Enhancing Saugeye (Sander vitreus x S. canadensis) Production Through the Use of
Assisted-Reproduction Technologies

THESIS

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By

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Abstract

The overall objective of this thesis was to increase the efficiency of saugeye (Sander vitreus x S. canadensis) production in the Ohio Department of Natural Resources hatchery system through the study of sauger (Sander canadensis) sperm. In the first experiment, we investigated the efficacy of sauger sperm cryopreservation in addition to determining the effects of base extender osmolality on sperm cryosurvival. Aliquots of sperm from ten male sauger were diluted with extenders with osmolalities of 350, 500 and 750 mOsm/kg (extender 350, 500 and 750, respectively) to a final concentration of $5.0 \times 10^8$ sperm/mL in extender containing 10% DMSO. Samples were placed at 3 cm above liquid nitrogen for 10 minutes, plunged into liquid nitrogen, and then thawed for 30 seconds at 21°C. Sperm parameters (total motility, progressive motility and velocity) were objectively assessed at different steps of the cryopreservation process. Viability was determined for thawed sperm. Cryoprotectant addition decreased sperm velocity in all extenders, but increased progressive motility in extender 350 and 500. Total motility was not affected by CPA addition in extender 350 and 500 but decreased in extender 750. All parameters measured, except progressive motility, were significantly affected by cryopreservation. Extender 500 produced the highest post-thaw progressive motility ($32.20 \pm 1.20\%$) and velocity ($84.97 \pm 5.32 \mu m/s$) while both extender 350 and 500
displayed the highest total motility (65.30 ± 1.40 and 68.70 ± 2.00%) and viability (80.60 ± 1.50 and 78.80 ± 1.20%), respectively. Extender 750 produced the lowest post-thaw velocity (38.17 ± 2.11 µm/s), viability (71.80 ± 1.20%), total (12.10 ± 1.60%) and progressive motility (1.60 ± 0.60%). The purpose of our second study was to decrease sauger sperm limitation during saugeye production in fish hatcheries. Towards this end, we investigated the effect of hCG administration on sperm production, the effect of a fertilization solution and sperm-to-egg ratios on egg fertilization rates and validated the use of a commercially available densimeter to determine sauger sperm cell concentration. We found that hCG increased sperm volume relative to controls (hCG 0.64 ± 0.19 mL/kg; control: 0.31 ± 0.05 mL/kg) but sperm cell concentration and total sperm production were unaffected. Fertilization rates similar among the three sperm-to-egg ratios tested (20,000/egg: 82.67 ± 3.39%, 50,000/egg: 91.33 ± 2.67% and 100,000/egg: 88.67 ± 3.44%) but were significantly decreased when an activation solution (16.00 ± 2.70%) compared to hatchery water (87.56 ± 2.04%). Lastly, we compared the use of a densimeter versus a hemocytometer and determined that a high correlation exists between the two methods used for calculation of sperm concentration (R² = 96.37%). These results can be used by hatchery staff working with sauger and saugeye to more efficiently quantify and utilize sauger sperm for improved saugeye production efficiency. We conclude that the results of this thesis can be implemented in the state hatchery system to preserve high quality sauger sperm in the long term while also standardizing the use of fresh sperm in the short term with the goal of increasing saugeye production.
Dedication

I dedicate this thesis to my parents Joe and Dana Blawut for always believing in me, supporting me, and pushing me to achieve my goals in life.
Acknowledgments

Without the love and support of my family, I would not have been able to accomplish all that I have in life. I thank them for bestowing me with a strong work ethic and a compassionate upbringing. My parents, Joseph and Dana, have always been there for me and provided the necessary financial and supportive means to achieve my goals in life. I also thank my sisters Leslie and Katie for dragging me to the zoo to volunteer every day as a teenager to foster my love of animals, which has led me to my current path.

Next, I would like to thank Dr. Barbara Wolfe for taking a chance on me as one of her first mentees at Ohio State University and Dr. Marco Coutinho da Silva for agreeing to transition from a committee member into the role of co-advisor. Together the two of you have provided me with the guidance and support that I needed to complete my research, coursework and thesis. It has been a great experience to explore the fields of fish reproduction and sperm cryopreservation together. Through our interactions and your guidance, I have become a better scientist and a better professional.

I owe thanks to Dr. Stuart Ludsin for participating as a committee member, as well as to Brian Kitchen, Doug Sweet, Scott Hale, and Rich Zweifel of the ODNR-DOW-DOW for their technical and financial support during the saugeye production season. Dr. Ludsin’s expertise in statistical analysis and fisheries were a huge help in
completing this project. Brian and Doug shared their years of knowledge and experience with me and were a crucial asset during the fertilization and incubation of saugeye eggs and collection of sauger sperm, respectively. Dr. Rich Zweifel was extremely kind and generous during the planning periods of my research and we could not have orchestrated these studies without him.

Any recollection of positive influences in my life would be incomplete without acknowledging the importance of Dr. Joe Greathouse and the Oglebay Good Zoo. I volunteered at the zoo at a young age in both education and animal care. Dr. Joe Greathouse took a chance on hiring me as a part-time keeper, despite my young age (15). The dedicated and passionate staff at the Good Zoo taught me the very definition of selflessness, hard work, and determination. In addition to animal care responsibilities, I also helped conduct population surveys for the eastern hellbender (*Cryptobranchus alleganiensis*) in West Virginia, which endeared me to the study of wildlife and fisheries. Several years of full day surveys in the rain and cold all while being told to “stop looking for fish” has contributed to my love and dedication for wildlife and science.

I would be remiss if I did not thank the two most important figures in my home life. Thank you Angela for joining me while I pursue my academic passions, for being there when I am uncertain about what the future holds, and for not yelling at me for leaving books, papers, and equipment all over the apartment. And last but not least, I want to give a big thanks to our dog Ellie, who wakes me up every morning and welcomes me home every evening with joyful exuberance. Even the most frustrating
days during my time at OSU were tolerable knowing that her wagging tail was waiting for me to come home.
Vita

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Fields of Study

Major Field: Comparative and Veterinary Medicine
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Chapter 1: Introduction

1.1 Ohio Department of Natural Resources – Division of Wildlife Saugeye Production

The Ohio Department of Natural Resources – Division of Wildlife (ODNR-DOW) aims to provide recreational fishing opportunities within the state of Ohio. Angler pursuit of recreational fisheries contributes hundreds of millions of dollars to the state economy each year in the form of license fees, tackle, travel, and other equipment related to the sport. Maintaining these economically important fisheries is a primary goal of the ODNR-DOW.

Poor recruitment in walleye (*Sander vitreus*) populations during the 1970’s started a search for a sport fish better suited for stocking in shallow, productive, and turbid lakes in Ohio (Hale, Degan, Renwick, Vanni, & Stein, 2008). Saugeye, a hybrid obtained by crossing of male sauger and female walleye (*S. vitreus x S. canadensis*) are fast growing, have high survival and angler catch rates comparable to walleye, and a tolerance for shallow, turbid habitat conditions (Flammang & Willis, 1994; Quist, Stephen, Lynott, Goeckler, & Schultz, 2010a). As a result, saugeye have been stocked extensively in the Midwest United States by state agencies (Quist et al., 2010a). The goal for saugeye stocking each year in Ohio is approximately 13 million fry and 7 million fingerling (ODNR-DOW Saugeye standard operating procedures (SOP)) to maintain these economically and recreationally valuable fisheries.
Saugeye are extensively produced through assisted reproduction by the ODNR-DOW. Up to 300 sexually mature male sauger are collected from the Ohio River during mid-February through mid-March each year and maintained for the remainder of the breeding season at the London State Fish Hatcher’s Isolation Facility. Milt is collected, extended, and transported to walleye collection sites such as the Maumee River, Berlin Reservoir or Mosquito Reservoir for fertilization. Eggs are collected from female walleye and extended sauger sperm is used to fertilize eggs at a fixed ratio of 0.5 mL of milt per quart of eggs. Fertilized eggs are treated with tannic acid and iodine before being transported to one of the state’s hatcheries for a 21 day incubation period. After hatching, a majority of the fry are stocked in lakes and reservoirs around the state while the remaining fry are maintained in earthen ponds at the hatchery before stocking to allow offspring to attain a larger size to increase their chances of survival.

Periodically, the ODNR-DOW is unable to meet saugeye production goals (figure 1.1). Elevated flows on the Ohio River during the spring can partially or entirely prevent the collection of male sauger broodstock. In addition to the lack of broodstock, the volume of milt produced by each male is small. As a results, the amount of sauger sperm available for the production of saugeye is often the limiting factor in saugeye production. Currently, the methodology used in saugeye production do not address long term storage of sperm, standardized sperm to egg ratios or sperm quality and quantity that could help alleviate sperm shortages. Sperm limitation in the ODNR-DOW hatchery system needs to be addressed in order to improve saugeye production independent of climate conditions and male availability.
Sperm limitation can be addressed by long-term storage of sperm achieved through cryopreservation. Cryopreservation of gametes is beneficial because it allows for long-term storage of different genetic lines, out-of-season spawning, hybridization of different species, protection of stock from natural disasters, easy stock transportation among hatcheries, and enhanced sperm economy (Cabrita et al., 2010; Cloud, Miller, & Levanduski, 1990; Muchlisin, 2005). Below the surface of liquid nitrogen (~ -196°C), cells can be stored for an indeterminate amount of time. A frozen supply of high quality

Figure 1.1 Yearly saugeye fry and fingerling production by the ODNR-DOW, 2006 – 2015, with reference lines for yearly production goals (grey = fry, red= fingerling).
sauger sperm can help ensure that male gametes are available when climate conditions and/or limited male broodstock hinder saugeye production.

1.2 Fish Sperm Cryopreservation

Cryopreservation is the process of cooling cells to a low enough temperature that all biologically important interactions cease, thus allowing it to be stored for an indefinite amount of time. Despite its many benefits, cryopreservation has yet to become a widely used technique in the management of fish species due to a lack of well-defined cryopreservation protocols, poor sperm cryosurvival, quality assurance, and species-specific differences in sperm physiology (Torres, Hu, & Tiersch, 2016a). Shortly after the discovery of cryoprotectants and the successful cryopreservation of fowl and bovine sperm during the early 1950’s, Blaxter (Blaxter, 1953) successfully cryopreserved sperm from the Atlantic herring (Clupea harengus) with post-thaw fertilization rates of 80%.

