories) slants.
- 5) Incubate the slants in a 22 °C incubator or at room temperature (22-24 °C).
- 6) Ship slants to the Louisiana Aquatic Animal Disease Diagnostic Laboratory at Louisiana State University for identification to at least the genus level.

SOP 5 - Sperm collection

- 1) Place the male ventral side up on the riverbank or hold the fish out of the water by the tail.
- 2) Dry the urogenital area with a towel to minimize contamination from water or mucus.
- 3) Sperm can be collected by two methods.

- 1) Use a 60-ml syringe and latex tubing.

Attach a small latex tube (1/4 inch outer diameter) to a 60-ml syringe.

Insert the tubing into the urogenital opening.

Slowly withdraw the plunger.

- 2) Use an aspirator (adapted from David et al., 2000).

Connect 8 mm (O.D.) latex tubing to one hole of a #6 stopper .

Connect 3 mm (O.D.) rigid plastic tubing to another hole in the #6 stopper.

Insert the stopper into the opening of a 50-ml centrifuge tube.

Insert the centrifuge tube into pre-drilled hole in a #11 stopper.

Fill the bottle with water (16 °C)

Insert the stopper into a 500-ml Nalgene wide-mouth bottle

Insert the rigid plastic tubing into the urogenital opening

Apply gentle suction to the latex tubing.

Stop suction when sperm reaches 40-ml mark on tube.

- 4) Transfer the sperm to 50-ml tubes.
- 5) Store undiluted sperm at 4 °C until used in experiments.

SOP 6 - Ingredients for 100 mOsmol/kg modified Hanks' balanced salt solution (1 L)

40 ml concentrate (H4385, Sigma Diagnostics Inc., St Louis, Missouri)

Ingredients – 80.000 g/L NaCl

4.000 g/L KCl

0.600 g/L KH_2PO_4

0.475 g/L Na_2HPO_4

0.170 g/L Phenol Red

960 ml de-ionized water

Adjust pH to 8.0 with HCl.

SOP 7 - Ingredients for 100 mOsmol/kg Hanks' balanced salt solution (1 L)

1000 ml de-ionized water

2.667 g NaCl

0.133 g KCl

0.053 g $\text{CaCl}_2 \bullet 2 \text{H}_2\text{O}$

0.067 g $\text{MgSO}_4 \bullet 7 \text{H}_2\text{O}$

0.020 g KH_2PO_4

0.020 g Na_2HPO_4

0.117 g NaHCO_3

0.333 g $\text{C}_6\text{H}_{12}\text{O}_6$ (Glucose)

Adjust pH to 8.0 with HCl.

SOP 8 - Ingredients for 500 ml of 10% buffered formalin

10 g Borax

200 g Sucrose

450 ml de-ionized water

50 ml formalin (37% formaldehyde)

SOP-9 Fertilization trials

- 1) Thaw sperm were thawed in a 40 °C water bath for 9 sec.
- 2) Estimate motility according to SOP 4.
- 3) Apply sperm to ~300 eggs in a bowl.
- 4) Add 250 ml of water to activate the gametes and initialize fertilization.
- 5) After 5 min, decant excess water and sperm.
- 6) Add a suspension of Fuller's earth (Humco, Texarkana, Texas) to reduce egg adhesiveness.
- 7) Mix the eggs were mixed in the Fuller's earth suspension for 30 minutes.
- 8) Wash the eggs were washed to remove Fuller's earth from bowl.
- 9) Place in incubator jars and incubate at 16 °C (Figures SOP 9-1, SOP 9-2).
- 10) After 6 hours, remove the eggs and place in glass tubes.
- 11) Preserve eggs in 10% buffered formalin (SOP 10) for the determination of percent fertilization at the 4-to 8-cell stage (Figure SOP 9-3).



Figure SOP 9-1. A 2" diameter acrylic hatching jar and plastic aquarium for incubating sturgeon eggs. The eggs are incubated in the bottom of the jar, and after hatching swim out of the jar into the aquarium.



Figure SOP 9-2. A closed recirculating incubation system for the incubation of sturgeon eggs. The system consists of 76 hatching jars and plastic aquariums, which allows the simultaneous incubation of numerous samples. A 1-hp heat pump is used to maintain the system at a constant temperature. A UV-filter is used to reduce the possibility of pathogen transfer between samples. The system was built into an enclosed trailer to make the system more portable and to reduce the effects of environmental variables.

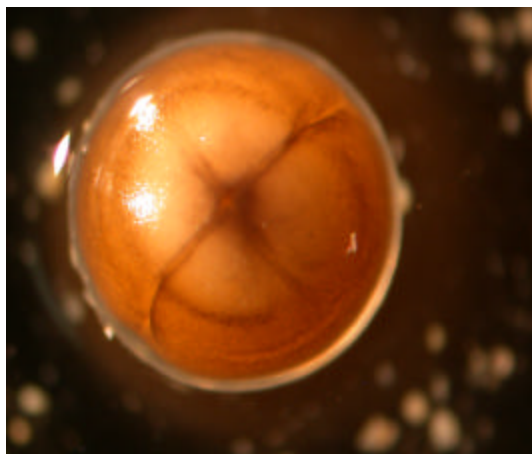


Figure SOP 9-3. Unfertilized and fertilized sturgeon eggs after 6 hr incubation at 16 °C. The picture at the top is an unfertilized egg, which shows no signs of division. The bottom two pictures are of fertilized eggs. The picture on the right is of an egg at the 4-cell stage, and the picture on the left is of an egg at the 8-cell stage.

SOP-10 Hatch trials

- 1) Thaw sperm were thawed in a 40 °C water bath for 9 sec.
- 2) Estimate motility according to SOP 4.
- 3) Apply sperm to ~300 eggs in a bowl.
- 4) Add 250 ml of water to activate the gametes and initialize fertilization.
- 5) After 5 min, decant excess water and sperm.
- 6) Add a suspension of Fuller's earth (Humco, Texarkana, Texas) to reduce egg adhesiveness.
- 7) Mix the eggs were mixed in the Fuller's earth suspension for 30 minutes.
- 8) Wash the eggs were washed to remove Fuller's earth from bowl.
- 9) Place in incubator jars and incubate at 16 °C (Figures SOP 9-1, SOP 9-2).
- 10) After 6 hours, remove half of the eggs and place in glass tubes.
- 11) Preserve eggs in 10% buffered formalin (SOP 10) for the determination of percent fertilization at the 4-cell stage (Figure SOP 9-3).
- 12) Count remaining eggs in the jars.
- 13) Incubate until hatch, approximately 5-7 days at 16 °C. (Figure SOP 10-1).
- 14) After all fry have hatched, count fry remaining in jars and in aquariums to determine percent hatch.



Figure SOP 10-1. Hatched sturgeon larvae. Sturgeon eggs will typically hatch after 5-7 days when incubated at 16 °C

SOP 11 - Ingredients for modified Tsvetkova solution (1 L)

8.01 g $\text{C}_{12}\text{H}_{22}\text{O}_{11}$ (sucrose)

3.63 g $\text{C}_4\text{H}_{11}\text{NO}_3$ (tris)

0.07 g KCl

985 ml deionized H_2O

Adjust to pH 8.0 with HCl

SOP 12 – Freezing in nitrogen-vapor shipping dewars.

- 1) Load sperm into 0.5-ml straws (bovine medium straws, IMV International Corporation, Minneapolis, Minnesota).
- 2) Seal the straws with PVC powder (IMV).
- 3) Place 5 straws into a 10-mm plastic goblet (IMV).
- 4) Insert a type-T thermocouples (Omega Engineering Inc., Stamford, Connecticut) into additional 0.5-ml straws and place straws in goblets.
- 5) Attach the goblets to the bottom position of a 10-mm aluminum canes (IMV).
- 6) Lower canes into a nitrogen-vapor shipping dewar (CP100, TS-Scientific, Perkasie, Pennsylvania).
- 7) Measure mean freezing rate using a datalogger (OM-550, Omega engineering Inc.)
- 8) After 30 min, transfer the canes to another nitrogen-vapor shipping dewar containing liquid nitrogen or a storage dewar (Model 8038, TS-Scientific).

Appendix B – Unanalyzed Data
Chapter 3 - Development of Methods to Verify the Field Identification of Species
Unanalyzed Data

ACD anticoagulant concentration study – Day 1

Contents	ACD Concentration	Standard Peak	Standard CV	Unknown Peak	Unknown CV	DNA Mass (pg)
Human		701	2.27			
CCF and Human		703	2.37	203	2.32	2.063
CCF and Human		697	2.42	204	2.23	2.073
CCF and Human		696	2.48	203	2.35	2.063
CCF and Rooster		399	2.37	513	2.04	2.606
CCF and Rooster		402	2.33	516	1.93	2.621
CCF and Rooster		402	2.39	517	2.00	2.627
CCF and Hen		400	2.32	503	2.35	2.510
CCF and Hen		397	2.35	501	2.39	2.500
CCF and Hen		397	2.35	500	2.38	2.495
CCF and PSN	10%	405	2.49	794	2.14	4.034
CCF and PSN	25%	401	2.52	784	1.99	3.983
CCF and PSN	50%	405	2.43	793	2.02	4.029
CCF and PSN	10%	406	2.28	793	1.85	4.029
CCF and PSN	25%	405	2.37	789	1.96	4.008
CCF and PSN	50%	402	2.90	785	2.28	3.988
CCF and PSN	10%	404	2.09	784	2.02	3.983
CCF and PSN	25%	404	2.08	787	1.95	3.998
CCF and PSN	50%	404	2.09	785	1.94	3.988
CCF and PSN	10%	408	2.35	793	1.91	4.029
CCF and PSN	25%	405	2.35	788	1.88	4.003
CCF and PSN	50%	403	2.39	789	1.93	4.008
CCF and PSN	10%	407	2.40	789	2.20	4.008
CCF and PSN	25%	398	2.35	774	2.13	3.932
CCF and PSN	50%	404	2.34	785	2.12	3.988
CCF and Rooster		402	2.38	520	1.96	2.642
CCF and Rooster		399	2.39	517	1.98	2.627
CCF and Rooster		397	2.36	513	1.96	2.606
CCF and Hen		402	2.38	505	2.28	2.520
CCF and Hen		399	2.40	499	2.38	2.490
CCF and Hen		400	2.37	502	2.31	2.505
CCF and Human		704	2.18	204	2.11	2.073
CCF and Human		704	2.16	206	2.17	2.093
CCF and Human		705	2.12	206	2.24	2.093

ACD anticoagulant study – Day 2

Contents	ACD Concentration	Standard Peak	Standard CV	Unknown Peak	Unknown CV	DNA Mass (pg)
Human		696	2.09			
CCF and Human		698	2.13	204	2.33	2.045
CCF and Human		696	2.06	205	2.28	2.055
CCF and Human		698	2.16	205	2.29	2.055
CCF and Rooster		401	2.41	518	1.94	2.596
CCF and Rooster		402	2.36	513	1.96	2.571
CCF and Rooster		398	2.40	515	1.89	2.581
CCF and Hen		401	2.42	498	2.19	2.496
CCF and Hen		396	2.42	500	2.19	2.506
CCF and Hen		398	2.48	496	2.24	2.486
CCF and PSN	10%	403	2.30	793	2.10	3.974
CCF and PSN	25%	400	2.26	785	1.97	3.934
CCF and PSN	50%	401	2.34	783	2.10	3.924
CCF and PSN	10%	404	2.34	784	1.89	3.929
CCF and PSN	25%	404	2.38	784	1.86	3.929
CCF and PSN	50%	400	2.37	785	2.07	3.934
CCF and PSN	10%	398	2.11	781	1.91	3.914
CCF and PSN	25%	396	2.07	777	1.82	3.894
CCF and PSN	50%	396	2.02	780	1.99	3.909
CCF and PSN	10%	401	2.28	792	1.90	3.969
CCF and PSN	25%	400	2.32	785	1.84	3.934
CCF and PSN	50%	400	2.35	783	1.89	3.924
CCF and PSN	10%	402	2.37	785	2.17	3.934
CCF and PSN	25%	400	2.30	785	2.09	3.934
CCF and PSN	50%	402	2.32	784	2.10	3.929
CCF and Rooster		398	2.40	519	1.93	2.601
CCF and Rooster		398	2.39	514	1.85	2.576
CCF and Rooster		399	2.39	517	1.85	2.591
CCF and Hen		396	2.40	498	2.20	2.496
CCF and Hen		397	2.38	493	2.24	2.471
CCF and Hen		400	2.38	498	2.22	2.496
CCF and Human		698	2.04	205	2.20	2.055
CCF and Human		702	2.03	206	2.21	2.065
CCF and Human		701	2.09	206	2.25	2.065

ACD anticoagulant study – Day 3

Contents	ACD Concentration	Standard Peak	Standard CV	Unknown Peak	Unknown CV	DNA Mass (pg)
Human		698	2.06			
CCF and Human		692	2.12	204	2.07	2.045
CCF and Human		702	2.22	205	2.23	2.055
CCF and Human		705	2.05	205	2.14	2.055
CCF and Rooster		397	2.37	519	1.99	2.601
CCF and Rooster		399	2.34	519	1.93	2.601
CCF and Rooster		400	2.40	519	1.96	2.601
CCF and Hen		395	2.45	502	2.26	2.516
CCF and Hen		397	2.43	502	2.30	2.516
CCF and Hen		398	2.46	505	2.26	2.531
CCF and PSN	10%	404	2.30	788	1.99	3.949
CCF and PSN	25%	400	2.31	783	1.87	3.924
CCF and PSN	50%	402	2.29	796	2.54	3.989
CCF and PSN	10%	402	2.37	785	2.08	3.934
CCF and PSN	25%	401	2.43	785	1.84	3.934
CCF and PSN	50%	398	2.44	791	2.45	3.964
CCF and PSN	10%	404	2.03	784	1.98	3.929
CCF and PSN	25%	400	2.02	782	1.82	3.919
CCF and PSN	50%	402	2.13	790	2.18	3.959
CCF and PSN	10%	402	2.36	789	1.86	3.954
CCF and PSN	25%	401	2.25	786	1.73	3.939
CCF and PSN	50%	402	2.31	784	2.05	3.929
CCF and PSN	10%	402	2.37	786	2.29	3.939
CCF and PSN	25%	404	2.32	786	1.99	3.939
CCF and PSN	50%	400	2.35	787	2.08	3.944
CCF and Rooster		398	2.39	518	2.01	2.596
CCF and Rooster		397	2.41	512	2.12	2.566
CCF and Rooster		399	2.41	516	2.09	2.586
CCF and Hen		399	2.45	503	2.32	2.521
CCF and Hen		398	2.41	505	2.35	2.531
CCF and Hen		400	2.47	504	2.31	2.526
CCF and Human		691	2.42	201	2.42	2.015
CCF and Human		699	2.46	209	2.41	2.095
CCF and Human		702	2.41	202	2.61	2.025

Shovelnose sturgeon blood storage – Day 0

Contents	Standard Peak	Standard CV	Unknown Peak	Unkown CV	DNA Mass (pg)
CCF and Goldfish	404	3.54	694	3.17	3.49
CCF and Goldfish	405	3.83	700	3.28	3.52
CCF and Goldfish	405	3.67	702	3.28	3.53
CCF and Human	202	3.84	698	3.1	2.03
CCF and Human	200	3.74	698	3.3	2.01
CCF and Human	201	4.00	705	3.42	2.02
CCF and Rooster	602	5.08	764	3.69	2.56
CCF and Rooster	694	4.81	793	4.37	2.66
CCF and Goldfish	204	6.04	353	6.15	3.55
CCF and SSN	401	3.07	788	2.85	3.96
CCF and SSN	402	3.05	781	2.85	3.93
CCF and SSN	401	3.23	788	2.98	3.96
CCF and SSN	399	3.10	781	2.7	3.93
CCF and SSN	402	2.98	777	2.65	3.91
CCF and SSN	398	2.93	788	2.53	3.96
CCF and SSN	397	2.74	775	2.52	3.90
CCF and SSN	399	2.71	782	2.39	3.93
CCF and SSN	397	2.75	776	2.37	3.90
CCF and SSN	402	2.79	778	2.33	3.91
CCF and SSN	397	2.63	779	2.35	3.92
CCF and SSN	402	2.58	783	2.57	3.94
CCF and Human	200	2.62	695	2.57	2.01
CCF and Human	200	2.69	688	2.6	2.01
CCF and Human	199	2.76	692	2.55	2.00
CCF and Rooster	396	2.57	511	2.21	2.57
CCF and Hen	395	2.63	497	2.48	2.50
CCF and Rooster	396	2.57	509	2.2	2.56
CCF and SSN	398	2.59	784	2.11	3.94
CCF and Hen	393	2.44	498	2.39	2.50
CCF and SSN	396	2.48	769	2.28	3.87
CCF and SSN	398	2.53	778	2.21	3.91
CCF and SSN	401	2.52	777	2.27	3.91
CCF and SSN	391	2.45	765	2.3	3.85
CCF and SSN	388	2.30	763	2.03	3.84
CCF and SSN	393	2.28	764	2.14	3.84
CCF and SSN	392	2.29	770	2.04	3.87
CCF and SSN	394	2.34	764	2.06	3.84
CCF and SSN	393	2.38	774	2.23	3.89
CCF and SSN	394	2.30	762	2.05	3.83

(table cont.)

