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Evaluation of Sperm Cryopreservation for Yellow Perch (Perca Flavescens) Broodstock Management

Sonya Ponzi
University of Wisconsin-Milwaukee

EVALUATION OF SPERM CRYOPRESERVATION FOR YELLOW PERCH (*PERCA FLAVESCENS*) BROODSTOCK MANAGEMENT

by

Sonya Ponzi

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ABSTRACT

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Sonya Ponzi

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The demand for yellow perch in the Great Lakes region has withstood time despite declining yellow perch populations in the Great Lakes. Today aquaculture can aid in meeting the demand. However, the technologies such as cryopreservation which would allow for large-scale, year-round broodstock operations is underdeveloped. Cryopreservation of fish gametes has many practical applications for use in aquaculture including assistance in asynchronized spawns, preservation of genetic resources with known pedigree and quality, and simplification of gamete transportation. This thesis evaluated the most current yellow perch semen cryopreservation protocol to determine whether fresh and cryopreserved semen differ in their ability to fertilize eggs and whether the motion and kinetics of sperm differ before and after cryopreservation. Cryopreservation had a negative impact on the percentage of viable eggs after two days of incubation ($P = 0.0206$) and a negative impact on the percentage of viable embryos after twelve days of incubation ($P < 0.0001$). Cryopreservation significantly reduced the percentage of motile sperm ($P = 0.0015$) and the curvilinear velocity of sperm ($P = 0.0116$), but it did not impact the average path velocity, straight line velocity, linearity, wobble or progression of sperm. The results imply a need for additional research on immobilizing solutions, cryoprotectants, activating solutions, freezing vessels and freezing methods specifically for yellow perch if cryopreservation is to be a viable broodstock management technique for this species.

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CHAPTER 1: INTRODUCTION

1.1 Current State of Fisheries and Aquaculture

Novel and improved food production techniques centered on sustainability and efficiency are needed to strengthen food security as natural resources (e.g., water, arable land, energy) become limited with population growth. Aquaculture has the potential to provide high quality sources of animal protein for human consumption. It can be done in a controlled manner, optimizing both sustainability and efficiency by utilizing water conservation techniques and alternative fish feeds. It can also ensure food security by growing fish in farm settings rather than relying upon marine fishery resources, which have continued to decline since the 1970's.

Marine fish stocks within biologically sustainable levels have decreased from 1974 to 2015 from 90 percent to 66.9 percent (FAO 2018). Greater reliance upon aquaculture rather than wild fish stocks could aid in the recovery of wild fish stocks. Aquaculture can also contribute to food security as human activities disrupt the functioning of ecosystems. Overfishing, pollution, and climate change are factors contributing to the decimation of fish populations. For example, climate change is expected to alter fish abundance and distributions as the global oceans experience changes in physical and chemical characteristics such as temperature, pH, vertical mixing, and salinity (Brander 2010). The future of wild fish stocks remains uncertain in light of human activities that cause environmental degradation and climate change. Aquaculture may prove to be a more reliable and sustainable seafood production technique.

Aquaculture is now a major producer of fish for human consumption. Global population continues to grow, but aquaculture has aided in keeping up with the demand for fish. Per capita fish consumption grew from 9.0 kg in 1961 to 20.2 kg in 2015 (1.5% increase per year) and fish made up about 17% of all the animal protein consumed by the global population in 2015 (FAO

2018). Capture fishery production has remained relatively static since the late 1980's, but 2016 saw a peak global fish production of 171 million tonnes (FAO 2018). Aquaculture production has been responsible for the growth in the supply of fish for human consumption and it now accounts for over 50% of the seafood consumed throughout the world (FAO 2018). Aquaculture allows for expanded fish production for human consumption even as human activities alter ecosystem functioning and the global population grows.

Although the world at large is increasingly utilizing aquaculture for fish production, the United States is not following that trend. In 2016, the United States caught 4,919,741 metric tons live weight of fish, crustaceans, and mollusks, while the country only produced 444,369 metric tons live weight using aquaculture practices in the same year (National Marine Fisheries Service 2018). The majority of seafood consumed in the United States is imported, rather than produced domestically. In 2016, seafood imports exceeded exports by almost four-fold and NOAA estimates that 84 percent of the seafood consumed in the United States is imported (National Marine Fisheries Service 2018). It is likely that demand for seafood in the United States will continue to increase, because from 1910 until 2017, per capita consumption in edible pounds of seafood rose from 11.2 to 16.0 (National Marine Fisheries Service 2018). Aquaculture can strengthen the United States' domestic supply of edible seafood and reduce the trade gap between imported and exported seafood. Fish grown domestically can also reduce issues related to food safety, quality, and authenticity if the proper regulations are applied and enforced.

1.2 Importance of Yellow Perch in the Great Lakes Region

Yellow perch (*Perca flavescens*) have ecological importance in the Great Lakes, because they serve as an important prey for predatory fish such as northern pike *Esox Lucius* (Blackwell,

Soupir, and Brown 1999), walleye *Sander vitreus* (Forney 1974; Hansen et al. 1998) and smallmouth bass *Micropterus dolomieu* (Dembkowski et al. 2015). Over the last century, the Great Lakes' ecology has changed dramatically due to anthropogenic perturbances, which can be broadly classified as eutrophication, contamination, and establishment of exotic species (Munawar et al. 2005). Munawar and coworkers (2005) argue non-indigenous species are the most serious threat to the integrity of the Laurentian Great Lakes, because they have affected all trophic levels. The alewife *Alosa pseudoharangus,* white perch *Morone americana*, Eurasian ruffe *Gymnocephalus cernuus*, and zebra mussels *Dreissena polymorpha* have been associated with significant changes in yellow perch populations in Lakes Michigan and Erie (Munawar et al. 2001). Today, yellow perch remain ecologically important, but their spawning habitat, and subsequently their populations, throughout the Great Lakes are reduced.

Yellow perch are economically important because of their history as a commercially and recreationally fished species in the Great Lakes. The harvesting of fish from the Great Lakes for sustenance has occurred for centuries, first by Native Americans and later by Euro-American immigrants (Hudson and Ziegler 2014). Historically, the majority of all yellow perch came from Great Lakes capture fisheries in the U.S. and Canada (Malison 2000), with commercial fishing occurring in Wisconsin, Indiana, and Illinois prior to 1997 (Marsden and Robillard 2004). These capture fisheries saw peak harvests of more than 15 million kg/year in the 1950s and 1960s, but harvests declined to 5-8 million kg/year in the 1980s and 1990s (Malison 2000). Due to the altered ecology of the Great Lakes, it is predicted that the historical peak harvests will never be reached again (Malison 2000). Today one commercial yellow perch fishery remains in Green Bay, Lake Michigan (Wilberg et al. 2005), while Lake Erie continues to support a successful commercial yellow perch fishery (Hudson and Ziegler 2014).

Yellow perch are also a highly sought-after sport fish that are accessible to shoreline anglers. In the early 1990s they comprised 85% of the recreational harvest by weight (Marsden and Robillard 2004; Kaemingk, Graeb, and Willis 2014). Both commercial and recreational fishing create jobs and bring substantial revenues to local businesses.

The demand for yellow perch in the Great Lakes region has withstood time and 70% of the yellow perch sales in the U.S. occur within 80 km of the Great Lakes, a region with over 40 million residents (Malison 2000). The long history of yellow perch in the region has developed strong consumer fidelity; these fish are the traditional fish used in the "Friday night fish fry" of Wisconsin communities. The yellow perch's fillet is prized for its firmness, high protein and low fat, which allows it to have a long shelf life, resistance to freeze damage and minimal off-flavor (Hart, Garling, and Malison 2006). The demand for yellow perch remains high in the face of declining supply (Malison 2000).

Aquaculture can aid in meeting the demand for yellow perch in the Great Lakes region. There is a fledgling industry contributing about 90,000 kg/year of yellow perch (Malison 2000). However, the technology which would allow for large-scale, year-round broodstock operations is still being researched and developed. Yellow perch aquaculture has the potential to provide a high-quality animal protein for human consumption in a controlled manner and it can be optimized for sustainability and efficiency. The use of innovative aquaculture production methods to produce yellow perch within the Great Lakes region would limit problems concerning food safety, quality, and authenticity. Yellow perch aquaculture would also strengthen the domestic supply of edible fish, reducing the trade gap between imported and exported seafood.

1.3 Gamete Cryopreservation in Fish

Over the last 70 years, research on cryopreservation technologies has established global industries for development, maintenance, and distribution of livestock genetic resources (Blaxter 2011). Research was performed for the cryopreservation of aquatic animal gametes at the same time, but today research efforts are still focused on protocol development for optimal cryopreservation (Torres, Hu, and Tiersch 2016). Nevertheless, sperm cryopreservation has been studied in more than 200 fish species (Liu et al. 2007).

The lag in commercial-scale cryopreservation technologies and germplasm repositories for fish is due to the lack of standardization, quality assurance, and quality control (Torres, Hu, and Tiersch 2016). Torres, Hu, and Tiersch (2016) identify four main stages in the cryopreservation process that require standardization to move forward in large-scale and widespread use in aquatic species: source, housing, and conditioning of fish; sample collection and preparation; freezing and cryogenic storage of samples; and egg collection and use of thawed sperm samples. Additionally, standardization in terminology and reporting of results is missing and the literature for cryopreservation is found across many different journals and disciplines. Standardization in all these areas must be sought for each species.

Cryopreservation of fish gametes has many practical applications for use in aquaculture. Cryopreserved fish semen can be kept in cryogenic storage to aid in spawns when males and females are asynchronized. It can also provide semen for egg fertilization in out-of-cycle spawning regimes of all-female broodstocks. Larvae and fingerlings could then be grown year-round and the need to maintain live males would be eliminated, simplifying hatchery operations. Cryopreservation can preserve genetic resources with known pedigree and quality, allowing for the protection of valuable genetic lineages of improved farm strains. Cryopreservation simplifies

the transportation of gametes between aquaculture facilities, because cryopreserved milt could be transported rather than live animals. Finally, cryopreservation is an important component in the domestication of animals in production systems (e.g., cattle, sheep, horses). Domestication allows for increased control of an animal's life cycle, including the animal's feeding, movement, protection and breeding. Cryopreservation of fish semen would give humans increased control of reproduction, which would further the domestication of fish used in aquaculture.

1.4 Common Cryopreservation Methodologies for Fish Semen

Cryopreservation is a method of preserving intact living cells by using chemical protectants and very low storage temperatures. To ensure optimum cell survival, every step of the cryopreservation process must be optimized and controlled. Firstly, semen sample collection must be done in a manner that avoids contamination from feces, urine, skin mucus and blood (Torres, Hu, and Tiersch 2016). Yellow perch semen samples are most commonly obtained by dry stripping or catheter insertion. Urine and feces contamination is common with dry stripping, because the fish may release these waste products along with the semen when the animal is firmly grasped. In Eurasian Perch (*Perca fluviatilis*), even a ten percent contamination of semen with urine can have negative effects on sperm motility (Król et al. 2018). Contamination can be largely avoided by inserting a catheter into the sperm duct of the fish and collecting the semen directly into a Eppendorf tube (Król et al. 2018; Judycka et al. 2019).

Secondly, the immobilizing solution formula and its ratio to the semen must be determined. The purpose of the immobilizer is to keep the sperm cells in a quiescent state after being released from the sperm duct. Freshwater species inhabit an environment of 0-50 mOsm and have semen osmolarity between 230 and 320 mOsm (Kopeika, Kopeika, and Zhang 2007).

Many fish are external fertilizers, so upon entering water, the sperm cells experience a large osmotic change that triggers their activation. Depending on the species, sperm often do not remain motile for more than sixty seconds. Sperm of freshwater fish are immotile in the testis and inhibition is mainly due to the osmolality of the seminal plasma (Alavi et al. 2007; Lahnsteiner et al. 1995). Therefore, the extender should contain solutes which increase the osmolality of the solution so that it matches the osmolality of the species' semen that is being cryopreserved. The composition of the immobilizing solution depends on the seminal plasma composition and the main components of the seminal plasma preventing sperm motility. If formulated properly, the immobilizing solution should preserve sperm motility for fertilization.

In addition to the composition of immobilizing solution, the ratio of semen to immobilizing solution must also be optimized. Semen is a viscous substance and requires a large dilution with immobilizer to create a homogenous solution (Billard and Cosson 1992). The volume of dilutent ends up determining the dynamics of sperm motility and thus, the fertilization capability of the sperm. A relatively high dilution ratio is required for simultaneous motility of 100 percent of spermatozoa (Alavi et al. 2008). The immobilizing solution also allows for the recovery of ATP within sperm cells in the case they were prematurely activated due to contamination. As long as the cells remain metabolically active while in the queiscent state within the immobilizing solution, they can recover ATP and be activated again for successful fertilization (Cosson 2004).

In 2007, Alavi and coworkers observed the highest sperm velocities of Eurasian perch when semen was diluted in immobilzing solution at a ratio of 1:100, but the maximum sperm velocity and the highest percentage of motile sperm were observed at 30 and 45 seconds post-

activation when the sperm was diluted in immobilizing solution at 1:50. Therefore, they argued that the optimum dilution ratio of Eurasian perch semen to immobilizing solution was 1:50.

Next, an appropriate cryoprotectant needs to be added to the solution of semen and immobilizer. Adding a cryoprotectant increases the total concentration of all solutes in the system, reducing the amount of ice formed when the sample is later cooled and frozen (Pegg 2015). A cryoprotectant must be able to penetrate into the sperm cells and have a low toxicity. The most commonly used compounds are glycerol, dimethyl sulfoxide, ethanediol, and propanediol. Proper application of the cryoprotectant is species dependent, so the compound used and its concentration varies. Methanol is the cryoprotectant of choice when freezing Eurasian perch semen, because it was found to not cause any effect on sperm motility parameters (Sarosiek et al. 2014).

Once the semen is mixed with immobilizing solution and the cryoprotectant, the solution is ready to be loaded into vessels for freezing. Packaging is important for standardizing the cooling rate and identifying samples (Yang and Tiersch 2009). Currently, the most common freezing vessels for yellow and Eurasian perch are pellets, plastic cryovials and straws. The different materials and shapes of these containers results in different heat transfer properties during freezing and thawing. Even the same packaging produced by different companies can vary, further complicating standardization.

Pellets are the least costly method. Small holes are drilled into dry ice and small volumes of semen are pipetted into the holes. The frozen pellets are transferred to a secondary container for storage. Cryovials are made in various volumes and they have threaded tops. They are still used in yellow perch semen cryopreservation, but they are not preferred in Eurasian perch semen cryopreservation. Eurasian perch semen is cryopreserved in straws (Judycka et al. 2019). Straws

are arguably the best option for cryopreservation. Straws can be used with automated straw filling and sealing equipment, permanently labeled with the printing of alpha-numeric labels or barcodes, completely sealed to ensure sample biosecurity. Finally, cryopreservation in straws are used to standardize the freezing and cooling process (Yang and Tiersch 2009).

After samples are loaded into freezing vessels, a freezing method is chosen. There are several freezing methods and each method produces a different cooling rate. The cooling rate of samples is a crucial factor in cryopreservation, because it affects the osmotic and pH balance of intracellular and extracellular solution (Yang and Tiersch 2009). Placement of semen samples on dry ice (the pellet method) and suspension in liquid nitrogen are inexpensive methods, but it is difficult to quantify and control the cooling rate (Yang and Tiersch 2009). Utilization of a controlled rate freezer is arguably the best option for cryopreservation, because the cooling rate can be controlled and reproduced, but controlled rate freezers are expensive. The optimum freezing method and cooling rate still need to be determined for many species used in aquaculture. After cryopreservation, cryopreserved sperm can be stored in liquid nitrogen for a long period of time without any detectable decline in sperm quality (Kopeika, Kopeika, and Zhang 2007).