Fish sperm cryopreservation has progressed slowly since its inception with several factors hindering its application beyond the laboratory (Torres, Hu, & Tiersch, 2016b). Since this time, sperm from more than 200 species of fish have been cryopreserved successfully (Billard, Cosson, Crim, & Suquet, 1995). However, species-specific differences in sperm characteristics and the vast diversity of fish, approximately 30,000 species, has led to an ever increasing number of publications in new species (Cabrita et al., 2010). Among all fish, marine species have shown significantly higher cryosurvival which is generally attributed to a higher cholesterol content in the plasma membrane and natural tolerance to hypertonic environments (E. Kopeika & Kopeika, 2008). To further complicate matters, fish sperm show such a high level of diversity in
spermatozoal biochemical and morphological characteristics (Mattei, 1991) that cryopreservation protocols are typically species-specific with close relatives diverging on optimal conditions. An example of this diversity is provided in table 1.1 where the current protocols for prominent members of family Percidae are displayed. Ultimately, extrapolation of an existing, specific protocol to an unstudied species of fish is unlikely to produce desirable results; a new protocol must be developed for each new species studied.
<table>
<thead>
<tr>
<th>Species</th>
<th>Source</th>
<th>Dilution Ratio</th>
<th>Extender</th>
<th>Cryoprotectant</th>
<th>Protein Source</th>
<th>Cooling Method</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Sander vitreus</em></td>
<td>(Alan A. Moore, 1987)</td>
<td>1:2</td>
<td>Rathbun¹</td>
<td>7% DMSO</td>
<td>7.5mg/mL ProFam, 4mg/mL BSA</td>
<td>Dry Ice</td>
<td>43.2-83.2% fertilization</td>
</tr>
<tr>
<td></td>
<td>(Bergeron, Vandenberg, Proulx, &amp; Bailey, 2002)</td>
<td>1:15</td>
<td>Rathbun¹</td>
<td>7% DMSO</td>
<td>7.5mg/mL ProFam, 4mg/mL BSA</td>
<td>Dry Ice</td>
<td>46% post thaw motility</td>
</tr>
<tr>
<td><em>Perca fluviatilis</em></td>
<td>(Bernáth et al., 2015)</td>
<td>1:20</td>
<td>Tanaka Extender²</td>
<td>10% Methanol</td>
<td>-</td>
<td>Controlled Rate Freezer</td>
<td>50-72% post that progressive motility</td>
</tr>
<tr>
<td><em>Perca flavescens</em></td>
<td>(Glogowski, Ciereszko, &amp; Dabrowski, 1999)</td>
<td>1:4</td>
<td>0.45 M sucrose</td>
<td>15% DMSO, 15%DMA</td>
<td>10% Egg yolk, None</td>
<td>Dry Ice</td>
<td>42.5-47.2% fertilization</td>
</tr>
<tr>
<td></td>
<td>(Ciereszko, Ramseyer, &amp; Dabrowski, 1993)</td>
<td>1:4,1:8</td>
<td>125mM sucrose, 6.5 mM reduced glutathione and 100mM potassium bicarbonate</td>
<td>8% DMSO</td>
<td>10% Egg yolk</td>
<td>Dry Ice</td>
<td>23.2% fertilization</td>
</tr>
</tbody>
</table>

Continued
Table 1.1 continued

<table>
<thead>
<tr>
<th>Species</th>
<th>Extender Details</th>
<th>Extender Ratio</th>
<th>Glucose Concentration</th>
<th>Ammonium Concentration</th>
<th>Post-thaw Motility</th>
<th>Hatching Success</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Sander lucioperca</em></td>
<td>(Zoltan Bokor, Horvath, Horvath, &amp; Urbany, 2008)</td>
<td>1:1</td>
<td>350 mM glucose and 30 mM Tris</td>
<td>10% Methanol</td>
<td>-</td>
<td>53% post-thaw motility, 47-87 % hatching success</td>
</tr>
<tr>
<td><em>Sander lucioperca</em></td>
<td>(Z. Bokor et al., 2007)</td>
<td>1:9</td>
<td>350 mM glucose and 30 mM Tris</td>
<td>10% DMSO</td>
<td>-</td>
<td>23% post-thaw motility, 43% fertilization</td>
</tr>
<tr>
<td><em>Sander volgensis</em></td>
<td>(Z. Bokor et al., 2007)</td>
<td>1:1</td>
<td>350 mM glucose</td>
<td>10% Methanol</td>
<td>-</td>
<td>60% hatching success</td>
</tr>
</tbody>
</table>

¹Rathbun Extender: 0.234g Calcium Chloride dihydrate, 0.267g Magnesium chloride, 0.472g Sodium phosphate dibase, 3.744g Potassium chloride, 13.155g Sodium Chloride, 20.000g Glucose, 0.200g Citric acid monohydrate, 0.508g Sodium hydroxide, 2.12 g Bicine, and 2.0 liters of distilled water.
²Tanaka Extender: 137mM NaCl and 76.2 NaHCO₃
Cells are typically frozen using one of two methods: equilibrium (conventional cryopreservation) or non-equilibrium cooling (vitrification). Conventional cryopreservation allows cells to cool slowly in order to reach an equilibrium in terms of the tonicity of the intra- and extra-cellular environments through cellular dehydration. This process deters the formation of intracellular ice. Vitrification uses solutions with very high concentrations of cryoprotectants and ultra-fast freezing rates to bypass the formation of ice completely en route to a state in which water exits in a glass-like state. While vitrification has shown promise in the field of fish sperm cryopreservation, conventional equilibrium cooling techniques are still the most widely applied.

During the cryopreservation process, sperm cells are exposed to a variety of abnormal, and potentially lethal conditions. First, sperm cells must be cooled to a low temperature before the addition of cryoprotectants to reduce osmotic stress and cryoprotectant toxicity. Cryoprotectants are then added to the solution to help cells maintain cell volume while cooling and to colligatively lower the freezing temperature and ice formation, at any given point during cooling (H. T. Meryman, 1971). Cells are then cooled in the vapor or liquid nitrogen or on dry ice. Pure water forms ice crystals in the extracellular environment as the temperature begins to fall below -5°C which causes the osmolality of the remaining solution to rise. This disparity in tonicity draws water out of the cell in attempt to equilibrate with the extracellular environment. Dehydration caused by this process is essential to the success of a cryopreservation protocol (Chao & Liao, 2001). When cells are cooled to a target temperature/state, a plunge into liquid nitrogen causes the transformation of the unfrozen liquid fraction to a vitrified state.
During thawing, cells are exposed to an increasingly hypotonic solution, which leads to water re-entering the cells until pre-freeze isosmolality can be achieved.

Owing to these unnatural conditions, most samples exhibit motility of 50% or less following cryopreservation (table 1.1). Lipid phase-transition and reactive oxygen species are responsible for a loss of membrane integrity during the initial phase of cooling, commonly referred to as cold shock (Holt, 2000; Sabeti, Pourmasumi, Rahiminia, Akyash, & Talebi, 2016). Cryoprotectants have been shown to be toxic at molar concentrations and volume changes beyond osmotically tolerable limits, and hence are implicated in a loss of membrane integrity (Doyong Gao & Critser, 2000; Hammerstedt, Graham, & Nolan, 1990; J. Kopeika, Kopeika, Zhang, & Rawson, 2003; Parks & Graham, 1992; Woods, Benson, Agca, & Critser, 2004). However, the formation and dissolution of ice is by far the most damaging process during cryopreservation (Parks & Graham, 1992; Pegg, 2002). Intracellular ice formation can damage cell membranes as well as the flagella and organelles of cells (figure 1.2). Cells exposed to increasingly hypertonic environments lose a considerable amount of volume which can lead to plasma membrane fusion and loss that manifests as losses in viability through the lysis of plasma membrane as cells attempt to return to isosmotic volume (Doyong Gao & Critser, 2000; Harold T. Meryman, 1974). The remaining cells that do survive this process may still bear some form of sub-lethal damage such as damaged organelles or DNA fragmentation.
Figure 1.2 Illustration of the techniques used in cryopreservation of gametes, as well as the two main sources of damage to cells during cooling. Cryoprotectant (black), solutes (white), and ice crystals (stars) are presented as colored dots and shapes. The two most common types of cryopreservation A) conventional and B) vitrification are illustrated at the top of the diagram. Panels in C) shows the possible negative effects of cooling at a rate that is too slow and results in solution effects with concentrated solutes within the cell. Panels in D) shows the negative consequences of rapid cooling with ice crystals forming within the cell. Figure taken from (T. R. Tiersch & C. C. Green, 2011)

To ameliorate the negative effects of cryopreservation, studies have been conducted to improve the post-thaw quality of fish sperm. A majority of these studies have focused on creating protocols for new species, fine tuning cryoprotectants, cooling/thawing rates, container types, sperm-to-egg ratios, activation solutions, and attempting to use vitrification. Despite the plethora of new studies, many species of freshwater fish still show low cryosurvival with few attempts to go beyond the traditional avenues of cryosurvival improvement. Sperm modulation, subpopulation analysis,
genetic improvement, DNA damage monitoring, reactive oxygen species formation, mitochondrial function and metabolism have all been suggested as possible areas for improvement in the field of fish sperm cryopreservation (Cabrita et al., 2010; Herráez, Cabrita, & Robles, 2012). Techniques to increase cryosurvival of freshwater fish sperm could aid in the conservation of endangered species and production of economically valuable species as well as increase the efficacy of incorporating cryopreserved sperm into routine hatchery practices (Vajta & Kuwayama, 2006).

Modulation of dehydration through extender manipulation could have significant effects on post-thaw quality in freshwater fish. Enhancing dehydration during cooling by increasing the osmolality of the base extender prior to freezing has been investigated in a mammalian species whose sperm shows an atypically poor cryosurvival rate, the ram (*Ovis aires*). Several studies on ram sperm cryopreservation using trehalose to increase base extender osmolality have shown increased cryosurvival leading to a doubling in lambing rates (E. G. Aisen, Medina, & Venturino, 2002). This technique has also been utilized in the anadromous striped bass (*Morone saxitilis*) with a hypertonic base extenders (~ 600 mOsm/kg) producing the highest sperm quality after thawing (He & Woods, 2003). While this technique has proven successful in these two species, further research should be codirected on the efficacy of hypertonic extenders and specifically their effects on a freshwater fish species.
1.3 Project Goals

The primary goal of this project is to increase the efficiency of saugeye production in Ohio’s hatcheries. The overarching goals of this thesis are to: 1.) develop a protocol for successful cryopreservation of sauger sperm, and 2.) increase the efficiency of saugeye production by improving sperm management in the hatchery system. In Chapter 2, I describe a study on the use of hypertonic base extenders during sauger sperm cryosurvival to increase post-thaw quality. Chapter 3 describes a series of studies on the effects of human chorionic administration on sperm production, validating the use of a commercially available equine densimeter to quickly and accurately quantify sperm cell concentration in ejaculates and determine the effect of sperm-to-egg ratio and two fertilization solutions on egg fertilization rates. Finally, in Chapter 4 I provide an overview of the results and conclusions of the studies we have completed throughout the 2015 and 2016 sampling season and future directions for this research.
1.4 References


Chapter 2: Use of Hypertonic Medium to Cryopreserve Sauger (Sander canadensis)

Spermatozoa

2.1 Abstract

Male sauger (Sander canadensis) are routinely crossed with walleye (S. vitreus) to produce saugeye (S. vitreus x S. canadensis) sport fish that contribute millions of dollars annually to state economies. Periodically, agencies are unable to meet saugeye production goals primarily due to heavy precipitation during the breeding season, resulting in low broodstock availability. Cryopreservation offers a means to store sperm for future use when male broodstock are in short supply, but no protocol exists for this species. While freshwater fish sperm has shown poor resistance to cryopreservation, base extenders hypertonic to the seminal plasma have improved post-thaw sperm quality in other species with poor cryosurvival. The objective of this study was to determine the effect of extender osmolality on post-thaw sperm quality in sauger. Fresh milt from 10 male sauger was diluted using extenders with osmolalities of 350, 500, or 750 mOsm/kg (extender 350, 500 and 750, respectively) containing 10% DMSO, frozen in LN2 vapor, and stored for 3 mos before being thawed and analyzed. Sperm parameters (total motility, progressive motility, velocity, and viability) were objectively assessed at different steps of the cryopreservation process (extended, equilibrated, and post-thaw). Cryoprotectant
(CPA) addition decreased sperm velocity in all extenders (p<0.001), but increased progressive motility in extender 350 and 500 (p<0.001). Total motility was unaffected by CPA addition in extender 350 and 500 but decreased in extender 750 (p<0.001). All parameters measured, except progressive motility, were significantly reduced by cryopreservation. Extender 500 yielded the highest post-thaw progressive motility (32.20 ± 1.20%) and velocity (84.97 ± 5.32 µm/s) whereas both extender 350 and 500 displayed the highest total motility (65.30 ± 1.40 and 68.70 ± 2.00%) and viability (80.60 ± 1.50 and 78.80 ± 1.20%), respectively. By contrast, extender 750 yielded the lowest post-thaw velocity, viability, total, and progressive motility. In conclusion, the use of a hypertonic extender with osmolality of 500 mOsm/kg resulted in higher sperm velocity and progressive motility post-thaw compared to an isosmotic extender. The improvements in sauger sperm cryosurvival obtained in our study lay the foundation for future experiments evaluating the fertilizing capacity of freshwater fish sperm cryopreserved in hypertonic extenders.