Contents	Standard Peak	Standard CV	Unknown Peak	Unkown CV	DNA Mass (pg)
CCF and PSN	396	2.36	772	2.37	3.88
CCF and PSN	394	2.32	763	1.95	3.84
CCF and Rooster	391	2.37	504	2.02	2.53
CCF and Rooster	390	2.35	505	2.05	2.54
CCF and Rooster	393	2.40	506	2.02	2.54
CCF and Hen	393	2.39	493	2.17	2.48
CCF and Hen	391	2.41	493	2.23	2.48
CCF and Hen	391	2.30	492	2.17	2.47

Shovelnose sturgeon blood storage – Day 1

Contents	Standard Peak	Standard CV	Unknown Peak	Unkown CV	DNA Mass (pg)
CCF and Human	204	2.41	696	2.81	2.05
CCF and Human	204	2.43	699	2.80	2.04
CCF and Human	205	2.53	702	2.83	2.04
CCF and Rooster	397	2.53	508	2.07	2.56
CCF and Rooster	394	2.44	506	2.02	2.57
CCF and Rooster	393	2.51	504	2.03	2.56
CCF and Hen	392	2.48	490	2.24	2.50
CCF and Hen	396	2.53	490	2.20	2.47
CCF and Hen	391	2.50	490	2.31	2.51
CCF and SSN	395	2.44	766	2.08	3.88
CCF and SSN	394	2.48	766	2.10	3.89
CCF and SSN	393	2.45	762	2.22	3.88
CCF and SSN	392	2.46	766	1.97	3.91
CCF and SSN	393	2.55	767	1.98	3.90
CCF and SSN	391	2.46	771	1.86	3.94
CCF and SSN	392	2.48	769	2.00	3.92
CCF and SSN	394	2.55	765	1.95	3.88
CCF and SSN	391	2.48	767	2.01	3.92
CCF and SSN	394	2.49	768	1.96	3.90
CCF and SSN	393	2.46	765	2.04	3.89
CCF and SSN	396	2.49	769	2.28	3.88
CCF and Human	203	2.29	689	2.44	2.06
CCF and Human	202	2.42	689	2.50	2.05
CCF and Human	202	2.45	690	2.50	2.05
CCF and Rooster	390	2.54	504	2.09	2.58
CCF and Rooster	393	2.48	506	2.12	2.58
CCF and Rooster	394	2.49	506	2.05	2.57
CCF and Hen	390	2.50	489	2.23	2.51
CCF and Hen	390	2.52	488	2.31	2.50
CCF and Hen	390	2.57	491	2.27	2.52
CCF and SSN	393	2.51	770	1.98	3.92
CCF and SSN	388	2.47	756	2.08	3.90
CCF and SSN	392	2.50	772	2.11	3.94
CCF and SSN	392	2.54	763	2.06	3.89
CCF and SSN	393	2.60	772	2.37	3.93
CCF and SSN	392	2.65	770	2.18	3.93
CCF and SSN	395	2.62	763	2.32	3.86
CCF and SSN	395	2.62	771	2.08	3.90
CCF and SSN	397	2.58	773	2.14	3.89

(table cont.)

Contents	Standard Peak	Standard CV	Unknown Peak	Unkown CV	DNA Mass (pg)
CCF and SSN	391	2.56	761	2.36	3.89
CCF and SSN	390	2.49	766	2.01	3.93
CCF and PSN	395	2.55	767	2.30	3.88
CCF and PSN	393	2.52	763	2.01	3.88
CCF and Rooster	386	2.59	501	1.96	2.60
CCF and Rooster	392	2.58	502	2.04	2.56
CCF and Rooster	393	2.58	503	2.00	2.56
CCF and Hen	390	2.59	483	2.30	2.48
CCF and Hen	390	2.71	485	2.25	2.49
CCF and Hen	387	2.65	488	2.20	2.52
CCF and Human	201	2.54	688	2.59	2.05
CCF and Human	202	2.59	686	2.63	2.06
CCF and Human	202	2.61	692	2.65	2.04

Shortnose sturgeon blood storage – Day 2

Contents	Standard Peak	Standard CV	Unknown Peak	Unkown CV	DNA Mass (pg)
Human	--	--	703	4.02	7.03
Human	--	--	700	4.18	7.00
Human	--	--	693	4.19	6.93
CCF and Human	202	3.64	695	4.21	2.03
CCF and Human	201	3.62	690	4.33	2.04
CCF and Human	200	3.42	693	4.22	2.02
CCF and Rooster	397	2.23	513	2.06	2.58
CCF and Rooster	403	2.43	517	2.09	2.57
CCF and Rooster	405	2.4	516	2.04	2.55
CCF and Hen	404	2.36	501	2.41	2.48
CCF and Hen	401	2.34	506	2.40	2.52
CCF and Hen	405	2.37	505	2.38	2.49
CCF and SSN	401	2.35	782	2.06	3.90
CCF and SSN	401	2.33	794	2.23	3.96
CCF and SSN	403	2.35	792	2.30	3.93
CCF and SSN	401	2.35	784	2.01	3.91
CCF and SSN	402	2.4	784	2.07	3.90
CCF and SSN	402	2.43	794	2.04	3.95
CCF and SSN	404	2.36	788	2.13	3.90
CCF and SSN	404	2.49	785	2.23	3.89
CCF and SSN	399	2.35	779	1.97	3.90
CCF and Human	199	2.61	688	2.54	2.02
CCF and Human	199	2.46	690	2.56	2.02
CCF and Human	202	2.61	693	2.52	2.04
CCF and Rooster	398	2.4	517	2.23	2.60
CCF and Rooster	397	2.33	516	2.19	2.60
CCF and Rooster	399	2.3	516	2.08	2.59
CCF and Hen	398	2.33	494	2.40	2.48
CCF and Hen	394	2.32	493	2.38	2.50
CCF and Hen	395	2.27	495	2.38	2.51
CCF and SSN	394	2.34	782	1.99	3.97
CCF and SSN	398	2.28	777	2.03	3.90
CCF and SSN	398	2.27	781	2.27	3.92
CCF and SSN	399	2.27	783	1.91	3.92
CCF and SSN	396	2.32	775	2.04	3.91
CCF and SSN	396	2.24	780	1.96	3.94
CCF and SSN	397	2.3	771	1.98	3.88
CCF and SSN	400	2.31	784	2.16	3.92
CCF and SSN	393	2.23	767	1.96	3.90

(table cont.)

Contents	Standard Peak	Standard CV	Unknown Peak	Unkown CV	DNA Mass (pg)
CCF and SSN	397	2.24	779	2.05	3.92
CCF and SSN	397	2.22	782	1.91	3.94
CCF and SSN	397	2.22	774	2.10	3.90
CCF and SSN	396	2.27	774	2.20	3.91
CCF and SSN	396	2.28	774	2.07	3.91
CCF and PSN	396	2.25	779	2.17	3.93
CCF and PSN	396	2.28	780	2.04	3.94
CCF and Rooster	398	2.38	505	2.16	2.54
CCF and Rooster	391	2.23	504	2.02	2.58
CCF and Rooster	390	2.18	504	1.95	2.58
CCF and Hen	391	2.22	492	2.32	2.52
CCF and Hen	392	2.17	492	2.25	2.51
CCF and Hen	393	2.22	492	2.29	2.50

Shovelnose sturgeon blood storage – Day 3

Contents	Standard Peak	Standard CV	Unknown Peak	Unkown CV	DNA Mass (pg)
CCF and Human	197	3.03	703	3.18	1.99
CCF and Human	197	3.07	694	3.23	1.99
CCF and Human	194	3.39	698	3.12	1.96
CCF and Rooster	399	2.50	518	2.16	2.61
CCF and Rooster	401	2.81	521	2.50	2.63
CCF and Rooster	401	2.78	522	2.52	2.63
CCF and Hen	399	2.65	506	2.64	2.55
CCF and Hen	396	2.65	507	2.64	2.56
CCF and Hen	401	2.62	504	2.55	2.54
CCF and SSN	400	2.53	791	2.19	3.99
CCF and SSN	402	2.60	817	2.28	4.12
CCF and SSN	400	2.55	791	2.42	3.99
CCF and SSN	402	2.56	796	2.25	4.01
CCF and SSN	397	2.47	791	2.10	3.99
CCF and SSN	400	2.46	796	1.96	4.01
CCF and SSN	398	2.47	795	1.97	4.01
CCF and SSN	398	2.43	790	2.09	3.98
CCF and SSN	396	2.39	785	2.10	3.96
CCF and SSN	396	2.44	789	2.11	3.98
CCF and SSN	400	2.42	785	2.03	3.96
CCF and SSN	401	2.44	790	2.19	3.98
CCF and Human	197	2.47	695	2.15	1.99
CCF and Human	197	2.45	692	2.31	1.99
CCF and Human	197	2.70	696	2.25	1.99
CCF and Rooster	397	2.42	516	2.18	2.60
CCF and Rooster	397	2.44	515	2.25	2.60
CCF and Rooster	396	2.58	514	2.30	2.59
CCF and Hen	398	2.48	502	2.50	2.53
CCF and Hen	397	2.41	498	2.52	2.51
CCF and Hen	396	2.46	501	2.36	2.53
CCF and SSN	398	2.43	796	2.31	4.01
CCF and SSN	396	2.50	788	1.95	3.97
CCF and SSN	392	2.38	778	1.81	3.92
CCF and SSN	397	2.49	785	2.11	3.96
CCF and SSN	396	2.46	776	2.02	3.91
CCF and SSN	398	2.46	784	2.19	3.95
CCF and SSN	398	2.50	784	2.10	3.95
CCF and SSN	399	2.54	789	2.23	3.98
CCF and SSN	396	2.59	795	2.19	4.01

(table cont.)

Contents	Standard Peak	Standard CV	Unknown Peak	Unkown CV	DNA Mass (pg)
CCF and SSN	397	2.42	789	2.01	3.98
CCF and SSN	396	2.45	780	2.07	3.93
CCF and SSN	394	2.48	772	2.03	3.89
CCF and PSN	394	2.45	785	2.25	3.96
CCF and PSN	390	2.21	767	1.78	3.87
CCF and Rooster	393	2.35	504	2.06	2.54
CCF and Rooster	391	2.30	506	2.15	2.55
CCF and Rooster	389	2.22	509	2.05	2.57
CCF and Hen	388	2.30	489	2.38	2.47
CCF and Hen	392	2.38	494	2.44	2.49
CCF and Hen	389	2.34	489	2.44	2.47
CCF and Human	197	2.61	695	1.95	1.99
CCF and Human	197	2.42	694	1.89	1.99
CCF and Human	196	2.28	690	1.90	1.98

Shovelnose sturgeon blood storage – Day 4

Contents	Standard Peak	Standard CV	Unknown Peak	Unkown CV	DNA Mass (pg)
CCF and Human	695	3.00	200	2.87	2.01
CCF and Human	695	3.05	201	2.96	2.02
CCF and Human	692	3.02	200	2.98	2.01
CCF and Rooster	399	2.79	515	2.28	2.59
CCF and Rooster	393	2.85	513	2.32	2.58
CCF and Rooster	401	2.75	523	2.23	2.63
CCF and Hen	401	2.72	511	2.62	2.57
CCF and Hen	402	2.69	507	2.67	2.55
CCF and Hen	400	2.72	508	2.66	2.55
CCF and SSN	403	2.70	797	2.17	4.01
CCF and SSN	401	2.72	819	2.39	4.12
CCF and SSN	404	2.65	794	2.35	3.99
CCF and SSN	408	2.64	820	2.29	4.12
CCF and SSN	402	2.69	792	2.03	3.98
CCF and SSN	402	2.67	796	1.93	4.00
CCF and SSN	403	2.58	792	2.10	3.98
CCF and SSN	399	2.68	788	2.18	3.96
CCF and SSN	396	2.57	788	2.09	3.96
CCF and SSN	397	2.71	782	2.07	3.93
CCF and SSN	398	2.72	781	2.14	3.93
CCF and SSN	401	2.74	798	2.33	4.01
CCF and Human	701	2.29	197	2.95	1.98
CCF and Human	688	2.38	197	2.77	1.98
CCF and Human	690	2.38	198	2.65	1.99
CCF and Rooster	396	2.76	514	2.21	2.58
CCF and Rooster	400	2.67	509	2.22	2.56
CCF and Rooster	400	2.73	518	2.26	2.60
CCF and Hen	395	2.68	501	2.62	2.52
CCF and Hen	394	2.82	496	2.73	2.49
CCF and Hen	396	2.76	499	2.77	2.51
CCF and SSN	400	2.69	791	1.98	3.98
CCF and SSN	394	2.58	779	2.03	3.92
CCF and SSN	399	2.69	790	2.10	3.97
CCF and SSN	399	2.67	782	1.98	3.93
CCF and SSN	399	2.70	796	2.33	4.00
CCF and SSN	401	2.65	786	2.17	3.95
CCF and SSN	398	2.64	785	2.15	3.95
CCF and SSN	397	2.74	805	2.19	4.05
CCF and SSN	400	2.73	787	2.25	3.96

(table cont.)

Contents	Standard Peak	Standard CV	Unknown Peak	Unkown CV	DNA Mass (pg)
CCF and SSN	397	2.75	782	2.57	3.93
CCF and SSN	400	2.74	787	2.17	3.96
CCF and PSN	397	2.74	786	2.36	3.95
CCF and PSN	398	2.74	786	2.28	3.95
CCF and Rooster	397	2.78	511	2.27	2.57
CCF and Rooster	393	2.68	510	2.13	2.56
CCF and Rooster	394	2.62	509	2.09	2.56
CCF and Hen	394	2.55	496	2.62	2.49
CCF and Hen	393	2.56	496	2.44	2.49
CCF and Hen	394	2.56	497	2.59	2.50
CCF and Human	703	2.18	199	2.66	2.00

Shovelnose sturgeon blood storage – Day 7

Contents	Standard Peak	Standard CV	Unknown Peak	Unkown CV	DNA Mass (pg)
CCF and Human	691	3.09	205	3.74	2.06
CCF and Human	705	3.04	205	4.48	2.06
CCF and Human	697	3.00	205	3.35	2.06
CCF and Rooster	401	2.82	516	2.48	2.60
CCF and Rooster	400	2.88	513	2.45	2.58
CCF and Rooster	399	2.81	517	2.14	2.60
CCF and Hen	399	2.84	504	2.45	2.54
CCF and Hen	399	2.77	498	2.47	2.51
CCF and Hen	396	2.77	497	2.36	2.50
CCF and SSN	403	2.74	784	2.94	3.95
CCF and SSN	405	2.87	854	2.28	4.30
CCF and SSN	405	2.73	785	2.32	3.95
CCF and SSN	407	2.89	857	2.28	4.31
CCF and SSN	402	2.75	782	2.52	3.94
CCF and SSN	403	2.75	795	1.95	4.00
CCF and SSN	394	2.81	804	2.50	4.05
CCF and SSN	401	2.66	786	3.16	3.96
CCF and SSN	398	2.62	781	2.78	3.93
CCF and SSN	399	2.64	791	2.80	3.98
CCF and SSN	397	2.72	777	1.99	3.91
CCF and SSN	403	2.76	806	2.84	4.06
CCF and Human	692	2.44	201	2.49	2.02
CCF and Human	691	2.58	200	2.58	2.01
CCF and Human	693	2.56	201	2.49	2.02
CCF and Rooster	397	2.76	509	2.34	2.56
CCF and Rooster	398	2.75	513	2.34	2.58
CCF and Rooster	400	2.72	510	2.28	2.57
CCF and Hen	398	2.66	496	2.44	2.50
CCF and Hen	394	2.60	492	2.39	2.48
CCF and Hen	398	2.70	497	2.57	2.50
CCF and SSN	399	2.72	778	2.19	3.92
CCF and SSN	400	2.73	780	2.40	3.93
CCF and SSN	396	2.72	780	2.09	3.93
CCF and SSN	400	2.64	776	2.17	3.91
CCF and SSN	398	2.64	787	2.97	3.96
CCF and SSN	394	2.71	784	2.50	3.95
CCF and SSN	397	2.84	812	2.95	4.09
CCF and SSN	398	2.73	825	2.17	4.15
CCF and SSN	400	2.64	778	2.07	3.92

(table cont.)