When Eurasian perch semen was cryopreserved by either suspension in liquid nitrogen or controlled rate freezer, samples cryopreserved in liquid nitrogen showed significantly lower progressive motility and curvilinear velocity when compared to the fresh control semen, but semen cryopreserved in the controlled rate freezer did not have reduced progressive motility and curvilinear velocity when compared to the fresh control semen (Bernáth, Bokor, et al. 2015). Controlled rate freezers are a good solution for commercial scale cryopreservation, because the

equipment stabilizes and controls many variables, including the cooling rate and the extrinsic environment around the sample vessel.

Finally, the cryopreserved semen is thawed and activated. Thawing of semen samples should be done at a controlled rate to the greatest extent possible. Important steps in the thawing process include the time lapse between sample retrieval from liquid nitrogen and the start of thawing protocol, thawing temperature, thawing duration, and final temperature after thawing (Torres, Hu, and Tiersch 2016). Usually, frozen samples are plunged into a water bath having a known temperature for a set amount of time. The sample rapidly thaws and is immediately mixed with the activating solution and the final solution is mixed with eggs for fertilization. In Eurasian perch, it is common for cryopreserved semen straws to be thawed for 10 to 13 seconds in a 40°C water bath (Bernáth, Zarski, et al. 2015; Judycka et al. 2019). Judycka and coworkers (2019) observed a decrease in post-thaw sperm motility over time and suggested using semen for fertilization within two minutes after thawing.

The last step in cryopreservation of fish sperm is activation. Alavi and coworkers (2007) found that an increasing concentration of potassium ions in an activating solution increased sperm velocity and the highest sperm velocity was observed when 50 mM K+ was present in the activating solution, but K+ did not significantly affect the percentage of motile sperm. The researchers also found that 2.5 mM Ca+2 has a positive effect by increasing sperm velocity and the percentage of motile sperm. Potassium may be involved in activating sperm through changes in plasma membrane potential. Potassium, calcium and magnesium ions improve motility duration at concentrations less than 1.5 mM (Lahnsteiner 2014). Neither calcium, potassium, or magnesium ions alone are sufficient to initiate Eurasian perch sperm motility (Alavi et al. 2007; Franz Lahnsteiner 2014).

The highest sperm motility of Eurasian perch was observed in a solution of NaCl, glucose, or sucrose with an osmolality of 100, so a hypo-osmotic shock is the predominant requirement for sperm motility (Alavi et al. 2007; Lahnsteiner 2014). Activating solutions have osmolalities comparable to the osmolality of freshwater. Typically Eurasian perch sperm are only motile for about one minute, but Lahnsteiner (2011) found their spermatozoa have the ability to swim for more than two hours in saline solution.

European nations have sought to develop commercial aquaculture practices for the Eurasian perch and the cryopreservation process for Eurasian perch semen has been researched consistently since 2006. The Eurasian and yellow perch are closely related, but they are distinct species. It is possible that many of the aforementioned steps in the cryopreservation process for Eurasian perch are similar for yellow perch, but this has not been experimentally determined.

1.5 Yellow Perch Sperm Biology and Cryopreservation

Yellow perch reproductive biology was first researched by Turner (1919). He observed that the testes of yellow perch are two elongated bodies that fuse at their posterior ends and are situated in the posterior part of the body cavity ventral to the swim bladder. In 1982, Wicker and Huish used scanning electron microscopy to observe the spermatozoa of yellow perch and they

Table 1. Dimensional characteristics of yellow perch spermatozoa. Reproduced from Wicker and Huish (1982).

Measurement	Sample size	Mean (μm)	Minimum (μm)	Maximum (μm)	Variance	Coefficient of variation
Head length	120	1.68	1.25	2.34	0.06	14.85
Head width	119	1.61	1.25	2.26	0.05	13.50
Midpiece length	6	0.92	0.65	1.49	0.10	34.53
Midpiece width	6	0.92	0.63	1.31	0.06	25.54
Flagellum length	66	19.97	13.11	32.44	17.84	21.15
Flagellum width	116	0.16	0.10	0.27	0.002	29.92

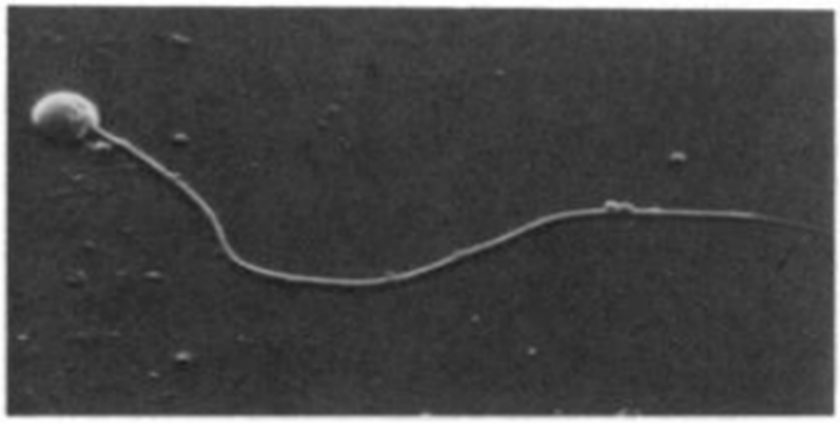


Figure 1. Scanning electron micrograph of a yellow perch spermatozoan. Reproduced from Wicker and Huish (1982).

characterized them as uniflagellate, acrosomeless, primitive aquasperm. The sperm head is spherical and contains genomic material. They have a small undifferentiated midpiece pervaded by the cytoplasmic channel (Alavi et al. 2015). It contains mitochondria and proximal and distal centrioles (Alavi et al. 2015). Wicker and Huish (1982) observed that midpiece structures were absent from some or many spermatozoa likely due to heterogeneities within sperm populations of the species. The flagellum consists of an axoneme with a "9+2" microtubule structure surrounded by a plasma membrane (Alavi et al. 2015). Sperm concentration of perch is one of the highest for freshwater fishes (Dabrowski et al. 1996).

The composition of yellow perch seminal plasma was measured a single time by Dabrowski and coworkers (1996). Osmolality and pH of seminal plasma were measured to be 316.7 mOsm/kg and 8.30. Inorganic ions are the main compounds of yellow perch seminal plasma. Seminal plasma contains 115 mM sodium, which is high for teleosts, and 12 mM potassium, which is low for teleosts. Protein concentration is higher than that of most other teleost species and amounted to 3.18 g/L. Organic substances found in seminal plasma that are likely important for energy include lipids, free amino acids, and carbohydrates. Cholesterol was

found and it is possibly a precursor for steroid hormones (Dabrowski et al. 1996). Additional studies on yellow perch seminal plasma composition would aid in formulating the most effective immobilizing and activating solutions.

Cryopreservation methods for yellow perch semen have been investigated intermittently for several decades. Yellow perch semen was first cryopreserved in 1993 by Ciereszko, Ramseyer, and Dabrowski. Semen was cryopreserved with either an extender containing 125 mM sucrose, 6.5 mM reduced glutathione, 100 mM potassium bicarbonate, 10% egg yolk and 8% dimethyl sulfoxide (DMSO) cryoprotectant in distilled water or an extender containing 0.3 M glucose and 20% glycerol cryoprotectant in distilled water. Two dilution ratios (4x or 8x) were tested. The pellet method was used for cryopreservation. Three or four frozen pellets were transferred into 3 mL of physiological saline at room temperature for activation before fertilization. The best egg fertilization rates were obtained when the milt was diluted 8x with the sucrose-based extender containing DMSO cryoprotectant (Ciereszko, Ramseyer, and Dabrowski 1993). Due to the experimental design, there is no way to determine whether the extender or the cryoprotectant contributed more to the results.

In 1999, a second study of yellow perch semen cryopreservation was completed by Glogowski, Ciereszko, and Dabrowski. Milt samples were diluted with one of four extenders and fertilization rates were observed. Each extender consisted of 0.45 M sucrose and were supplemented with either 15 percent DMSO, 15 percent DMSO and hen's egg yolk, 15 percent dimethylacetamide (DMA), or 15 percent DMA and 10 percent egg yolk as cryoprotectant. The samples were frozen using the pellet method.

The authors did not observe significantly different fertilization rates among the extenders tested and each treatment produced about 70 percent fertilization, but cryopreservation success

differed significantly between milt samples from individual males. Thus, the authors determined simple extenders and DMSO or DMA are effective for yellow perch semen cryopreservation, but they note their improved fertilization rates could be due to the osmolality of their activating solution which contained 0.5 percent NaCl rather than 0.7 percent NaCl used previously. Semen in this experiment was not pooled, so it is also possible that individual male variation contributed to the results. This research simplified yellow perch semen cryopreservation, but elements of cryopreservation have yet to be explored for optimum freezing and thawing.

The cryopreservation of yellow perch semen was tested again in 2018 by Miller and coworkers, almost two decades after the prior publication. The authors sought to simplify cryopreservation techniques. They observed the effect of absence versus presence of salmon seminal plasma in cryoprotectant solutions. They also tested whether DMSO or methanol (MeOH) was a better cryoprotectant and whether the pellet or vial cryopreservation method produced better results. Their extender consisted of 300 mM glucose and 7 mM NaCl and their activator consisted of 75 mM NaCl.

In their first experiment, semen samples were cryopreserved with either DMSO or MeOH as a cryoprotectant and the extender lacked salmon seminal plasma. The researchers did not observe sperm motility after cryopreservation and activation. Therefore, their following experiments included the addition of salmon seminal plasma along with cryoprotectant. There was no statistically significant difference in sperm motility found between freezing methods with either cryoprotectant, but between treatments, the DMSO vial method had the highest 24-hour survival of any cryopreserved group.

The lack of standardization between experiments does not allow for direct comparisons. Additionally, it is not clear which steps in the cryopreservation process are responsible for the

results or whether male-to-male variation exists. In the future, it would be beneficial to utilize experimental designs that simply test the effect of differing immobilizing solutions, cryoprotectants and activating solutions either by making use of computer assisted sperm analysis (CASA) or by fertilizing eggs. CASA software tracks the movement of sperm heads in consecutive frames of video to calculate their trajectories and describe their swimming characteristics (Fauvel, Suquet, and Cosson 2010). CASA software objectively describes sperm movement and is helpful in determining the effects of immobilizing solutions, cryoprotectants, activating solutions, and freezing on sperm motility. Yellow perch sperm motility analysis has only been done subjectively using the human eye (Miller et al. 2018) and CASA has not yet been implemented in experiments with this species.

1.6 Research Objectives and Hypotheses

This thesis aims to further technologies that aid in efficiently managing broodstocks of yellow perch. Specifically, cryopreservation methods are being evaluated with a fertilization experiment and the use of CASA to address the burden of maintaining numerous duplicated broodstock cohorts for year-round fingerling production. Cryopreservation can preserve genetic resources with known pedigree and quality. It is essential for the maintenance of neomale semen to produce all female, monosex progeny for more uniform and faster growth (Rodina et al. 2008). It can also allow for fertilization in out-of-cycle spawning regimens of all-female broodstocks, which would produce larvae and fingerlings year-round. Finally, cryopreservation can simplify the transportation of gametes between aquaculture facilities.

Objective 1: Determine the fertilizing capability of cryopreserved yellow perch sperm using the most recently published cryopreservation methods.

Hypotheses:

H_O: Fresh semen and cryopreserved semen do not differ in their ability to fertilize eggs.

H_A: Fresh semen and cryopreserved semen differ in their ability to fertilize eggs.

Rationale: Miller and coworkers (2018) recently updated the methods for yellow perch semen cryopreservation, making it more effective and straightforward. They observed poor egg fertilization with cryopreserved semen but also subjectively observed high percentages (75%) of motile sperm. In the fertilization trial I completed in March 2019, I followed their methods to attain optimal egg fertilization by cryopreserved semen. Therefore, I completed an egg fertilization trial to determine whether semen, cryopreserved with the most updated methods, can produce high fertilization rates of eggs. If my results agree with the results from their 2018 study, then additional experiments will need to be conducted to improve cryopreservation methods.

Objective 2: Evaluate sperm motion and kinetics before and after cryopreservation.

Hypotheses:

H_O: The motion and kinetics of sperm do not differ before and after cryopreservation.

H_A: The motion and kinetics of sperm differ before and after cryopreservation.

Rationale: A computer assisted semen analysis (CASA) plugin has been developed and improved for ImageJ, an opensource software (Purchase and Earle 2012). CASA has

been utilized in Eurasian perch sperm studies to study variables including progressive motility, curvilinear velocity, average path velocity, rate of change of direction, percent sperm motile and amplitude of lateral head displacement (Bernáth et al. 2015; Judycka et al. 2019; Lahnsteiner, 2014; Rodina et al. 2008). CASA has not yet been applied to observe the motion and kinetics of fresh and cryopreserved sperm of yellow perch. Therefore, I will record videos of fresh sperm and cryopreserved sperm. The cryopreservation methods will match those from Miller and others (2018). I will analyze the videos to compare the percentage of motile sperm, curvilinear velocity, average path velocity, straight line velocity, linearity, wobble and progression of the sperm from treatment and control samples.

CHAPTER 2: METHODS

2.1 Semen Cryopreservation and Egg Fertilization Trial

Semen samples were obtained from male broodstock of the Perquimans strain yellow perch housed in the University of Wisconsin-Milwaukee School of Freshwater Sciences aquaculture lab. Perquimans strain yellow perch originate from the Perquimans River, a coastal waterway in Northeastern North Carolina. The broodstock is 2014-year class, F4 generation and was born and raised in captivity. The broodstock is housed in a 2562-liter flow through tank at a density of 45-60 kg/m3 and experiences seasonal thermal regimes to stimulate natural spawns. At the time of sampling, the broodstock was held in dechlorinated water between 6.3°C and 7.3°C with a flow of about 40 liters per minute.

Following the methods of Miller and coworkers (2018), an immobilizing solution was prepared by dissolving 108 grams of D-glucose in 1 liter of phosphate buffered saline to make a 0.6 molar glucose solution. Fifty-mL falcon tubes held about 20 mL immobilizing solution. A funnel was attached to the top of a falcon tube before sample collection to direct the milt stream into the tube. It was estimated that 5 mL of semen would be collected from each fish, so the ratio of semen to immobilizing solution in the tube was about 1:4. The relatively small ratio limited semen dilution to ensure high sperm densities and fertilization capability after cryopreservation.

When a fish was dry stripped, its urogenital opening was rinsed with immobilizing solution and then wiped dry to prevent sperm activation. The fish was positioned on top of the funnel so that the milt was directed into the tube and immediately mixed with the immobilizing solution. The sides of the tube were rinsed with immobilizing solution and the tube was swirled to thoroughly incorporate the solution. Samples (one falcon tube per fish) were held on wet crushed ice in a Styrofoam cooler for about one hour.

After sample collection, 1.6 mL of the semen solution and 0.16 mL dimethyl sulfoxide (10 percent cryoprotectant) (Miller et al. 2018) was pipetted into a 2-mL vial with a screw cap and the vial was inverted to ensure complete mixing. The vials were buried in dry ice inside a Styrofoam cooler for 1 hour and the solution within the vials froze solid within that time. The vials were then transferred to an ultra-low freezer set at -150°C. The samples were held in the freezer for 6 to 8 days depending on when the milt was collected. Samples were not pooled, so the milt from each fish had four replicate 2-mL vials for a total of 40 vials.

