OPTIMIZING PROTOCOLS FOR THE CRYOPRESERVATION OF SHORTNOSE STURGEON (*Acipenser brevirostrum*) GERMPLASM

BY

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Abstract

Cryopreservation of germplasm is a valuable *ex-situ* conservation strategy, as the genetic material of threatened species can be preserved indefinitely and protected from external threats. There has yet to be standard protocols for the acquisition of gonadal material for cryopreservation. The purpose of my experiment was twofold: to refine laparoscopic surgery protocols, and to optimize the freezing rate and cryoprotectant type for the cryopreservation of shortnose sturgeon (*Acipenser brevirostrum*) germ cells. Two methods of laparoscopic surgery were performed on the sturgeon, with and without saline insufflation. One sturgeon was sacrificed, and its gonad was cut into ~0.1g pieces. Gonadal tissue fragments were exposed to one of two cryoprotectants, dimethyl sulfoxide (DMSO) or ethylene glycol (EG) and cooled at a rate of either -1, -5 or -10°C/min in a Kryo 360 automated freezer. Post-thaw viability was assessed using a trypan blue stain. The highest post-thaw viability (± SD) was observed with a cryoprotectant of DMSO and a cooling rate of -1^{O}C/min (62.3% ± 8.9%), and the next highest viability was seen with a cryoprotectant of EG and a cooling rate of -5°C/min (61.5 ± 7.5%). No significant differences in viability were present between -1 and -5°C/min with DMSO as a cryoprotectant. The high viability results with a freezing rate of -5°C/min indicate that we can cryopreserve shortnose sturgeon gonadal tissue at a rate 5 times faster than what is typically seen in the literature, which will accelerate the construction of cryobanks.

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CHAPTER I: LITERATURE REVIEW

INTRODUCTION TO CRYOPRESERVATION OF GERMPLASM AS A CONSERVATION STRATEGY FOR THE THREATENED SHORTNOSE STURGEON (*ACIPENSER BREVIROSTRUM*)

Introduction to Sturgeon

The order Ascipenserformes is an ancient order of fish that evolved around 250 million years ago (Keyvanshokooh and Gharaei 2010). The order has two extant families: Polyodontidae (paddlefish) and Acipenseridae (sturgeon), both of which are distributed throughout the Northern Hemisphere (Billard and Lecointre 2000). Sturgeon are sometimes referred to as "living fossils", as their basic body plans from the early Cretaceous period have remained relatively unchanged for millions of years (Bemis et al. 1997). Historically, sturgeon were revered and known as 'Royal Fish' in the Middle Ages (Vecsei 2005). Their image was engraved on coins in 600 BC in modern day Tunisia, which gives insight into how much they were valued (Bronzi et al. 2011). Sturgeon were valued primarily for their eggs, known as caviar, which are harvested as a luxury food. The trading of sturgeon caviar has been prevalent for centuries, but increased significantly in the mid-1800s with the invention of the refrigerator (Ludwig 2008). Caviar of the Beluga sturgeon (*Huso huso*) has been sold for US$4290/kg (Ludwig 2008). The processing of sturgeon for their caviar and meat provides means of employment and income for people living in regions where sturgeon are prevalent, and is integral to food systems around the world (COSEWIC, 2015).

Conservation Status

Sturgeon are the most endangered vertebrate group in the world, as all 25 species are listed on the International Union on the Conservation of Nature (IUCN) Red List, and 63%

are noted as Critically Endangered (IUCN, 2010). This is primarily due to overfishing and exploitation, as sturgeon are highly sought after in the commercial aquaculture industry for their meat and caviar (Bronzi et al. 2011; Alavi et al. 2012). There is prominent legal and illegal trade of caviar, the illegal trade putting the species more at risk since it is unregulated (Pikitch et al. 2005). In 1997, all species of sturgeon were listed on the Convention on International Trade in Endangered Species of Fauna and Flora (CITES), which limits the conditions under which trade can happen and seeks to protect the fish, thereby aiding in conservation efforts (Pikitch et al. 2005). Habitat degradation and in particular damming, are two additional factors that are causing sturgeon populations to drastically decline (IUCN, 2010).