Contents	Standard Peak	Standard CV	Unknown Peak	Unkown CV	DNA Mass (pg)
CCF and SSN	395	2.72	775	2.57	3.90
CCF and SSN	397	2.65	778	2.31	3.92
CCF and PSN	399	2.60	781	2.24	3.93
CCF and PSN	396	2.53	777	2.05	3.91
CCF and Rooster	391	2.60	505	2.19	2.54
CCF and Rooster	398	2.66	509	2.30	2.56
CCF and Rooster	392	2.66	510	2.28	2.57
CCF and Hen	396	2.68	497	2.43	2.50
CCF and Hen	395	2.68	492	2.44	2.48
CCF and Hen	396	2.73	494	2.54	2.49
CCF and Human	692	2.63	200	2.43	2.01
CCF and Human	689	2.65	201	2.55	2.02
CCF and Human	686	2.66	199	2.53	2.00

Shovelnose sturgeon blood storage – Day 8

Contents	Standard Peak	Standard CV	Unknown Peak	Unkown CV	DNA Mass (pg)
CCF and Human	695	3.23	203	2.69	2.06
CCF and Human	693	3.19	202	2.73	2.05
CCF and Human	696	2.23	203	2.74	2.06
CCF and Rooster	401	2.89	519	2.25	2.64
CCF and Rooster	405	2.73	522	2.22	2.65
CCF and Rooster	404	2.71	520	2.26	2.64
CCF and Hen	404	2.70	506	2.57	2.57
CCF and Hen	405	2.63	504	2.56	2.56
CCF and Hen	397	2.69	500	2.45	2.54
CCF and SSN	405	2.66	805	3.20	4.09
CCF and SSN	407	2.72	855	2.39	4.34
CCF and SSN	406	2.71	792	2.43	4.02
CCF and SSN	406	2.79	872	2.27	4.43
CCF and SSN	404	2.75	791	2.76	4.02
CCF and SSN	398	2.70	793	2.02	4.03
CCF and SSN	400	2.76	814	2.44	4.14
CCF and SSN	400	2.72	792	3.25	4.02
CCF and SSN	397	2.55	784	2.75	3.98
CCF and SSN	401	2.62	795	2.75	4.04
CCF and SSN	397	2.69	777	2.09	3.95
CCF and SSN	399	2.61	789	2.74	4.01
CCF and Human	680	2.50	197	2.41	2.00
CCF and Human	684	2.60	197	2.48	2.00
CCF and Human	685	2.58	198	2.59	2.01
CCF and Rooster	400	2.71	509	2.32	2.59
CCF and Rooster	399	2.73	516	2.39	2.62
CCF and Rooster	391	2.71	508	2.23	2.58
CCF and Hen	396	2.63	493	2.45	2.50
CCF and Hen	397	2.65	492	2.43	2.50
CCF and Hen	394	2.67	494	2.38	2.51
CCF and SSN	400	2.63	785	2.19	3.99
CCF and SSN	391	2.57	770	2.75	3.91
CCF and SSN	396	2.68	780	2.14	3.96
CCF and SSN	398	2.57	774	2.26	3.93
CCF and SSN	397	2.58	791	2.86	4.02
CCF and SSN	392	2.65	780	2.57	3.96
CCF and SSN	386	2.68	798	2.80	4.05
CCF and SSN	394	2.80	832	2.11	4.23
CCF and SSN	397	2.67	780	2.08	3.96

(table cont.)

Contents	Standard Peak	Standard CV	Unknown Peak	Unkown CV	DNA Mass (pg)
CCF and SSN	393	2.69	773	2.57	3.93
CCF and SSN	400	2.60	777	2.15	3.95
CCF and PSN	397	2.65	776	2.17	3.94
CCF and PSN	400	2.61	774	1.91	3.93
CCF and SSN	399	2.72	840	2.33	4.27
CCF and SSN	394	2.76	842	2.21	4.28
CCF and Rooster	394	2.61	505	2.13	2.57
CCF and Rooster	395	2.61	511	2.16	2.60
CCF and Rooster	396	2.71	510	2.21	2.59
CCF and Hen	394	2.60	492	2.39	2.50
CCF and Hen	390	2.65	490	2.43	2.49
CCF and Hen	393	2.60	490	2.39	2.49
CCF and Human	674	2.41	197	2.42	2.00
CCF and Human	678	2.53	199	2.43	2.02
CCF and Human	674	2.47	197	2.44	2.00

Shovelnose sturgeon blood storage – Day 11

Contents	Standard Peak	Standard CV	Unknown Peak	Unkown CV	DNA Mass (pg)
CCF and Rooster	400	2.24	513	2.11	2.56
CCF and Rooster	397	2.24	515	2.12	2.57
CCF and Rooster	400	2.24	518	2.09	2.59
CCF and Hen	400	2.23	498	2.42	2.49
CCF and Hen	397	2.30	497	2.35	2.48
CCF and Hen	397	2.30	497	2.36	2.48
CCF and SSN	402	2.24	834	2.98	4.17
CCF and SSN	405	2.23	863	2.15	4.31
CCF and SSN	399	2.22	783	2.51	3.91
CCF and SSN	406	2.30	886	2.34	4.43
CCF and SSN	400	2.28	817	2.86	4.08
CCF and SSN	399	2.23	801	2.19	4.00
CCF and SSN	402	2.32	856	2.41	4.28
CCF and SSN	398	2.36	806	3.13	4.03
CCF and SSN	401	2.26	801	3.17	4.00
CCF and SSN	401	2.28	809	2.87	4.04
CCF and SSN	400	2.22	786	2.17	3.93
CCF and SSN	402	2.22	819	2.72	4.09
CCF and Human	704	2.19	205	2.18	2.05
CCF and Human	702	2.17	204	2.23	2.04
CCF and Human	704	2.26	206	2.21	2.06
CCF and Rooster	401	2.20	517	2.03	2.58
CCF and Rooster	402	2.26	514	2.08	2.57
CCF and Rooster	401	2.23	517	2.12	2.58
CCF and Hen	400	2.21	502	2.38	2.51
CCF and Hen	399	2.28	500	2.28	2.50
CCF and Hen	399	2.23	499	2.30	2.49
CCF and SSN	403	2.19	821	2.49	4.10
CCF and SSN	401	2.14	809	3.18	4.04
CCF and SSN	405	2.19	800	2.24	4.00
CCF and SSN	399	2.28	800	2.39	4.00
CCF and SSN	402	2.24	823	2.47	4.11
CCF and SSN	400	2.35	817	2.62	4.08
CCF and SSN	400	2.37	838	2.57	4.19
CCF and SSN	401	2.40	860	2.22	4.30
CCF and SSN	402	2.25	785	1.82	3.92
CCF and SSN	400	2.42	801	2.85	4.00
CCF and SSN	401	2.28	785	2.04	3.92
CCF and PSN	405	2.25	796	1.81	3.98

(table cont.)

Contents	Standard Peak	Standard CV	Unknown Peak	Unknown CV	DNA Mass (pg)
CCF and PSN	402	2.36	789	1.68	3.94
CCF and Rooster	401	2.35	515	1.96	2.57
CCF and Rooster	400	2.25	512	1.99	2.56
CCF and Rooster	402	2.28	516	2.01	2.58
CCF and Hen	398	2.37	502	2.26	2.51
CCF and Hen	398	2.34	502	2.32	2.51
CCF and Hen	399	2.36	500	2.34	2.50
CCF and Human	692	2.07	203	2.11	2.03
CCF and Human	702	2.02	205	2.23	2.05
CCF and Human	705	2.04	205	2.13	2.05

Genome Size Analysis

Sample	CCF Mode Channel	CCF CV	Unknown Mode Channel	Unknown CV	DNA Value (pg)
Human	696	2.65			
CCF and Hen	401	2.43	503	2.40	2.545
CCF and Hen	400	2.48	503	2.29	2.545
CCF and Hen	397	2.55	505	2.37	2.556
CCF and Hen	396	2.47	501	2.33	2.535
CCF and Hen	397	2.47	498	2.28	2.520
CCF and Hen	398	2.46	504	2.34	2.550
CCF and Hen	400	2.41	502	2.25	2.540
CCF and Hen	400	2.43	503	2.27	2.545
CCF and Hen	396	2.49	497	2.27	2.515
CCF and Human	697	2.65	202	2.36	1.022
CCF and Human	697	2.62	202	2.32	1.022
CCF and Human	694	2.79	201	2.41	1.017
CCF and Human	687	2.92	201	2.20	1.017
CCF and Human	688	3.25	201	2.45	1.017
CCF and Human	693	3.36	202	2.52	1.022
CCF and Human	687	2.60	201	2.25	1.017
CCF and Human	684	2.20	201	2.20	1.017
CCF and Human	692	2.44	201	2.23	1.017
CCF and Pallid	402	2.43	792	2.05	4.008
CCF and Pallid	404	2.41	786	2.12	3.978
CCF and Pallid	403	2.35	791	2.00	4.003
CCF and Pallid	407	2.35	792	2.22	4.008
CCF and Pallid	402	2.38	789	2.11	3.993
CCF and Pallid	403	2.45	784	2.13	3.968
CCF and Pallid	401	2.38	788	1.89	3.988
CCF and Pallid	395	2.26	774	2.10	3.917
CCF and Pallid	404	2.30	784	1.89	3.968
CCF and Pallid	402	2.37	789	2.38	3.993
CCF and Pallid	405	2.40	789	1.97	3.993
CCF and Pallid	403	2.04	788	1.83	3.988
CCF and Pallid	402	2.57	782	1.95	3.958
CCF and Pallid	402	2.54	781	2.07	3.953
CCF and Pallid	405	2.40	794	2.14	4.018
CCF and Pallid	401	2.35	783	2.03	3.963
CCF and Pallid	398	2.12	784	1.89	3.968
CCF and Pallid	400	2.26	779	1.88	3.942
CCF and Pallid	401	1.98	781	1.72	3.953
CCF and Pallid	399	2.23	781	1.71	3.953
CCF and Rooster	401	2.51	519	2.04	2.626
CCF and Rooster	403	2.45	522	2.00	2.642

(table cont.)

Sample	CCF Mode Channel	CCF CV	Unknown Mode Channel	Unknown CV	DNA Value (pg)
CCF and Rooster	394	2.45	512	1.93	2.591
CCF and Rooster	396	2.56	512	2.07	2.591
CCF and Rooster	397	2.47	513	2.05	2.596
CCF and Rooster	402	2.40	516	2.00	2.611
CCF and Rooster	399	2.46	514	2.00	2.601
CCF and Rooster	400	2.45	515	1.99	2.606
CCF and Roster	401	2.49	519	2.01	2.626
CCF and Shovel	408	2.35	798	1.93	4.039
CCF and Shovel	406	2.36	792	2.37	4.008
CCF and Shovel	403	2.37	783	1.89	3.963
CCF and Shovel	407	2.39	784	1.87	3.968
CCF and Shovel	401	2.35	785	1.76	3.973
CCF and Shovel	406	2.36	793	2.00	4.013
CCF and Shovel	404	2.35	780	2.15	3.947
CCF and Shovel	400	2.34	780	2.10	3.947
CCF and Shovel	404	2.38	780	2.18	3.947
CCF and Shovel	400	2.42	777	1.81	3.932
CCF and Shovel	405	2.37	786	1.87	3.978
CCF and Shovel	406	2.36	790	1.95	3.998
CCF and Shovel	402	2.38	783	1.95	3.963
CCF and Shovel	401	2.52	782	1.98	3.958
CCF and Shovel	405	2.45	789	1.96	3.993
CCF and Shovel	398	2.46	783	2.00	3.963
CCF and Shovel	402	2.41	782	2.41	3.958
CCF and Shovel	402	2.34	785	1.87	3.973
CCF and Shovel	405	2.36	785	1.81	3.973
CCF and Shovel	403	2.42	791	2.14	4.003

Chapter 4 - Bacterial Contamination of Sperm Samples Unanalyzed Data

Shortnose Sturgeon Storage Study

Fish #	Antibiotic	Concentration (µg/ml)	Motility (%)																						
			Time (d)			3	4	5	6	7	8	10	11	12	13	14	15	16	17	18	19	20	21	22	23
			0	1	2																				
1	Enrofloxacin	1	90	90	85	75	60	60	55	40	65	40	60	0	0	0	0	0	0	0	0	0	0	0	0
2	Enrofloxacin	1	95	80	80	85	85	85	85	85	75	80	70	70	65	60	65	50	40	30	0	0	0	0	0
3	Enrofloxacin	1	95	90	90	90	90	85	80	75	75	70	70	70	60	60	65	60	35	30	25	5	0	0	0
4	Enrofloxacin	1	90	90	90	65	70	70	50	50	65	5	0.5	0	0	0	0	0	0	0	0	0	0	0	0
5	Enrofloxacin	1	90	75	75	45	45	50	60	30	45	25	35	35	20	1	0	0	0	0	0	0	0	0	0
1	Enrofloxacin	5	90	90	85	75	75	55	65	60	50	45	30	25	20	0	0	0	0	0	0	0	0	0	0
2	Enrofloxacin	5	95	70	70	75	75	80	85	70	70	70	70	65	55	55	40	35	35	40	20	1	0.5	0	0
3	Enrofloxacin	5	95	90	90	90	85	85	75	80	70	80	70	65	70	70	65	65	55	35	35	30	10	0	0
4	Enrofloxacin	5	90	90	85	75	60	75	75	50	65	50	50	30	20	1	0	0	0	0	0	0	0	0	0
5	Enrofloxacin	5	90	75	70	45	50	65	60	55	45	20	35	45	25	20	10	0	0	0	0	0	0	0	0
1	Enrofloxacin	10	90	90	90	80	75	75	60	60	35	40	50	25	20	0	0	0	0	0	0	0	0	0	0
2	Enrofloxacin	10	95	80	80	90	85	80	85	80	80	75	60	70	70	60	60	60	40	35	0	0	0	0	0
3	Enrofloxacin	10	95	90	90	90	85	85	75	75	80	75	60	70	65	65	70	60	40	45	30	20	20	0	0
4	Enrofloxacin	10	90	90	90	70	60	75	50	50	50	45	50	10	10	0	0	0	0	0	0	0	0	0	0
5	Enrofloxacin	10	90	75	60	45	50	35	50	35	35	30	25	25	25	15	0.5	0	0	0	0	0	0	0	0
1	Control	--	90	90	95	70	65	60	60	50	55	40	0.5	0	0	0	0	0	0	0	0	0	0	0	0
2	Control	--	95	90	95	90	80	90	85	80	70	70	65	75	45	60	65	35	35	30	0	0	0	0	0
3	Control	--	95	90	90	85	85	90	75	75	70	75	75	75	65	65	70	70	45	50	35	25	15	0	0
4	Control	--	90	95	85	65	65	65	60	70	55	40	15	0.5	0	0	0	0	0	0	0	0	0	0	0
5	Control	--	90	80	70	65	50	70	70	50	40	25	25	50	25	10	0	0	0	0	0	0	0	0	0
1	Gentamycin	2	90	90	85	75	70	80	60	60	65	35	50	35	55	25	0	0	0	0	0	0	0	0	0
2	Gentamycin	2	95	85	80	85	90	90	85	80	75	80	60	65	55	70	65	60	60	50	35	15	5	0	0
3	Gentamycin	2	95	90	80	90	85	85	80	80	80	75	70	50	70	60	65	70	35	50	35	20	15	0	0
4	Gentamycin	2	90	95	90	75	65	65	50	40	55	50	50	25	1	0	0	0	0	0	0	0	0	0	0
5	Gentamycin	2	90	85	75	75	60	65	50	45	50	25	30	40	40	25	20	10	0	0	0	0	0	0	0

(table cont.)