The fertilization trial began on 26 February 2019 and eggs were harvested from the Perquimans strain perch. Ten Perquimans males in total were randomly selected as semen donors and dry stripped for semen in this experiment. Five of the males were dry stripped on 18 February 2019 and their milt was cryopreserved as described previously. Five of the males were dry stripped on 26 February 2019 and their milt was used fresh as described below. Six Perquimans females donated eggs on 26 February 2019. Each egg ribbon was divided into 5 segments and each segment, originating from 1 female, was fertilized by a different male. The six egg ribbons served as replicates: three egg ribbons fertilized by cryopreserved milt and three egg ribbons fertilized by fresh milt. See figure 2 for a visual representation of the experimental setup.

The semen samples were cryopreserved for 6 days and then removed from the -150°C freezer and placed on wet, crushed ice inside a Styrofoam cooler until it was needed for egg fertilization. As the eggs were being harvested, the cryopreserved semen was plunged into a water bath of 9.5°C, which matched the water temperature of the broodstock holding tank. Once thawed (about 10 -15 seconds), the 2 mL semen vials were inverted and added to a 50 mL falcon tube containing 75 mM NaCl activator solution or tank water. The contents of the 50 mL falcon

tube were added to the eggs for the cryopreservation treatment. Fresh semen was also harvested and held in 50 mL falcon tubes with immobilizing solution and placed on wet, crushed ice in a Styrofoam cooler for no longer than an hour and then activated with tank water. The fresh activated semen was mixed with eggs for fertilization.

After the addition of semen, a bentonite clay suspension, comprised of clean dechlorinated water and dry bentonite, was added to the eggs to prevent the eggs from sticking. The eggs were rinsed with clean dechlorinated water and placed in a zip lock bag. The bag was filled with clean dechlorinated water having a temperature matching that of the broodstock tank (9.5°C). The bag floated in the broodstock tank before being transported for incubation.

Finally, each portion of fertilized eggs was incubated in an individual 3.5 L bucket. Each bucket was individually supplied with water via tubing attached to a water diverter and each bucket had an outlet for continuous flow through water. Water originated from one of two head sump tanks and the temperature for each was independently adjusted. Buckets were grouped by female, so the 5 segments of one female's egg ribbon were held in one large tank liner (Figure 2). Ribbon segments were pinned to plastic grates with zip ties to ensure eggs were constantly submerged in water. The grates were weighted with lead to prevent them from floating and exposing the eggs to air.

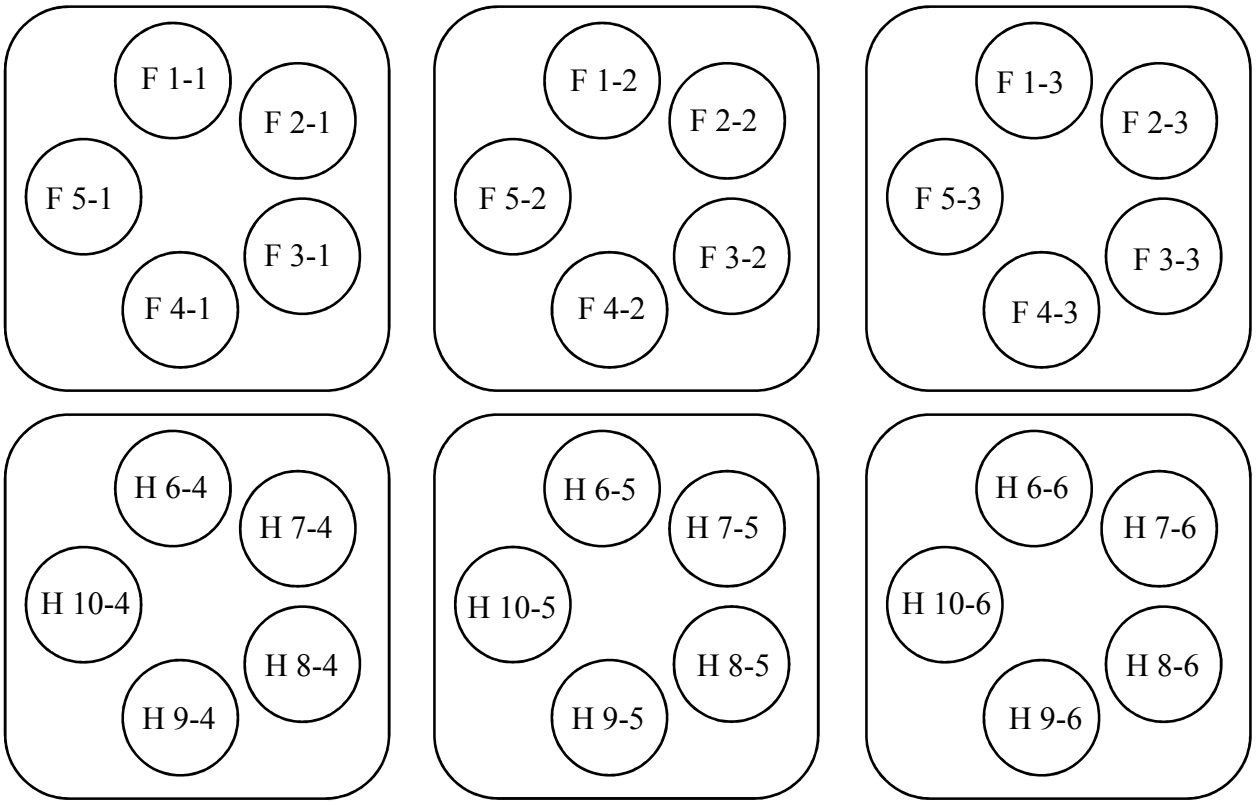


Figure 2. Experimental setup for the semen cryopreservation and egg fertilization experiment. Each square indicates a tank liner that held five smaller buckets, which are indicated by small circles. Each small circle incubates one segment of egg ribbon. "F" stands for cryopreserved semen. "H" stands for fresh semen. The first number indicates the male that fertilized the egg ribbon segment. The second number indicates the female from which the eggs originate. Thus, there were 6 females and 10 males used in this study.

On the first day of incubation, the water temperature in the experimental setup matched the water temperature of the tank from which the female fish were housed (9.5°C). On the last day of incubation, the water temperature was 15°C. The water temperature increased about 1°C every two days. After the first 24 hours of incubation, formalin (40% formaldehyde by volume) was added to each bucket to prevent fungal growth on eggs. Application increased as the experiment progressed (Table 2). Incubation stopped and the experiment was terminated when larvae began hatching out on day 13.

Table 2. Water temperatures and formalin dose (40% formaldehyde by volume) for egg incubation during the semen cryopreservation and egg fertilization trial. Formalin was dosed either once per day (X1) in the morning or twice per day (X2), once in the morning and once in the evening.

Date	Target Temperature Range (°C)	Head Tank 1 Temp. (°C)	Head Tank 2 Temp. (°C)	Formalin per Bucket (mL)
2/26/19	9-10	9.3	9.3	
2/27/19	9-10	10.0	9.6	0.90 x1
2/28/19	10-11	10.0	9.5	0.90 x2
3/1/19	10-11	10.8	10.7	0.90 x2
3/2/19	11-12	11.0	11.7	0.90 x2
3/3/19	11-12	11.0	11.0	0.90 x2
3/4/19	12-13	12.0	11.7	0.90 x2
3/5/19	12-13	12.0	12.0	0.90 x1 & 1.00 x1
3/6/19	13-14	12.1	12.7	1.00 x1 & 2.00 x1
3/7/19	13-14	13.0	13.7	3.00 x2
3/8/19	14-15	14.0	14.7	3.00 x2
3/9/19	14-15	14.0	14.7	3.00 x1
3/10/19	15-16	15.0	15.3	
3/11/19	15-16	15.0	15.3	

After the eggs were incubated for about 48 hours, the mass of each ribbon segment was determined. A 3.5 L bucket was filled with 1.5 L of dechlorinated water and weighed (1592.92 g) to maintain consistency for each ribbon segment. The egg segment, including its grate, zip ties and lead weights, were placed into the bucket containing 1.5 L dechlorinated water and weighed. When the experiment was completed, the same bucket was filled with 1.5 liters of water and the grate, zip ties and lead weights were placed into the bucket and weighed on the scale. The mass of the egg segment was the difference between the first and second masses. An estimated egg ribbon mass was determined by adding the five segment masses for each female.

Sampling of the eggs occurred after 48 hours (28 February 2019) and after 12 days (10 March 2019) of incubation. About 3 grams of ribbon from each bucket was sampled and split

into three 1-gram pieces. Each 1-gram sample was placed on a labeled petri dish and spread out to create one layer of eggs. Each petri dish sample was photographed by setting the petri dish on top of a green background. A Canon EOS RebelT5i camera was mounted nadir to the petri dish. A ruler was included in each photo along with the petri dish label for later computer analyzation in ImageJ image analysis software (National Institute of Health, Bethesda, MD; http://imagej.nih.gov/ij). All 3 1-gram pieces from each bucket were preserved in one bottle with formalin (40% formaldehyde by volume). This procedure was repeated on the 12th day of incubation when larvae started hatching out of the eggs. Two-day incubation photographs were

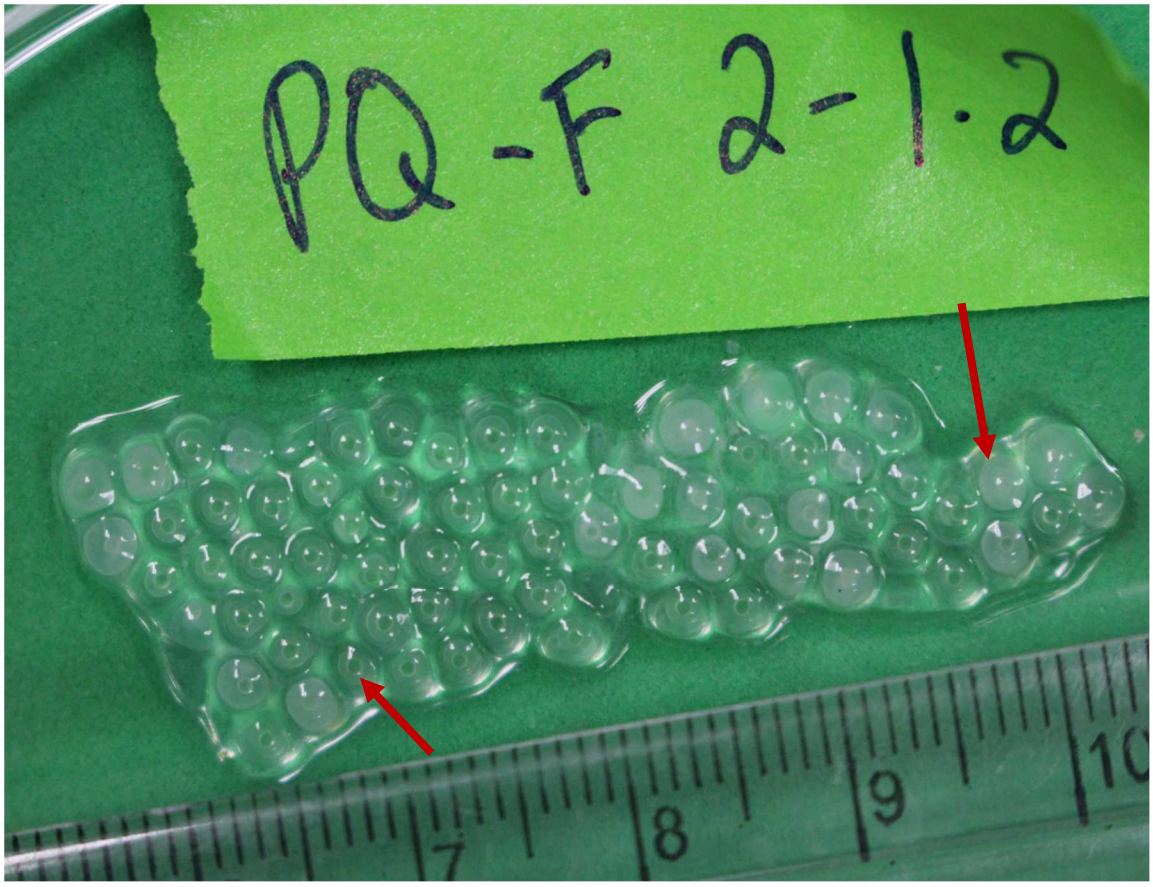


Figure 3. Example of a 2-day incubation photograph. Dead eggs are opaque (right arrow). Live eggs are clear (left arrow). The oil drop inside of live eggs is the small, clear, inner circle and its diameter was measured using ImageJ. A label is present for sample identification and a ruler is visible for scale setting within ImageJ.

analyzed for the number of eggs, the diameter of the oil droplet (used in calculation for volume), the percentage of fertilized eggs (clear), and the percentage of dead eggs (opaque). The twelve-day incubation photographs were analyzed for the percentage of dead eggs (opaque), the percentage of dead embryos (eyed but white) and the percentage of viable embryos (eyed and

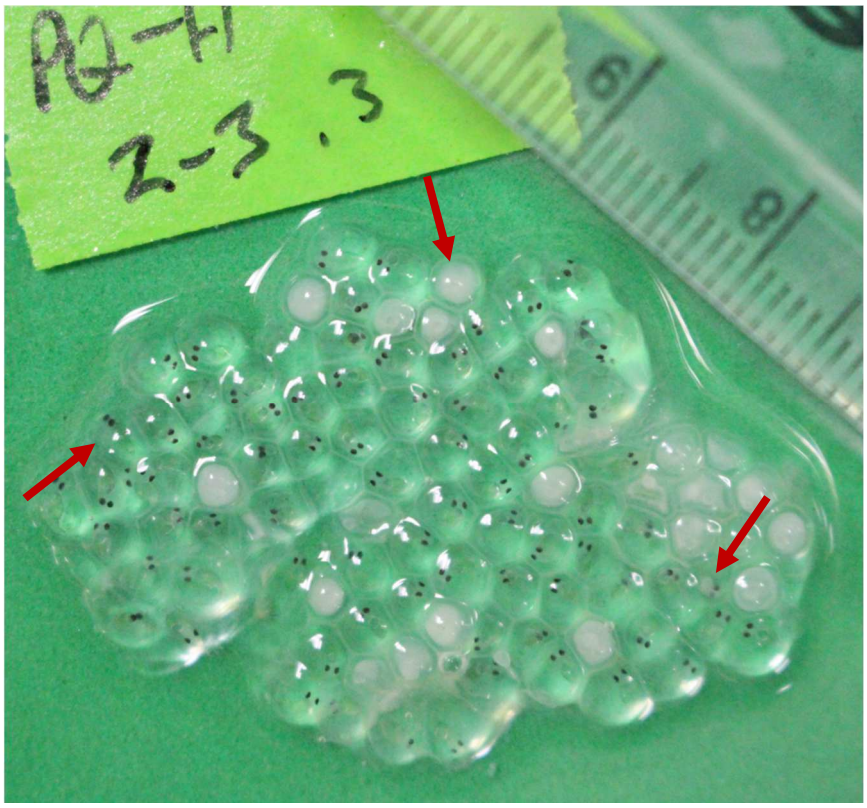


Figure 4. Example of a 12-day incubation photograph. Dead eggs are opaque (center arrow). Viable larvae are clear and have black eyes (left arrow). Dead larvae are opaque and have black eyes (right arrow). A label is present for sample identification and a ruler is visible for scale setting within ImageJ.

living).