Morphology

Sturgeon have a cartilaginous skeleton, a long cylindrical leathery body with a heterocercal tail, and five rows of bony plates called scutes (Wayman 2003). The scutes and tough leathery skin provide protection, and lead to lower mortality rates in juveniles (COSEWIC, 2015). They are benthic feeders and use their rostrum and tactile barbels on the front of their mouth to find and collect food. They have small eyes, and thus don't primarily rely on vision to catch prey (Billard and Lecointre 2000). Sturgeon can range in length from 1 meter, to over 6 meters depending on the species; the Beluga sturgeon (*H. huso)* is currently know as the world's largest freshwater fish (Pikitch et al. 2005).

Life History and Reproduction

Many species of sturgeon are anadromous, and spend the majority of their life in marine environments, but spawn in freshwater (COSEWIC, 2015). Sturgeon are late maturing and have a long reproductive cycle, which poses a difficulty for conserving them in their natural environment (Billard and Lecointre 2000; Pikitch et al. 2005; Pšenička et al. 2015). Most females do not mature until they are between 11-21 years of age, and most males mature between 9-12 years of age (Moghim et al. 2002). They spawn on hard substrates such as gravel or rock at the bottom of rivers. Sturgeon spawn in spring to early summer, and do not spawn every year, as females spawn every 2-11 years and males spawn every 1-6 years

(Billard and Lecointre 2000). Their life history renders them susceptible to over-harvesting and makes recovery difficult.

Sexing Methods

Sturgeon were once thought to be sexually monomorphic, where males and females appear the same morphologically (Dadswell 1979). However, recent research has shown that there are some observable differences in head morphology between sexes. The male head is narrower and more elongate, whereas females have shorter and wider head shapes (Balazadeh and Litvak 2018). Additionally, Vecsei et al. (2003) showed with 82% accuracy that sturgeon sex can be determined by looking at the shape of the urogenital opening. Males were shown to have Y-shaped urogenital openings, and females had O-shaped openings (Vecsei et al. 2003). However, this method is controversial as the sample size used in the study was low, and it has yet to be investigated in other species of sturgeon (Balazadeh and Litvak 2018). There continues to be a need for quick, non-invasive, reliable methods of sex determination in sturgeon.

Ultrasound

Despite evidence of slight morphological differences, it is difficult to quickly distinguish between males and females based on external features. Ultrasonography is a non-invasive way to determine sex that reduces handling stress (Kohn et al. 2013). An ultrasound transducer sends out acoustic energy waves at different frequencies that penetrate tissue. As the sound waves pass through tissue, they are either absorbed or reflected (Martin-Robichaud and Rommens 2001). The degree of reflection is referred to as echogenicity. Hyperechoic structures reflect more of the ultrasound waves, and appear lighter in the ultrasound image, whereas hypoechoic structures appear darker. Using the ultrasound, the gonads can be visualized and sex can be determined (Figure 1). Accuracy for sexing sturgeon using ultrasonography ranges from 68-98% (Moghim et al. 2002; Colombo et al. 2004; Webb et al. 2019). However, a limitation to using ultrasonography is the limited effectiveness in distinguishing immature females from males, due to underdeveloped gonads (Wildhaber et al. 2005). Additionally, the effectiveness of using ultrasonography to determine sex depends on

the time of year and the developmental stage of the sturgeon, as accuracy decreases in immature individuals (Chiotti et al. 2016).

Laparoscopic Surgery

A minimally invasive way of confirming the sex is through laparoscopic surgery, by using an endoscope to view the gonads. Divers et al. (2009, 2013) have established protocols for laparoscopic surgery on sturgeon. There are two main techniques of endoscopic surgery: coelioscopy and gonoductoscopy. Coelioscopy is when the endoscope is inserted through a small incision on the ventral side of the fish, and gonoductoscopy is when the endoscope is inserted through the urogenital pore, and the gonads are viewed through the gonoduct wall (Divers et al. 2009). The limitations of using endoscopy to determine sex is that it is more invasive and thus requires anesthetic to be used, and it can be difficult to distinguish between gonads and fat (Wildhaber et al. 2005). Nevertheless, using an endoscope permits a high degree of accuracy for sexing sturgeon. Endoscopic techniques for sexing mature sturgeon have been shown to be >93% accurate (Wildhaber et al. 2005; Hurvitz et al. 2007; Divers et al. 2009). Wildhaber et al. (2005) found that incision endoscopy (coelioscopy) provided more accurate results compared to urogenital duct endoscopy (gonoductoscopy) and ultrasonography.