Fish #	Antibiotic	Concentration (µg/ml)	Motility (%)																						
			Time (d)			3	4	5	6	7	8	10	11	12	13	14	15	16	17	18	19	20	21	22	23
			0	1	2																				
1	Gentamycin	10	90	90	85	75	70	65	55	65	30	30	35	35	40	35	35	25	10	10	1	0	0	0	0
2	Gentamycin	10	95	75	80	85	75	85	80	80	80	60	50	50	65	65	50	50	40	30	0	0	0	0	0
3	Gentamycin	10	95	90	90	90	85	90	75	80	80	75	75	70	65	65	60	65	40	50	40	35	20	10	10
4	Gentamycin	10	90	90	90	70	60	70	50	75	60	45	50	25	1	0.5	0	0	0	0	0	0	0	0	0
5	Gentamycin	10	90	80	75	60	60	50	55	50	40	25	30	30	35	30	25	10	0	0	0	0	0	0	0
1	Gentamycin	20	90	85	90	75	75	75	60	65	60	25	40	55	40	40	5	0	0	0	0	0	0	0	0
2	Gentamycin	20	95	80	75	85	80	85	80	80	75	80	60	65	60	50	60	55	50	45	40	25	20	0.5	0
3	Gentamycin	20	95	85	95	90	90	80	80	80	75	75	65	70	60	70	65	70	40	40	40	20	10	0	0
4	Gentamycin	20	90	90	90	75	75	65	70	50	60	55	50	50	40	20	1	0	0	0	0	0	0	0	0
5	Gentamycin	20	90	65	75	70	65	45	40	55	55	30	20	40	35	35	15	10	0	0	0	0	0	0	0
1	Oxytetracyclin	6	90	85	90	70	70	70	70	70	60	40	40	35	30	0.5	0	0	0	0	0	0	0	0	0
2	Oxytetracyclin	6	95	75	80	85	85	90	70	80	80	70	65	65	55	60	60	55	35	35	0	0	0	0	0
3	Oxytetracyclin	6	95	90	90	85	75	80	75	75	85	70	65	60	60	50	60	70	50	45	35	35	20	0	0
4	Oxytetracyclin	6	90	80	90	60	45	60	50	50	60	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0
5	Oxytetracyclin	6	90	60	70	45	60	70	50	35	65	25	10	25	25	10	0	0	0	0	0	0	0	0	0
1	Oxytetracyclin	30	90	90	90	75	70	65	40	50	40	35	30	5	0	0	0	0	0	0	0	0	0	0	0
2	Oxytetracyclin	30	95	80	80	90	85	85	80	80	75	75	65	55	65	65	35	40	20	20	0	0	0	0	0
3	Oxytetracyclin	30	95	95	90	85	90	85	80	75	75	80	60	75	70	60	65	70	40	40	30	35	20	15	5
4	Oxytetracyclin	30	90	90	90	65	70	60	50	45	50	10	0.5	0	0	0	0	0	0	0	0	0	0	0	0
5	Oxytetracyclin	30	90	80	75	55	50	45	30	20	50	20	25	20	20	0.5	0	0	0	0	0	0	0	0	0
1	Oxytetracyclin	60	90	90	80	85	70	75	55	60	65	40	30	0.5	0	0	0	0	0	0	0	0	0	0	0
2	Oxytetracyclin	60	95	90	85	85	85	85	80	80	70	70	70	65	50	55	60	60	30	30	0	0	0	0	0
3	Oxytetracyclin	60	95	95	90	90	90	90	85	75	80	75	70	65	70	45	70	65	45	35	40	25	10	0	0
4	Oxytetracyclin	60	90	95	90	50	40	40	45	50	55	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0
5	Oxytetracyclin	60	90	70	70	60	40	55	40	40	60	20	30	30	35	10	0.5	0	0	0	0	0	0	0	0

Shortnose Sturgeon Fertilization Trial

Fish #	Antibiotic	Concentration (µg/ml)	Motility (%)	Fertilization (%)	Hatch (%)
272B	Control *	--	95	86	43
272B	Control *	--	95	86	22
3	No Antibiotic	--	15	23	7
3	Enrofloxacin	5	10	24	21
3	Enrofloxacin	10	20	15	8
3	Gentamycin	2	15	38	20
2	Gentamycin	2	5	1	1
3	Gentamycin	10	20	76	22
2	Gentamycin	20	20	46	17
3	Gentamycin	20	10	6	5
3	Oxytetracyclin	6	20	81	45
3	Oxytetracyclin	30	20	77	36
3	Oxytetracyclin	60	10	41	22

* Fresh sperm control.

Pallid Sturgeon Storage Study

Fish	Treatment	Concentration (µg/ml)	Motility (%)																		
			Time (d)			3	4	5	6	8	9	10	11	12	13	15	16	17	18	19	20
			0	2	3																
1	Enrofloxacin	10	90	5	35	55	35	50	40	35	25	25	25	10	10	5	0.5	0	0	0	0
2	Enrofloxacin	10	90	85	85	50	70	70	70	65	60	40	45	40	35	5	0.5	0	0	0	0
3	Enrofloxacin	10	85	80	85	80	75	45	55	50	40	35	35	25	30	10	1	0	0	0	0
1	Enrofloxacin	2	90	40	75	40	45	45	40	35	30	35	25	20	25	15	10	5	5	1	1
2	Enrofloxacin	2	90	90	80	65	80	75	70	65	60	60	45	40	25	5	1	0	0	0	0
3	Enrofloxacin	2	85	50	85	85	70	70	55	35	50	35	40	15	30	10	0.5	0	0	0	0
1	Enrofloxacin	20	90	50	5	55	50	50	50	25	25	15	5	10	10	0	0	0	0	0	0
2	Enrofloxacin	20	90	60	75	70	80	65	75	60	65	60	25	35	20	1	0	0	0	0	0
3	Enrofloxacin	20	85	85	85	80	75	55	65	35	40	30	30	20	30	5	0.5	0	0	0	0
1	Gentamycin	--	85	90	85	80	85	65	50	30	35	30	35	20	30	0	0	0	0	0	0
2	Gentamycin	--	90	35	80	70	75	40	65	55	60	35	40	25	25	10	5	5	1	1	1
3	Gentamycin	--	90	90	45	65	65	65	75	60	55	45	35	20	10	0	0	0	0	0	0
1	Gentamycin	10	90	35	70	50	10	60	50	45	35	45	25	20	10	1	0	0	0	0	0
2	Gentamycin	10	90	65	80	80	60	80	75	60	55	50	45	25	30	5	0	0	0	0	0
3	Gentamycin	10	85	60	75	75	70	70	60	40	55	30	30	25	25	20	5	1	0	0	0
1	Gentamycin	2	90	65	50	35	55	50	30	35	25	20	25	15	5	0.5	0	0	0	0	0
2	Gentamycin	2	90	90	80	85	65	65	75	45	55	50	30	30	15	0	0	0	0	0	0
3	Gentamycin	2	85	70	85	80	85	60	60	40	40	25	25	10	30	5	5	1	0.5	0	0
1	Gentamycin	20	90	70	15	60	50	55	60	50	30	30	25	20	25	5	1	0	0	0	0
2	Gentamycin	20	90	50	85	80	80	60	80	70	65	55	50	25	35	0	0	0	0	0	0
3	Gentamycin	20	85	80	75	75	70	60	55	40	50	35	40	30	35	10	5	0	0	0	0
1	Oxytetracyclin	10	90	65	65	50	35	40	60	40	45	30	30	25	20	10	5	1	1	1	1
2	Oxytetracyclin	10	90	10	85	70	70	75	70	65	50	55	35	35	10	1	0	0	0	0	0
3	Oxytetracyclin	10	85	15	85	80	75	65	70	45	40	45	30	30	25	10	10	0.5	0	0	0
1	Oxytetracyclin	2	90	75	65	65	40	40	45	30	25	25	20	15	10	15	5	5	1	1	0.5
2	Oxytetracyclin	2	90	75	70	40	65	65	70	70	65	50	45	30	30	1	0	0	0	0	0
3	Oxytetracyclin	2	85	75	80	75	75	65	60	50	45	20	30	30	25	10	1	0	0	0	0
1	Oxytetracyclin	20	90	60	60	65	50	40	30	30	25	20	25	30	15	10	5	1	0.5	0	0
2	Oxytetracyclin	20	90	20	80	65	60	80	75	50	60	40	55	30	30	5	0.5	0	0	0	0
3	Oxytetracyclin	20	85	60	85	80	70	65	55	50	35	35	35	30	25	5	5	0	0	0	0

Shovelnose Sturgeon Bacterial Survival

Sample	Bacterial colonies per plate									
	Plate 10 ⁰	Plate 10 ⁻¹	Plate 10 ⁻²	Plate 10 ⁻³	Plate 10 ⁻⁴	Plate 10 ⁻⁵	Plate 10 ⁻⁶	Plate 10 ⁻⁷	Plate 10 ⁻⁸	Plate 10 ⁻⁹
4-3	TNTC *	TNTC	TNTC	TNTC	207	16	1	0	0	0
5-4	TNTC	TNTC	TNTC	TNTC	271	27	2	0	0	0
5-6	TNTC	TNTC	TNTC	359	34	4	1	0	0	0
5-7	TNTC	TNTC	TNTC	TNTC	TNTC	160	27	1	0	0
7-1	TNTC	TNTC	406	41	6	0	0	0	0	0
12-4	TNTC	TNTC	TNTC	TNTC	TNTC	255	35	7	0	0
14-4	TNTC	TNTC	TNTC	TNTC	TNTC	116	16	0	0	0
19-1	TNTC	TNTC	TNTC	TNTC	TNTC	265	40	4	0	0

* TNTC = Too numerous to count.

Chapter 5 – Development of Refrigerated Storage Methods Unanalyzed Data

Blood and Seminal Plasma osmolality

Lake sturgeon

Date	Fish #	Osmolality
	989	89
	4385	131
	4384	90
	4382	105
	4381	113
05/04/2001	1	92
	2	127
	3	121
	4	157
	5	101
	6	124
	7	89
	8	120
Mean		112.23
SD		20.44

Shortnose sturgeon

Date	Fish #	Osmolality (mOsmol/kg)	
		Seminal	Blood
3/2/1999	625F	27	272
	813	77	266
	5A51	17	268
	706C	93	269
	2C40	57	266
	6C09	50	273
	6028	33	270
	638	30	284
3/9/1999	3C12	84	267
	7E7C	63	255
	1863	63	263
	256C	74	258
3/16/1999	3C12	82	272
	5D2F	107	272
	813	56	266
	256C	158	270
	706C	68	263
	6C09	70	268

Pallid Sturgeon

Date	Fish #	Osmolality (mOsmol/kg)	
		Seminal	Blood
6/23/2000	453A	30	260
	4552	29	258
	3A65	41	261
	3350	51	266
	194B	62	250
	4773	36	254
	6E31	117	250

Shovelnose Sturgeon

Date	Fish #	Osmolality (mOsmol/kg)	
		Seminal	Blood
6/6/2001	1	77	281
	3	96	286
	5	78	273
	8	69	277
	9	70	283
	10	77	277
	11	85	281
	12	84	286
	13	76	277

Blood and seminal plasma composition

Lake sturgeon

Seminal plasma

	Glucose	Magnesium	Phosphorus	Calcium	Sodium	Potassium	Chloride
Fish #	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)
4385	0.44	0.45	2.58	0.07	41.00	5.45	36.30
4381	0.50	0.41	2.52	0.05	25.40	4.25	59.90
989	0.72	0.45	3.91	0.07	32.70	3.51	32.60
4382	0.56	0.45	3.55	0.15	33.70	6.12	36.90
4384	0.61	0.41	2.74	0.05	37.30	5.48	37.60

Blood plasma

	Glucose	Magnesium	Phosphorus	Calcium	Sodium	Potassium	Chloride
Fish #	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)
988	10.82	0.78	1.80	0.65	80.00	4.79	70.50
993	13.21	1.15	1.98	0.97	131.30	4.94	95.90
4381	12.32	1.07	2.56	1.10	114.00	3.19	88.10

Pallid Sturgeon

Seminal Plasma							
Fish #	Glucose (mM)	Magnesium (mM)	Phosphorus (mM)	Calcium (mM)	Sodium (mM)	Potassium (mM)	Chloride (mM)
3350	0.000	0.420	2.267	0.0	27	1.17	31
4552	0.000	0.436	0.894	0.0	16	1.10	19
3A65	0.218	0.469	1.146	0.1	27	2.83	28
194B	0.218	0.399	1.214	0.1	34	2.67	32
453A	0.048	0.407	1.860	0.0	23	1.49	28
4773	0.000	0.276	0.601	0.0	22	1.81	25
6E31	1.044	0.576	2.189	0.1	61	3.32	51

Blood Plasma							
Fish #	Glucose (mM)	Magnesium (mM)	Phosphorus (mM)	Calcium (mM)	Sodium (mM)	Potassium (mM)	Chloride (mM)
1579	37.139	1.156	1.714	1.023	123	2.20	86
4552	39.044	1.341	1.831	1.193	126	2.64	86
194B	35.124	1.164	1.582	1.110	122	2.47	84
3350	39.645	1.403	2.089	1.245	131	2.66	90
3A65	46.385	1.247	1.621	1.290	128	2.73	89
453A	39.645	1.333	2.205	1.255	128	2.59	89
4773	35.891	1.148	1.814	1.013	124	2.20	88
6E31	36.575	1.123	1.291	1.075	121	2.39	88
755B	37.750	1.255	1.921	1.228	126	2.57	89

Shortnose sturgeon

Seminal plasma

Fish #	Glucose (mM)	Magnesium (mM)	Phosphorus (mM)	Calcium (mM)	Sodium (mM)	Potassium (mM)	Chloride (mM)
1	5.32	0.21	0.22	0.06	22.10	0.78	23.20
2	5.37	0.44	0.66	0.24	42.00	3.13	32.60
3	5.35	0.23	0.36	0.06	16.10	0.22	22.20
4	5.35	0.30	3.06	0.11	51.50	2.35	36.70
5	5.40	0.30	5.10	0.07	35.20	2.96	22.70
6	5.43	0.44	4.00	0.07	33.60	2.26	22.40
7	5.46	0.24	4.13	0.06	22.60	3.22	20.40
8	5.4.3	0.27	1.64	0.05	20.00	0.60	22.90

Blood plasma

Fish #	Glucose (mM)	Magnesium (mM)	Phosphorus (mM)	Calcium (mM)	Sodium (mM)	Potassium (mM)	Chloride (mM)
1	8.18	0.74	1.53	0.95	135.00	2.66	111.00
2	8.78	0.74	1.87	1.14	126.10	2.89	100.20
3	16.04	0.69	0.19	0.86	132.90	2.51	110.20
4	9.52	0.62	1.76	0.86	133.20	3.05	107.80
5	8.12	0.64	1.71	1.34	130.00	3.11	108.10
6	8.53	0.86	1.94	1.31	139.90	2.24	110.80
7	8.18	1.02	2.40	0.19	138.10	2.44	110.90

White sturgeon

Seminal plasma

Fish #	Glucose (mM)	Magnesium (mM)	Phosphorus (mM)	Calcium (mM)	Sodium (mM)	Potassium (mM)	Chloride (mM)
25-5	0.44	0.70	2.03	0.17	43	7.92	41
4	0.39	0.45	1.23	0.15	37	5.31	34
11	0.56	0.62	3.49	0.02	25	3.13	33