Statistical analyses were carried out with JMP Pro 15 (SAS Institute, Cary, North Carolina). A Shapiro-Wilk test was used to check for normality and a Levene's test was used to check for equal variances. If data were normal and had equal variances, a one-way ANOVA was conducted. If data were non-normal and/or had unequal variances, the nonparametric Welch test

was used (Lantz 2013). One-way ANOVAs or Welch tests were used to determine whether significant differences existed between average egg parameters by treatment, female, and male. Following a significant one-way ANOVA, a Tukey's HSD post hoc test was conducted and following a significant Welch test, a Games-Howell post hoc test was conducted (Ruxton and Beauchamp 2008). Covariance matrices were conducted to determine whether relationships existed between egg ribbon mass, egg count, average mass per egg, average oil drop volume, and average percent viable.

A nested ANOVA was conducted to determine the effects from female, male and treatment on the percentage of viable eggs after 2 days of incubation (Schielzeth and Nakagawa 2013). Similarly, nested ANOVAs were conducted to determine the effects from female, male and treatment on the percentage of viable embryos, the percentage of dead embryos, and the percentage of dead eggs after 12 days of incubation (Schielzeth and Nakagawa 2013). The treatment served as a fixed factor. Females and males were both nested within treatment and were random factors. The male by female interaction was also nested within treatment and served as the error mean square (residuals).

A paired sample t-test was conducted to determine whether the percentage of dead eggs after two days of incubation differed from the percentage of dead eggs after twelve days of incubation. Additionally, a paired sample t-test was conducted to determine whether the percentage of dead eggs fertilized with fresh semen after two days of incubation differed from the percentage of dead eggs fertilized with fresh semen after twelve days of incubation. Finally, a paired sample t-test was conducted to determine whether the percentage of dead eggs fertilized with cryopreserved semen after two days of incubation differed from the percentage of dead eggs

fertilized with cryopreserved semen after twelve days of incubation. For all statistical analyses, means were considered statistically significant when $P < 0.05$.

2.2 Semen Cryopreservation and Computer Assisted Sperm Analysis

On 12 March 2020, semen samples were collected from Choptank River strain perch. Choptank strain yellow perch originate from the Choptank River, a major tributary of the Chesapeake Bay. The male fish that were netted and freely releasing milt were euthanized in a solution of 200 mg/L of MS-222 and hatchery water. Once the fish was euthanized, it was rinsed in clean water and dried to avoid contamination. Semen samples were collected by dry stripping directly into a clean falcon tube. The volume of milt collected was recorded and then mixed with

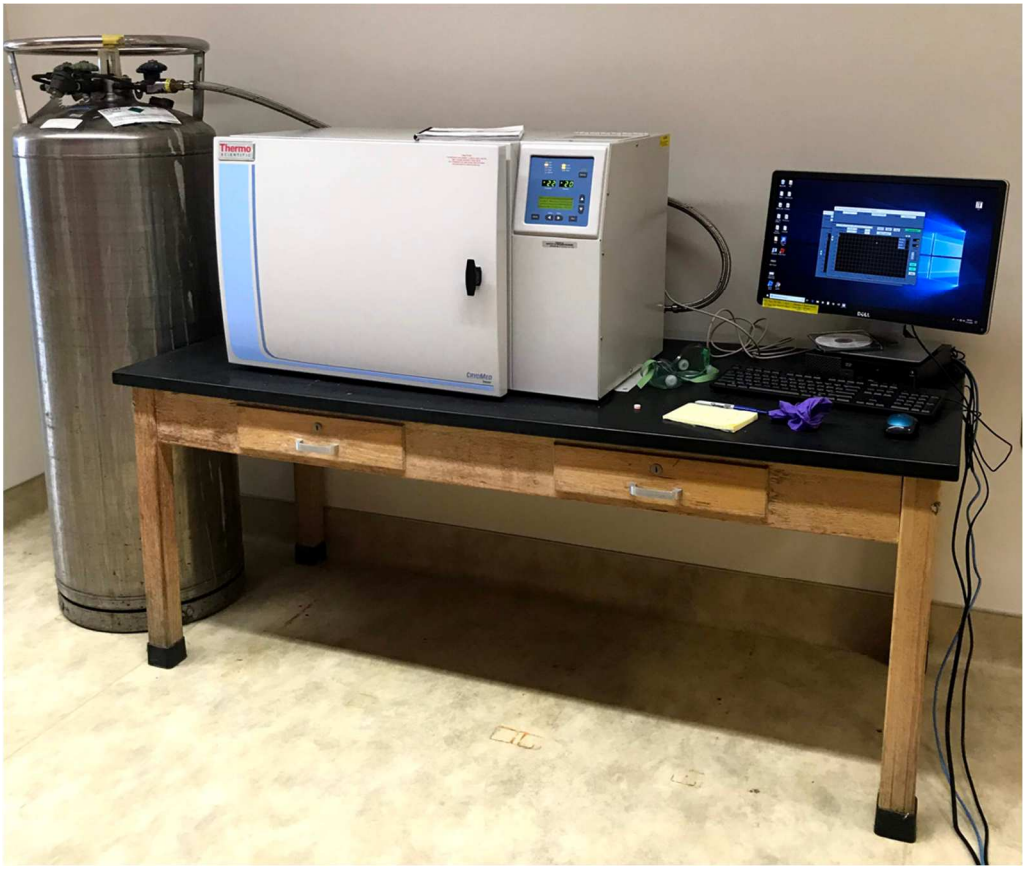


Figure 5. Semen cryopreservation laboratory setup. From left to right: liquid nitrogen tank, control rate freezer, computer running control rate freezer software.

immobilizing solution at ratio of 1:20 (Bernáth, Zarski, et al. 2015; Bernáth, Bokor, et al. 2015; Bernáth et al. 2016). The immobilizing solution consisted of 137 mM NaCl and 76.2 mM $NaHCO_3$ in deionized water (modified Tanaka) (Bernáth, Zarski, et al. 2015; Bernáth, Bokor, et al. 2015; Bernáth et al. 2016). Samples were held on crushed ice. The euthanized fish were weighed, measured for total length, and dissected. The gonads were removed and weighed.

An OMAX phase contrast trinocular compound LED microscope and a HAYEAR 14 MP microscope camera (Shenzhen Hayear Electronics Company, Futian, Shenzhen, China) with video recording capability were used to capture sperm movement and kinetics. Four aliquots from the original falcon tube holding one fish's milt was transferred into four separate snap cap centrifuge tubes. Each aliquot contained three microliters. Before sperm motion analysis, 47 microliters of activating solution were added to an aliquot and shaken to incorporate. The activating solution consisted of 50 mM NaCl and 0.1g/L BSA (bovine serum albumin, MP Biomedicals, Solon, Ohio) (Bernáth et al. 2016). The BSA was added to prevent sperm cells from clumping and sticking to the slide (Rodina et al. 2008). Countess cell counting chamber slides having a 10-microliter capacity were used for sperm viewing. Ten microliters of activated semen were pipetted into the chamber slide immediately after activation. Video recording began immediately after loading the sample into the chamber. Videos were 30 seconds in duration. Each semen sample had four aliquots taken from it and each aliquot had one video recording, so each semen sample had a total of four video recordings.

Once video recording was completed, the remaining inactivated semen samples were cryopreserved. 1.8 mL of semen which had been previously diluted with immobilizing solution was mixed with 0.18 mL DMSO (Bernáth, Zarski, et al. 2015; Rodina, Policar, and Linhart 2008; Rodina et al. 2008; Miller et al. 2018). The cryopreservation vessels used were 2-mL

screw top vials. A Thermo Fisher Scientific Model 7452 Series CryoMed controlled rate freezer was used for cryopreservation (Fig. 3) and it was programmed to follow the cooling rate developed by Bernáth, Bokor, et al. (2015). Specifically, freezing began at 7.5°C and dropped to -160°C at 56°C/minute. Cryopreserved samples were stored in a -150°C freezer.

For sperm motility analysis of cryopreserved semen, samples were thawed in a 40°C water bath for 10 seconds (Judycka et al. 2019). Four aliquots from each cryopreserved semen sample were activated and had video recorded as previously described. Samples were activated and recorded less than two minutes after thawing.

To prepare videos for CASA analyses, they were first converted from mp4 videos to uncompressed AVI videos and cropped using VirtualDub. All videos were then edited within ImageJ. First, the "conversion options" were set to "weighted RGB conversions". Next, each video frame was converted from RGB to 8-bit. Then each frame was processed to "find edges", which outlined the body of each sperm. The frames were processed using fast Fourier transform (FFT) bandpass filter to filter out structures larger than 50 pixels and structures smaller than 15 pixels. Finally, the frames were adjusted using a threshold. Pixels having values between 130

and 255 were considered foreground (sperm) and all other pixels that did not have values within this range were considered background. This resulted in a binary image of white sperm heads on

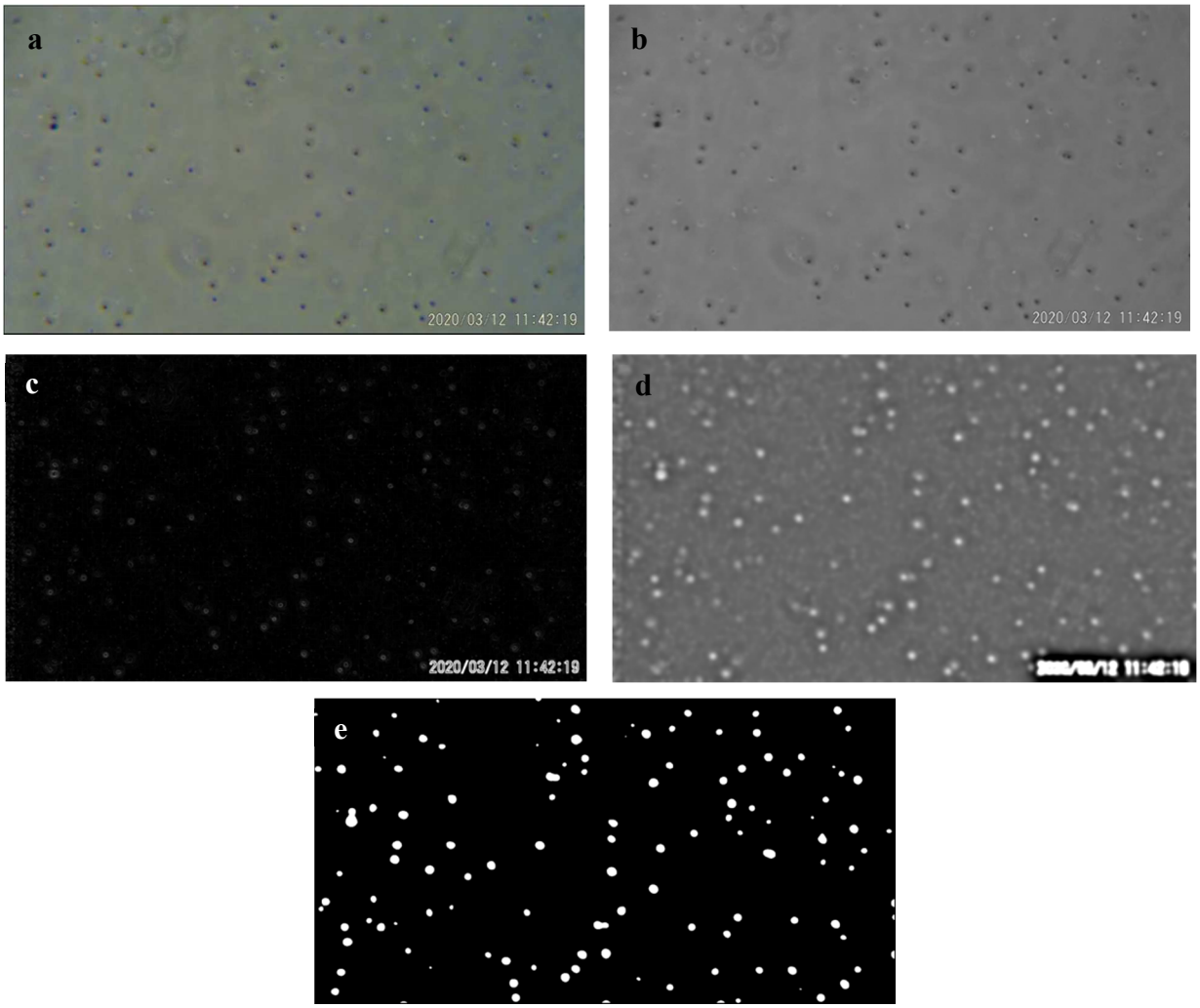


Figure 6. A still frame extracted from a video and edited for computer assisted sperm analysis (CASA) within ImageJ. Image 1: raw photo of sperm cells as would be seen with a microscope. Image 2: image converted from RGB to 8-bit. Image 3: image processed by "finding edges". Image 4: image processed using a fast Fourier transform (FFT) bandpass filter. Image 5: threshold applied to image so sperm cells are white on a black background.; image prepared for CASA.

a black background. The frames were cropped one last time to remove the date and time stamp.

Videos were analyzed in ImageJ image analysis software (National Institute of Health, Bethesda, MD, http://imagej.nih.gov/ij) utilizing a CASA plugin (https://imagej.nih.gov/ij/plugins/casa.html). The plugin gathered data from each video to

determine progressive motility, curvilinear velocity, average path velocity, rate of change of direction, percent sperm motile and amplitude of lateral head displacement (Wilson-Leedy and Ingermann 2011, Table 3). See table 4 for the specific settings used within the plugin to gather data.

The paired sample t-test is robust to small sample sizes, skewed population distributions, and unequal variances when within-pair correlation is high (Winter 2013). Therefore, paired sample t-tests were conducted to determine whether motility characteristics differed when the semen sample was fresh versus when the semen sample was cryopreserved. Means were considered statistically significant when $P < 0.05$.

Table 3. Sperm motility characteristics measured in the computer assisted sperm analysis (CASA) plugin for ImageJ. Modified from Wilson-Leedy and Ingermann (2011).

Characteristic	Abbreviation	Description
percent motile	% Motile	The percentage of sperm moving in a manner fitting motility determination parameters
velocity curvilinear	VCL	Point to point velocity (total distance traveled) per second
velocity average path	VAP	Point to point velocity on a path constructed using a roaming average; the number of points in the roaming average is 1/6th of the frame rate of the video used
velocity straight line	VSL	Velocity measured using the first point and the velocity average path and the point reached that is furthest from this origin during the measured time period
linearity	LIN	VSL/VAP; describes path curvature
wobble	WOB	VAP/VCL; describes side to side movement of the sperm head
progression	PROG	The average distance of the sperm from its origin on the average path during all frames analyzed

Table 4. Parameters and their values required to run the computer assisted sperm analysis (CASA) plugin in ImageJ. The values were experimentally chosen for yellow perch sperm and for the unique settings present in the videos from this experiment. Modified from Wilson-Leedy and Ingermann (2011).

CASA Parameter	**Value**
Minimum sperm size (pixels)	2
Maximum sperm size (pixels)	1000
Minimum track length (frames)	100
Maximum sperm velocity between frames (pixels)	60
Minimum VSL for motile (um/s)	3
Minimum VAP for motile (um/s)	20
Minimum VCL for motile (um/s)	25
Low VAP speed (um/s)	5
Maximum percentage of path with zero VAP	1
Maximum percentage of path with low VAP	25
Low VAP speed 2 (um/s)	25
Low VCL speed (um/s)	35
High WOB (percent VAP/VCL)	80
High LIN (percent VSL/VAP)	80
High WOB 2 (percent VAP/VCL)	50
High LIN 2 (percent VSL/VAP)	60
Frame rate (frames per second)	60
Microns per 1000 pixels	1075

CHAPTER 3: RESULTS

3.1 Semen Cryopreservation and Egg Fertilization Trial

3.1.1 Effect of cryopreservation on egg fertilization after 2-day incubation period

A nested ANOVA was conducted to determine the effects of treatment, female and male on the percentage of viable eggs after two days of incubation. The treatment was either fresh or cryopreserved semen (fixed factors). Females and males were both nested within treatment and were random factors. The male by female interaction was also nested within treatment and served as the error mean square (residuals). See figure 2 for clarification of the experimental design.