2.2 Introduction

Spermatozoa from > 200 species of fish have been cryopreserved successfully (Billard et al., 1995). Cryopreservation of gametes is beneficial by allowing for long-term storage of different genetic lines, out-of-season spawning, creation of hybrid stocks, protection of a species or stock from natural disasters, easy stock transportation among hatcheries, and precise sperm allocation (Cabrita et al., 2010; Cloud et al., 1990; Muchlisin, 2005). Despite the numerous benefits of cryopreservation, the process can have negative impacts on post-thaw sperm quality (Cabrita et al., 2010), especially in
freshwater fishes. Marine species have shown substantially higher cryosurvival rates, which is generally attributed to a higher cholesterol content in the sperm’s plasma membrane and natural tolerance to hypertonic environments (E. Kopeika & Kopeika, 2008). Except for a few notable exceptions, most freshwater species exhibit a 50% or greater reduction in motility following cryopreservation (Suquet, Dreanno, Fauvel, Cosson, & Billard, 2000). The poor cryosurvival of freshwater fish thus far has hindered the use of cryopreserved sperm beyond the laboratory. Improvements to current cryopreservation protocols that lead to increased post-thaw sperm quality would benefit both laboratory and commercial application of cryopreserved sperm for freshwater species.

To prevent damage to the plasma membrane during the process of cryopreservation, sperm cells must lose water to their environment. Exposure to an increasingly hypertonic environment during cooling results in dehydration as water is drawn from the cell in response to this gradient (Hammerstedt et al., 1990; Holt, 2000; Parks & Graham, 1992). The use of base extenders hypertonic to the seminal plasma to increase dehydration have been investigated in striped bass (Morone saxatilis). In this anadromous species, an extender isotonic to the seminal plasma was less effective in cryopreserving bass sperm than a hypertonic base extender of 600 mOsm/kg (He & Woods, 2003). However, motility was prematurely activated by extenders at both the isotonic and highest osmolalities tested. Therefore, the effects of extender osmolality and cryopreservation could not be separated from the inability to keep sperm immotile.
Our study addresses knowledge gap by investigating hypertonic base extenders in sauger (*Sander canadensis*), a freshwater fish with known motility activation mechanisms, to determine the effects of enhanced dehydration on post-thaw quality. Male sauger have been routinely used to produce a hybrid fish, saugeye (*S. vitreus x S. canadensis*), through cross-fertilization with female walleye (*S. vitreus*). Saugeye are stocked extensively in the Midwestern United States by state agencies (Lynch, Johnson, & Schell, 1982) and comprise an economically and recreationally valuable fishery by contributing millions of dollars annually to local economies. Saugeye are fast growing, and have angling rates similar to walleye while also being more tolerant of eutrophic systems, thus making them a viable option for lake and reservoirs where walleye stocking has not been successful in the past (Flammang & Willis, 1994; Lynch et al., 1982; Quist, Stephen, Lynott, Goeckler, & Schultz, 2010b).

Saugeye are produced during a short period in spring when the sauger and walleye breeding season overlap. Frequently, heavy spring rainfall and snowmelt prevent the collection of a sufficient number of male broodstock to meet saugeye production demands. Long-term storage of sauger sperm through a successful cryopreservation protocol resulting in high post-thaw quality could allow for optimal saugeye production regardless of climate conditions and male availability. However, to date, there are no published reports of cryopreservation in sauger spermatozoa.

Herein, we quantified the effect of hypertonic base extenders on cryosurvival and post-thaw sperm parameters of sauger spermatozoa. Our specific objectives were to cryopreserve sauger sperm using extenders with different osmolalities (350, 500 and 750...
mOsm/kg) and compare sperm quality (total motility, progressive motility and sperm velocity) among base extenders through the completion of the cryopreservation process. We hypothesized that the use of hypertonic base extenders would result in higher sperm motility characteristics and viability post-thaw by enhancing dehydration during cooling and reducing intracellular ice crystal formation.

2.3 Materials and Methods

Chemicals and Media

Rathbun extender (Alan A. Moore, 1987) was used as the isotonic base medium [350 mOsm/kg, sauger seminal plasma (mean ± SEM): 331.49 ± 10.36 (BJB, unpublished data)] and two hypertonic media (500 and 750 mOsm/kg) were prepared by increasing the amount of solute per volume of Rathbun by a fixed factor (Table 2.1). Prior to cryopreservation, extenders were supplemented with 4 mg/mL bovine serum albumin (BSA), which resulted in a pH of 7.6. Extender osmolality was confirmed using a Vapro 5600 vapor pressure osmometer (Wescor, Puteaux, FR).
Table 2.1 Composition of extenders used during cryopreservation modified from Rathbun Extender

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Extender</th>
<th>350</th>
<th>500</th>
<th>750</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium chloride dihydrate (CaCl₂·2H₂O)</td>
<td></td>
<td>0.234g</td>
<td>0.334g</td>
<td>0.501g</td>
</tr>
<tr>
<td>Magnesium chloride (MgCl₂·6H₂O)</td>
<td></td>
<td>0.267g</td>
<td>0.381g</td>
<td>0.572g</td>
</tr>
<tr>
<td>Sodium phosphate diabase (Na₂HPO₄)</td>
<td></td>
<td>0.472g</td>
<td>0.674g</td>
<td>1.011g</td>
</tr>
<tr>
<td>Potassium chloride (KCl)</td>
<td></td>
<td>3.744g</td>
<td>5.349g</td>
<td>8.023g</td>
</tr>
<tr>
<td>Sodium chloride (NaCl)</td>
<td></td>
<td>13.155g</td>
<td>18.793g</td>
<td>28.189g</td>
</tr>
<tr>
<td>Glucose</td>
<td></td>
<td>20.000g</td>
<td>28.571g</td>
<td>42.857g</td>
</tr>
<tr>
<td>Citric acid monohydrate (HOCCOOH [CH₂COOH]₂·H₂O)</td>
<td></td>
<td>0.200g</td>
<td>0.286g</td>
<td>0.429g</td>
</tr>
<tr>
<td>Sodium hydroxide (NaOH)</td>
<td></td>
<td>0.508g</td>
<td>0.726g</td>
<td>1.089g</td>
</tr>
<tr>
<td>Bicine</td>
<td></td>
<td>2.120g</td>
<td>3.029g</td>
<td>4.543g</td>
</tr>
<tr>
<td>Distilled Water</td>
<td></td>
<td>2000 ml</td>
<td>2000 ml</td>
<td>2000 ml</td>
</tr>
<tr>
<td>Osmolarity</td>
<td></td>
<td>350 mOsm/kg</td>
<td>500 mOsm/kg</td>
<td>750 mOsm/kg</td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td>8.5</td>
<td>8.5</td>
<td>8.5</td>
</tr>
</tbody>
</table>

Prior to cryopreservation, extenders were supplemented with 4mg/mL BSA which resulted in a pH of 7.6

Broodstock and Gamete Collection

Mature male sauger were collected from the Ohio River near the Greenup, KY, USA dam during February and March 2016 via electrofishing by Ohio Department of Natural Resources – Division of Wildlife personnel. They were transported by truck for ~2 hours in a 0.5% salt solution to the London State Fish Hatchery isolation facility (London, OH, USA) and then maintained in a ~2840 L recirculating system at 4-5°C with a flow rate of 45-57 L/hour through the end of the study. Photoperiod was not controlled during this experiment. Fish were allowed to acclimate for at least one week before milt collection. All procedures and animal use were approved by The Ohio State University.
Milt was obtained from 20 males. Upon capture, individuals were dried with a towel to prevent premature activation of spermatozoa with water. Milt was collected using abdominal massage and a 1.0 mL rubberless syringe at the opening of the cloaca. The total volume of milt produced was determined to the nearest 0.01 mL and milt was immediately extended 1:2 with 350 mOsm/kg Rathbun extender and maintained at 5° C.

After collection, initial sperm motility was assessed subjectively for each sample using a compound microscope at 100 X magnification. An aliquot of one µL of milt was diluted in 199 µL of Rathbun extender. Samples were activated by further diluting 1 µL of diluted milt in 9 µL of distilled water supplemented with 1% BSA. Samples exhibiting a total motility of ≥80% (n=10) were selected for this study.

Sperm Evaluation, Processing, and Cryopreservation

In the laboratory, sperm concentration of each sample was determined using an Equine 590b densimeter (Animal Reproductive Systems, Chino, CA, USA) that was previously validated for sauger sperm in our laboratory (Blawut, unpublished data). Aliquots of sperm from each sample were diluted to a concentration of 1.0 x10⁹ sperm/mL using Rathbun extender prepared at different osmolalities [350 mOsm/kg (extender 350); 500 mOsm/kg (extender 500); and 750 mOsm/kg (extender 750)] and equilibrated at 5° C for 10 min. Each sample was then diluted 1:1 (v: v) with the respective extender containing 20% dimethyl sulfoxide (DMSO) for a final concentration of 10% DMSO and 5 x 10⁸ sperm/mL. Samples were then loaded into 0.5mL French
straws (Agtech, Manhattan, KS, USA) and re-equilibrated at 5° C for 10 min before being placed at 3 cm above the surface of liquid nitrogen and allowed to freeze for 10 min. Straws were then plunged into LN₂ and stored at -196°C until analysis.

Sperm motility parameters were evaluated at three different time points: after dilution of sperm with extender (Extended); after addition of extender containing DMSO (Equilibrated); and after thawing (Post-Thaw). At each time point, sperm samples were allowed to equilibrate at 5° C for 10 min before samples were activated as previously described (section 2.2). After activation, an aliquot (2 µL) of activated sperm was quickly transferred to a Cytonix microchamber (20 µm depth, Cytonix LLC, Beltsville, MD, USA) and analyzed within 10-15 s post activation using a Ceros II computer assisted sperm analysis (CASA) system (Hamilton Thorne, Beverly, MA, USA). Images were captured at a rate of 60 Hz for 30 frames. Progressively motile sperm were considered to have an average path velocity (VAP) of >50 µm/s and a straightness of > 80.0%. A minimum VAP of 20 µm/s was used as a criterion for motile cells to reduce the effects of drift (Park, Egnatchik, Bordelon, Tiersch, & Monroe, 2012). Other CASA settings included: exposure = 80 Ms; gain = 300; minimum head brightness = 152; min head size = 1 µm²; max head size = 19 µm²; capillary correction = 1.3; max photometer = 70; min photometer = 60; and minimum total cell count = 200. Experimental endpoints included total motility (%), progressive motility (%) and average path velocity (µm/s).