Shovelnose sturgeon

Blood plasma

Fish #	Glucose (mM)	Magnesium (mM)	Phosphorus (mM)	Calcium (mM)	Sodium (mM)	Potassium (mM)	Chloride (mM)
2	23.32	1.22	2.21	1.15	130	2.33	92
4	24.56	1.21	2.54	1.11	132	2.61	94
6	28.29	1.39	2.57	1.38	130	2.71	93
10	28.00	1.24	2.58	1.03	127	2.65	92
11	25.33	1.23	2.48	1.21	133	3.04	95
14	23.80	1.22	0.28	1.92	125	2.22	87
15	23.17	1.42	3.22	3.04	129	2.09	94
17	25.57	1.48	3.19	1.30	132	2.29	94
18	24.79	1.67	3.29	3.69	127	2.52	86
1	19.63	1.13	2.32	1.14	127	2.35	91
12	21.28	1.19	2.67	1.11	131	2.82	95
12B	23.48	1.16	2.32	1.15	126	2.35	89
13	22.81	1.30	3.00	2.49	131	2.12	91
16	22.27	1.12	2.58	1.62	127	2.70	89
3	27.15	1.01	2.51	1.39	133	2.81	92
5	23.20	1.16	2.56	1.29	126	2.67	88
7	22.11	1.11	2.95	1.10	129	2.54	92
8	23.80	1.19	2.67	1.11	136	2.48	93
9	25.16	1.16	2.42	1.25	136	3.22	96

Motility Characterization

Lake sturgeon

Fish #	Time (min)													
	0	0.25	0.5	0.75	1	1.5	2	2.5	3	3.5	4	4.5	5	6
1	95	95	95	95	90	80	35	10	1	0.5	0.5	0.5	0.5	0
2	95	95	90	85	70	40	20	10	1	0.5	0	0	0	0
Mean	95	95	93	90	80	60	28	10	1	1	0	0	0	0
SD	0	0	4	7	14	28	11	0	0	0	0	0	0	0

Shovelnose sturgeon

Fish #	Time (min)																				
	0	0.25	0.5	0.75	1	1.25	1.5	1.75	2	2.25	2.5	2.75	3	3.25	3.5	3.75	4	4.25	4.5	4.75	5
1	95	95	95	90	90	85	80	70	40	25	15	10	5	1	0.5	0	0	0	0	0	0
2	95	95	95	90	90	85	85	80	75	70	60	40	35	25	15	5	1	0.5	0	0	0
3	95	95	95	95	90	85	75	60	50	40	35	25	20	20	15	10	5	1	0.5	0	0
4	95	95	95	90	85	75	65	60	50	50	45	35	25	15	5	1	1	0.5	0	0	0
5	95	95	90	85	85	85	85	75	60	40	30	25	15	10	5	1	1	0.5	0	0	0
6	95	95	90	90	90	85	80	75	60	35	25	15	10	10	5	5	1	1	1	0.5	0
7	95	95	95	95	90	85	75	70	65	50	40	25	15	10	5	1	0.5	0	0	0	0
8	95	95	90	60	60	45	40	35	35	25	25	20	15	10	5	1	1	1	0.5	0	0
9	95	95	90	75	75	75	60	50	25	10	1	0.5	0	0	0	0	0	0	0	0	0
10	95	95	95	90	90	85	80	75	65	50	50	40	40	30	25	15	10	5	1	1	0.5

Osmotic Activation

Lake sturgeon

Fish #	Solution #	HBSS		Sucrose		NaCl	
		Motility (%)	Osmolality (mOsmol/kg)	Motility (%)	Osmolality (mOsmol/kg)	Motility (%)	Osmolality (mOsmol/kg)
1	1	95	63	95	62	95	67
	2	95	89	95	83	95	86
	3	90	115	95	107	90	108
	4	5	140	80	127	80	133
	5	0.5	164	30	157	25	158
	6	0	198	0.5	179	0.5	187
	7	0	222	0	204	0	214
	8	0	247	0	246	0	238
	9	0	274	0	255	0	262
	10	0	300	0	278	0	292
2	1	95	71	95	68	95	59
	2	50	93	95	83	95	85
	3	25	115	95	105	95	111
	4	5	143	95	127	75	136
	5	1	170	90	158	25	160
	6	0.5	193	50	183	0.5	193
	7	0	221	0.5	211	0	214
	8	0	253	0	237	0	239
	9	0	270	0	266	0	263
	10	0	311	0	296	0	339
3	1	95	51	95	51	95	58
	2	95	78	95	74	95	79
	3	90	101	95	95	95	103
	4	75	123	95	127	95	134
	5	50	161	95	150	95	161
	6	30	192	90	183	90	211
	7	5	224	70	215	75	233
	8	0	248	5	247	0.5	254
	9	0	279	0	279	0	282
	10	0	314	0	311	0	317

(table cont.)

Fish #	Solution #	HBSS		Sucrose		NaCl	
		Motility (%)	Osmolality (mOsmol/kg)	Motility (%)	Osmolality (mOsmol/kg)	Motility (%)	Osmolality (mOsmol/kg)
4	1	95	65	95	57	95	58
	2	95	91	95	75	95	82
	3	90	123	95	102	95	106
	4	70	144	95	129	95	142
	5	40	167	90	154	90	172
	6	0.5	211	50	186	10	208
	7	0.5	235	10	221	0	233
	8	0	257	0	256	0	254
	9	0	285	0	277	0	273
	10	0	310	0	302	0	312
5	1	95	67	95	69	95	65
	2	90	96	95	85	95	90
	3	40	120	95	109	75	114
	4	5	149	95	135	75	143
	5	0.5	172	75	160	15	168
	6	0	207	5	192	0	205
	7	0	237	0.5	220	0	237
	8	0	257	0	243	0	256
	9	0	301	0	277	0	287
	10	0	328	0	306	0	320

Pallid Sturgeon

Fish #	Solution #	mHBSS		HBSS		NaCl		Sucrose	
		Motility (%)	Osmolality (mOsmol/kg)	Motility (%)	Osmolality (mOsmol/kg)	Motility (%)	Osmolality (mOsmol/kg)	Motility (%)	Osmolality (mOsmol/kg)
1	DI	95	6	95	4	95	10	95	6
	1	90	36	95	33	95	36	95	29
	2	50	46	--	--	--	--	--	--
	3	10	62	90	62	95	60	95	49
	4	1	89	35	93	95	90	95	76
	5	0.5	120	5	117	95	118	95	91
	6	0	156	0.5	147	90	142	95	114
	7	0	166	0	176	90	178	95	137
	8	0	203	0	202	70	204	95	172
	9	0	230	0	239	50	230	80	191
	10	0	253	0	260	30	265	75	208
	11	0	285	0	291	0.5	288	10	246
2	DI	95	10	95	6	95	1	95	3
	1	85	29	95	32	95	29	95	23
	2	15	42	90	60	--	--	--	--
	3	0.5	57	65	75	95	57	95	44
	4	0.5	86	35	97	95	84	95	67
	5	0.5	115	5	114	90	113	95	89
	6	0	142	0.5	142	80	146	95	112
	7	0	175	0	164	60	174	95	134
	8	0	198	0	203	70	197	85	166
	9	0	232	0	229	20	231	80	183
	10	0	254	0	253	5	255	70	202
	11	0	282	0	287	0.5	288	0.5	248

Fish #	Solution #	mHBSS		HBSS		NaCl		Sucrose	
		Motility (%)	Osmolality (mOsmol/kg)	Motility (%)	Osmolality (mOsmol/kg)	Motility (%)	Osmolality (mOsmol/kg)	Motility (%)	Osmolality (mOsmol/kg)
3	DI	95	6	95	12	95	8	95	8
	1	60	30	95	32	95	32	95	32
	2	1	43	80	59	--	--	--	--
	3	0.5	62	75	76	95	59	95	46
	4	0.5	89	50	92	95	86	95	67
	5	0	118	20	119	95	115	95	91
	6	0	146	1	144	95	148	95	116
	7	0	176	0.5	175	90	167	95	135
	8	0	202	0	203	80	200	90	157
	9	0	224	0	230	80	225	75	189
	10	0	258	0	253	70	245	60	204
	11	0	284	0	283	10	275	40	245
4	DI	95	12	95	7	95	14	95	6
	1	70	38	95	31	95	33	95	29
	2	30	47	80	61	--	--	--	--
	3	0.5	59	45	84	95	60	95	46
	4	0.5	93	15	88	95	89	95	68
	5	0.5	115	0.5	117	95	116	95	95
	6	0	142	0.5	143	95	142	95	116
	7	0	174	0	174	90	170	95	136
	8	0	201	0	202	80	197	85	164
	9	0	230	0	227	25	227	50	192
	10	0	257	0	259	10	243	25	209
	11	0	285	0	290	1	281	10	246

Shortnose sturgeon

Fish #	Solution #	mHBSS		NaCl		Sucrose	
		Motility (%)	Osmolality (mOsmol/kg)	Motility (%)	Osmolality (mOsmol/kg)	Motility (%)	Osmolality (mOsmol/kg)
1	0	90	0	90	0	90	0
	10	90	6	--	--	--	--
	20	70	16	90	18	90	13
	30	10	25	--	--	--	--
	40	1	38	90	36	90	32
	60	1	52	90	55	90	53
	80	0.5	75	90	73	90	70
	100	0.5	95	90	94	90	88
	120	0.5	111	90	113	90	105
	140	0.5	131	90	133	90	132
	160	0.5	151	90	154	80	144
	180	0.5	176	80	174	80	169
	200	0	194	70	192	70	192
	250	0	247	20	239	25	235
	300	0.5	281	0.5	281	5	279
2	0	95	2	95	5	95	2
	10	95	19	--	--	--	--
	20	50	25	95	21	95	21
	30	25	30	--	--	--	--
	40	20	38	95	43	95	45
	60	5	56	95	61	95	58
	80	1	78	95	80	95	76
	100	1	99	95	106	95	94
	120	0.5	117	95	127	95	112
	140	0.5	138	95	141	95	135
	160	0.5	156	90	167	90	154
	180	0	179	80	184	90	175
	200	0	202	60	206	75	200
	250	0	251	10	250	20	243
	300	0	293	0.5	290	5	288

(table cont.)

Fish #	Solution #	mHBSS		NaCl		Sucrose	
		Motility (%)	Osmolality (mOsmol/kg)	Motility (%)	Osmolality (mOsmol/kg)	Motility (%)	Osmolality (mOsmol/kg)
3	0	90	0	90	0	90	0
	10	90	8	--	--		
	20	75	19	90	16	90	13
	30	50	26	--	--		
	40	20	34	90	38	90	34
	60	10	56	90	57	90	52
	80	1	72	90	78	90	71
	100	0.5	96	90	97	90	91
	120	0.5	115	90	119	90	108
	140	0.5	134	90	136	90	129
	160	0.5	153	90	158	90	151
	180	0	179	80	182	85	173
	200	0	195	60	201	75	192
	250	0	243	10	244	30	239
	300	0	289	5	289	5	286
4	0	95	9	85	8	95	8
	10	95	19	--	--	--	--
	20	90	23	95	26	95	24
	30	40	31	--	--	--	--
	40	15	46	95	41	95	37
	60	5	64	95	57	95	58
	80	1	86	95	77	95	71
	100	1	104	95	103	95	93
	120	0.5	124	95	124	95	109
	140	1	146	95	141	95	131
	160	0.5	170	95	163	95	149
	180	0.5	189	95	181	95	175
	200	0.5	209	85	205	90	195
	250	0	250	70	243	70	242
	300	0	298	30	287	35	284

(table cont.)

Fish #	Solution #	mHBSS		NaCl		Sucrose	
		Motility (%)	Osmolality (mOsmol/kg)	Motility (%)	Osmolality (mOsmol/kg)	Motility (%)	Osmolality (mOsmol/kg)
5	0	95	0	95	0	95	0
	10	95	0	--	--	--	--
	20	90	8	95	13	95	11
	30	60	22	--	--	--	--
	40	30	29	95	32	95	28
	60	0.5	48	95	50	95	49
	80	0.5	69	95	69	95	61
	100	0	88	95	88	95	83
	120	0.5	108	95	109	95	98
	140	0	129	95	131	95	120
	160	0	146	90	149	90	140
	180	0	168	70	172	80	161
	200	0	188	35	194	50	184
	250	0.5	232	20	221	10	228
	300	0	277	0.5	276	0.5	274

Shovelnose sturgeon

Fish #	Solution #	mHBSS		NaCl		Sucrose	
		Motility (%)	Osmolality (mOsmol/kg)	Motility (%)	Osmolality (mOsmol/kg)	Motility (%)	Osmolality (mOsmol/kg)
1	DI	95	4	--	--	--	--
	1	90	33	90	33	90	33
	2	60	61	90	59	90	59
	3	0.5	88	90	85	90	86
	4	0	122	90	109	90	115
	5	0	146	90	139	90	152
	6	0	175	80	162	90	168
	7	0	201	75	193	90	203
	8	0	234	75	229	0	232
	9	0	269	15	250	0	256
	10	0	312	0	287	0	289
2	DI	95	12	--	--	--	---
	1	95	41	95	48	95	48
	2	95	73	95	71	95	80
	3	50	110	95	92	95	99
	4	0.5	141	95	123	95	130
	5	0	149	95	155	90	162
	6	0	210	90	181	80	194
	7	0	206	85	207	0.5	228
	8	0	225	0.5	240	0	246
	9	0	272	0	265	0	274
	10	0	311	0	301	0	310
3	DI	95	11	--	--	--	--
	1	95	31	95	32	95	37
	2	25	59	95	58	95	67
	3	0	85	95	84	95	89
	4	0	113	95	108	80	124
	5	0	139	80	141	50	141
	6	0	165	50	167	5	168
	7	0	198	20	192	0.5	210
	8	0	223	0	217	0	228
	9	0	256	0	248	0	261
	10	0	288	0	275	0	283

(table cont.)