The ANOVA showed a statistically significant treatment effect ($F_{1,16} = 6.60$, $P = 0.021$), so cryopreserved semen had a negative impact on egg viability after two days of incubation (Table 5). There was no statistically significant female effect ($F_{4,16} = 1.52$, $P = 0.243$) or male effect ($F_{8,16} = 0.85$, $P = 0.572$) on egg viability (Table 5).

Table 5. Nested ANOVA results for the percentage of viable eggs after 2 days of incubation. "Female [Treatment]" and "Male [Treatment]" indicate that the female and male effects are nested within treatment.

Source	df	MS	F	P
Treatment	1	1011.3	6.60	0.021
Female [Treatment]	4	233.5	1.52	0.243
Male [Treatment]	8	130.8	0.85	0.572
Residual	16	153.2		

Each treatment (cryopreserved semen and fresh semen) fertilized similar egg segment masses ($F_{1,28} = 2.06$, $P = 0.162$) and each treatment fertilized eggs having similar oil drop volumes ($F_{1,28} = 1.53$, $P = 0.662$), but each treatment fertilized egg segments that differed in their

egg count ($F_{1,28} = 9.52$, $P = 0.005$) and average mass per egg ($F_{1,28} = 5.64$, $P = 0.025$). Most importantly, the treatment had a significant impact ($F_{1,28} = 6.39$, $P = 0.017$) on the percentage of viable eggs after two days of incubation with cryopreserved semen negatively impacting the percentage of viable eggs (Table 6).

Table 6. Egg parameters by treatment (mean ± standard deviation) after 2 days of incubation. Average segment mass is the segment of egg ribbon which was fertilized with cryopreserved semen or fresh semen. Average egg count per segment is the estimated number of eggs for each segment of egg ribbon fertilized by cryopreserved semen or fresh semen. Egg count, average mass per egg, average oil drop volume per egg, and average percent viable were calculated using ImageJ. Different superscript letters within the same column indicate a significant difference ($P < 0.05$). Columns absent of superscript letters indicate no significant difference.

Treatment	Avg. Segment Mass (g)	Avg. Egg Count per Segment	Avg. Mass per Egg (g)	Avg. Oil Drop Vol. (mm^3)	Avg. Percent Viable (%)
Cryopreserved	107.7 ± 28.0	8936 ± 2389[a]	0.012 ± 0.0019[a]	0.071 ± 0.017	34.9 ± 17.3[a]
Fresh	124.4 ± 35.3	11722 ± 2552[b]	0.011 ± 0.0018[b]	0.065 ± 0.017	46.5 ± 16.1[b]

Although the nested ANOVA indicated that females and males did not have a significant impact on the percentage of viable eggs after two days of incubation, there were significant variations between females and males. The female had a significant impact on the average mass per egg (ANOVA: $F_{5, 24} = 91.37$, $P < 0.0001$), average oil drop volume (Welch's test: $F_{5, 9.8543} = 8.98$, $P = 0.002$), and the average percentage of viable eggs (ANOVA: $F_{5,24} = 2.67$, $P = 0.047$) (Table 7). Covariance matrices did not reveal any relationships between egg ribbon mass, egg count, average mass per egg, average oil drop volume, or average percent viable.

Each male fertilized egg segments with similar masses (ANOVA: $F_{9,20} = 1.27$, $P = 0.314$), similar masses per egg (ANOVA: $F_{9,20} = 0.50$, $P = 0.856$), and similar average oil drop volumes (ANOVA: $F_{9,20} = 1.01$, $P = 0.464$), but males fertilized egg segments that differed in their egg

count ($F_{9, 20} = 4.09$, $P = 0.004$) (Table 8). Most importantly, the male did not impact the average percentage of viable eggs after two days of incubation (ANOVA: $F_{9,20} = 1.35$, $P = 0.274$).

Table 7. Egg parameters by female (mean ± standard deviation) after 2 days of incubation. Egg ribbon mass is the total mass of the egg ribbon harvested from each female. Egg count is the estimated number of eggs per egg ribbon. Egg count, average mass per egg, average oil drop volume per egg, and average precent viable were calculated using ImageJ. Different superscript letters within the same column indicate a significant difference ($P < 0.05$). Columns absent of superscript letters indicate no significant difference.

Female	Egg Ribbon Mass (g)	Egg Count	Avg. Mass per Egg (g)	Avg. Oil Drop Volume per Egg (mm^3)	Avg. Percent Viable (%)
1	600.7	42,600	0.014 (± 0.0025)^{a}	0.090 (± 0.013)^{a}	36.3 (± 14.2)^{ab}
2	445.8	45,300	0.010 (± 0.0007)^{d}	0.060 (± 0.004)^{b}	39.9 (± 17.5)^{ab}
3	569.0	43,800	0.013 (± 0.0009)^{ab}	0.064 (± 0.014)^{b}	28.4 (± 19.1)^{b}
4	645.6	55,400	0.012 (± 0.0011)^{c}	0.074 (± 0.017)^{ab}	41.8 (± 17.9)^{ab}
5	747.7	61,900	0.012 (± 0.0008)^{bc}	0.062 (± 0.013)^{b}	42.3 (± 13.7)^{ab}
6	473.2	56,900	0.008 (± 0.0005)^{e}	0.059 (± 0.018)^{ab}	55.3 (± 13.5)^{a}

Table 8. Egg parameters by male (mean ± standard deviation) after 2 days of incubation. Average segment mass is the segment of egg ribbon which was fertilized by a single male. Average egg count per segment is the estimated number of eggs for each segment of egg ribbon fertilized by a single male. Egg count, average mass per egg, average oil drop volume per egg, and average precent viable were calculated using ImageJ. Different superscript letters within the same column indicate a significant difference ($P < 0.05$). Columns absent of superscript letters indicate no significant difference.

Male	Avg. Segment Mass (g)	Avg. Egg Count per Segment	Avg. Mass per Egg (g)	Avg. Oil Drop Vol. (mm^3) per Egg	Avg. Percent Viable (%)
1	99.4 ± 43.4	8,154 ± 2,782^{b}	0.012 ± 0.002	0.075 ± 0.019	42.0 ± 15.1
2	94.1 ± 30.5	7,233 ± 1,458^{b}	0.013 ± 0.002	0.071 ± 0.021	35.4 ± 17.3
3	118.1 ± 33.6	9,641 ± 1,161^{ab}	0.012 ± 0.002	0.073 ± 0.010	37.1 ± 19.1
4	107.4 ±24.7	9,296 ± 2,961^{ab}	0.012 ± 0.002	0.070 ± 0.023	22.2 ± 14.1
5	119.5 ± 14.0	10,358 ± 3,278^{ab}	0.012 ± 0.002	0.067 ± 0.011	37.1 ± 16.9
6	100.1 ± 39.4	9,136 ± 1,896^{ab}	0.011 ± 0.002	0.071 ± 0.013	47.6 ± 21.9
7	112.4 ± 9.8	10,990 ± 1,628^{ab}	0.010 ± 0.002	0.055 ± 0.019	38.9 ± 15.0
8	107 ± 28.8	9,882 ± 923^{ab}	0.011 ± 0.002	0.057 ± 0.015	42.9 ± 15.8
9	151.3 ± 28.9	14,477 ± 940^{a}	0.010 ± 0.002	0.062 ± 0.017	52.8 ± 17.0
10	151.4 ± 41.0	14,124 ± 1,244^{a}	0.011 ± 0.002	0.081 ± 0.009	50.2 ± 6.2

3.1.2 Effect of cryopreservation on embryonic development after 12-day incubation period

Nested ANOVAs were conducted to determine the effects of treatment, female, and male after 12 days of incubation on the percentage of dead eggs, percentage of dead embryos, and the percentage of viable embryos. The treatment ($P < 0.0001$), female ($P = 0.026$), and male ($P = 0.006$) all had statistically significant effects on the percentage of dead eggs, but the treatment had the largest impact (Table 9). The treatment ($P = 0.009$) and the female ($P = 0.038$) had statistically significant effects on the percentage of dead embryos after twelve days of incubation, but treatment had the larger impact (Table 10). The treatment ($P < 0.0001$) and the male ($P = 0.003$) had statistically significant effects on the percentage of viable larvae, but the treatment had the larger impact (Table 11).

Table 9. Nested ANOVA results for the percentage of dead eggs after 12 days of incubation. "Female [Treatment]" and "Male [Treatment]" indicate that the female and male effects are nested within treatments.

Source	df	MS	F	P
Treatment	1	16878.7	133.16	<0.0001
Female [Treatment]	4	469.1	3.70	0.026
Male [Treatment]	8	547.5	4.32	0.006
Residual	16	126.8		

Table 10. Nested ANOVA results for the percentage of dead embryos after 12 days of incubation. "Female [Treatment]" and "Male [Treatment]" indicate that the female and male effects are nested within treatments.

Source	df	MS	F	P
Treatment	1	558.9	8.91	0.009
Female [Treatment]	4	206.5	3.29	0.038
Male [Treatment]	8	45.9	0.73	0.663
Residual	16	62.8		

Table 11. Nested ANOVA results for the percentage of viable embryos after 12 days of incubation. "Female [Treatment]" and "Male [Treatment]" indicate that the female and male effects are nested within treatments.

Source	df	MS	F	P
Treatment	1	12074.0	130.10	< 0.0001
Female [Treatment]	4	217.0	2.34	0.100
Male [Treatment]	8	464.4	5.00	0.003
Residual	16	92.8		

To better understand differences between treatments and variations between females and males, additional ANOVAs were conducted. The treatment had a statistically significant effect on the percentage of dead eggs (Welch's test: $F_{(1, 15.094)} = 57.04$, $P < 0.0001$), the percentage of dead larvae (Welch's test: $F_{(1, 14.288)} = 7.12$, $P = 0.018$), and the percentage of viable larvae (ANOVA: $F_{(1, 15.056)} = 55.71$, $P < 0.0001$). Egg segments fertilized with fresh semen had a smaller percentage of dead eggs than egg segments fertilized with cryopreserved semen. Egg segments fertilized with fresh semen had a larger percentage of dead larvae and of viable larvae then egg segments fertilized with cryopreserved semen. This indicates that fewer eggs developed to larvae when fertilized cryopreserved semen than fresh semen (Table 12).

Table 12. Embryonic development parameters by treatment (mean ± standard deviation) after 12 days of incubation. Different superscript letters within the same column indicate a significant difference ($P < 0.05$).

Treatment	Percent Dead Eggs (%)	Percent Dead Embryos (%)	Percent Viable Embryos (%)
Cryopreserved	97.1 ± 4.7^{a}	0.45 ± 1.3^{a}	2.50 ± 4.0^{a}
Fresh	49.7 ± 23.9^{b}	9.09 ± 12.5^{b}	42.6 ± 20.4^{b}

The female from which eggs originated had a statistically significant effect on the percentage of dead eggs (Welch's test: $F_{(5, 9.4013)} = 14.51$, $P = 0.0004$), the percentage of dead larvae (ANOVA: $F_{(4, 24)} = 4.85$, $P = 0.003$), and the percentage of viable larvae (ANOVA: $F_{(5,24)} = 11.95$, $P < 0.0001$), so individual female variation had a significant impact on the percentage of eggs that died, the percentage of eggs that developed into larvae but died, and the percentage of eggs that developed into viable larvae (Table 6). The male had a statistically significant effect on the percentage of dead eggs (ANOVA: $F_{(9, 20)} = 12.10$, $P < 0.0001$) and the percentage of viable larvae (ANOVA: $F_{(9, 20)} = 14.91$, $P < 0.0001$), so individual male variation had a significant

impact on the percentage of eggs that died and the percentage of eggs that developed into viable larvae (Table 7).

Table 13. Embryonic development parameters by female (mean ± standard deviation) after 12 days of incubation. Different superscript letters within the same column indicate a significant difference ($P < 0.05$).

Female	Percent Dead Eggs (%)	Percent Dead Embryos (%)	Percent Viable Embryos (%)
1	93.5 ± 6.8^{a}	1.1 ± 2.2^{b}	5.4 ± 5.7^{bc}
2	98.0 ± 2.3^{a}	0.3 ± 0.3^{b}	1.8 ± 2.4^{c}
3	99.8 ± 0.3^{a}	0^{b}	0.3 ± 0.42^{c}
4	36.3 ± 25.2^{b}	19.5 ± 17.7^{a}	48.1 ± 20.4^{a}
5	62.9 ± 13.5^{b}	4.9 ± 3.9^{ab}	32.3 ± 14.1^{ab}
6	49.8 ± 27.1^{ab}	2.8 ± 2.9^{b}	47.4 ± 25.4^{a}

Table 14. Embryonic development parameters by male (mean ± standard deviation) after 12 days of incubation. Different superscript letters within the same column indicate a significant difference ($P < 0.05$). Columns absent of superscript letters indicate no significant difference.

Male	Percent Dead Eggs (%)	Percent Dead Embryos (%)	Percent Viable Embryos (%)
1	100^{a}	0	0^{c}
2	98.4 ± 1.4^{a}	0.4 ± 0.4	1.1 ± 1.1^{c}
3	92.9 ± 7.1^{a}	1.6 ± 2.8	5.4 ± 4.5^{c}
4	95.2 ± 7.2^{a}	0	5.0 ± 7.0^{c}
5	99.0 ± 1.0^{a}	0.2 ± 0.4	0.8 ± 0.9^{c}
6	37.3 ± 15.7^{c}	18.4 ± 27.1	50.8 ± 2.9^{a}
7	41.9 ± 30.3^{bc}	7.4 ± 7.5	50.7 ± 23.1^{a}
8	35.8 ± 15.9^{c}	6.0 ± 3.3	58.4 ± 18.7^{a}
9	51.9 ± 18.3^{bc}	9.2 ± 9.6	38.9 ± 13.2^{ab}
10	81.5 ± 9.9^{ab}	4.4 ± 3.9	14.1 ± 6.2^{bc}

3.1.3 Effects of cryopreservation on dead eggs at 2-days incubation compared to 12-days incubation

When a paired sample t-test was conducted comparing the percentage of dead eggs after two days of incubation compared to the percentage of dead eggs after twelve days of incubation, there was a statistically significant difference between the percentage of dead eggs ($t_{(29)} = 2.70$, $P = 0.012$). Two F tests were run to determine whether the across groups (cryopreserved and fresh) values were different. The percentage of dead eggs within pairs ($F = 19.24$, $P = 0.0001$) and among pairs ($F = 64.11$, $P < 0.0001$) were both statistically significant. The percentage of dead eggs increased by a statistically significant amount from two days of incubation to twelve days of incubation.

Paired sample t-tests were conducted to determine whether the percentage of dead eggs after two days of incubation differed from the percentage of dead eggs after twelve days of incubation for each treatment. For the cryopreserved semen treatment, there was a statistically significant difference between the percentage of dead eggs after two days of incubation compared to after twelve days of incubation ($t_{(14)} = 9.57$, $P < 0.0001$). For the fresh semen treatment, there was not a statistically significant difference between the percentage of dead eggs after two days of incubation compared to after twelve days of incubation ($t_{(14)} = -0.51$, $P = 0.612$). Therefore, there were more dead eggs after twelve days of incubation compared to two days of incubation when eggs were fertilized with cryopreserved semen. When eggs were fertilized with fresh semen, there was not a statistically significant difference between the percentage of dead eggs for both time points. (Figure 7).