**Thawing Procedure and Post-thaw Analysis**

Two straws per treatment replicate for each male were thawed simultaneously in a water bath at 21° C for 30 s. The contents of both straws were combined in a
microcentrifuge tube, mixed thoroughly, and maintained at 5° C using a bench-top cooler (VWR International, Radnor, PA, USA). Motility was examined by CASA within 1-2 min of thawing. Plasma membrane integrity (i.e., viability) was determined using the Live/Dead® Sperm Viability kit (Molecular Probes, Eugene, OR, USA). Briefly, thawed sperm (67µL) was diluted to 1.0 mL using the isotonic extender, supplemented with 10% BSA and 10 mM HEPES, and then 2 µL (0.2 µM) and 5 µL (1.2 µM) of SYBR 14 and propidium iodide (PI) dye, respectively, were added to the solution. Samples were incubated at 5°C for 10 min and then visualized using an Accu-Scope 3025 (Accu-Scope Commack, NY, USA) fluorescent microscope at 1000 X. Spermatozoa (n=200) were evaluated and the percentage of cells with intact plasma membranes (SYBR positive, PI negative) was determined for each sample.

Activation Osmolarity

To determine if osmolarity of activation was affecting extender 750 post-thaw sperm parameters, we activated sperm at several osmolalities through increasingly larger activation dilutions. A single straw of sperm cryopreserved in extender 750 from 5 replicates was thawed as described in above. Then, two microliters of thawed sperm were activated in either a 10, 15 or 20 fold dilution in distilled water containing 1.0% BSA before analyzing sperm quality parameters at 10-15 seconds post-thaw using the CASA as described above.

Statistical Analysis

All statistical analyses were completed using Statistica 13 software (Dell Statistica Tulsa, OK USA) with an α-value of 0.05. Response variables were found to be
normal (Kolmogorov-Smirnoff tests) with variances being homogenous (Levene’s test). Thus, no data transformations were necessary. The effects of base extender treatment and differences among time points (i.e., Extended, Equilibrated, and Post-Thaw) on sperm quality were analyzed using repeated measures ANOVA followed by Fisher’s LSD post hoc test (n=6), when a significant treatment effect was found. Post-thaw sperm attributes were compared among the three base extender treatments using a one-way ANOVA and Fisher’s LSD post hoc tests (n=10). A one way ANOVA was used to determine the effect of the dilution ratio on post-thaw sperm from the extender 750 treatment (n=5).

2.4 Results

We found a large degree of variability in milt quality parameters among our study individuals. Only 50% of the sperm samples collected from the initial 20 sauger displayed an initial motility > 80% and were used in the study. No correlation of length or mass was found with any initial sperm quality parameters. Table 2.2 represents the detailed physical and reproductive parameters of the sauger sperm donors used for this study.
Table 2.2 Physical and reproductive parameters of the male sauger selected for the use in cryopreservation.

<table>
<thead>
<tr>
<th>FishID</th>
<th>Weight (g)</th>
<th>Standard Length (mm)</th>
<th>Volume (mL)</th>
<th>Concentration (Sperm/mL)</th>
<th>Total Sperm (#)</th>
<th>pH</th>
<th>Total Motility (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>217.4</td>
<td>305</td>
<td>0.25</td>
<td>7.96 x 10^{10}</td>
<td>1.99 x 10^{10}</td>
<td>8.2</td>
<td>90</td>
</tr>
<tr>
<td>2</td>
<td>255.1</td>
<td>319</td>
<td>0.6</td>
<td>3.93 x 10^{10}</td>
<td>2.36 x 10^{10}</td>
<td>8.2</td>
<td>99</td>
</tr>
<tr>
<td>3</td>
<td>354.6</td>
<td>363</td>
<td>0.3</td>
<td>7.13 x 10^{10}</td>
<td>2.14 x 10^{10}</td>
<td>8</td>
<td>93</td>
</tr>
<tr>
<td>4</td>
<td>354.2</td>
<td>360</td>
<td>0.26</td>
<td>8.82 x 10^{10}</td>
<td>2.29 x 10^{10}</td>
<td>8.5</td>
<td>80</td>
</tr>
<tr>
<td>5</td>
<td>251.3</td>
<td>320</td>
<td>0.25</td>
<td>5.22 x 10^{10}</td>
<td>1.31 x 10^{10}</td>
<td>7.9</td>
<td>90</td>
</tr>
<tr>
<td>6</td>
<td>369.3</td>
<td>343</td>
<td>0.16</td>
<td>3.69 x 10^{10}</td>
<td>5.90 x 10^{09}</td>
<td>7.3</td>
<td>80</td>
</tr>
<tr>
<td>7</td>
<td>389.5</td>
<td>358</td>
<td>0.3</td>
<td>8.12 x 10^{10}</td>
<td>2.43 x 10^{10}</td>
<td>8</td>
<td>97</td>
</tr>
<tr>
<td>8</td>
<td>251.6</td>
<td>357</td>
<td>0.18</td>
<td>7.96 x 10^{10}</td>
<td>1.43 x 10^{10}</td>
<td>8.5</td>
<td>97</td>
</tr>
<tr>
<td>9</td>
<td>289.3</td>
<td>340</td>
<td>0.23</td>
<td>9.30 x 10^{10}</td>
<td>2.14 x 10^{10}</td>
<td>8</td>
<td>96</td>
</tr>
<tr>
<td>10</td>
<td>216.4</td>
<td>316</td>
<td>0.31</td>
<td>8.82 x 10^{10}</td>
<td>2.73 x 10^{10}</td>
<td>8.2</td>
<td>95</td>
</tr>
</tbody>
</table>

Mean ± SEM

<table>
<thead>
<tr>
<th>Mean</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>294.9</td>
<td>20.9</td>
</tr>
<tr>
<td>338.1</td>
<td>6.8</td>
</tr>
<tr>
<td>0.284</td>
<td>0.04</td>
</tr>
<tr>
<td>7.10 x 10^{10}</td>
<td>6.54 x 10^9</td>
</tr>
<tr>
<td>1.94 x 10^{10}</td>
<td>6.45 x 10^9</td>
</tr>
<tr>
<td>8.12</td>
<td>0.11</td>
</tr>
<tr>
<td>91.7</td>
<td>2.16</td>
</tr>
</tbody>
</table>

The addition of the cryoprotectant, cooling/thawing, and base extender treatments affected sperm parameters. Cryoprotectant addition reduced sperm velocity in all three base extenders (Extended: 108.57 ± 5.82; Equilibrated: 71.45 ± 6.93 µm/s; p<0.05). Extenders 350 and 500 had higher velocities (97.76 ± 8.42 and 100.53 ± 8.63 µm/s, respectively) than extender 750 (71.71 ± 9.63 µm/s; p<0.05) after cryoprotectant addition. An interaction existed between time and treatment for total and progressive motility during cryoprotectant addition (Table 2.3). Both total and progressive motility decreased in extender 750 after cryoprotectant addition. By contrast, total motility was unaffected and progressive motility was significantly higher after cryoprotectant addition.
for extenders 350 and 500. Cryopreservation negatively affected total motility and sperm velocity across all treatments (Equilibrated: 61.97 ± 6.57% and 71.45 ± 6.93 µm/s; Post-thaw: 48.78 ± 6.35% and 58.03 ± 5.25 µm/s, respectively; p<0.05). Progressive motility was unaffected by cryopreservation (Equilibrated: 19.40 ± 2.81; Post-Thaw: 17.27 ± 3.08; p > 0.05).

Table 2.3 Effect of cryoprotectant addition on sauger sperm quality compared among three hypertonic base extender treatments (n=6 replicates per treatment). [Mean ± SEM]

<table>
<thead>
<tr>
<th>Sperm Parameter</th>
<th>Time</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>350 mOsm/kg</td>
</tr>
<tr>
<td>Total Motility (%)</td>
<td>Extended</td>
<td>86.50 ± 3.37</td>
</tr>
<tr>
<td></td>
<td>Equilibrated</td>
<td>82.15 ± 3.10 a</td>
</tr>
<tr>
<td>Progressive Motility (%)</td>
<td>Extended</td>
<td>10.98 ± 3.82 a</td>
</tr>
<tr>
<td></td>
<td>Equilibrated</td>
<td>24.28 ± 2.79 b</td>
</tr>
</tbody>
</table>

a,b Values for each sperm parameter with different superscripts differ.

The osmolality of the extender affected post-thaw sperm velocity, viability, total motility, and progressive motility (Table 2.4, all p < 0.05). Extenders 350 and 500 exhibited similar total motility and viability. Moreover, progressive motility and sperm velocity were higher after thawing for samples diluted in extender 500 than extender 350. Extender 750 exhibited the lowest total motility, progressive motility, sperm velocity and viability of any treatment.
Table 2.4 Comparison of post-thaw sperm quality characteristics among three base extender treatments (n=10). [Mean +/- SEM]

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>350 mOsm/kg</td>
</tr>
<tr>
<td>Total Motility (%)</td>
<td>65.30 ± 1.4 a</td>
</tr>
<tr>
<td>Progressive Motility (%)</td>
<td>19.50 ± 1.50 b</td>
</tr>
<tr>
<td>Viability (%)</td>
<td>80.60 ± 1.50 a</td>
</tr>
<tr>
<td>Velocity (µm/s)</td>
<td>62.60 ± 5.07 b</td>
</tr>
</tbody>
</table>

Values with different superscripts within rows differ (P<0.05).

The dilution used to activate post-thaw sperm from treatment 750 affected sperm velocity, total motility and progressive motility (Table 2.5). Sperm velocity was similar in dilutions 10 and 15 but significantly reduced in dilution 20. Total motility was highest in dilution 10 and progressive motility was similar between dilutions 10 and 15 but was altogether absent in the 20 fold dilution.

Table 2.5 Comparison of sperm parameters in sperm cryopreserved in extender 750 at different activation dilutions (n=5). [Mean +/- SEM].

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Activation Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:10</td>
</tr>
<tr>
<td>Velocity (µm/s)</td>
<td>37.03 ± 2.68 a</td>
</tr>
<tr>
<td>Total Motility (%)</td>
<td>12.38 ± 2.15 a</td>
</tr>
<tr>
<td>Progressive Motility (%)</td>
<td>1.32 ± 0.61 a</td>
</tr>
</tbody>
</table>

a,b Values with different superscripts within rows differ.
2.5 Discussion

We assessed the effects of hypertonic base extenders on sperm cryopreservation in the freshwater fish sauger. As with previous studies, cryopreserved sperm were of lower quality than fresh sperm for most variables (Linhart, Rodina, & Cosson, 2000). We demonstrated that an extender with an osmolality of 500 mOsm/kg was able to achieve higher post-thaw sperm velocity and progressive motility compared to isosmotic Rathbun extender (350 mOsm/kg). In previous studies, polysaccharides, namely trehalose, were used to raise solution osmolality and produced higher levels of dehydration while positively interacting with the sperm plasma membrane during cryopreservation (E. Aisen, Quintana, Medina, Morello, & Venturino, 2005; Richards et al., 2002). Our study used ionic extenders that do not interact with elements of the plasma membrane. Therefore, we speculate that the positive effects of hypertonic base extenders on sperm cryosurvival were a result of increased dehydration during the cooling process.