Fish #	Solution #	mHBSS		NaCl		Sucrose	
		Motility (%)	Osmolality (mOsmol/kg)	Motility (%)	Osmolality (mOsmol/kg)	Motility (%)	Osmolality (mOsmol/kg)
4	DI	95	8	--	--	--	--
	1	95	42	95	34	90	37
	2	5	67	95	61	85	73
	3	0	96	90	87	80	90
	4	0	116	90	115	75	124
	5	0	148	25	136	10	144
	6	0	177	0.5	164	0	176
	7	0	193	0	193	0	201
	8	0	232	0	216	0	230
	9	0	257	0	234	0	251
	10	0	307	0	273	0	283
5	DI	95	15	--	--	--	--
	1	0.5	41	95	37	95	38
	2	0	62	95	59	95	59
	3	0	88	95	86	85	88
	4	0	115	90	107	50	113
	5	0	142	25	135	5	136
	6	0	168	0	157	0.5	159
	7	0	215	0	182	0	192
	8	0	227	0	214	0	221
	9	0	271	0	242	0	248
	10	0	283	0	269	0	278

Ionic Activation

Lake sturgeon

Fish #	Solution	Motility (%)				
		Concentration (mM)				
		0.4	0.8	1.2	1.6	2.0
1	NaHCO ₃	95	95	95	95	95
	K ₂ HPO ₄	35	0.5	0.5	0.5	0.5
	CaCl ₂	95	95	95	85	75
	KCl	10	0.5	0.5	0.5	0.5
	Glucose	95	95	95	95	95
	NaH ₂ PO ₄	95	95	95	90	85
	MgSO ₄	95	95	95	95	95
	NaCl	95	95	95	95	95
2	NaHCO ₃	95	95	95	95	95
	K ₂ HPO ₄	95	5	0.5	0	0
	CaCl ₂	90	90	90	60	75
	KCl	40	1	0	0	0
	Glucose	95	95	95	95	95
	NaH ₂ PO ₄	95	95	95	95	90
	MgSO ₄	95	90	95	90	90
	NaCl	95	95	95	95	95

Pallid Sturgeon

Fish #	Solution	Motility (%)					
		Concentration (mM)					
		0	0.4	0.8	1.2	1.6	2.0
4773	MgCl	90	90	90	90	90	90
	KCl	90	1	0.5	1	0.5	0.5
	NaCl	90	90	90	90	90	90
	CaCl	90	90	90	90	90	80
	Sucrose	90	90	90	90	90	90
453A	MgCl	90	90	90	90	90	90
	KCl	90	5	0.5	0	0	0
	NaCl	90	90	90	90	90	90
	CaCl	90	90	90	90	75	65
	Sucrose	90	90	90	90	90	90
194B	MgCl	90	90	90	90	90	90
	KCl	90	5	1	0.5	0.5	0.5
	NaCl	90	90	90	90	90	90
	CaCl	90	90	90	90	90	80
	Sucrose	90	90	90	90	90	90

Shortnose sturgeon

Fish #	Solution	Concentration (mM)		Motility (%)				
		0.1	0.2	0.3	0.4	0.5	0.75	1.0
1	KCl	90	90	80	50	10	1	0.5
	NaCl	90	90	90	90	90	90	90
	CaCl	90	90	95	90	90	90	90
2	KCl	95	90	50	25	20	3	1
	NaCl	95	95	95	95	95	95	95
	CaCl	95	95	95	95	95	95	95
3	KCl	95	95	75	40	10	1	0.5
	NaCl	95	95	95	95	95	95	95
	CaCl	95	95	95	95	95	95	95
4	KCl	90	80	50	25	10	1	0.5
	NaCl	90	90	90	90	90	90	90
	CaCl	90	90	90	90	90	85	85
5	KCl	95	95	80	50	30	1	0.5
	NaCl	95	95	95	95	95	95	95
	CaCl	95	95	95	95	95	95	95

Shovelnose sturgeon

Fish #	Solution	Motility (%)					
		Concentration (mM)					
		0	0.4	0.8	1.2	1.6	2.0
1	NaH ₂ PO ₄	95	95	95	95	95	95
	Glucose	95	95	95	95	95	95
	MgSO ₄	95	95	95	95	95	95
	NaCl	95	95	95	95	95	95
	CaCl ₂	95	95	90	75	65	20
	K ₂ PO ₄	95	30	0.5	0	0	0
	NaHCO ₃	95	95	95	95	95	95
	KCl	95	15	0	0	0	0
2	NaH ₂ PO ₄	95	95	95	95	95	95
	Glucose	95	95	95	95	95	95
	MgSO ₄	95	95	950	90	95	95
	NaCl	95	95	95	95	95	95
	CaCl ₂	95	95	80	60	50	35
	K ₂ PO ₄	95	80	40	15	1	0.5
	NaHCO ₃	95	95	95	95	95	95
	KCl	95	95	30	5	0.5	0.5
3	NaH ₂ PO ₄	95	95	95	95	95	95
	Glucose	95	95	95	95	95	95
	MgSO ₄	95	95	95	95	95	95
	NaCl	95	95	95	95	95	95
	CaCl ₂	95	95	85	15	10	10
	K ₂ PO ₄	95	70	0	0	0	0
	NaHCO ₃	95	95	95	95	95	95
	KCl	95	10	0	0	0.5	0
4	NaH ₂ PO ₄	95	95	95	95	95	95
	Glucose	95	95	95	95	95	95
	MgSO ₄	95	95	95	95	95	95
	NaCl	95	95	95	95	95	95
	CaCl ₂	95	95	90	85	65	90
	K ₂ PO ₄	95	70	0	0	0	0
	NaHCO ₃	95	95	95	95	95	95
	KCl	95	60	0	0	0	0

(table cont.)

Fish #	Solution	Motility (%)					
		Concentration (mM)					
		0	0.4	0.8	1.2	1.6	2.0
5	NaH ₂ PO ₄	95	95	95	95	95	95
	Glucose	95	95	95	95	95	95
	MgSO ₄	95	95	95	95	95	95
	NaCl	95	95	95	95	95	95
	CaCl ₂	95	90	85	30	15	10
	K ₂ PO ₄	95	10	0	0	0	0
	NaHCO ₃	95	95	95	95	95	95
	KCl	95	0.5	0	0	0	0

Storage studies

Pallid Sturgeon – Pre-activation motility

Fish #	Solution	Osmolality (mOsmol/kg)	Motility (%)	Fish #	Solution	Osmolality (mOsmol/kg)	Motility (%)
A65	HBSS	100	40	A65	NaCl	100	95
16A			50	16A			95
F2B			75	F2B			95
14F			0.5	14F			95
A65		200	0	A65		200	50
16A			0.5	16A			5
F2B			0	F2B			80
14F			0	14F			80
A65		300	0	A65		300	0.5
16A			0	16A			0.5
F2B			0	F2B			0.5
14F			0	14F			0.5
A65	mHBSS	100	0.5	A65	Sucrose	100	95
16A			1	16A			95
F2B			0.5	F2B			95
14F			0	14F			95
A65		200	0	A65		200	50
16A			0	16A			80
F2B			0	F2B			90
14F			0	14F			80
A65		300	0	A65		300	1
16A			0	16A			1
F2B			0	F2B			10
14F			0	14F			10

Pallid Sturgeon – Storage Study

Fish #	Solution	Osmolality (mOsmol/kg)	Motility (%) at day																
			0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	HBSS	100	95	90	90	85	75	25	15	0.5	0	0	0	0	0	0	0	0	0
		200	95	80	80	60	30	5	0.5	0	0	0	0	0	0	0	0	0	0
		300	95	0.5	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	mHBSS	100	95	95	90	90	75	75	70	55	5	0.5	0	0	0	0	0	0	0
		200	95	55	15	20	40	15	5	1	0	0	0	0	0	0	0	0	0
		300	95	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	NaCl	100	95	35	25	30	10	1	0.5	0	0	0	0	0	0	0	0	0	0
		200	95	25	10	25	10	0	0	0	0	0	0	0	0	0	0	0	0
		300	95	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Sucrose	100	95	85	35	50	30	5	1	0.5	0	0	0	0	0	0	0	0	0
		200	95	35	20	25	1	0	0	0	0	0	0	0	0	0	0	0	0
		300	95	15	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	HBSS	100	95	80	75	75	40	50	25	60	50	25	25	30	5	0	0	0	0
		200	95	45	80	75	60	30	15	40	15	10	1	10	1	0	0	0	0
		300	95	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	mHBSS	100	95	90	85	85	85	65	50	70	65	60	50	40	20	15	10	5	0
		200	95	10	35	35	40	40	20	20	30	10	5	15	10	1	0	0	0
		300	95	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	NaCl	100	95	70	30	15	15	5	0	0	0	0	0	0	0	0	0	0	0
		200	95	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
		300	95	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Sucrose	100	95	80	60	65	35	35	25	10	1	0.5	0	0	0	0	0	0	0
		200	95	35	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		300	95	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

(table cont.)

Pallid sturgeon sperm storage study – continued

		Osmolality	Motility (%) at day																
Fish #	Solution	(mOsmol/kg)	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
3	HBSS	100	95	90	90	85	70	65	70	55	10	0	0	0	0	0	0	0	0
		200	95	95	80	75	50	25	15	10	0	0	0	0	0	0	0	0	0
		300	95	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	mHBSS	100	95	95	80	85	90	60	65	50	35	15	10	5	0	0	0	0	0
		200	95	75	45	20	45	20	30	5	1	0	0	0	0	0	0	0	0
		300	95	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	NaCl	100	95	40	15	20	20	10	10	5	0	0	0	0	0	0	0	0	0
		200	95	0.5	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		300	95	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Sucrose	100	95	90	75	80	60	25	0	0	0	0	0	0	0	0	0	0	0
		200	95	25	5	1	0	0	0	0	0	0	0	0	0	0	0	0	0
		300	95	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	HBSS	100	95	90	85	80	70	20	0	0	0	0	0	0	0	0	0	0	0
		200	95	90	85	75	65	5	0.5	0	0	0	0	0	0	0	0	0	0
		300	95	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	mHBSS	100	95	90	85	75	80	65	50	40	25	10	5	25	5	0.5	0	0	0
		200	95	40	60	60	25	15	45	10	0	0	0	0	0	0	0	0	0
		300	95	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	NaCl	100	95	40	20	30	30	15	5	10	0.5	0	0	0	0	0	0	0	0
		200	95	5	5	15	10	0	0	0	0	0	0	0	0	0	0	0	0
		300	95	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Sucrose	100	95	75	75	75	75	35	55	25	5	0.5	0	0	0	0	0	0	0
		200	95	0.5	15	5	0	0	0	0	0	0	0	0	0	0	0	0	0
		300	95	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Shortnose sturgeon sperm storage study

Fish #	Solution	Osmolality (mOsmol/ kg)	Motility (%) at day																
			0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
0813	mHBSS	100	90	80	70	50	35	35	50	60	25	40	20	25	15	25	15	10	5
		200	90	15	20	1	0	0	0	0	0	0	0	0	0	0	0	0	0
		300	90	0	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	HBSS	100	90	75	35	45	30	30	40	25	30	35	10	15	15	10	10	10	1
		200	90	45	50	25	20	20	20	15	15	10	10	10	10	10	5	0.5	0
		300	90	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Mounib	100	90	5	10	5	1	1	0	0	0	0	0	0	0	0	0	0	0
		200	90	0.5	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		300	90	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Sucrose	100	90	60	40	30	30	20	30	15	25	25	20	15	15	10	0.5	0	0
		200	90	40	15	1	0	0	0	0	0	0	0	0	0	0	0	0	0
		300	90	20	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	undil	---	90	70	75	50	50	50	65	50	30	30	40	10	25	20	15	0	0
706C	mHBSS	100	90	50	30	25	15	15	30	5	1	0	0	0	0	0	0	0	0
		200	90	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		300	90	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	HBSS	100	90	30	5	10	1	0	0	0	0	0	0	0	0	0	0	0	0
		200	90	15	10	5	1	0	0	0	0	0	0	0	0	0	0	0	0
		300	90	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Mounib	100	90	0.5	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		200	90	0.5	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		300	90	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Sucrose	100	90	25	10	5	1	0.5	0	0	0	0	0	0	0	0	0	0	0
		200	90	50	5	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0
		300	90	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	undil	----	90	40	20	15	15	10	10	5	5	5	0.5	0	0	0	0	0	0

(table cont.)

Shortnose sturgeon sperm storage study (continued)

Fish #	Solution	Osmolality (mOsmol/kg)	Motility (%) at day																
			0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
2C40	mHBSS	100	90	80	30	50	20	15	25	40	10	25	10	10	1	1	0	0	0
		200	90	0.5	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		300	90	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	HBSS	100	90	90	25	20	10	10	30	10	5	10	10	1	0	0	0	0	0
		200	90	10	5	5	5	10	10	10	1	0	0	0	0	0	0	0	0
		300	90	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Mounib	100	90	1	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		200	90	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		300	90	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Sucrose	100	90	70	30	25	20	10	20	5	0.5	0	0	0	0	0	0	0	0
		200	90	50	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		300	90	15	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	undil	---	90	90	75	60	35	35	20	20	25	20	20	0	0	0	0	0	0
6C09	mHBSS	100	95	75	50	10	10	10	20	20	10	10	15	0.5	0	0	0	0	0
		200	95	1	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		300	95	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	HBSS	100	95	90	20	15	20	10	10	15	10	10	5	0	0	0	0	0	0
		200	95	5	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		300	95	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Mounib	100	95	5	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		200	95	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		300	95	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Sucrose	100	95	60	10	1	0	0	0	0	0	0	0	0	0	0	0	0	0
		200	95	15	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		300	95	20	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	undil	---	95	95	90	80	50	50	35	25	10	15	5	0	0	0	0	0	0

Shovelnose sturgeon sperm storage study

Fish #	Solution	Osmolality (mOsmol/kg)	Motility (%) at day																					
			0	1	2	3	4	5	6	7	8	9	10	12	13	14	15	16	17	18	19	20	21	23
1	HBSS	100	95	95	90	90	85	75	80	35	80	65	70	60	45	35	40	25	35	30	20	15	10	0
		200	95	90	65	35	25	35	30	20	15	15	25	10	10	10	10	5	5	1	1	0	0	0
		300	95	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	mHBSS	100	95	95	95	90	95	85	90	75	75	80	75	65	60	50	65	50	40	20	30	20	15	0
		200	95	20	25	5	20	20	10	10	25	25	20	20	15	20	15	10	10	5	1	1	0	0
		300	95	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	NaCl	100	95	95	40	5	5	5	20	35	25	15	20	10	40	30	10	20	15	5	5	10	1	0
		200	95	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		300	95	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Sucrose	100	95	90	85	80	85	55	60	30	55	35	40	20	15	15	1	1	0.5	0	0	0	0	0
		200	95	85	40	25	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		300	95	65	1	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Undiluted	---	95	95	95	95	90	90	90	90	90	85	85	75	75	50	35	50	35	15	20	20	5	0
2	HBSS	100	95	95	90	90	85	65	60	40	35	25	25	15	20	15	15	15	15	10	5	5	5	0
		200	95	75	70	45	70	45	35	20	35	35	35	25	25	25	25	20	15	10	10	5	1	0.5
		300	95	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	mHBSS	100	95	95	90	85	85	65	60	40	30	25	25	20	20	15	10	15	1	0	0	0	0	0
		200	95	35	35	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		300	95	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	NaCl	100	95	90	75	15	20	20	15	15	20	15	15	5	10	10	15	10	10	5	5	15	5	0
		200	95	10	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		300	95	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Sucrose	100	95	95	90	80	85	85	65	40	50	35	35	25	15	25	20	25	15	5	1	1	0.5	0
		200	95	70	25	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		300	95	35	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Undiluted	---	95	95	75	35	35	25	20	20	15	50	0	0	0	0	0	0	0	0	0	0	0	0

(table cont.)

Shovelnose sturgeon sperm storage study (continued)

Fish #	Solution	Osmolality (mOsmol/kg)	Motility (%) at day																					
			0	1	2	3	4	5	6	7	8	9	10	12	13	14	15	16	17	18	19	20	21	23
3	HBSS	100	95	90	90	90	90	85	80	75	65	35	30	25	25	20	15	15	10	5	5	1	0	0
		200	95	65	50	35	50	25	35	30	30	15	25	15	25	20	15	15	3	5	5	5	0.5	0
		300	95	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	mHBSS	100	95	95	95	95	90	90	90	85	75	65	45	40	30	25	25	10	20	10	10	5	5	0.5
		200	95	15	45	30	45	35	20	20	30	25	30	20	30	20	20	20	10	1	1	0.5	0	0
		300	95	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	NaCl	100	95	95	80	10	10	20	25	50	20	25	25	20	35	35	20	15	10	5	10	10	1	0
		200	95	10	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		300	95	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Sucrose	100	95	95	85	80	85	70	70	30	50	35	35	35	30	35	20	25	20	10	5	5	5	0
		200	95	55	40	5	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		300	95	25	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Undiluted	---	95	95	70	85	60	70	65	30	50	25	45	25	35	30	25	25	5	0	0	0	0	0
4	HBSS	100	95	95	85	65	50	60	40	30	35	20	25	20	15	10	0	0	0	0	0	0	0	0
		200	95	50	85	50	65	60	60	35	35	20	20	25	15	15	10	5	0.5	0	0	0	0	0
		300	95	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	mHBSS	100	95	95	90	50	75	80	40	45	25	30	25	25	20	20	25	20	20	25	15	10	20	1
		200	95	25	15	25	35	30	10	15	15	20	20	10	5	1	0.5	0	0	0	0	0	0	0
		300	95	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	NaCl	100	95	90	50	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		200	95	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		300	95	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Sucrose	100	95	85	75	50	60	30	50	25	45	20	20	0	0	0	0	0	0	0	0	0	0	0
		200	95	30	30	25	5	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		300	95	40	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Undiluted	---	95	85	90	35	20	20	15	15	20	15	10	0	0	0	0	0	0	0	0	0	0	0

(table cont.)