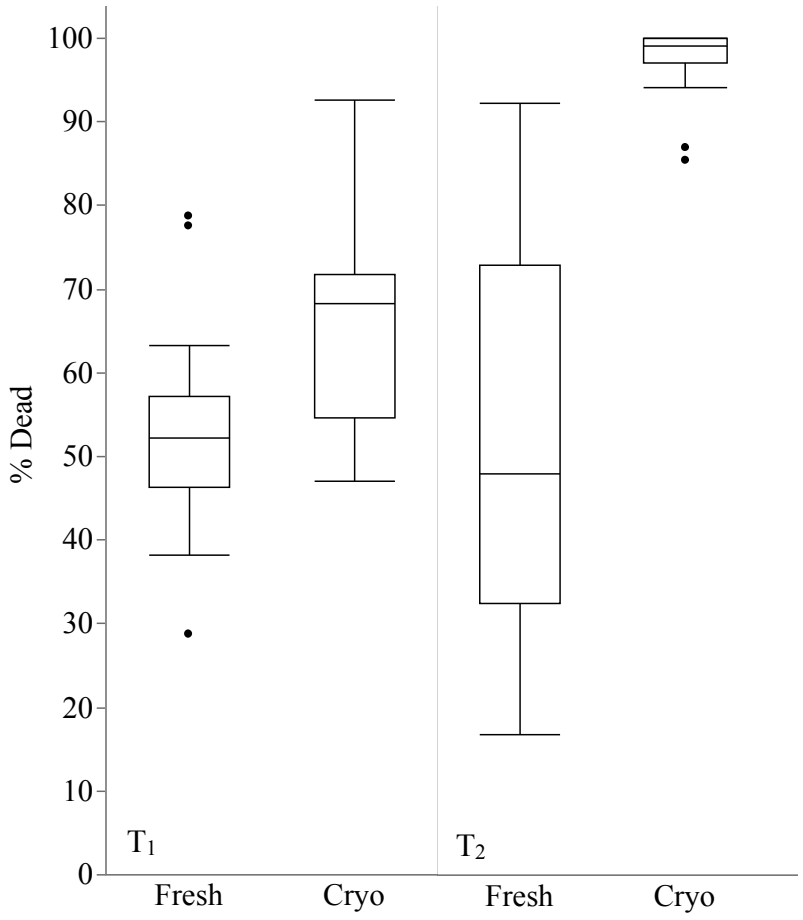
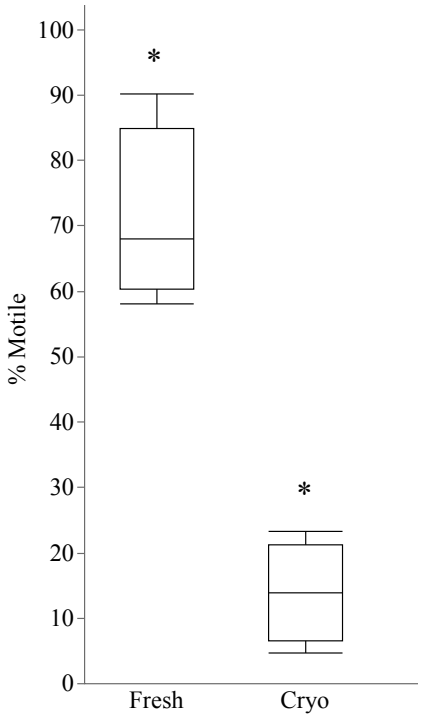
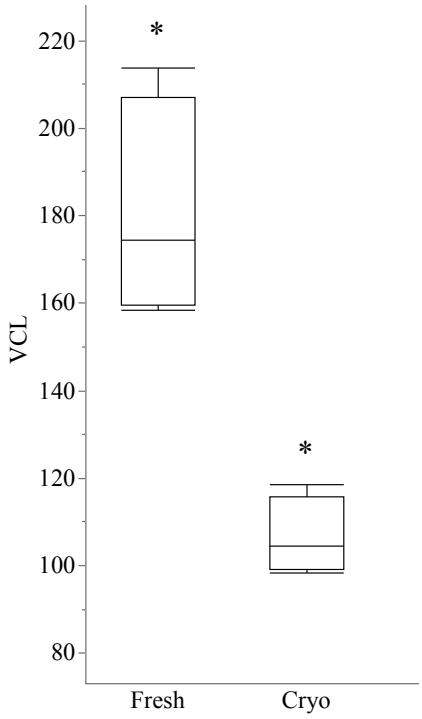


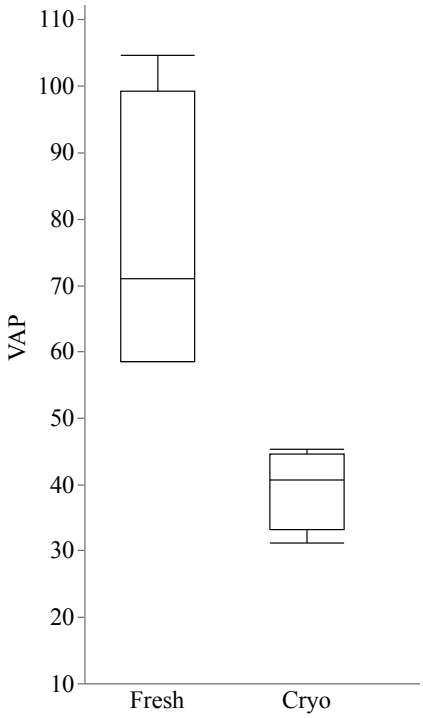
Figure 7. Box plots for each treatment indicating the percentage of dead eggs at T1 (2 days of incubation) and T2 (12 days of incubation). A paired t-test indicated a statistically significant difference in the percentage of dead eggs between each time point for the cryopreserved treatment, but a paired t-test did not indicate a statistically significant difference in the percentage of dead eggs between each time point for the fresh treatment. Boxes indicate the first and third quartiles. The center line in each box is the median. Whiskers are drawn 1.5 times the interquartile range from the box. Circles indicate outliers.

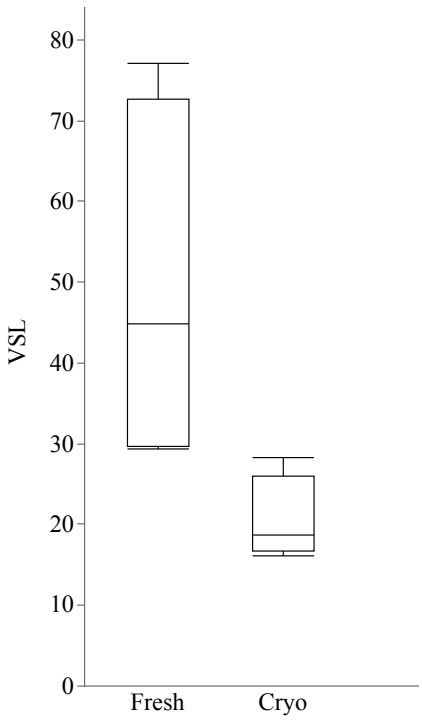
3.2 Semen Cryopreservation and Computer Assisted Sperm Analysis

The CASA motility results of the fresh semen (0.71 ± 0.14) and the cryopreserved semen (0.14 ± 0.08) indicated that cryopreservation decreased the percentage of motile sperm ($P < 0.01$). There was a significant decrease in the curvilinear velocity of sperm ($P = 0.01$) from fresh semen (180.30 ± 25.57) compared to sperm from cryopreserved semen (106.51 ± 8.88). There was not a statistically significant difference ($P = 0.07$) between the average path velocity of fresh sperm (76.33 ±22.19) and cryopreserved sperm (39.50 ± 6.08). Cryopreservation did not have a significant effect ($P = 0.12$) on the straight line velocity of sperm from fresh semen (49.06 ± 23.31) compared to sperm from cryopreserved semen (20.47 ± 3.59). Cryopreservation did not have a significant impact ($P = 0.58$) on the linearity of fresh sperm tracks (0.51 ± 0.06) compared to cryopreserved sperm tracks (0.54 ± 0.04). Cryopreservation did not have a significant impact ($P = 0.81$) on the wobble of fresh sperm (0.40 ± 0.02) tracks compared to cryopreserved sperm tracks (0.39 ± 0.04). Cryopreservation did not have a significant impact ($P = 0.31$) on the progression of sperm from fresh semen (62.38 ± 4.44) compared to sperm from cryopreserved semen (148.82 ± 140.94). See figure 8 for box plots of each motility characteristics and table 15 for paired t-test results.


% Motile
100
90
80
70
60
50
40
30
20
10
0
*
*
Fresh
Cryo


VCL
220
200
180
160
140
120
100
80
*
*
Fresh
Cryo


VAP
110
100
90
80
70
60
50
40
30
20
10
Fresh
Cryo


VSL
80
70
60
50
40
30
20
10
0
Fresh
Cryo

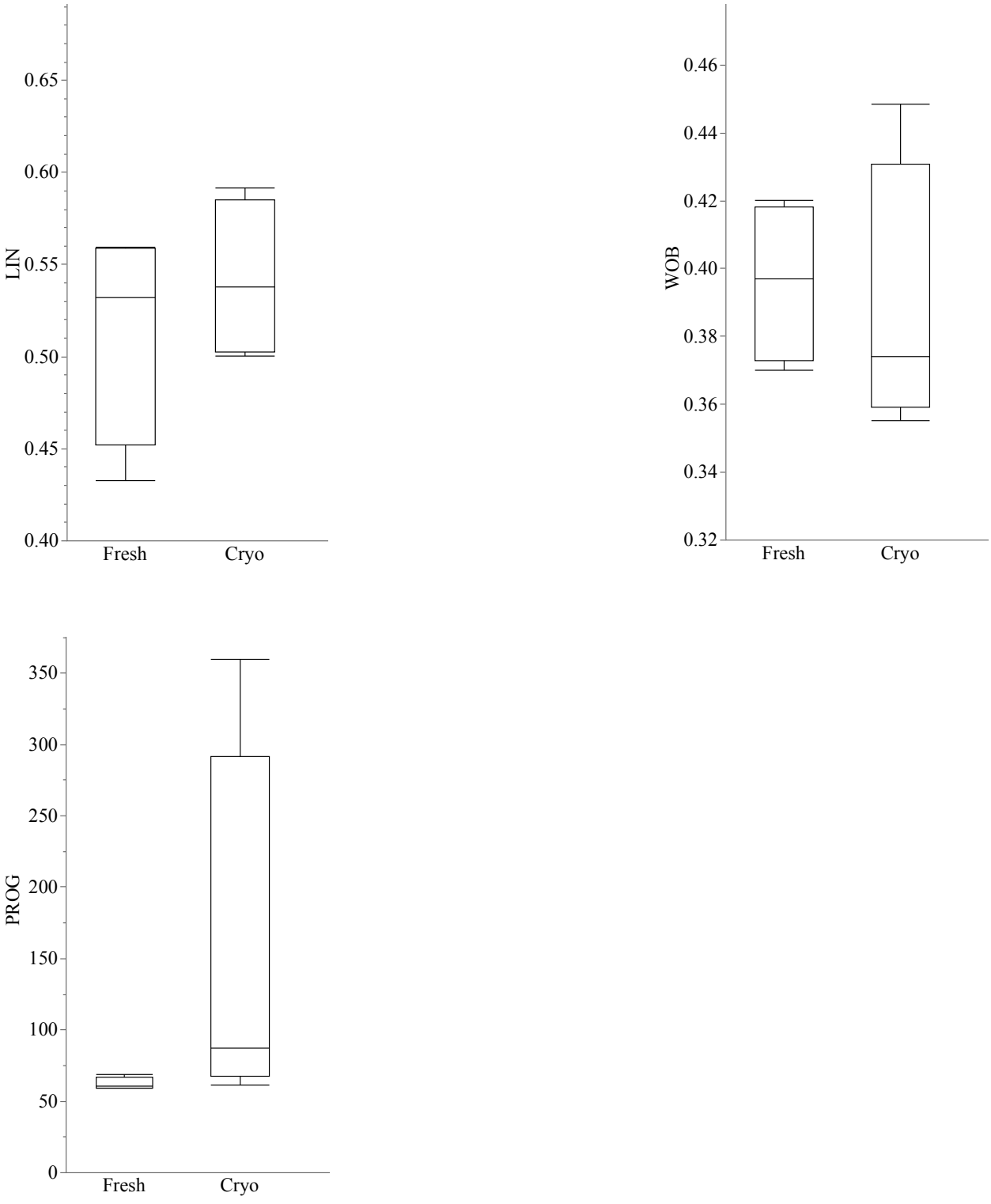


Figure 8. Box plots for the CASA characteristics of fresh semen and cryopreserved semen (pages 45 and 46). The presence of asterisks indicates statistically significant differences. Boxes indicate the first and third quartiles. The center line in each box is the median. Whiskers are drawn 1.5 times the interquartile range from the box.

Table 15. Paired t-test results for the CASA motility characteristics of fresh and cryopreserved semen.

Motility Characteristic	t	df	P
Percent Motile	-11.23	3	< 0.01
Velocity Curvilinear	-5.55	3	0.01
Velocity Average Path	-2.71	3	0.07
Velocity Straight Line	-2.12	3	0.12
Linearity	0.61	3	0.58
Wobble	-0.26	3	0.81
Progression	1.21	3	0.31

CHAPTER 4: DISCUSSION

Cryopreservation of yellow perch semen had a negative impact on egg viability after two days of incubation (P = 0.021, Table 5). 34.9% of eggs treated with cryopreserved semen were viable and 46.5% of eggs treated with fresh semen were viable and the two groups differed by statistically significant amounts (P = 0.017, Table 6).

The two-day egg viability results from the current study are larger, but similar, to the viability results attained by Miller and coworkers (2018). The aforementioned group tested two cryoprotectants (MeOH and DMSO) and two freezing methods (pellet and vial) and each treatment was supplemented with Atlantic salmon seminal plasma. At three hours post fertilization, they observed a fertilization rate of 28.2% for their pooled cryopreservation treatments and a fertilization rate of 60.2% for their pooled fresh treatments. At two days post fertilization, they observed 16.4% survival for cryopreserved treated eggs and 47.0% survival for fresh treated eggs. Overall, they found that the vial method with DMSO as a cryoprotectant produced the most viable eggs and subsequent larvae.

The current study provides evidence that Atlantic salmon seminal plasma is not a necessary extender supplement for yellow perch semen cryopreservation, which simplifies cryopreservation protocol. Historically, egg yolk was used in an original yellow perch sperm cryopreservation extender by Ciereszko et al. (1993) and in conjunction with DMSO fertilization rates of 23.2% were achieved. Miller and coworkers (2018) used easily accessible seminal plasma from another species of fish, the Atlantic salmon, to supplement the extender. Prior studies have found that supplementing extender solutions with seminal plasma reduced harmful effects from cryoprotectants, since seminal plasma is itself a cryoprotectant and antioxidant. Cryopreservation dilutes the protective seminal plasma, so additional seminal plasma or

exogenous antioxidants are sometimes added for extra sperm cell protection (reviewed in Sandoval-Vargas et al. 2020).