The use of the 750 mOsm/kg extender resulted in detrimental effects on all sperm parameters evaluated throughout the cryopreservation process. Despite similar total motility after extension with the different extenders, addition of cryoprotectant resulted in a significant decrease in motility and velocity only for extender 750. We speculate that initial dilution of semen with a highly hypertonic extender (i.e., 750 mOsm/kg) resulted in substantial dehydration of sperm during equilibration. Addition of cryoprotectant resulted in further increase in the tonicity of the extender leading to excessive dehydration, resulting in potential cytoskeletal damage and fusion of plasma membrane. The potential fusion of plasma membrane leads likely led to a reduction in the osmotic
tolerance of the cell upon rehydration resulting in plasma membrane disruption and cell death (Rutllant, Pommer, & Meyers, 2003).

Despite these potential changes in plasma membrane function, sperm viability was similar between extenders after thawing. This finding could be explained by the fact that plasma membrane integrity was assessed in the post-thaw samples without dilution or activation. Thus, sperm cells were not exposed to osmotic stress changes that occur during the process of rehydration. These findings are in agreement with previous studies in which plasma membrane integrity was fairly well-maintained within hypertonic solutions but significantly diminished when returned to isosmolality (Dy Gao et al., 1993; Guthrie, 2002; Rutllant et al., 2003; Willoughby, Mazur, Peter, & Critser, 1996). Similar results have been seen in studies testing the osmotic tolerance of mammalian sperm cells. In these studies, damage to the sperm plasma membrane also occurred upon return to isosmotic conditions from hyperosmotic salt solutions (Dy Gao et al., 1993; Willoughby et al., 1996). Therefore, base extender osmolalities of 750 mOsm/kg or higher are not recommended for cryopreservation of sauger sperm.

Post-thaw sperm velocity and progressive motility were higher in extender 500 compared to extender 350, and these changes could potentially translate into differences in fertility. Motility (total and progressive) and velocity have all been positively correlated with fertilization success (Rurangwa, Kime, Ollevier, & Nash, 2004). Casselman et al. (Casselman, Schulte-Hostedde, & Montgomerie, 2006) were able to demonstrate with walleye that, when sperm concentration was held constant, velocity was able to account for 90% of the variability in fertility with higher velocities producing
higher fertility. The increased post-thaw sperm quality in the 500 mOsm/kg extender seen in this study could translate into an increase in fertilization rate and increase the efficacy of using cryopreserved sperm in the large scale production of saugeye. The post-thaw motility rates of cryopreserved sperm obtained in this study are higher than in typical freshwater species and more similar to the rates seen in marine fish where relatively small amounts of sperm can be used to achieve fertilization rates comparable to fresh sperm (Suquet et al., 2000). Therefore, the improvements in sperm cryosurvival obtained in our study will lay the foundation for future experiments evaluating the fertilizing capacity of freshwater fish sperm cryopreserved in hypertonic extenders.

2.6 Conclusion

We demonstrated that extender osmolality affected post-thaw sperm quality. Cryopreservation of sperm in extender 500 was capable of producing total motility and viability similar to that of traditional Rathbun extender (350 mOsm/kg) while also enhancing post-thaw velocity and progressive motility. Increased velocity may contribute to an increased fertilization rate using extender 500 for cryopreservation, potentially increasing the efficacy of integrating frozen sperm into routine hatchery production. Extender 750 was detrimental to sperm cryosurvival and is not recommended for fish sperm cryopreservation. Sauger sperm was successfully cryopreserved with acceptable post-thaw motility in two of the three treatments used. This study demonstrated that hypertonic base extenders can be used to improve the cryosurvival of freshwater fish sperm compared to traditional isotonic base extenders resulting in acceptable post-thaw parameters for potential commercial use. In turn, commercial development of hypertonic extenders...
base extenders would offer a means for fishery management agencies to ensure availability of saugeye sperm during years when male broodstock are inaccessible.

2.7 Funding and Acknowledgments

This work was supported by the Federal Aid in Sport Fish Restoration Program (F-69-P, Fish Management in Ohio, project FADR72), administered jointly by the U.S. Fish and Wildlife Service and the Ohio Department of Natural Resources-Division of Wildlife (ODNR-DOW). We would like to thank Scott Hale, Rich Zweifel, and the ODNR-DOW hatchery staff for their assistance in collecting and maintaining the sauger broodstock used for this experiment. We would also like to thank Emilee Copple, Laura Binkley and Jennifer Garret for all of their technical assistance.
2.8 References


tolerance, and results of multiple safety studies. Food and Chemical Toxicology 2002;40:871–98.


Chapter 3: Increasing Efficiency of Saugeye (*Sander vitreus* x *S. canadensis*)

Production in a Hatchery Setting

3.1 Abstract

Assisted reproductive technologies are utilized to increase fertilization rates in a number of animal species, but are limited in the sauger (*Sander canadensis*), one parental contributor in the production of the recreationally and economically important saugeye (*Sander vitreus* x *S. canadensis*). State fish management agencies are periodically unable to meet saugeye production goals due to the inability to collect sufficient numbers of sauger broodstock as well as a lack of protocols for standardized sperm use during production. Therefore, the need exists to overcome sauger sperm limitation during saugeye production season. The objectives of this study were to investigate the effect of human chorionic gonadotropin (hCG) on sperm production, determine an efficient sperm-to-egg ratio for fertilization, and validate the use of a densimeter to measure sauger sperm cell concentration for precise sperm addition during fertilization. The effect of hCG on sperm production was evaluated by comparing pre and post treatment milt parameters among fish treated with either hCG or saline (control). hCG increased sperm volume relative to saline controls (hCG 0.64 ± 0.19 mL/kg; control: 0.31 ± 0.05 mL/kg). However, sperm cell concentration and total sperm production were unaffected. To determine an efficient sperm-to-egg ratio, fresh milt from several individuals was pooled.
and used to fertilize walleye eggs at three sperm-to-egg ratios (20,000, 50,000, and 100,000 sperm/egg\(^1\)). Fertilization rates were not affected by the range of sperm-to-egg ratios tested in this study (20,000: 82.67 ± 3.38 %, 50,000: 91.33 ± 2.67 % and 100,000: 88.67 ± 3.44 %). Lastly, we validated the use of densimeter estimates to predict manual hemocytometer concentration estimates. Densimeter and hemocytometer estimates were compared using a number of serially diluted sperm samples using linear regression. Log transformed densimeter estimates were able to estimate log transformed hemocytometer estimates with a high degree of accuracy (\(R^2 = 0.96\)). In conclusion, we showed that hCG was not able to increase total sperm production, as few as 20,000 sperm per egg can be used for fertilization, and a densimeter can be used to rapidly and accurately measure sperm concentration. Our results provide tools and standards that can be implemented by hatchery staff in order to increase saugeye production efficiency and lay the foundation for future research of assisted reproductive technologies for sauger.

3.2 Introduction

Assisted reproduction technologies (ART) are routinely utilized in fish research to increase production of offspring. Common ARTs used include hormonal manipulation of breeding and gamete production and optimization of sperm-to-egg ratios during artificial insemination (Mylonas, Fostier, & Zanuy, 2010; Rurangwa et al., 1998). These techniques allow for an increased supply of high quality gametes and guidelines for the optimal application of those gametes to maximize reproductive potential. However, species specific optimization of these technologies is required for maximized ART efficacy (Moore & Thatcher, 2006).
The Ohio Department of Natural Resources – Division of Wildlife (ODNR-DOW) enhances inland recreational fishing opportunities by stocking hybrid offspring of male sauger (*Sander canadensis*) and female walleye (*Sander vitreus*). These saugeye (*S. vitreus x S. canadensis*) that are produced are fast-growing, have survival and angling rates comparable to walleye, and have an enhanced tolerance of the turbid and warm conditions in Ohio’s network of reservoirs (Flammang & Willis, 1994; Quist et al., 2010b). However, variability in the number of saugeye fry and fingerlings produced each year is high, in part due to a limitation in sauger sperm availability. Spring weather conditions tend to limit access to males and are the primary source of this variability. To minimize saugeye sperm limitation, the ODNR-DOW has been investigating ways to increase access to sperm. Several options exist to help reduce sperm limitation in hatchery settings including hormonal stimulation to increase sperm production, careful addition of sperm to eggs to avoid overuse and accurate, objective determination of sperm concentrations.

First, hormonal stimulation can be used to induce the production of more sperm. A variety of spawning aid products are available, but the only Food and Drug Administration approved agent for the use in fish that may enter the food supply is human chorionic gonadotropin (hCG). While hCG has proven to be a powerful promoter of spermiation in many tank cultured fish (Blecha, Kristan, Samarin, Rodina, & Policar, 2015; Guzmán, Ramos, Mylonas, & Mañanós, 2011; Schiavone, Zilli, Vilella, & Fauvel, 2006), there are conflicting reports about its efficiency. For example, in the common dace (*Leuciscus leuciscus*), hCG administration resulted in enhanced milt volume, whereas
total sperm production was unaffected (Cejko et al., 2012). Interestingly, sperm production significantly increased in European eel (*Anguilla anguilla*) treated with hCG (Asturiano et al., 2006). While the effect of hCG administration on sauger milt production has been tested previously, data was not collected for the concentration or quality of the milt (Bushman, Hansen, & Petges, 2003). Further investigation into the efficacy of hCG administration on sperm production in sauger is necessary to determine if administration could aid in reducing sperm limitation.

In addition to increasing sperm production, standardizing the number of sperm applied to each egg during fertilization can increase the fertilization potential of broodstock by preventing the overuse of sperm. Sperm that fertilize eggs in the external environment reach the oolemma through the micropyle, a single hole in the hard outer chorion, located at the animal pole (Coward, Bromage, Hibbitt, & Parrington, 2002). However, a single sperm placed on the opposite side of the egg from the micropyle cannot travel far enough in its short swimming period (~30 s) for fertilization to occur (Perchec, Cosson, Andre, & Billard, 1993). Therefore, successful fertilization relies on high concentrations of sperm around the egg. Many studies, however, have shown that beyond an optimal number of sperm per egg, the increases in fertilization in response to a higher sperm concentration around the egg do not justify the cost of applying more sperm (Lahnsteiner, Berger, & Weismann, 2003). The optimal sperm-to-egg ratio has been determined to be 25,000 sperm per egg in the walleye (Rinchard, Dabrowski, Van Tassell, & Stein, 2005), but no such guidelines exist for the production of saugeye, which uses sauger sperm to fertilize walleye eggs. Determining an optimal sperm-to-egg ratio in
saugeye production will maximize the fertilization potential of a limited supply of sperm, which is a common obstacle in Ohio fish hatcheries.