Gametogenesis

Gametogenesis is the process of cells becoming gametes and consists of spermatogenesis and oogenesis. The term germplasm refers to the reproductive tissue of both males and females, and primordial germ cells (PGCs) are the precursors to germ cells that make up germplasm (Pšenička et al. 2015). During embryonic development, PGCs migrate from their origin towards the gonadal ridge, where they differentiate into spermatozoa and oocytes (Saito et al. 2014). Sturgeon are gymnovarian, meaning that the peritoneum only covers part of the ovary, and mature oocytes are released right into the abdominal cavity (Coward et al. 2002). Sturgeon gametes differ from other fish in that their sperm has acrosomes and the eggs have multiple micropyles (Cherr and Clark 1985).

Spermatogenesis

Spermatogenesis is the male form of gametogenesis. It is the process of diploid spermatocytes becoming haploid spermatozoa. There are three main parts to spermatogenesis: 1) the mitotic phase, with the different phases of spermatogonia, 2) the meiotic phase, with the primary and secondary spermatocytes, and 3) the spermiogenic phase, where the spermatids differentiate into spermatozoa (Schulz et al. 2010). At the first meiotic division primary spermatocytes become secondary spermatocytes, which divide to become spermatids. At the second meiotic division, secondary spermatocytes divide to become spermatids. Spermatids then undergo spermiogenesis, where they gain a flagellum and release from Sertoli cells to become spermatozoa (Redding and Patino 1993).

Oogenesis

Oogenesis occurs in females and is the process in which a diploid oogonium becomes a haploid egg cell. There are four main phases: proliferation, primary growth phase, secondary growth phase, and maturation and ovulation (Kagawa, 2013). In the proliferation stage, oogonia multiply via mitosis and form nests, and eventually the cell grows larger and forms a primary oocyte. The oocyte is surrounded by follicle cells, with the inner layer being the granulosa cell layer and the outer being theca cells, separated by a basement membrane. Between the oocyte and the granulosa cells, there is an acellular layer called the zona radiata (Redding and Patino 1993). The primary growth phase contains a chromatin nucleolus stage and a perinucleolus stages, both of which contribute to an increase in cell size (Kagawa 2013b). At the first meiotic division, the primary oocyte divides, and forms a larger secondary oocyte and a degenerate polar body (Lubzens et al. 2010). Meiosis is arrested at two points during oogenesis: at prophase I and at metaphase II. In between these periods of arrested meiosis is when the oocyte matures (Redding and Patino 1993). During the secondary growth phase, also known as vitellogenesis, there is prominent oocyte growth, as it accumulates nutrients that are necessary for embryonic development (Kagawa 2013a). Vitellogenesis involves the uptake of vitellogenin, a glycolipophosphoprotein, which accounts for about 95% of the eggs final size (Coward et al. 2002). Vitellogenic growth begins when the sturgeon is 4-8 years old and lasts 16-18 months. After vitellogenesis,

oocytes increase in size to about 3.5mm, and change colour from yellow, to brown, to grey, and then finally to black (Doroshov et al. 1997).

Gonadal Development

Anatomical differentiation of the gonads can begin at 6 months old, but is variable with different species of sturgeon, with larger individuals differentiating later (Flynn and Benfey 2007). The timing of gonadal development depends on both abiotic and biotic factors, such as conspecific density, temperature and food availability (McGuire et al. 2019). Female Russian sturgeon and White sturgeon exhibit asynchronous ovarian development up to a certain stage (Doroshov et al. 1997; Hurvitz et al. 2007). Hurvitz et al. (2007) found evidence in Russian sturgeon that an ovarian follicle develops independently of surrounding follicles until it reaches a specific yolk stage. Thus, it is important to examine both sides of an ovary using an endoscope in order to determine the developmental stage of the ovarian cells. Additionally, identifying the sex of a sturgeon is difficult, as male and female gonads look very similar until differentiation occurs. Once differentiation is complete, females have clear ovarian grooves on their ovary (Doroshov et al. 1997).