However, Sarosiek et al. (2014) found that short term storage of Eurasian perch semen does not require antioxidant supplements and Kobayashi buffer solution was suitable for short term storage. Successful cryopreservation and high rates of fertilization (78%) of the closely related Eurasian perch has been achieved with a simple glucose extender (Judycka et al. 2019). Although there is the potential for improvements in cryopreservation results by utilizing additional seminal plasma or exogenous antioxidants in extender solution, there is currently no evidence that it benefits Eurasian or yellow perch semen cryopreservation. Additionally, yellow perch cryopreservation protocol is not yet at the point that this research can be effectively utilized, because the most effective extender, cryoprotectant and activating solution has not been determined.

The slightly higher rates of viability achieved in the current study are unlikely attributable to individual variations stemming from females and males. A nested ANOVA found that females ($P = 0.24$) and males ($P = 0.57$) had little impact on the viability of eggs after two days of incubation (Table 5). Still, significant individual variations existed even if they did not impact the overall results. For example, individual female variation had a significant impact on the average mass per egg ($P < 0.0001$) and the average oil drop volume ($P = 0.002$), egg parameters which may impact the health and survival of eggs and are maternally dependent (Table 7).

The percentage of viable eggs differed significantly between females ($P = 0.047$), but this is most likely attributed to the treatment received by each females' eggs, because females one through three were treated with cryopreserved semen and had a smaller percentage of viable eggs

compared to females four through six that were treated with fresh semen (Table 7). Additionally, female three, treated with cryopreserved semen, had the smallest percentage of viable eggs while female six, treated with fresh semen, had the largest percentage of viable eggs and these pairs differed by statistically significant amounts. All other females did not differ by statistically significant amounts (Table 7). Males did not impact the percentage of viable eggs after two days of incubation ($P = 0.27$). Therefore, cryopreservation had a significant impact on the percentage of viable eggs after two days of incubation, while female and male variation had much smaller and insignificant impacts.

Cryopreservation had a large impact on the percentage of dead eggs ($P < 0.0001$), the percentage of dead larvae ($P = 0.009$), and the percentage of viable larvae after twelve days of incubation ($P < 0.0001$). Although there were significant effects from females and males, cryopreservation had the largest impact on embryonic development after twelve days of incubation (Tables 9, 10, 11).

Only 2.5% of eggs fertilized with cryopreserved semen developed into viable larvae compared to 42.6% of eggs fertilized with fresh semen. The percentage of viable larvae attained in this study is smaller than the percentage of viable larvae attained by Miller and coworkers (2018), who did not find a significant difference between their cryopreserved semen treatments. They pooled the larvae from all cryopreserved treatments and attained 14.8% viable larvae.

In the current study, fungal growth may have contributed to the low percentage of viable larvae. Egg segments with many dead eggs had a lot of fungal growth. In this experiment, egg segments treated with cryopreserved semen tended to have more dead eggs and more fungal growth than egg segments treated with fresh semen. Dead eggs provide a good substrate for the

growth of Saprolegnia spores (aquatic fungi). The fungus can then spread to live eggs, suffocating them, killing them and accelerating the spread of the fungus (Rach et al. 2005).

Yellow perch eggs may be particularly susceptible to fungal growth, because the eggs are arranged in a ribbon, connecting all eggs to each other and facilitating spread. Abd El-Gawad, Shen, and Wang (2016) recommend dosing formalin at a concentration of 150-200 mg L^{-1} for 30 minutes to improve yellow perch hatchability. The current experiment dosed as much as 1 mg L^{-1} formalin in flow through conditions (Table 2). This was not an adequate treatment to slow down the growth of fungi on eggs. It is likely that as more eggs died and became infected, the fungi suffocated live eggs and killed them, creating a positive feedback loop.

After twelve days of incubation, the individual female had a significant impact on the percentage of dead eggs ($P = 0.026$) and the percentage of dead larvae ($P = 0.038$), while the individual male had a significant impact on percentage of dead eggs (0.006) and the percentage of viable larvae ($P = 0.003$). Overall, ANOVAs indicated that treatment was the biggest factor in the results, because females and males treated with cryopreserved semen had a larger percentage of dead eggs than females and males treated with fresh semen; females and males treated with cryopreserved semen had a smaller percentage of dead larvae than females and males treated with fresh semen; females and males treated with cryopreserved semen had a smaller percentage of viable larvae than females and males treated with fresh semen (Tables 13 and 14). In this experiment, the large variations between individual females and individual males are mostly attributable to the treatment or experimental limitations reduced statistical inference power to identify true individual effects. However, the cryopreservation treatment uniformly impacted embryonic development in a negative way.

After twelve days of incubation, the percentage of dead eggs for the cryopreservation treatment increased by a statistically significant amount compared to the percentage of dead eggs for the cryopreservation treatment after two days of incubation. This was not the case for eggs treated with fresh semen. The percentage of dead eggs after two days of incubation compared to the percentage of dead eggs after twelve days of incubation did not differ by a statistically significant amount for the fresh semen treatment (Figure 7). Additionally, the effect of cryopreservation increased from day two ($P = 0.021$) to day twelve ($P < 0.0001$), indicating that cryopreserved semen has a negative impact on embryonic development.

Egg viability was measured after two days of incubation and not immediately after fertilization. It is possible that if egg viability was measured immediately after the process of fertilization in the current study, there may have been a higher percentage of viable eggs for both treatments and a smaller treatment effect. Nevertheless, the current study achieved slightly higher rates of viability, but much smaller portion of viable larvae, than the prior study in 2018 by Miller et al.

Semen cryopreservation negatively impacted the percentage of motile sperm ($P < 0.01$) and the curvilinear velocity of sperm ($P = 0.01$). Semen cryopreservation did not impact the average path velocity ($P = 0.07$), the straight line velocity ($P = 0.12$), the linearity of sperm tracks ($P = 0.58$), the wobble of sperm tracks ($P = 0.81$), or the progression of sperm ($P = 0.31$). Variations from individual males did not impact any of the measured variables. Therefore, cryopreservation of yellow perch semen greatly impacts the percentage of motile sperm after thawing, but it does not impact the kinetics of the sperm after thawing.

Miller et al. (2018) subjectively observed the motility of sperm after cryopreservation. They did not see any motility of sperm after thawing when the extender did not contain Atlantic

salmon seminal plasma. When Atlantic salmon seminal plasma was included in the extender, thawed semen showed 15-25% motility, which is similar to the percentage of motile sperm objectively observed with CASA in this study. Miller et al. (2018) argue that "sperm motility data in the absence of the stimulating effect of ovarian fluid could be misleading as to its fertilizing ability". They cite a CASA study by Ciereszko et al. (1999) of muskellunge in which sperm showing 0.5% motility resulted in a 35% fertilization rate.

Why are fertilization rates of cryopreserved yellow perch semen significantly lower than fertilization rates of fresh yellow perch semen? The current study suggests that the kinetics of cryopreserved sperm are not altered, but the percentage of motile sperm are greatly reduced. Fresh sperm in the current study had 71.1% motility and 42.6% viable larvae while cryopreserved sperm had 13.9% motility and 2.5% viable larvae. If fungal infection is blamed for the poor larval survival in the current study and if it is accepted that current yellow perch cryopreservation protocol can yield 14.8% viable larvae at best, as was achieved by Miller et al. (2018), then the percentage of motile sperm is likely the culprit for low larvae viability.

Judycka et al. (2019) measured 73% motility of thawed sperm using CASA and achieved 79% fertilization. This suggests that motility has an important role in fertilization and that an increased percentage of motility means an increased potential of an egg being fertilized just due to an increased number of motile sperm. The problem of greatly lowered amounts of motile sperm could be partially bypassed without improved yellow perch semen cryopreservation protocol if the ratio of sperm cells to eggs is increased. But if semen is not available in large quantities, this does not solve the problem. In the current study, only four males were harvested for the CASA study because of a poor spawning season. The majority of males were not freely releasing semen and the males that were freely releasing did not produce a large volume of

semen. In this scenario, improved cryopreservation protocol could have improved egg viability and subsequent viable larvae, since it was not possible to fertilize eggs with an increased concentration of sperm cells.

Additional CASA on fresh and cryopreserved yellow perch sperm would be helpful in understanding how sperm motility and fertility are related. Kime and coworkers (2001) argue progressive movement and duration of movement are two of the most important factors in assessing sperm quality. In mammals, the straight line velocity is thought to be a reliable predictor of fertility, because it predicts how a sperm cell would travel in a reproductive tract, meet an egg and enter it via an acrosome reaction (Moore and Akhondi 1996). However, with fish, the sperm generally travel in curved paths rather than straight paths and the sperm of fish can move three dimensionally within water. The velocity of fish sperm rapidly decreases with time and often sperm do not remain motile for more than one minute. To further complicate things, fish sperm need to enter an egg through a single opening, the micropyle, to fertilize the egg (Kime et al. 2001).

For fish, the most important CASA motility characteristics for predicting fertilization may be percent motile, curvilinear velocity, and progression. The percentage of motile sperm is important for fertilization, because it is going to determine how many sperm are able to fertilize an egg. Semen with a greater percentage of motile sperm would likely fertilize with greater success than semen with a lesser percentage of motile sperm.

The curvilinear velocity may be biologically relevant for fertilization, because it describes the velocity of a sperm cell on a curved path. When a sperm cell is swimming to fertilize an egg, it is likely going to be traveling in a curved path as it circles the membrane of the

egg searching for the micropyle (Kime et al. 2001). A sperm cell that can do this quickly can reach the micropyle for fertilization before running out of ATP.

Finally, progression describes the distance that a sperm travels during the period it is analyzed. In aquaculture settings, fertilization is often done artificially, so eggs and semen are directly mixed with a small amount of aqueous solution present. Therefore, the sperm cells do not have too much volume to travel through to reach the egg. However, if the sperm cannot travel far enough to reach the micropyle, then fertilization will not be achieved. Thus, progression could explain the likelihood of a sperm cell reaching the micropyle. A sperm cell that can travel a further distance will likely have a better chance fertilizing an egg than a sperm cell that cannot travel as far.

Future yellow perch semen cryopreservation research should focus on standardization and repeatability. First, straws should be tested as a possible cryopreservation vessel for yellow perch semen. Straws are now routinely used for cryopreservation of Eurasian perch semen (Bernáth et al. 2016; Bernáth, Zarski, et al. 2015; Judycka et al. 2019; Bernáth, Bokor, et al. 2015). Straws allow the semen solution to be spread out unlike vials. In vials, the semen solution near the walls of the vial freeze first and the solution in the center of the vial will freeze last due to the large volume. In a 2 mL vial, the diameter of the vial may be 1.5 cm whereas with straws, the diameter may be 0.5 cm. Straws disperse this volume so freezing is uniform.

Secondly, a control rate freezer (CRF) should be utilized to control the freezing rate of sperm and so that the freezing procedure is repeatable. CRFs have been found to be the best method for semen cryopreservation in Eurasian perch (Bernáth, Bokor, et al. 2015). CRFs are expensive instruments, so if one is not available, straws should be frozen in liquid nitrogen (Judycka et al. 2019). In the current study, the freezing method and vessel used for

cryopreservation likely impacted the fertilization rate of eggs. For the egg fertilization trial, vials were frozen in dry ice. The center of the vials were the last to freeze and the rate of the freezing was not controlled, which can damage cells (Torres, Hu, and Tiersch 2016). For the CASA trial, vials were frozen in a CRF, but again, the sample was not uniformly frozen due to the nature of vials.

Third, sperm volume and density and seminal plasma indices (ionic contents and osmolality) should be characterized for yellow perch. Seminal plasma indices were characterized once in 1996 by Dabrowski et al., but these indices are likely to vary within the species and by strain. Experiments could be done to test the effect of dilution ratio, ions and osmolality on sperm motility parameters as has been done with Eurasian perch (Alavi et al. 2007, Lahnsteiner 2014). A clear understanding of sperm volume and density along with seminal plasma indices allows for the proper formulation of extender and activator solutions. Specific experiments can then be conducted to test the efficacy of extenders and activating solutions with differing ingredients. Finally, additional cryoprotectant tests should be completed to determine the effects these toxic solutions have on sperm motility and fertility and whether they impact eggs.

CHAPTER 5: CONCLUSIONS

The results from the semen cryopreservation and egg fertilization trial indicate that fresh and cryopreserved yellow perch semen differ in their ability to fertilize eggs. After two days of incubation, cryopreserved semen fertilized eggs at lower rates than fresh semen. After twelve days of incubation, eggs treated with cryopreserved semen experienced a greater percentage of dead eggs, a smaller percentage of dead embryos, and a smaller percentage of viable embryos than eggs treated with fresh semen.

The results from the semen cryopreservation and CASA suggest that the motion and kinetics of yellow perch sperm differ before and after cryopreservation. The percentage of motile sperm after cryopreservation is significantly lower than the percentage of motile sperm before cryopreservation and the curvilinear velocity of sperm after cryopreservation is significantly lower than the curvilinear velocity of sperm before cryopreservation. The average path velocity, straight line velocity, linearity wobble and progression of sperm were not significantly different before and after cryopreservation.

The results from these experiments imply that the methods for cryopreservation of yellow perch semen are not optimal for broodstock management or aquaculture practice. The poor fertilization and embryo development rates of eggs treated with cryopreserved semen could be partially attributed to the low percentages and low curvilinear velocities of motile sperm.

Future research should utilize fertilization trials and CASA in tandem. Studies that include the results of both provide more information and context on the effects of cryopreservation. The toxicity of cryoprotectants to sperm cells and eggs, the efficacy of different freezing vessels and cooling rates, and the extent of cellular damage due to freezing and thawing are important areas for further research.

References

Abd El-Gawad, Eman A., Zhi-Gang Shen, and Han-Ping Wang. 2016. "Efficacy of Formalin, Iodine and Sodium Chloride in Improvement of Egg Hatching Rate and Fry Survival Prior to the Onset of Exogenous Feeding in Yellow Perch." *Aquaculture Research* 47 (8): 2461–69. https://doi.org/10.1111/are.12694.

Alavi, Sayyed Mohammad Hadi, Andrzej Ciereszko, Azadeh Hatef, Jiří Křišťan, Boris Dzyuba, Sergei Boryshpolets, Marek Rodina, Jacky Cosson, and Otomar Linhart. 2015. "Sperm Morphology , Physiology , Motility , and Cryopreservation in Percidae." In *Biology and Culture of Percid Fishes: Principles and Practices*, 163–91. https://doi.org/10.1007/978-94-017-7227-3.

Alavi, Sayyed Mohammad Hadi, Otomar Linhart, Kevin Coward, and Marek Rodina. 2008. "Fish Spermatology: Implications for Aquaculture Management." In *Fish Spermatology*, 397–460.

Alavi, Sayyed Mohammad Hadi, Marek Rodina, Tomas Policar, Pavel Kozak, Martin Psenicka, and Otomar Linhart. 2007. "Semen of Perca Fluviatilis L.: Sperm Volume and Density, Seminal Plasma Indices and Effects of Dilution Ratio, Ions and Osmolality on Sperm Motility." *Theriogenology* 68 (2): 276–83. https://doi.org/10.1016/j.theriogenology.2007.05.045.

Bernáth, G., Z. Bokor, E. Kása, L. Várkonyi, T. Hegyi, T. Kollár, B. Urbányi, D. Zarski, J. Radóczi Ifj., and T. Horváth. 2015. "Comparison of Two Different Methods in the Cryopreservation of Eurasian Perch (Perca Fluviatilis) Sperm." *Cryobiology* 70 (1): 76–78. https://doi.org/10.1016/j.cryobiol.2014.12.003.