Lastly, the ability to accurately and rapidly assess the concentration of sperm in each ejaculate will assist in maximizing fertilization potential. A hemocytometer is the most commonly used method for determining sperm cell concentration (Brito et al., 2016). While this method is accurate with reliable replication, the time required for analysis and the need for an experienced technician is sometimes prohibitive. To streamline this aspect of sample analysis, several other tools have been used to address sperm cell concentration reliably and rapidly. While a wide variety of products are available, many are expensive, not portable and/or need to be calibrated for the species of interest to make accurate measurements. Commercially available densimeters calibrated for livestock offer a potentially fast, reliable, easy to use, portable, and cost efficient alternative. While these instruments have primarily been used with equine semen, the possibility of using a densimeter with non-mammalian sperm (Donoghue, Thistithwaite, Donoghue, & Kirby, 1996) could provide hatchery personnel with a cost effective and reliable means to control sperm-to-egg ratios.

Toward helping overcoming sperm limitation and more effective and efficient procedures to produce milt, the objectives of this study are to 1) Investigate the effects of human chorionic gonadotropin administration on the production of sperm in male sauger, 2) Determine an optimal sperm-to-egg ratio for the hybridization of male sauger and female walleye, and 3) Validate the use of a densimeter to estimate the concentration of sperm in sauger ejaculates. We hypothesize that hCG will increase sperm production in
sauger, fertility will plateau as the number of sperm applied to each egg increases, and that the densimeter will provide an accurate estimate of manual hemocytometer sperm concentration estimates.

3.3 Materials and Methods

3.3.1 Experiment 1: Human chorionic gonadotropin

Broodstock and Gamete Collection

Mature, wild-caught sauger broodstock were used for this study. All procedures and animal use were approved by The Ohio State University (OSU) Institutional Animal Care and Use Committee. Male sauger were collected via electrofishing from the Ohio River in mid-March 2015 by ODNR-DOW personnel. Broodstock were transported to the London Isolation Facility (London, OH, USA) by truck in a 0.5% NaCl solution for approximately three hours where they were maintained in an 750 gallon recirculating system at 4-5°C with a 12 -15 gallons/per hour flow rate through the end of the study.

At time of collection, individual sauger were removed from the holding tank and dried with a towel to prevent premature activation of sperm. Milt was collected from 10 males each week for four weeks using abdominal pressure and a 1.0 mL syringe placed at the opening of the cloaca. The volume of milt collected was recorded for each male to the nearest 0.01mL. Samples were extended 1:3 using Rathbun extender (Alan A. Moore, 1987) and maintained at 5°C.

Evaluation Sperm Concentration and Production

Extended sperm (20 µL) was preserved a 3% glutaraldehyde solution and a hemocytometer (Hausser Bright-Line Hemocytometer, Thermo Fisher Scientific,
Waltham, MA, USA) with a light microscope (Accu-Scope 3025, Accu-Scope, Commack, NY, USA) at 200x was used to manually count the number of sperm in 5 of the 25 grids of the counting surface on each side of the device. The follow equation was used to determine the concentration of sperm in each sample per manufacturer’s instruction:

\[
Sperm \text{ mL}^{-1} = (\text{Dilution Factor}) \times (\text{Average Count in 5 squares}) \times (0.05 \times 10^6)
\]

The total sperm produced by each male was determined using the following equation:

\[
\text{Total Sperm} = \text{Ejaculate Volume (mL)} \times \text{Sperm Concentration (sperm/mL)}
\]

Treatment Administration

After the initial collection, broodstock were randomly assigned to one of the following treatment groups: 1) intramuscular administration of 550 IU/kg hCG (CHORULON, Merck Animal Health Madison, NJ, USA); or 2) an equal volume of physiological saline solution (control). Each fish was tagged with a passive integrated transponder (PIT) tag (Biomark, Boise, Idaho, USA) in the muscle ventral to the first dorsal fin for identification. Tagged individuals were kept separate from the remaining individuals for ease of recapture using floating mesh cages (3’ height x 1.4’ diameter). Milt was collected from these same fish a second time 5 days later and evaluated as described in section 2.1.1 - 2.1.2.

3.3.2 Experiment 2: Sperm-to-Egg ratio

Evaluation of Sperm Motility and Concentration

In order to determine optimal sperm to egg ratios milt was collected, as described in section 2.1.1, from 24 sauger over three days. Extended milt (2µL) was diluted 1:200
in Rathbun extender and mixed thoroughly before activating 1 µL in 10 µL of 5°C
distilled water containing 1% bovine serum albumin. Motility was determined
subjectively using a compound microscope (Zeiss International, Oberkochen, GER) at
10X magnification. Samples with motility >70% were pooled together and stored in a
10.0 mL culture flask and maintained overnight at 5°C. The concentration of each of
sperm in each pool was determined by a hemocytometer as described in section 2.1.2.

Female Broodstock, Fertilization and Incubation

Pooled milt was transported to Berlin Reservoir (Berlin Center, OH, USA) the
following morning and maintained on site at 5°C. Female walleye were retrieved by
ODNR-DOW personnel each morning from trap nets placed in Berlin Reservoir
overnight. Female broodstock were maintained for less than 2 hours in 100 gallon aerated
holding tanks containing reservoir water. Fish were captured, dried, and their ova were
collected via abdominal pressure. Approximately 150,000 oocytes (506 g) from two or
more females were pooled and then 3 x 10^6, 7.5 x 10^6, or 15 x 10^6 sperm diluted to 1.0
mL with Rathbun extender and were mixed with the eggs resulting in 20,000, 50,000 and
100,000 sperm per egg. One liter of hatchery water or activation solution (Table 2.1) was
added to the egg mass to initiate fertilization and then stirred for 2 min. Next, the mixture
was treated with a 500 ppm tannic acid solution for 4 min to remove the outer layer of the
eggs that leads to adhesion and poor incubation. Then the eggs were briefly exposed to a
50 ppm ovadine solution (Western Chemical, Ferndale, WA, USA) for 30 s with agitation
to remove organic material. Eggs were rinsed with hatchery water then exposed to a
second 50 ppm ovadine solution for 30 min to eliminate viral and bacterial pathogens.
Table 3.1 Fertilization solution formulas for both the activation solution and hatchery water.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Lahnsteiner Solution (2011)</th>
<th>Hatchery Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl (mmol/L)</td>
<td>75</td>
<td>-</td>
</tr>
<tr>
<td>KCl (mmol/L)</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>MgSO4 (mmol/L)</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>CaCl2 (mmol/L)</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Trizma (mmol/L)</td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>pH *</td>
<td>8.6</td>
<td>7.65</td>
</tr>
<tr>
<td>Osmolarity (mOsm/kg)</td>
<td>170</td>
<td>31.5</td>
</tr>
</tbody>
</table>

* HCl to pH to 8.6

Treated eggs were transported back to the Hebron State Fish Hatchery (Hebron, OH, USA), exposed to an additional 100ppm ovadine solution for 10 minutes, and incubated for eight days in McDonald Style incubation jars (Pentair Aquatic-Ecosystems, Apopka, FL, USA). Prophylactic treatments of 1667 ppm formaldehyde for 15 min were administered daily to prevent fungal outbreaks. After eight days of incubation, the contents of each incubation jar were mixed thoroughly and a random sample was taken and evaluated using a Meiji dissection scope (Rocky Mountain Microscope Company, Ft. Collins, CO, USA) to determine the percentage of eyed embryos for each treatment. Two samples were evaluated from each jar (n=3 per treatment) and a total of 100 eggs were evaluated from each sample.
3.3.3 Experiment 3: Equine 509a Densimeter validation

Gamete Collection, Dilution and Concentration Analysis

Milt was collected from three males for use in validating the equine densimeter. Following collection, samples were immediately extended (1:4) using Rathbun extender and maintained at 5°C before use. One milliliter aliquots of milt from each sample were diluted 1:20 using Rathbun extender, mixed thoroughly and maintained at 5° in a bench top cooling block (VWR International, Randor, PA). Two hundred microliter aliquots from this dilution were serially diluted with Rathbun extender resulting in 20-200 fold dilutions. Additionally, 200µl of extended milt was taken from each of the 10 dilutions and fixed in 3% glutaraldehyde solution. A hemocytometer was used to estimate sperm concentration in glutaraldehyde preserved samples as described in 2.1.2. Per manufacturer instructions, diluted sperm (180 µL) was mixed with formalin (3420 µL) in a cuvette and analyzed using the 590b equine densimeter (Assisted Reproduction Systems, Chino, CA) to estimate the concentration of sperm in each dilution.

3.3.4 Statistical Analysis

All statistical analyses were completed in Statistica 13 (Dell, Tulsa, OK, USA) using an alpha value of 0.05. Normality and homogeneity of variance of each dependent variable was confirmed using Kolmogorov-Smirnov and Levene’s tests, respectively. All results are represented as means ± SEM.

Human Chorionic Gonadotropin

Seven of the original 40 fish were removed from the analysis (4 from the hCG treatment and 3 from the control group). Six were excluded because mortality prevented a
second collection event and one individual was removed because it did not produce milt upon the initial collection. Both milt volume (mL) and total sperm production (number of sperm) measurements were standardized using donor body mass (kg). A t-test was used to determine if the average body mass of individuals differed between the treatment groups. Gains scores were calculated for all three response variables (milt volume, sperm concentration, and total sperm production) by subtracting the initial measurement from the final measurement. Prior to the beginning of the study, a HOBO Water Temperature Pro v2 Data Logger (Onset, Bourne, MA, USA) was placed inside the holding tank to monitor temperature during the study. Average weekly temperatures varied from 5.76 °C to 9.81°C. Consequently, temperature effects were quantified as the average temperature of the water 5 d before the second collection event and were included in our statistical analysis. Two-way ANOVA was used to determine the effects of treatment, week (temperature), and their interaction on ejaculate quality gains scores with a Tukey’s HSD post hoc analysis.

*Sperm-to-egg Ratio*

A two-way ANOVA was used to compare the fertilization rates among each sperm-to-egg ratio (20000, 50000, and 100000 sperm per egg) as well as between fertilization media. Individual means were compared by Tukey’s HSD post hoc analysis. All results are represented as means ± SEM.

*Densimeter Validation*

Data were transformed using the natural logarithm to satisfy the requirements for homogeneity of variance and normality prior to analysis. One data point was removed
(leaving 29 observations) owing to human error. Least squares linear regression was used to fit a regression line to the relationship between manual hemocytometer counts and densimeter estimates and 95% confidence intervals for the slope were calculated and plotted with the regression line.

3.4 Results

3.4.1 Experiment 1: Human chorionic gonadotropin

The average mass and total lengths of fish used in this study were 0.302 ± 0.02 kg and 330 ± 5 mm, respectively. No differences in average body mass was observed between groups (t-test, t= -1.70, df = 31, p = 0.099). Additionally there was no effect of treatment on sperm cell concentration (F 1, 25 = 3.25, p = 0.083) or total sperm production (F 1, 25 = 1.92, p = 0.18) (Table 1). Sauger treated with hCG produced a higher volume of milt at the post-treatment sampling than fish treated with the saline control (F 1, 25 = 7.80, p = 0.01). Temperature was not significant in any models, but showed a trend (F 3, 25 = 2.84, p = 0.058) in its effect on sperm cell concentration with a decrease in concentration occurring during week two (5.7°C, data not shown), the lowest average weekly temperature.
Table 3.2 Comparison of sauger milt characteristics pre and post treatment (hCG or saline) administration (hCG n=16, control = 17). Gains scores (Δ) were calculated by subtracting the initial measurement from the final measurement. Values scores within the same row with different superscripts (a, b) differ (>0.05). [Mean ± SEM].