Shovelnose sturgeon sperm storage study (continued)

Fish #	Solution	Osmolality (mOsmol/kg)	Motility (%) at day																					
			0	1	2	3	4	5	6	7	8	9	10	12	13	14	15	16	17	18	19	20	21	23
5	HBSS	100	95	95	90	85	85	75	65	65	70	40	40	40	40	35	20	25	25	10	10	15	5	0
		200	95	40	25	15	35	25	15	5	10	15	10	10	5	5	5	1	0	0	0	0	0	0
		300	95	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	mHBSS	100	95	95	95	90	90	90	70	80	75	80	80	70	65	55	60	50	40	35	25	25	15	5
		200	95	10	5	5	10	15	10	15	10	20	20	15	20	15	15	15	10	5	5	5	5	1
		300	95	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	NaCl	100	95	85	75	5	5	10	10	15	25	1	35	20	35	20	25	25	15	15	10	15	5	1
		200	95	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		300	95	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Sucrose	100	95	90	60	80	80	65	75	25	65	45	60	45	25	30	30	30	10	1	0	0	0	0
		200	95	75	70	20	10	15	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		300	95	25	15	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Undiluted	---	95	95	90	85	75	50	70	50	40	30	30	30	25	30	20	15	0	0	0	0	0	0

Chapter 6 – Development of Cryopreservation Techniques

Unanalyzed Data

Shortnose sturgeon freezing rate study.

Freezing Rate – 5 °C/min		Motility		Hatch (%)
Fish #	Treatment	Equilibration (%)	Thaw (%)	
4D6F	Control	--	90	26
4D6F	Control	--	90	9
1	DMSO 10	50	5	0
2	DMSO 10	65	10	0
3	DMSO 10	80	10	0
1	DMSO 5	80	0.5	0
2	DMSO 5	90	1	0
3	DMSO 5	90	1	0
1	MeOH 10	85	0	0
2	MeOH 10	90	0.5	1
3	MeOH 10	95	0.5	0
1	MeOH 5	85	0.5	0
2	MeOH 5	90	0	0
3	MeOH 5	95	0	0

Freezing Rate – 10 °C/min		Motility		Hatch (%)
Fish #	Treatment	Equilibration (%)	Thaw (%)	
4A66	Control	--	90	14
4A66	Control	--	90	15
1	DMSO 10	60	10	0
2	DMSO 10	50	15	0
3	DMSO 10	70	20	0
1	DMSO 5	75	0.5	0
2	DMSO 5	85	5	0
3	DMSO 5	95	5	0
1	MeOH 10	60	0	0
2	MeOH 10	75	0	0
3	MeOH 10	90	1	0
1	MeOH 5	90	0	0
2	MeOH 5	90	0	0
3	MeOH 5	95	1	0

Shortnose sturgeon freezing rate study (cont.)

Freezing Rate – 20 °C/min		Motility		Hatch (%)
Fish #	Treatment	Equilibration (%)	Thaw (%)	
457C	Control	--	95	36
457C	Control	--	95	61
1	DMSO 10	50	15	0
2	DMSO 10	60	20	0
3	DMSO 10	80	10	0
1	DMSO 5	70	10	0
2	DMSO 5	80	10	0
3	DMSO 5	90	5	0
1	MeOH 10	75	0	0
2	MeOH 10	80	0	0
3	MeOH 10	90	0.5	0
1	MeOH 5	85	0	0
2	MeOH 5	90	0.5	4
3	MeOH 5	95	1	0

Freezing Rate – 30 °C/min		Motility		Hatch (%)
Fish #	Treatment	Equilibration (%)	Thaw (%)	
706C	Control	--	90	38
706C	Control	--	90	70
1	DMSO 10	70	10	0
2	DMSO 10	55	15	0
3	DMSO 10	85	20	0
1	DMSO 5	80	1	0
2	DMSO 5	75	5	0
3	DMSO 5	90	5	0
1	MeOH 10	85	0.5	0
2	MeOH 10	80	0.5	0
3	MeOH 10	90	15	0
1	MeOH 5	90	0.5	0
2	MeOH 5	95	0.5	0
3	MeOH 5	90	0.5	0

Shortnose sturgeon freezing rate study (cont.)

Freezing Rate – 40 °C/min		Motility		Hatch (%)
Fish #	Treatment	Equilibration (%)	Thaw (%)	
272B	Control	--	95	19
272B	Control	--	95	26
1	DMSO 10	50	20	0
2	DMSO 10	50	15	0
3	DMSO 10	75	15	0
1	DMSO 5	85	10	0
2	DMSO 5	75	5	0
3	DMSO 5	90	5	0
1	MeOH 10	90	0	0
2	MeOH 10	85	0	0
3	MeOH 10	95	0.5	0
1	MeOH 5	90	0	0
2	MeOH 5	80	0.5	0
3	MeOH 5	95	0.5	0

Shortnose sturgeon dewar freezing rate

Fish #	Treatment	Motility		Fertilization (%)	Hatch (%)
		Equilibration (%)	Post-thaw (%)		
mTsvetkova extnder				0	0
1	MeOH 5%	95	5	0	0
	MeOH 10%	95	1	0	0
	DMSO 5%	95	35	0	0
	DMSO 10%	35	25	0	0
2	MeOH 5%	95	0.5	0	0
	MeOH 10%	95	0.5	0	0
	DMSO 5%	90	30	0	0
	DMSO 10%	30	10	0	0
3	MeOH 5%	90	25	0	0
	MeOH 10%	90	0.5	0	0
	DMSO 5%	75	25	0	0
	DMSO 10%	10	1	0	0
	Control	--	95	35	25
	Control	--	95	46	45
MHBSS extender`					
1	MeOH 5%	95	0.5	0	0
	MeOH 10%	95	0	0	0
	DMSO 5%	95	30	0	0
	DMSO 10%	35	10	0	0
2	MeOH 5%	95	0	0	0
	MeOH 10%	95	0.5	0	0
	DMSO 5%	95	10	0	0
	DMSO 10%	20	5	0	0
3	MeOH 5%	90	0.5	0	0
	MeOH 10%	90	0	0	0
	DMSO 5%	70	5	0	0
	DMSO 10%	10	0.5	0	0
	Control	--	95	89	69
	Control	--	95	72	67

Shortnose sturgeon sperm frozen at LSU Dairy Improvement Center

Fish #	Cryoprotectant	Motility		Fertilization (%)
		Equilibration (%)	Post-thaw (%)	
1	Methanol	80	5	4
2	Methanol	80	1	2
3	Methanol	85	1	3
4	Methanol	95	1	3
1	Glycerol	0.5	0.5	1
2	Glycerol	0.5	1	0
3	Glycerol	0.5	0.5	0
4	Glycerol	0.5	0	1
1	DMSO	70	15	
2	DMSO	70	30	
3	DMSO	80	20	2
4	DMSO	70	10	0
2A40	Fresh	--	75	89
2A40	Fresh	--	75	93
2A40	Fresh	--	75	88

Lake Sturgeon sperm crypreserved with mHBSS extender

Fish #	Cryoprotectant	Concentration (%)	Motility		Fertilization (%)
			Equilibration (%)	Post-thaw (%)	
1	DMSO	5	90	25	1
2	DMSO	5	90	25	2
3	DMSO	5	95	0.5	1
4	DMSO	5	95	1	0
5	DMSO	5	90	5	1
1	DMSO	10	75	30	2
2	DMSO	10	80	20	0
3	DMSO	10	65	5	1
4	DMSO	10	75	10	0
5	DMSO	10	65	20	0
1	MeOH	5	90	15	62
2	MeOH	5	90	1	39
3	MeOH	5	95	10	66
4	MeOH	5	95	5	35
5	MeOH	5	90	5	39
1	MeOH	10	80	5	66
2	MeOH	10	85	1	28
3	MeOH	10	95	5	41
4	MeOH	10	95	20	59
5	MeOH	10	85	10	40
Control				75	96
Control				75	93

Lake sturgeon sperm cryopreserved with mTsvetkova extender.

Fish #	Treatment	Motility	
		Equilibration (%)	Post-thaw (%)
1	MeOH 5%	85	15
	MeOH 10%	90	60
	DMSO 5%	90	20
	DMSO 10%	80	10
2	MeOH 5%	95	40
	MeOH 10%	95	70
	DMSO 5%	95	25
	DMSO 10%	10	1
3	MeOH 5%	95	15
	MeOH 10%	95	20
	DMSO 5%	95	20
	DMSO 10%	50	15
4	MeOH 5%	95	60
	MeOH 10%	95	50
	DMSO 5%	95	20
	DMSO 10%	15	1

Pallid sturgeon sperm cryopreservation.

Fish #	Cryoprotectant	Concentration (%)	Motility (%)		Fertilization (%)	Hatch (%)
			Equilibration (%)	Post-thaw (%)		
1	Methanol	5	95	50	94	82
2	Methanol	5	95	50	96	82
3	Methanol	5	90	30	85	73
4	Methanol	5	95	40	89	71
1	Methanol	10	90	35	86	78
2	Methanol	10	90	30	92	66
3	Methanol	10	95	25	98	86
4	Methanol	10	90	50	93	79
1	Methanol	15	90	40	28	28
2	Methanol	15	90	10	14	0
3	Methanol	15	90	10	2	3
4	Methanol	15	90	20	5	8
1	Glycerol	5	20	0	--	--
2	Glycerol	5	40	1	--	--
3	Glycerol	5	50	0.5	--	--
4	Glycerol	5	40	0.5	--	--
1	Glycerol	10	10	0	--	--
2	Glycerol	10	5	0.5	--	--
3	Glycerol	10	15	0	--	--
4	Glycerol	10	5	0	--	--
1	Glycerol	15	0.5	0	--	--
2	Glycerol	15	0	0	--	--
3	Glycerol	15	0	0	--	--
4	Glycerol	15	0	0	--	--
1	DMSO	5	95	20	27	35
2	DMSO	5	95	55	44	35
3	DMSO	5	95	65	42	37
4	DMSO	5	95	75	39	30
1	DMSO	10	95	35	0	0
2	DMSO	10	40	15	0	0
3	DMSO	10	90	25	6	9
4	DMSO	10	90	60	0	0
1	DMSO	15	15	0	0	0
2	DMSO	15	10	5	0	0
3	DMSO	15	85	5	0	4
4	DMSO	15	70	10	7	0
Control	--	--	--	95	95	65
Control	--	--	--	95	98	68
Control	--	--	--	95	97	75

Shovelnose sturgeon sperm cryopreserved with mHBSS extender.

Fish #	Cryoprotectant	Concentration (%)	Motility		Fertilization (%)	Hatch (%)
			Equilibration (%)	Post-thaw (%)		
1	Methanol	5	95	5	11	11
2	Methanol	5	95	20	78	55
3	Methanol	5	95	40	86	66
4	Methanol	5	95	10	56	45
5	Methanol	5	95	25	86	69
1	Methanol	10	95	5	7	3
2	Methanol	10	95	35	85	69
3	Methanol	10	95	60	89	64
4	Methanol	10	95	15	45	36
5	Methanol	10	95	35	59	50
Control	--	--	--	95	82	51
Control	--	--	--	95	74	62
1	DMSO	5	95	15	9	5
2	DMSO	5	90	30	28	23
3	DMSO	5	95	40	66	51
4	DMSO	5	95	20	56	43
5	DMSO	5	95	15	41	31
1	DMSO	10	80	10	3	3
2	DMSO	10	85	25	37	29
3	DMSO	10	95	50	75	60
4	DMSO	10	90	30	26	25
5	DMSO	10	95	15	41	31
Control	--	--	--	95	100	81
Control	--	--	--	95	95	57

Shovelnose sturgeon sperm cryopreserved with mTsvetkova extender.

Fish #	Cryoprotectant	Concentration (%)	Motility		Fertilization (%)	Hatch (%)
			Equilibration (%)	Post-thaw (%)		
1	Methanol	5	95	45	75	56
2	Methanol	5	95	50	98	93
3	Methanol	5	95	40	91	68
4	Methanol	5	95	60	96	64
5	Methanol	5	95	40	99	85
1	Methanol	10	95	50	77	58
2	Methanol	10	95	55	97	71
3	Methanol	10	95	50	25	17
4	Methanol	10	95	50	100	50
5	Methanol	10	95	45	94	59
Control	--	--	--	95	96	77
Control	--	--	--	95	95	73
1	DMSO	5	95	25	14	23
2	DMSO	5	95	55	32	16
3	DMSO	5	95	40	11	11
4	DMSO	5	95	50	17	10
5	DMSO	5	95	40	15	5
1	DMSO	10	95	25	1	1
2	DMSO	10	90	55	24	18
3	DMSO	10	90	40	4	1
4	DMSO	10	90	50	6	1
5	DMSO	10	85	40	3	1
Control	--	--	--	95	98	71
Control	--	--	--	95	99	68

Chapter 7 – Determination of Methods for the Objective Determination of Sperm Quality Unanalyzed Data

Lake Sturgeon Sperm Viability Staining – Controls

Fish #	Treatment	Label	Events	Gated (%)	Viable (%)
1	0	Viable	8	0.08	0.080112
1	0	Dead	9978	99.78	
1	25	Viable	673	6.73	6.742811
1	25	Dead	9308	93.08	
1	50	Viable	1087	10.87	10.89724
1	50	Dead	8888	88.88	
1	75	Viable	2100	21	21.04419
1	75	Dead	7879	78.79	
1	100	Viable	2938	29.38	29.47728
1	100	Dead	7029	70.29	
2	0	Viable	15	0.15	0.150225
2	0	Dead	9970	99.7	
2	25	Viable	568	5.68	5.685117
2	25	Dead	9423	94.23	
2	50	Viable	1385	13.85	13.90004
2	50	Dead	8579	85.79	
2	75	Viable	2055	20.55	20.62218
2	75	Dead	7910	79.1	
2	100	Viable	2644	26.44	26.50627
2	100	Dead	7331	73.31	

Lake Sturgeon Sperm Viability Staining – mHBSS extender

Fish#	Treatment	Label	Events	Gated (%)	Viable Cells (%)	Motility (%)
1	DMSO 5%	Viable	2795	27.95	28.2	35
		Non-viable	7129	71.29		
2	DMSO 5%	Viable	3366	33.66	33.8	35
		Non-viable	6594	65.94		
3	DMSO 5%	Viable	1416	14.16	14.2	5
		Non-viable	8521	85.21		
4	DMSO 5%	Viable	1368	13.68	13.8	10
		Non-viable	8527	85.27		
5	DMSO 5%	Viable	4332	43.32	44.0	20
		Non-viable	5517	55.17		
1	DMSO 10%	Viable	5794	57.94	58.6	30
		Non-viable	4097	40.97		
2	DMSO 10%	Viable	4209	42.09	42.3	25
		Non-viable	5741	57.41		
3	DMSO 10%	Viable	5572	55.72	56.1	5
		Non-viable	4369	43.69		
4	DMSO 10%	Viable	5484	54.84	63.1	5
		Non-viable	3201	32.01		
5	DMSO 10%	Viable	6598	65.98	66.6	20
		Non-viable	3314	33.14		
1	MeOH 5%	Viable	834	8.34	8.4	10
		Non-viable	9128	91.28		
2	MeOH 5%	Viable	468	4.68	4.7	5
		Non-viable	9510	95.1		
3	MeOH 5%	Viable	2076	20.76	21.0	15
		Non-viable	7803	78.03		
4	MeOH 5%	Viable	2539	25.39	25.9	20
		Non-viable	7263	72.63		
5	MeOH 5%	Viable	2594	25.94	26.2	20
		Non-viable	7290	72.9		
1	MeOH 10%	Viable	1414	14.14	14.2	15
		Non-viable	8524	85.24		
2	MeOH 10%	Viable	555	5.55	5.6	5
		Non-viable	9406	94.06		
3	MeOH 10%	Viable	3380	33.8	34.9	15
		Non-viable	6308	63.08		
4	MeOH 10%	Viable	3555	35.55	36.0	30
		Non-viable	6319	63.19		
5	MeOH 10%	Viable	4279	42.79	43.3	35
		Non-viable	5594	55.94		