Ovarian development can be classified into seven maturity stages. The first stage (Fv) denotes a juvenile virgin female, where the ovarian tissue is beige, and oocytes are transparent and roughly 0.05 mm in diameter (Bruch et al. 2001). In stage I (F1), there is a fissure on the lateral side of the gonad, where clusters of generative cells lay. On an ultrasound image, the ovary appears as an irregular form, and moderately echogenous. This is a very early stage of gonadal development which often leads to misidentification of female sturgeon, so the margins of the gonad must be carefully examined (Chebanov and Galich 2013). In F2, fat begins to accumulate in the germinal part of the gonad, making it appear yellow, and ovigerous lamellae (folds) begin to appear on the ovary. Eggs are 1-2 mm in diameter (Bruch et al. 2001). Stage II semi-fatty (F2sf) occurs next, and is characterized by increased fat deposition, starting from the ovigerous lamellae. There must be enough fat to cover more than half of the ovary's width to be at this stage. On an ultrasound, fat appears darker than the ovarian tissue. The oocytes are small and white at this stage. At F2-3, the oocytes have begun to undergo vitellogenesis and are increasing in size. Less fat is apparent,

and yellow oocytes are visible from the lateral part of the ovary. F3 is when the ovigerous lamella thicken, white and grey oocytes are 2.5 mm in diameter, and the gonad increases in size. This stage is difficult to detect on ultrasounds, and is very short in duration (Chebanov and Galich 2013). F4 is when the ovaries fill the majority of the body cavity, and black eggs reach their final size, about 3mm in diameter. In F5, the female sturgeon is spawning, and her eggs free in the body cavity to be ovulated (Bruch et al. 2001). F6 describes the period after spawning, where the ovaries appear pink with little fat. The ovaries resemble how they appeared in stage II of their development (Chebanov and Galich 2013).

Testicular development can be divided into five main stages. In stage 1 (M1), testes are thin, white and mainly comprised of connective tissue, and are indiscernible on the ultrasound. In M2, testes appear pinkish or white, and appear hyperechoic on the ultrasound. As the testes begin to accumulate fat, the volume of the gonad expands, which can be visualized on the ultrasound. M3 is a short stage where fat is used for sex cell development, and blood vessels start to appear in a netting pattern on the surface of the testes. On the ultrasound, testes appear as lobular hyperechoic structures, exhibiting a light grey or white colour. M4 is marked by completion of spermatogenesis, and the testes appear a milky light colour with little fat. In the ultrasound image they are well-defined, homogenous, hyperechoic structures, and are at their brightest in this stage. M5 is the final stage of testicular development, and the release of seminal fluid causes a reduction in echogenicity in the ultrasound image (Chebanov and Galich 2013).

Shortnose sturgeon

Shortnose sturgeon (*Acipenser breviostrum*) are distributed among 25 rivers along the east coast of North America, and the only Canadian population resides in the lower Saint John River and its tributaries in New Brunswick (COSEWIC, 2015). Shortnose sturgeon are one of the smaller species of sturgeon, with an average length of 91 cm and a maximum reported length of 143 cm (COSEWIC, 2015). As their name suggests, their snout is shorter and more blunt than other species (Scott and Scott 1988). Shortnose sturgeon remain in their natal streams throughout their adult life, which leaves them vulnerable as they have a slow recolonization rate if their river populations were to be extirpated (Kynard 1997). Females

first spawn at about 18 years old, and spawn again every three years, producing up to 200 000 eggs in each spawning period. Males spawn earlier, at about 12 years old, and spawn every other year (Dadswell et al., 1984; COSEWIC, 2015).