<table>
<thead>
<tr>
<th>Quality Parameter</th>
<th>Pre</th>
<th>Post</th>
<th>Δ</th>
<th>Pre</th>
<th>Post</th>
<th>Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milt Volume (mL/kg)</td>
<td>0.64 ± 0.15</td>
<td>0.93 ± 0.19</td>
<td>0.28 ± 0.27a</td>
<td>0.94 ± 0.14</td>
<td>0.31 ± 0.05</td>
<td>-0.63 ± 0.12b</td>
</tr>
<tr>
<td>Sperm Concentration (Sperm/mL)</td>
<td>5.42 x 10^{10} ±</td>
<td>3.95 x 10^{10} ±</td>
<td>-4.54 x 10^{10} ±</td>
<td>4.99 x 10^{10} ±</td>
<td>5.66 x 10^{10} ±</td>
<td>1.20 x 10^{10} ±</td>
</tr>
<tr>
<td></td>
<td>6.15 x 10^9</td>
<td>3.66 x 10^9</td>
<td>1.89 x 10^{10}a</td>
<td>6.00 x 10^9</td>
<td>6.27 x 10^9</td>
<td>3.31 x 10^{10}a</td>
</tr>
<tr>
<td>Sperm Production (Total Sperm/kg)</td>
<td>4.11 x 10^{10} ±</td>
<td>3.11 x 10^{10} ±</td>
<td>-1.00 x 10^{10} ±</td>
<td>5.21 x 10^{10} ±</td>
<td>1.68 x 10^{10} ±</td>
<td>-3.53 x 10^{10} ±</td>
</tr>
<tr>
<td></td>
<td>1.23 x 10^10</td>
<td>4.67 x 10^9</td>
<td>1.50 x 10^{10}a</td>
<td>1.15 x 10^10</td>
<td>3.44 x 10^9</td>
<td>1.28 x 10^{10}a</td>
</tr>
</tbody>
</table>
3.4.2 Experiment 2: Sperm-to-egg ratio

The average weight and total length of sauger used in this experiment were 0.285 ± 0.016 kg and 334.71 ± 6.75 mm, respectively. Figure 3.2 shows the fertilization rates of each activation solution at each sperm-to-egg ratio. The type of fertilization solution had an effect on fertility of saugeye eggs (p <0.05). The percentage of eyed embryos twelve days after fertilization was higher in eggs fertilized with hatchery water (87.56 ± 2.04 %) than in the activation solution (16.00 ± 2.70 %). There was no effect of sperm-to-egg ratio on fertility rates (p = 0.52).

Figure 3.1 Comparison of day twelve fertilization rates using eyed-stage embryo percentage among three sperm to egg ratios (20,000, 50,000 and 100,000) using either hatchery water or activation solution (Lahnsteiner 2011) as the fertilization media (n=3). Values with different superscripts differ. [Mean +/-SEM]
3.4.3 Experiment 3: Equine 509a Densimeter validation

The mass and total length of the sauger used in this experiment was 0.271 ± 0.03 kg and 330 ± 10 mm, respectively. Log transformed densimeter estimates were a useful predictor of the log transformed hemocytometer estimates and the two variables showed a strong positive relationship ($F_{1,27} = 715.82$, $p = 5.70 \times 10^{-21}$, $R^2 = 0.96$; Figure 2). The intercept of the regression differed significantly from 0 ($\beta_0 = -6.41 \pm 0.93$, t-test, df = 27, $p = 2.17 \times 10^{-7}$). The confidence interval of the regression slope was significantly different from 1 ($\beta_1 = 1.41 \pm 0.05$, 95% CI: 1.31 – 1.52), indicating more than a 1:1 relationship between the two estimates.

![Figure 3.2](image)

Figure 3.2. Least squares linear regression of natural log transformed hemocytometer counts on natural log transformed 590b equine densimeter counts (n=29) with 95% confidence intervals (grey lines). The regression coefficient and equation are also provided.
3.5 Discussion

*Human Chorionic Gonadotropin*

The effect of human chorionic gonadotropin (hCG) on sperm volume, concentration, and total sperm production was evaluated. A larger volume of sperm was produced in the hCG-treated fish compared to saline controls after treatment administration while sperm concentration and production were unaffected by hCG treatment. A previous study that investigated the effects of hCG in sauger showed an increase in milt volume but failed to quantify sperm concentration and total sperm production (Bushman et al., 2003). Several other species showed an increase in the average volume of milt produced but also showed that the concentration of sperm decreased as a result, and that overall sperm production was not affected by the treatment (Blecha et al., 2015; Cejko et al., 2012; Viveiros & Godinho, 2008; Zadmajid, 2016). The large increase in volume and decrease in concentration in these studies suggests that hCG increases the hydration of milt by stimulating the Sertoli cells in an LH-like manner to produce the hormone 17,20β dihydroxypregn-4-en-3-one, which is linked to increased milt pH and fluidity (Clemens & Grant, 1965; Scott, Sumpter, & Stacey, 2010). While we found that hCG did not increase the amount of sperm cells produced relative to controls, milt volume increased. This may be useful to hatchery personnel in that increasing the volume of milt produced can make collection and handling of milt easier in species that typically produce small volumes of milt.
**Sperm to Egg Ratio**

In this experiment, the effect of three sperm-to-egg ratios on saugeye egg fertilization was tested. Fertilization rates did not differ when the number of sperm applied to each egg ranged from $2.0 \times 10^4$ – $1.0 \times 10^5$. In walleye, an optimal sperm-to-egg ratio with ~60% fertilization was determined to be 25,000 sperm per egg (Rinchard et al., 2005). We were able to achieve a fertilization rate of $82.67 \pm 3.38\%$ using a smaller amount of sauger sperm to fertilize walleye eggs. Other species have seen suitable sperm-to-egg ratios ranging from 700 to 36,000 (Butts, Roustaian, & Litvak, 2012; Yasui, Arias-Rodriguez, Fujimoto, & Arai, 2009). Variation in the fertilization rates among species can be affected by both sperm and egg characteristics and variation within a single species can be a result of the methods used in the fertilization process (Chereguini, La Banda, García, Rasines, & Fernandez, 1999). Compared to the sperm-to-egg ratio used in blue catfish (*Ictalurus furcatus*) x channel catfish (*Ictalurus punctatus*) hybridization (Bart & Dunham, 1996), saugeye production required far fewer sperm and yielded a higher fertilization rate. As is seen in many species, the percentage of eyed embryos did not increase in response to an increase the number of sperm suggesting a threshold value of sperm around the micropyle exists beyond which is fertilization does not increase or even decreases (Rosenthal, Klumpp, & Willführ, 1988). Our results provide a standardized number of quality sperm cells that can be incorporated into hatchery systems to prevent the over application of sauger milt.

Low fertility of the eggs fertilized using the activation solution was a contradiction to our hypothesis. One study has shown that while the activation solution
was optimal for sperm motility, fertility rates were superior in water where sperm produced the lowest motility characteristics (Lahnsteiner et al., 2003). The source of this low fertility could lie in the formula of the solution. Previous activation solution attempts by the ODNR-DOW have been similarly unsuccessful when using Tris buffered solutions (ODNR-DOW, personal communication). Future investigation into the use of activation solutions that increase sperm motility without negatively affecting the egg is warranted to further minimize the sperm-to-egg ratio thus increasing the efficiency of sperm use (Adames et al., 2015). Future studies should focus on the effects of these solutions on both the male and female gametes.

*Densimeter Validation*

We also demonstrated that the 590b equine densimeter specifically designed for equine sperm analysis can be adapted to use in determining fish sperm concentration. While the hemocytometer is the most commonly employed technique for sperm concentration analysis, setup and analysis time combined can require 10-15 min per sample while the densimeter can determine concentration in as little as 1-2 min. This offers a faster, easy to use and accurate assessment of sperm concentration in the sauger.

Some limitations to this method exist, however. The densimeter requires disposable cuvettes and a formalin solution for analysis of samples. Supply costs will rise with the number of samples that are processed. In addition, a relatively large volume of sperm is required, which can be problematic in species that do not produce a large volume of sperm. However, sperm diluted for motility analysis can be repurposed to determine concentration, provided a representative sample of sperm is taken at the start.
Similar to other methods, clumping and contamination of a sample with a debris of any kind may also skew measurements (T. R. Tiersch & C. C. Green, 2011). Overall, we provided a tool for hatchery staff to use to accurately estimate the concentration of sperm in an ejaculate, which will aid in implementing quality control standards.

### 3.6 Conclusion

Altogether, this research has provided many new assisted reproductive technologies for use in sauger in a hatchery setting. We demonstrated that hCG was able to induce an increase the volume of expressible milt, but that it was unable to increase total sperm production relative to the saline control. Furthermore, we do not recommend the use of hCG to increase sperm production in sauger but it may be useful as a tool to increase milt volume to aid in collection and handling of milt. Our results showed 20,000 sperm per egg could achieve the same fertility rates 50,000 and 100,000 sperm per egg, effectively reducing the amount of sperm used for fertilization by two third, relative to current protocols. We recommend 20,000 sperm per egg for routine fertilization of walleye eggs with sauger sperm in hatchery settings. At this time, activation of gametes with hatchery water is recommended in lieu of the activation solution that favors sperm motility characteristics. We also demonstrated the efficacy of using a commercially available livestock densimeter for fast and reliable fish sperm concentration evaluation. This work is applicable to aquaculture personnel working with sauger sperm to enhance saugeye production but also has the potential to be applied to other species of interest.
3.7 Funding and Acknowledgments

This work was supported by the Federal Aid in Sport Fish Restoration Program (F-69-P, Fish Management in Ohio, project FADR72), administered jointly by the U.S. Fish and Wildlife Service and the Ohio Department of Natural Resources-Division of Wildlife (ODNR-DOW). We would like to thank Scott Hale, Rich Zweifel, and the ODNR-DOW hatchery staff for their assistance in collecting and maintaining the sauger broodstock used for this experiment. We would also like to thank Jennifer Garret, Laura Binkley, and Emilee Copple for their technical assistance.
3.8 References


agonist (GnRHa) treatments on the stimulation of male Senegalese sole (Solea senegalensis) reproduction. Aquaculture 316, 121–128.


Chapter 4: Conclusions

The primary goal of this thesis was to increase the efficiency of saugeye production in Ohio hatchery settings. The overarching goals of this thesis were to: 1.) develop a protocol for successful cryopreservation of sauger sperm, and 2.) increase the efficiency of saugeye production by improving sperm management in the hatchery system. These projects were completed through our collaboration with the ODNR-DOW, DOW hatchery staff and biologists. Our results support the potential increase in saugeye production efficiency using fresh sperm as well as the potential use of using cryopreserved sauger sperm in routine production.