Bernáth, G., Z. Bokor, D. Zarski, L. Várkonyi, A. Hegyi, A. Staszny, B. Urbányi, J. Radóczi Ifj.,

and A. Horváth. 2016. “Commercial-Scale out-of-Season Cryopreservation of Eurasian Perch (Perca Fluviatilis) Sperm and Its Application for Fertilization.” *Animal Reproduction Science* 170: 170–77. https://doi.org/10.1016/j.anireprosci.2016.05.005.

Bernáth, G., D. Zarski, S. Krejszeff, K. Palińska-Zarska, Z. Bokor, J. Król, T. Kollár, D. Kucharczyk, B. Urbányi, and Horváth. 2015. “Optimization of Conditions for the Cryopreservation of Eurasian Perch (Perca Fluviatilis Linnaeus, 1758) Sperm.” *Journal of Applied Ichthyology* 31 (S1): 94–98. https://doi.org/10.1111/jai.12740.

Bernáth, Gergely, Zoltán Bokor, Eszter Kása, Levente Várkonyi, Árpád Hegyi, Tímea Kollár, Béla Urbányi, Daniel Zarski, János Radóczi Ifj., and Ákos Horváth. 2015. “Comparison of Two Different Methods in the Cryopreservation of Eurasian Perch (Perca Fluviatilis) Sperm.” *Cryobiology* 70 (1): 76–78. https://doi.org/10.1016/j.cryobiol.2014.12.003.

Billard, Roland, and Marie Paule Cosson. 1992. “Some Problems Related to the Assessment of Sperm Motility in Freshwater Fish.” *Journal of Experimental Zoology* 261 (2): 122–31. https://doi.org/10.1002/jez.1402610203.

Blackwell, B. G., C. A. Soupir, and M. L. Brown. 1999. “Seasonal Diets of Walleye and Diet Overlap with Other Top-Level Predators in Two South Dakota Lakes.” *Fisheries Division Report*. Pierre: South Dakota Department of Game, Fish, and Parks.

Blaxter, J. H. S. 2011. “Seminal Studies in the Cryopreservation of Fish Sperm.” In *Cryopreservation in Aquatic Species*, edited by Terrence R. Tiersch and C. C. Green, 2nd ed., 886–89. Baton Rouge: World Aquaculture Society.

Brander, Keith. 2010. “Impacts of Climate Change on Fisheries.” *Journal of Marine Systems* 79 (3–4): 389–402. https://doi.org/10.1016/j.jmarsys.2008.12.015.

Ciereszko, A, K Dabrowski, F Lin, S. A. Christ, and G. P. Toth. 1999. “Effects of Extenders and

Time of Storage before Freezing on Motility and Fertilization of Cryopreserved Muskellunge Spermatozoa." *Transactions of the American Fisheries Society* 128 (3): 542–47. https://doi.org/10.1577/1548-8659(1999)128.

Ciereszko, A, L Ramseyer, and K Dabrowski. 1993. "Cryopreservation of Yellow Perch Semen." *The Progressive Fish-Culturist* 55 (4): 261–64. https://doi.org/10.1577/1548-8640(1993)055<0261:coyps>2.3.co;2.

Cosson, Jacky. 2004. "The Ionic and Osmotic Factors Controlling Motility of Fish Spermatozoa." *Aquaculture International* 12: 69–85.

Dabrowski, K., R. E. Ciereszko, A. Ciereszko, G. P. Toth, S. A. Christ, D. El-Saidy, and J. S. Ottobre. 1996. "Reproductive Physiology of Yellow Perch (Perca Flavescens): Environmental and Endocrinological Cues." *Journal of Applied Ichthyology* 12 (3–4): 139–48. https://doi.org/10.1111/j.1439-0426.1996.tb00079.x.

Dembkowski, Daniel J., D. W. Willis, B. G. Blackwell, S. R. Chipps, T. D. Bacula, and M. R. Wuellner. 2015. "Influence of Smallmouth Bass Predation on Recruitment of Age-0 Yellow Perch in South Dakota Glacial Lakes." *North American Journal of Fisheries Management* 35: 736–47. https://doi.org/10.1080/02755947.2015.1044629.

FAO. 2018. "The State of World Fisheries & Aquaculture 2018 - Meeting Sustainable Development Goals." Rome. http://www.fao.org/state-of-fisheries-aquaculture.

Fauvel, C, M Suquet, and J Cosson. 2010. "Evaluation of Fish Sperm Quality." *Journal of Applied Ichthyology* 26: 636–43. https://doi.org/10.1111/j.1439-0426.2010.01529.x.

Forney, John L. 1974. "Interactions Between Yellow Perch Abundance, Walleye Predation, and Survival of Alternate Prey in Oneida Lake, New York." *Transactions of the American Fisheries Society* 103 (1): 15–24. https://doi.org/10.1577/1548-

8659(1974)103<15:IBYPAW>2.0.CO;2.

Glogowski, Jan, Andrzej Ciereszko, and Konrad Dabrowski. 1999. “Cryopreservation of Muskellunge and Yellow Perch Semen.” *North American Journal of Aquaculture* 61 (3): 258–62. https://doi.org/10.1577/1548-8454(1999)061<0258:comayp>2.0.co;2.

Hansen, Michael J., Michael A. Bozek, Justine R. Newby, Steven P. Newman, and Michael D. Staggs. 1998. “Factors Affecting Recruitment of Walleyes in Escanaba Lake, Wisconsin, 1958–1996.” *North American Journal of Fisheries Management* 18: 764–74. https://doi.org/10.1577/1548-8675(1998)018<0764:farowi>2.0.co;2.

Hart, Steven D., Donald L. Garling, and Jeffrey A. Malison, eds. 2006. *Yellow Perch (Perca Flavescens) Culture Guide*. *North Central Regional Aquaculture Center*. Vol. 103.

Hudson, John C., and Susy S. Ziegler. 2014. “Environment, Culture, and The Great Lakes Fisheries.” *Geographical Review* 104 (4): 391–413. https://doi.org/10.1111/j.1931-0846.2014.12041.x.

Judycka, Sylwia, Daniel Żarski, Mariola A. Dietrich, Katarzyna Palińska-Żarska, Halina Karol, and Andrzej Ciereszko. 2019. “Standardized Cryopreservation Protocol of European Perch (Perca Fluviatilis) Semen Allows to Obtain High Fertilization Rates with the Use of Frozen/Thawed Semen.” *Aquaculture* 498 (August 2018): 208–16. https://doi.org/10.1016/j.aquaculture.2018.08.059.

Kaemingk, Mark A., Brian D.S. Graeb, and David W. Willis. 2014. “Temperature, Hatch Date, and Prey Availability Influence Age-0 Yellow Perch Growth and Survival.” *Transactions of the American Fisheries Society* 143 (4): 845–55. https://doi.org/10.1080/00028487.2014.886622.

Kime, D. E., K. J.W. Van Look, B. G. McAllister, G. Huyskens, E. Rurangwa, and F. Ollevier.

2001. “Computer-Assisted Sperm Analysis (CASA) as a Tool for Monitoring Sperm Quality in Fish.” *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology* 130 (4): 425–33. https://doi.org/10.1016/S1532-0456(01)00270-8.

Kopeika, Eugeny, Julia Kopeika, and Tiantian Zhang. 2007. “Cryopreservation of Fish Sperm.” In *Methods in Molecular Biology*, edited by J. G. Day and G. N. Stacey, 2nd ed., 368:203–17. Totowa, NJ: Human Press Inc.

Król, Jarosław, Daniel Żarski, Gergely Bernáth, Katarzyna Palińska-Żarska, Sławomir Krejszeff, Artur Długoński, and Ákos Horváth. 2018. “Effect of Urine Contamination on Semen Quality Variables in Eurasian Perch Perca Fluviatilis L.” *Animal Reproduction Science* 197 (August): 240–46. https://doi.org/10.1016/j.anireprosci.2018.08.034.

Lahnsteiner, F., B. Berger, T. Weismann, and R. Patzner. 1995. “Fine Structure and Motility of Spermatozoa and Composition of the Seminal Plasma in the Perch.” *Journal of Fish Biology* 47 (3): 492–508. https://doi.org/10.1111/j.1095-8649.1995.tb01917.x.

Lahnsteiner, Franz. 2014. “The Effect of K+, Ca2+, and Mg2+ on Sperm Motility in the Perch, Perca Fluviatilis.” *Fish Physiology and Biochemistry* 40 (2): 469–80. https://doi.org/10.1007/s10695-013-9858-7.

Lantz, Björn. 2013. “The Impact of Sample Non-Normality on ANOVA and Alternative Methods.” *British Journal of Mathematical and Statistical Psychology* 66 (2): 224–44. https://doi.org/10.1111/j.2044-8317.2012.02047.x.

Liu, Qing Hua, Jun Li, Zhi Zhong Xiao, Fu Hong Ding, Dao De Yu, and Xi Zhang Xu. 2007. “Use of Computer-Assisted Sperm Analysis (CASA) to Evaluate the Quality of Cryopreserved Sperm in Red Seabream (Pagrus Major).” *Aquaculture* 263 (1–4): 20–25. https://doi.org/10.1016/j.aquaculture.2006.11.017.

Malison, Jeffrey A. 2000. “A White Paper on the Status and Needs of Yellow Perch Aqauculture in the North Central Region.” *North Central Regional Aquaculture Center*. http://lib.dr.iastate.edu/cgi/viewcontent.cgi?article=1004&context=ncrac_whitepapers.

Marsden, J Ellen, and Steven R Robillard. 2004. “Decline of Yellow Perch in Southwestern Lake Michigan , 1987 – 1997.” *North American Journal of Fisheries Management* 24 (3): 952–66.

Miller, Mackenzie E., Megan Kemski, John D. Grayson, Kristen Towne, and Konrad Dabrowski. 2018. “Yellow Perch Sperm Motility, Cryopreservation, and Viability of Resulting Larvae and Juveniles.” *North American Journal of Aquaculture* 80 (1): 3–12. https://doi.org/10.1002/naaq.10001.

Moore, H, and A Akhondi. 1996. “Fertilizing Capacity of Rat Spermatozoa Is Correlated With Decline in Straight-Line Velocity Measured by Continuous Computer-Aided Sperm Analysis” 17 (1): 50–60.

Munawar, M., I. F. Munawar, N. E. Mandrak, M. Fitzpatrick, R. Dermott, and J. Leach. 2005. “An Overview of the Impact of Non-Indigenous Species on the Food Web Integrity of North American Great Lakes: Lake Erie Example.” *Aquatic Ecosystem Health and Management* 8 (4): 375–95. https://doi.org/10.1080/14634980500411606.

Munawar, M, R Dermott, J Leach, S Nepszy, D V Wesoloh, D Graham, S Carou, H Niblock, and O Johannsson. 2001. “Zooplankton, Benthos, Fish, Colonial Waterbirds and Exotic Species.” In *The Great Lakes of the World (GLOW): Food-Webs, Health and Integrity*, edited by M Munawar and R Hecky, 277–307. Leiden, the Netherlands: Ecovision World Monograph Series, Backhuys.

Pegg, David. 2015. “Principles of Cryopreservation.” In *Cryopreservation and Freeze-Drying*

Protocols, edited by Willem F Wolkers and Harriëtte Oldenhof, Third. Vol. 1257. Humana Press. https://doi.org/10.1007/978-1-4939-2193-5_24.

Rach, Jeff, Steven Redman, Dale Bast, and Mark Gaikowski. 2005. "Efficacy of Hydrogen Peroxide versus Formalin Treatments to Control Mortality Associated with Saprolegniasis on Lake Trout Eggs." *North American Journal of Aquaculture* 67 (2): 148–54. https://doi.org/10.1577/a04-062.1.

Rodina, M., T. Policar, O. Linhart, and C. Rougeot. 2008. "Sperm Motility and Fertilizing Ability of Frozen Spermatozoa of Males (XY) and Neomales (XX) of Perch (Perca Fluviatilis)." *Journal of Applied Ichthyology* 24 (4): 438–42. https://doi.org/10.1111/j.1439-0426.2008.01137.x.

Rodina, Marek, Tomas Policar, and Otomar Linhart. 2008. "Cryopreservation of Sperm of Testicular Neomales and Stripped Normal Males of European Perch (Perca Fluviatilis L.)." *Cybium* 32 (2 SUPPL.): 214.

Ruxton, Graeme D., and Guy Beauchamp. 2008. "Time for Some a Priori Thinking about Post Hoc Testing." *Behavioral Ecology* 19 (3): 690–93. https://doi.org/10.1093/beheco/arn020.

Sandoval-Vargas, Leydy, Mauricio Silva Jiménez, Jennie Risopatrón González, Elías Figueroa Villalobos, Elsa Cabrita, and Iván Valdebenito Isler. 2020. "Oxidative Stress and Use of Antioxidants in Fish Semen Cryopreservation." *Reviews in Aquaculture*, no. i: 1–23. https://doi.org/10.1111/raq.12479.

Sarosiek, Beata, Katarzyna Dryl, Dariusz Kucharczyk, Daniel Żarski, and Radosław K. Kowalski. 2014. "Motility Parameters of Perch Spermatozoa (Perca Fluviatilis L.) during Short-Term Storage with Antioxidants Addition." *Aquaculture International* 22 (1): 159–65. https://doi.org/10.1007/s10499-013-9679-9.

Schielzeth, Holger, and Shinichi Nakagawa. 2013. "Nested by Design: Model Fitting and Interpretation in a Mixed Model Era." *Methods in Ecology and Evolution* 4 (1): 14–24. https://doi.org/10.1111/j.2041-210x.2012.00251.x.

Service, National Marine Fisheries. 2018. "Fisheries of the United States, 2017." https://www.fisheries.noaa.gov/content/fisheries-united-states-2017.

Torres, Leticia, E. Hu, and Terrence R. Tiersch. 2016. "Cryopreservation in Fish: Current Status and Pathways to Quality Assurance and Quality Control in Repository Development." *Reproduction, Fertility and Development* 28 (8): 1105. https://doi.org/10.1071/rd15388.

Turner, Clarence Lester. 1919. "The Seasonal Cycle in the Spermary of the Perch." University of Wisconsin.

Wicker, Anton M., and Melvin T. Huish. 1982. "Morphology of Bluegill (Lepomis Macrochirus), Chain Pickerel (Esox Niger) and Yellow Perch (Perca Flavescens) Spermatozoa, as Determined by Scanning Electron Microscopy." *Copeia* 1982 (4): 955. https://doi.org/10.2307/1444106.

Wilberg, Michael J., James R. Bence, Bradley T. Eggold, Daniel Makauskas, and David F. Clapp. 2005. "Yellow Perch Dynamics in Southwestern Lake Michigan during 1986–2002." *North American Journal of Fisheries Management* 25 (3): 1130–52. https://doi.org/10.1577/m04-193.1.

Wilson-Leedy, Jonas G., and Rolf L. Ingermann. 2011. "Computer Assisted Sperm Analysis Using ImageJ; Description of Necessary Components and Use of Free Software." http://rsbweb.nih.gov/ij/plugins/docs/CASAinstructions.pdf.

Winter, J. C.F. 2013. "Using the Student's t-Test with Extremely Small Sample Sizes." *Practical Assessment, Research and Evaluation* 18 (10): 1–12.

Yang, Huiping, and Terrence R. Tiersch. 2009. “Current Status of Sperm Cryopreservation in Biomedical Research Fish Models: Zebrafish, Medaka, and Xiphophorus.” *Comparative Biochemistry and Physiology. Part C: Toxicology Pharmacology* 149 (2): 224–32. https://doi.org/10.1016/j.cbpc.2008.07.005.