Shortnose sturgeon are listed as vulnerable by the IUCN, and Aboriginal traditional knowledge suggests that populations in the Saint John River have been in decline since the construction of the Mactaquac Dam in 1968 (COSEWIC, 2015). They are listed as a species of special concern by the Committee on the Status of Endangered Wildlife in Canada, and are listed in Appendix I of CITES, where trade is only permitted under certain circumstances (COSEWIC, 2015). The trading of wild shortnose sturgeon is prohibited, but commercial trade of captive sturgeon from registered aquaculture facilities is permitted (Government of Canada 2009). The Saint John River population undergoes a small spawning migration in the spring, with the majority of spawning occurring near the Mactaquac Dam. They have distinct cultural and historical significance to Indigenous populations in Canada, as coastal communities have relied on them for meat, roe, and oil (Government of Canada 2009). It is clear that conservation action needs to be taken with these fish, but their late age of sexual maturity poses a difficulty in conserving them in their natural environment. Researchers are now turning to *ex-situ* conservation methods, such as the cryopreservation of their germplasm, as a tool for protecting this species.

Introduction to Cryopreservation

Cryopreservation is a technique in which cells and tissues are frozen at extremely low temperatures (i.e -80°C). The cells/tissues can be stored for months to years, and when thawed, the cells/tissue are still viable (Tsai and Lin 2012). Biological samples are most often stored in liquid nitrogen (LN_2), which has a temperature of -196°C, for an indefinite period of time. Exposure to high solute concentrations and intracellular ice formation are the two main sources of damage to a cell during cryopreservation (Stachecki et al. 1998). Cryopreservation protocols are centered around protecting the cell from intracellular ice formation and excessive cell dehydration (Marques et al. 2019). Cryopreservation has applications in aquaculture, and also as an important *ex situ* conservation strategy. Cryopreservation of germplasm and establishing cryobanks are valuable *ex situ* conservation

strategies, as genetic material can be stored indefinitely, thus preserving valuable genetic lineages (Guan et al. 2008; Tsai and Lin 2012; Rivers et al. 2020).

Methods of Cryopreservation

There are a number of protocols already established for the cryopreservation of fish sperm, but few for the cryopreservation of fish eggs and embryos (Diwan et al. 2020). This is largely due to difficulties such as the low membrane permeability and larger cell size of eggs and embryos compared to sperm (Mayer 2019). The high surface to volume ratios of oocytes makes it more difficult for protective agents to penetrate the cell, thus putting it more at risk for damage. Spermoatozoa on the other hand, have a smaller size and a higher resistance to chilling, which makes them an easier and more accessible candidate for cryopreservation (Asturiano et al. 2017). However, cryopreserving spermatozoa only preserves the paternal genome. This is a valuable conservation method when the fish species of interest has male heterogamety. However, this is not sufficient strategy for sturgeon, who exhibit female heterogmety (ZW/ZZ) (Van Eenennaam 1999). Solely focusing on cryopreserving male germ cells will result in the exclusion of maternal genetic information, and would only enable male ZZ individuals to be preserved (Van Eenennaam 1999). Thus, preserving genetic material of the female, the oogonial cells, is particularly important to preserve genetic diversity for sturgeons. Primordial germ cells (PGCs) contain both maternal and paternal genomes, which makes them a valuable material to cryopreserve, to contribute to the conservation of endangered species (Robles et al. 2017).

Cryopreservation of gonadal tissue is a newer alternative to preserving the genetic lineage of species. Gonadal tissue contains germline stem cells, like spermatogonia and oogonia (Ye et al. 2021). Cryopreservation of intact tissue is superior to cells, since it can retain all of its potential, and can be cultured in small pieces (Anil 2013). Psenicka et al. (2016) found that cryopreserving whole tissue promoted higher post-thaw viability, compared to disassociating cells prior to the freezing process.