4.1. Use of Hypertonic Medium to Cryopreserve Sauger (Sander canadensis) Spermatozoa

Traditionally, sperm cryopreservation has resulted in post-thaw motility of 50% or less for freshwater species, with the notable exceptions of the commercially valuable species (Suquet et al., 2000). In an attempt to increase cryosurvival of sperm using base extenders hypertonic to the seminal fluid (E. G. Aisen et al., 2002; He & Woods, 2003), we preserved sauger sperm using three base extenders (350 mOsm/kg, 500 mOsm/kg and 750 mOms/kg) and compared quality objectively through the process of cryopreservation. Our results showed that extenders 350 and 500 responded similarly to
cryoprotectant addition and freeze/thaw with the hypertonic extender 500 producing increased rates of progressive motility and higher velocities post-thaw. On the other hand, sperm diluted in extender 750 showed a marked decrease in quality in response to cryoprotectant addition and freeze/thaw. We speculate that the elevated extender osmolality was responsible for the differential effects seen during the study. Enhanced cellular dehydration in extender 500 is likely responsible for the increase in progressive motility and velocity. However, dehydration was also responsible for the large reduction in sperm quality seen in extender 750. Other studies have shown that removal of water beyond the cells osmotic tolerance limits results in large decreases in sperm quality when returned to isosmolality or activation (Rutllant et al., 2003). Future research should focus on how the effects of elevated dehydration in extender 500 altered cell cryosurvival and how they affect fertility.

The results of this experiment have promising implications for saugeye production using cryopreserved sperm. We were able to more successfully preserve progressive motility and velocity through cryopreservation by utilizing a base extender hypertonic to the seminal plasma. Progressive motility and velocity have been shown to be accurate predictors of fertilization success and velocity has been shown to account for 90% of the variation in walleye fertilization rates when other parameters have been standardized (Casselman et al., 2006). Subsequent experiments using sperm cryopreserved in extenders 350 and 500 are needed to determine if the altered post-thaw quality affects fertilization success. Increasing the fertility of cryopreserved sperm could
increase the efficacy with which cryopreserved sperm is utilized in hatchery level production.

In addition to demonstrating that base extender osmolality can influence post-thaw sperm quality, this is the first reported attempt to cryopreserve sauger sperm. Close relatives including the walleye, yellow perch, Eurasian perch, and pike-perch have all been successfully cryopreserved in the past (Bergeron et al., 2002). Using a protocol for cryopreservation similar to that in walleye (Alan A. Moore, 1987; Bergeron et al., 2002) we were able to successfully cryopreserve sauger sperm with higher post-thaw motilities and viabilities than seen in previous walleye studies. Future studies should focus on optimizing a sauger-specific protocol through manipulation of cryoprotectants, additives, and the rates of both cooling and thawing.

4.2 Increasing Efficiency of Saugeye (*Sander vitreus x S. canadensis*) Production in a Hatchery Setting

Saugeye production in the Ohio hatchery system has seen tremendous variability in fish production each year in response to climate conditions leading to sperm limitation. In an attempt to ameliorate the effects of sperm shortages, we attempted to increase the amount of sperm produced from each reproductively mature male captured, validate techniques to determine the number of sperm in ejaculates and to standardize the amount of sperm that is applied to each egg to better utilize this limiting resource.

Increasing sperm production from the available sauger broodstock using hCG administration would be beneficial regardless of male availability. By increasing the total number of sperm a fish produces, excess sperm could be cryopreserved for long-term
storage or used in the short-term to fertilize more walleye eggs. Variability in broodstock numbers could be counteracted simply with the direct effect of hCG administration on sperm production or indirectly through the use of cryopreserved sperm from years past when hCG was used to create a sperm surplus. Despite the promising expectations for the process, we found that hCG administration did not in fact increase sperm production. Similar to another study on sauger, we found that the total volume of sperm produced was higher in hCG treated fish than in controls (Bushman et al., 2003). However, when examining the concentration and total production of sperm, we saw that no differences existed between the two treatments, consistent with studies on carp, dace and pike-perch (Blecha, Samarin, Křišt’an, & Policar, 2016; Cejko et al., 2012). We conclude that there is no benefit of hCG administration on total sperm production in male sauger and it should not be utilized for that purpose.

While sperm production was the main purpose of the hCG treatment, the increase in milt volume is still beneficial. Sauger males typically produce a small volume of milt with a high concentration of sperm cells and the volume of milt produced during subsequent collections decreases (ODNR-DOW, personal communication). Small milt volumes can be hard to collect and handle without losing sperm along the way to accidents or adhesion to containers and even the donor fish itself. The hCG treatment was able to increase sperm volume relative to control which could be helpful for working with the small volumes this species produces. Hormonal stimulation using hCG in male sauger broodstock, while ineffective at increasing total sperm production, can still be used as a spawning aid to facilitate sperm handling.
We were able to validate the use of the commercially available Equine 590B densimeter (Animal Reproductive Systems, Chino, CA, USA) to evaluate sperm concentration in sauger. This tool allows for a fast and reliable measure of sperm concentration without requiring much expertise or expensive equipment. When applied in the fish hatchery, the densimeter will allow hatchery staff to standardize the dose of sperm for the number of eggs being fertilized. Preventing the use of excess sperm will allow for more efficient fertilization and help decrease sperm limitation that hinders the ODNR-DOW’s ability to reliably produce the desired number of saugeye fry and fingerlings each year.

Fertilization rates are ultimately decided by the number of competent sperm applied to each egg during fertilization. However, most attempts to enumerate the optimal sperm-to-egg ratio have shown that fertilization plateaus at a certain ratio and the cost of applying more sperm does not produce much benefit to fertility rates (Babiak et al., 2012; Butts, Trippel, & Litvak, 2009; Rinchard et al., 2005). Therefore, we attempted to determine an optimal sperm-to-egg ratio for using in saugeye production by varying the number of sperm applied to each egg (20,000, 50,000, and 100,000 sperm/egg). We found no significant differences among sperm to egg ratios in this experiment. This finding suggests that the levels of sperm per egg that we tested were within the plateau region and it is possible that fewer sperm can be applied to reach a similar fertilization rate. Combined with the results of the densimeter experiments, we have provided a useful and effective way to quantify the amount of available sperm and guidelines for the standardization of gamete ratios to maximize fertility as well as to prevent the overuse of
sperm. Such protocol changes will likely lead to reduced sperm limitation each year as well as increase the overall hatchery production. Future studies should focus on testing lower sperm-to-egg ratios to determine if a more conservative ratio exists, that will give similar fertilization results which would likely further reduce sperm limitation.

The Lahnsteiner (2011) solution used to fertilized eggs resulted in unexpectedly low fertilization rates. While this solution was previously reported to have positive benefits for sperm attributes, its effect on the egg was not described. Personnel at the ODNR-DOW have reported low fertilization rates in saugeye eggs when Tris was included in the formula (ODNR-DOW, personal communication). However, at this time we are unable to determine the exact cause of the low fertilization success that we observed due to differences in chemical composition (pH, buffer constituent, and osmolality) between the two solutions. Future studies should focus on developing an activation media that has positive effects on both the sperm and the egg.

4.3 Implications, Future Directions and Closing Remarks.

The results of this study are useful to hatchery staff that are propagating sauger or saugeye in human care. Yearly variability in the amount of sauger sperm available is primarily affected by male availability due to low catch rates or climate conditions. These sport fish have a high economic value and in particular, the saugeye fishery is maintained almost entirely through stocking due to low natural hybridization rates (BILLINGTON & SLOSS, 2011; Lynch et al., 1982). In the face of climate change and decreasing populations of sauger throughout their range (Nelson & Walburg, 1977; Schneider, Newman, Card, Weisberg, & Pereira, 2010), sauger sperm limitation may become more
prevalent. These changes will likely make the yearly production of saugeye hybrids more variable and these fisheries could suffer marked declines.

Our work demonstrated the ability of surplus sperm to be stored indefinitely through cryopreservation. This will provide a frozen supply of high quality sperm for years when saugeye production demands have not been met due to climate conditions or male shortages. We also demonstrated that fresh sperm can be utilized more efficiently using the densimeter to quantify sperm concentration and applying a standardized dose of sperm cells to egg pools during fertilization. Standardization of sperm usage using this process will lead to increase fertilization capabilities for each male’s gametes, ultimately reducing the negative effects of limited sperm availability. While hCG has been shown to be successful in inducing spermiation and increasing sperm production in other species (Asturiano et al., 2006; Guzmán et al., 2011), we were unable to show a benefit to sperm production in the sauger contrary to the results of a similar study on sauger (Bushman et al., 2003). Future work should focus on the use of other spawning aids in increasing sauger sperm production to further reduce the negative effects of male and sperm limitation on saugeye production.

To incorporate cryopreserved sperm into the hatchery system’s current protocols, a number of factors need to be investigated. Cryopreservation reduces the quality of thawed sperm relative to that of fresh sperm. Consequently, the sperm-to-egg ratio used in fresh sperm may not be applicable to frozen/thawed sperm. Fertilization experiments testing a similar range of sperm-to-egg ratios will result in determining an optimal ratio for cryopreserved sperm. Additionally, fertilization/activation solutions have been used in
many species to increase fertilization rates without increasing the number of sperm applied per egg. Formulating a solution for fresh and cryopreserved sperm with this effect would be beneficial to saugeye production by further reducing the negative effects of sperm limitation.

Secondly, the concentration of sperm used for cryopreservation in this experiment would result in a prohibitively large number of straws being required to fertilize a typical batch of 450,000 walleye eggs, per ODNR-DOW protocol. The total number of straws required per fertilization event would require extensively large storage spaces and would pose a practical handling obstacle. Increasing the concentration of sperm in each straw would reduce the amount of straws required per egg batch and further increase the efficacy with which cryopreserved sperm can be incorporated into the hatchery system (Table 4.1).
Table 4.1 Saugeye fertilization scenarios using cryopreserved sperm at different cell concentrations.

<table>
<thead>
<tr>
<th>Cell Concentration (billion per milliliter)</th>
<th>Sperm per straw (Billion Sperm)</th>
<th>Eggs per Jar</th>
<th>Sperm required</th>
<th>Straws per fertilization</th>
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<tr>
<td>0.50</td>
<td>0.25</td>
<td>450,000</td>
<td>22,500,000,000</td>
<td>90</td>
</tr>
<tr>
<td>1.00</td>
<td>0.50</td>
<td>450,000</td>
<td>22,500,000,000</td>
<td>45</td>
</tr>
<tr>
<td>1.50</td>
<td>0.75</td>
<td>450,000</td>
<td>22,500,000,000</td>
<td>30</td>
</tr>
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<td>1.00</td>
<td>450,000</td>
<td>22,500,000,000</td>
<td>23</td>
</tr>
<tr>
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<td>1.25</td>
<td>450,000</td>
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</tr>
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<td>450,000</td>
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</tr>
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</tr>
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<td>2.50</td>
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<td>9</td>
</tr>
</tbody>
</table>

All calculations displayed assume 0.5ml straws with a sperm to egg ratio of 50,000 sperm per egg.

In conclusion, we have demonstrated that sauger sperm can be successfully cryopreserved and that hypertonic base extenders were able to increase post-thaw sperm.
quality. Additionally, we provided a valuable tool and derived standards for sauger sperm
doses during walleye egg fertilization. Both methods will contribute to decreasing the
negative effects of sauger sperm limitation in the present and future. This information is
valuable to hatchery staff that are interested in sauger and saugeye production and will
aid in the production of both species.
4.4 References


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