Lake Sturgeon Sperm Viability Staining – mTsvetkova extender

Fish #	Treatment*	Label	Events	Gated (%)	Viable Cells (%)	Motility (%)
1	DMSO 5%	Viable	5836	58.36	58.9	20
		Non-viable	4064	40.64		
2	DMSO 5%	Viable	5817	58.17	58.7	25
		Non-viable	4096	40.96		
3	DMSO 5%	Viable	5425	54.25	54.7	20
		Non-viable	4496	44.96		
4	DMSO 5%	Viable	5168	51.68	52.2	20
		Non-viable	4731	47.31		
1	DMSO 10%	Viable	7352	73.52	74.1	10
		Non-viable	2570	25.7		
2	DMSO 10%	Viable	7216	72.16	73.0	1
		Non-viable	2675	26.75		
3	DMSO 10%	Viable	6953	69.53	69.9	15
		Non-viable	2988	29.88		
4	DMSO 10%	Viable	7956	79.56	81.3	1
		Non-viable	1830	18.3		
1	MeOH 5%	Viable	2863	28.63	28.9	15
		Non-viable	7060	70.6		
2	MeOH 5%	Viable	4083	40.83	41.1	40
		Non-viable	5855	58.55		
3	MeOH 5%	Viable	3086	30.86	31.1	15
		Non-viable	6840	68.4		
4	MeOH 5%	Viable	6222	62.22	62.7	60
		Non-viable	3696	36.96		
1	MeOH 10%	Viable	6242	62.42	63.3	60
		Non-viable	3625	36.25		
2	MeOH 10%	Viable	6455	64.55	65.4	70
		Non-viable	3422	34.22		
3	MeOH 10%	Viable	3827	38.27	38.6	20
		Non-viable	6084	60.84		
4	MeOH 10%	Viable	6077	60.77	62.4	50
		Non-viable	3659	36.59		

Pallid Sturgeon Sperm Viability Staining - Controls

Fish #	Treatment	Label	Events	Gated (%)	Viable Cells (%)
1	0	Viable	5937	59.37	59.97
1	0	Non-viable	3963	39.63	
1	25	Viable	4334	43.34	43.64
1	25	Non-viable	5598	55.98	
1	50	Viable	3298	32.98	33.16
1	50	Non-viable	6648	66.48	
1	75	Viable	1325	13.25	13.29
1	75	Non-viable	8648	86.48	
1	100	Viable	46	0.46	0.46
1	100	Non-viable	9946	99.46	
2	0	Viable	6840	68.4	68.70
2	0	Non-viable	3117	31.17	
2	25	Viable	5766	57.66	58.12
2	25	Non-viable	4155	41.55	
2	50	Viable	4011	40.11	40.68
2	50	Non-viable	5849	58.49	
2	75	Viable	2492	24.92	25.23
2	75	Non-viable	7384	73.84	
2	100	Viable	206	2.06	2.09
2	100	Non-viable	9657	96.57	
3	0	Non-viable	4386	43.86	55.75
3	0	Viable	5526	55.26	
3	25	Non-viable	5829	58.29	41.35
3	25	Viable	4109	41.09	
3	50	Non-viable	7232	72.32	27.48
3	50	Viable	2741	27.41	
3	75	Non-viable	8531	85.31	14.55
3	75	Viable	1453	14.53	
3	100	Non-viable	9995	99.95	0.04
3	100	Viable	4	0.04	
4	0	Non-viable	3862	38.62	60.98
4	0	Viable	6036	60.36	
4	25	Non-viable	5815	58.15	41.57
4	25	Viable	4137	41.37	
4	50	Non-viable	7238	72.38	27.35
4	50	Viable	2725	27.25	
4	75	Non-viable	8755	87.55	12.33
4	75	Viable	1231	12.31	
4	100	Non-viable	10000	100	0.00
4	100	Viable	0	0	

Pallid Sturgeon Sperm Viability Staining – mHBSS extender

Fish #	Treatment	Label	Events	Gated (%)	Viable (%)
1	DMSO 5%	Non-viable	4796	47.96	51.66
		Viable	5125	51.25	
2	DMSO 5%	Non-viable	3258	32.58	66.77
		Viable	6546	65.46	
3	DMSO 5%	Non-viable	2934	29.34	70.49
		Viable	7009	70.09	
4	DMSO 5%	Non-viable	2714	27.14	72.74
		Viable	7242	72.42	
1	DMSO 10%	Non-viable	3174	31.74	68.05
		Viable	6759	67.59	
2	DMSO 10%	Non-viable	2437	24.37	75.50
		Viable	7510	75.1	
3	DMSO 10%	Non-viable	4261	42.61	57.17
		Viable	5687	56.87	
4	DMSO 10%	Non-viable	2095	20.95	78.93
		Viable	7847	78.47	
1	DMSO 15%	Non-viable	3501	35.01	64.74
		Viable	6429	64.29	
4	DMSO 15%	Non-viable	2468	24.68	75.16
		Viable	7467	74.67	
1	MeOH 5%	Non-viable	4556	45.56	54.20
		Viable	5392	53.92	
3	MeOH 5%	Non-viable	5075	50.75	48.99
		Viable	4875	48.75	
4	MeOH 5%	Non-viable	4658	46.58	53.07
		Viable	5268	52.68	
1	MeOH 10%	Non-viable	4020	40.2	59.33
		Viable	5864	58.64	
2	MeOH 10%	Non-viable	2889	28.89	70.86
		Viable	7025	70.25	
3	MeOH 10%	Non-viable	4535	45.35	53.99
		Viable	5321	53.21	
4	MeOH 10%	Non-viable	4050	40.5	59.05
		Viable	5841	58.41	
2	MeOH 15%	Non-viable	4771	47.71	51.96
		Viable	5160	51.6	
3	MeOH 15%	Non-viable	6175	61.75	37.35
		Viable	3681	36.81	

Shovelnose Sturgeon Viability Staining – Controls

Fish #	Treatment	Label	Events	Gated (%)	Viable (%)
1	0	Viable	4727	47.27	47.94
1	0	Non-viable	5134	51.34	
1	25	Viable	3163	31.63	31.98
1	25	Non-viable	6728	67.28	
1	50	Viable	2130	21.3	21.49
1	50	Non-viable	7781	77.81	
1	75	Viable	938	9.38	9.42
1	75	Non-viable	9021	90.21	
1	100	Viable	11	0.11	0.11
1	100	Non-viable	9983	99.83	
2	0	Viable	4204	42.04	42.77
2	0	Non-viable	5626	56.26	
2	25	Viable	3130	31.3	31.66
2	25	Non-viable	6755	67.55	
2	50	Viable	2026	20.26	20.44
2	50	Non-viable	7887	78.87	
2	75	Viable	979	9.79	9.83
2	75	Non-viable	8985	89.85	
2	100	Viable	14	0.14	0.14
2	100	Non-viable	9982	99.82	

Shovelnose Sturgeon Viability Staining – mHBSS extender

Fish #	Treatment	Label	Events	Gated (%)	Viable (%)	Motility (%)
1	DMSO5%	Viable	5206	52.06	52.06	52.26
		Non-viable	4756	47.56	47.56	
1	DMSO5	Viable	5385	53.85	53.85	54.40
		Non-viable	4513	45.13	45.13	
2	DMSO5	Viable	5879	58.79	58.79	59.28
		Non-viable	4038	40.38	40.38	
2	DMSO5	Viable	6036	60.36	60.36	60.85
		Non-viable	3884	38.84	38.84	
3	DMSO5	Viable	6557	65.57	65.57	66.08
		Non-viable	3366	33.66	33.66	
3	DMSO5	Viable	6484	64.84	64.84	65.28
		Non-viable	3449	34.49	34.49	
4	DMSO5	Viable	5115	51.15	51.15	51.71
		Non-viable	4776	47.76	47.76	
4	DMSO5	Viable	6264	62.64	62.64	63.11
		Non-viable	3661	36.61	36.61	
5	DMSO5	Viable	5355	53.55	53.55	54.12
		Non-viable	4539	45.39	45.39	
5	DMSO5	Viable	5399	53.99	53.99	54.60
		Non-viable	4490	44.9	44.9	
1	DMSO 10%	Viable	6815	68.15	68.15	68.58
		Non-viable	3122	31.22	31.22	
1	DMSO 10%	Viable	6722	67.22	67.22	67.60
		Non-viable	3222	32.22	32.22	
2	DMSO 10%	Viable	7867	78.67	78.67	79.30
		Non-viable	2054	20.54	20.54	
2	DMSO 10%	Viable	7932	79.32	79.32	79.86
		Non-viable	2001	20.01	20.01	
3	DMSO 10%	Viable	8251	82.51	82.51	83.17
		Non-viable	1670	16.7	16.7	
3	DMSO 10%	Viable	8136	81.36	81.36	82.09
		Non-viable	1775	17.75	17.75	
4	DMSO 10%	Viable	7125	71.25	71.25	71.85
		Non-viable	2791	27.91	27.91	
4	DMSO 10%	Viable	7244	72.44	72.44	73.13
		Non-viable	2662	26.62	26.62	
5	DMSO 10%	Viable	6600	66	66	66.61
		Non-viable	3309	33.09	33.09	
5	DMSO 10%	Viable	6378	63.78	63.78	64.31
		Non-viable	3539	35.39	35.39	

(table cont.)

Fish #	Treatment	Label	Events	Gated (%)	Viable (%)	Motility (%)
1	MeOH 5%	Viable	2567	25.67	25.67	25.81
		Non-viable	7380	73.8	73.8	
1	MeOH 5%	Viable	2555	25.55	25.55	26.10
		Non-viable	7233	72.33	72.33	
2	MeOH 5%	Viable	4876	48.76	48.76	49.90
		Non-viable	4896	48.96	48.96	
2	MeOH 5%	Viable	4803	48.03	48.03	49.18
		Non-viable	4964	49.64	49.64	
3	MeOH 5%	Viable	6769	67.69	67.69	68.77
		Non-viable	3074	30.74	30.74	
3	MeOH 5%	Viable	6705	67.05	67.05	67.97
		Non-viable	3159	31.59	31.59	
4	MeOH 5%	Viable	3436	34.36	34.36	35.25
		Non-viable	6311	63.11	63.11	
4	MeOH 5%	Viable	3772	37.72	37.72	38.71
		Non-viable	5972	59.72	59.72	
5	MeOH 5%	Viable	4037	40.37	40.37	41.58
		Non-viable	5673	56.73	56.73	
5	MeOH 5%	Viable	4727	47.27	47.27	47.94
		Non-viable	5134	51.34	51.34	
1	MeOH 10%	Viable	3387	33.87	33.87	34.93
		Non-viable	6309	63.09	63.09	
1	MeOH 10%	Viable	3578	35.78	35.78	36.69
		Non-viable	6173	61.73	61.73	
2	MeOH 10%	Viable	6819	68.19	68.19	69.45
		Non-viable	2999	29.99	29.99	
2	MeOH 10%	Viable	6212	62.12	62.12	63.07
		Non-viable	3638	36.38	36.38	
3	MeOH 10%	Viable	7935	79.35	79.35	80.57
		Non-viable	1914	19.14	19.14	
3	MeOH 10%	Viable	7742	77.42	77.42	78.29
		Non-viable	2147	21.47	21.47	
4	MeOH 10%	Viable	4829	48.29	48.29	49.54
		Non-viable	4918	49.18	49.18	
4	MeOH 10%	Viable	5242	52.42	52.42	53.47
		Non-viable	4562	45.62	45.62	
5	MeOH 10%	Viable	6317	63.17	63.17	64.11
		Non-viable	3537	35.37	35.37	
5	MeOH 10%	Viable	6628	66.28	66.28	67.06
		Non-viable	3256	32.56	32.56	

Shovelnose Sturgeon Viability Staining – mTsvetkova extender

Fish #	Treatment	Label	Events	Gated (%)	Live (%)	Motility (%)
1	DMSO 5%	Viable	4703	47.03	47.08	20
		Non-Viable	5286	52.86		
1	DMSO 5%	Viable	4811	48.11	48.17	20
		Non-Viable	5176	51.76		
2	DMSO 5%	Viable	4974	49.74	49.80	35
		Non-Viable	5014	50.14		
2	DMSO 5%	Viable	5225	52.25	52.27	40
		Non-Viable	4771	47.71		
3	DMSO 5%	Viable	3946	39.46	39.51	25
		Non-Viable	6041	60.41		
3	DMSO 5%	Viable	3761	37.61	37.67	20
		Non-Viable	6224	62.24		
3	DMSO 5%	Viable	5014	50.14	50.24	20
		Non-Viable	4966	49.66		
4	DMSO 5%	Viable	5769	57.69	58.02	60
		Non-Viable	4174	41.74		
4	DMSO 5%	Viable	3476	34.76	34.95	50
		Non-Viable	6471	64.71		
5	DMSO 5%	Viable	4288	42.88	42.95	20
		Non-Viable	5696	56.96		
5	DMSO 5%	Viable	5420	54.2	54.29	30
		Non-Viable	4564	45.64		
1	DMSO 10%	Viable	5042	50.42	50.41	35
		Non-Viable	4960	49.6		
1	DMSO 10%	Viable	4998	49.98	50.05	45
		Non-Viable	4988	49.88		
2	DMSO 10%	Viable	6272	62.72	62.74	20
		Non-Viable	3725	37.25		
2	DMSO 10%	Viable	6260	62.6	62.61	25
		Non-Viable	3739	37.39		
3	DMSO 10%	Viable	5027	50.27	50.34	10
		Non-Viable	4960	49.6		
3	DMSO 10%	Viable	5649	56.49	56.51	10
		Non-Viable	4347	43.47		
4	DMSO 10%	Viable	5088	50.88	50.84	50
		Non-Viable	4919	49.19		
4	DMSO 10%	Viable	5151	51.51	51.53	50
		Non-Viable	4845	48.45		
5	DMSO 10%	Viable	4522	45.22	45.35	15
		Non-Viable	5449	54.49		
5	DMSO 10%	Viable	4237	42.37	42.44	10
		Non-Viable	5746	57.46		

(table cont.)

Fish #	Treatment	Label	Events	Gated (%)	Live (%)	Motility (%)
1	MeOH 5%	Viable	3950	39.5	39.45	40
		Non-Viable	6063	60.63		
3	MeOH 5%	Viable	4499	44.99	44.82	50
		Non-Viable	5538	55.38		
4	MeOH 5%	Viable	5795	57.95	58.28	50
		Non-Viable	4149	41.49		
4	MeOH 5%	Viable	5983	59.83	60.10	60
		Non-Viable	3972	39.72		
5	MeOH 5%	Viable	4827	48.27	48.51	50
		Non-Viable	5124	51.24		
5	MeOH 5%	Viable	2961	29.61	29.72	40
		Non-Viable	7001	70.01		
3	MeOH 10%	Viable	2981	29.81	29.76	35
		Non-Viable	7036	70.36		
4	MeOH 10%	Viable	5754	57.54	57.61	75
		Non-Viable	4234	42.34		
4	MeOH 10%	Viable	5746	57.46	57.74	75
		Non-Viable	4206	42.06		
4	MeOH 10%	Viable	5855	58.55	58.71	65
		Non-Viable	4118	41.18		
5	MeOH 10%	Viable	3482	34.82	35.25	35
		Non-Viable	6395	63.95		
5	MeOH 10%	Viable	4072	40.72	41.12	35
		Non-Viable	5831	58.31		

Vita

William Rittenhouse Wayman was born on April 8, 1968, in Warner Robbins, Georgia, United States of America. He attended St. Martin's Episcopal High School in Metairie, Louisiana. Upon graduating, he attended Auburn University where he earned a Bachelor of Science degree in marine biology. He then worked for one year at Laboratory Technology Inc., a bioassay testing laboratory in Kenner, Louisiana. He enrolled in the graduate program in the School of Forestry, Wildlife and Fisheries at Louisiana State University in 1993. His primary area of focus was cryopreservation of sperm of marine fishes, but he also worked with the U. S. Fish and Wildlife Service to cryopreserve sperm from endangered fishes in Arizona. He earned a Master of Science degree in fisheries in May of 1996, and entered the doctoral degree program at Louisiana State University during the summer of the same year. In October of 1999, William entered the Student Career Experience Program with the U. S. Fish and Wildlife Service, and worked full time at the Warm Springs Fish Technology Center developing a cryopreservation program for endangered fishes. He received the Doctor of Philosophy degree in wildlife and fisheries science from the School of Renewable Natural Resources at Louisiana State University in August of 2003.