Cryopreservation Materials

Cryoprotective agents and extenders are often added to promote higher viability of cells following cryopreservation. Cryoprotective agents (CPAs) are chemicals that are added to protect the cells from damage during the freezing and thawing processes (Tiersch 2001). There are two types of CPAs, permeating and non-permeating. Permeating CPAs such as dimethyl sulfoxide (DMSO), ethylene glycol (EG), propylene glycol (PG), and methanol have lower molecular weights and penetrate the cell membrane. They displace water molecules inside the cell and form hydrogen bonds with DNA and RNA, lowering the freezing point of the cell (Stachecki et al. 1998; Tsai 2009; Anil 2013). Non-permeating CPAs have higher molecular weights, can't pass through the cell membrane, and are often sugars or egg yolk (Tsai 2009; Yamaner et al. 2016). Often a mixture of permeating and non-permeating CPAs are used. Psenicka et al. (2016) showed that 1.5M EG was the optimal cryoprotectant to use for Siberian sturgeon gonadal tissue, yielding a post-thaw viability of near 90%. Franek et al. (2019) showed that 1.5M DMSO gave highest viability for common carp ovarian tissue fragments, with a post thaw viability of near 65%.

The right concentration of CPA is essential to the viability of the tissue, as too high a concentration can cause toxicity and cell rupture (Tiersch et al. 2007). Too low of a concentration of CPA can result in intracellular ice formation, and ultimately cell death. Research has shown that CPAs are a necessary component of cryopreservation, as viability of thawed cells that are frozen without a CPA is close to 0 (Pšenička et al., 2016). Extenders are added as a diluent to obtain a larger volume of sample and to protect against CPA toxicity (Muchlisin 2005; Yamaner et al. 2016). Commonly used extenders are NaCl and KCl, and usually have a pH close to seminal plasma (Billard et al. 2004; Yamaner et al. 2016).

Slow Cooling

The conventional method of cryopreservation is slow cooling, in which the sample is cooled in a controlled fashion, often using an automated programmable freezer. With slow cooling there is a risk of water crystallizing and intracellular ice forming, which can cause cell membrane damage (Marques et al. 2019). Slow-cooling methods aim to create

extracellular ice, causing water to passively follow its concentration gradient out of the cell, completely dehydrating the cell (Stachecki et al. 1998). If the rate is too slow the "solution effect" may occur, where the tissue is exposed to high concentrations of CPAs for too long during dehydration (Tiersch et al. 2007). During the slow-cooling process supercooling can occur, which is when the sample cools to below its freezing point without transitioning to a solid state. The exothermic process of a liquid freezing causes heat to be released, and the solution warms up to its freezing point. This spontaneous freezing is called nucleation (Denniston et al. 2000). To control the freezing process and improve the tissue's post thaw viability, seeding can be performed. Seeding is induced ice nucleation at a temperature near the freezing point of the cryoprotective medium, normally a high sub-zero temperature (Huang et al. 2017). Seeding allows for control over when nucleation occurs, thus lessening the probability of intracellular ice forming (Tsai 2009). The optimal seeding temperature varies depending on the concentration of CPAs used. For 1.5 M concentration of CPA a seeding temperature of -7°C is common, -7.5^{O}C for 2 M, and -10°C for 3 M (Tsai 2009).

Vitrification

A newer method of cryopreservation is vitrification, or "fast-cooling". This is usually done by immersing the sample directly into liquid nitrogen, without any prior cooling (Cuevas-Uribe, 2011). The ultra-rapid cooling prevents the formation of crystals, and the sample transitions into an amorphous, glassy state (Marques et al. 2015). This method is beneficial as it is more cost effective because the need for an automated freezer is eliminated. Additionally, this method is useful as it can be done in the field (Cuevas-Uribe, 2011). However, vitrification requires higher concentrations of CPAs to be used to compensate for the sudden rapid drop in temperature (Franěk et al. 2019). Higher CPA concentrations put the cell at a higher risk for CPA toxicity and other cellular damage (Tiersch et al. 2007; Marques et al. 2019).

Thawing

Thawing following cryopreservation can either be done at a slow controlled rate, or be done very rapidly. Guan et al. (2008) found that rapid thawing (>300°C/min) was more

effective and promoted a higher viability of zebrafish (*Danio rerio*) oocytes when compared to thawing rates of +2^{O}C/min or +10°C/min. Rapid thawing is most often done by immersion in a water bath for a short period of time (<5 minutes). Following thawing, it is necessary to remove all the CPAs by washing the sample in a CPA-free medium (Tsai 2009). It is critical that CPAs are removed following cryopreservation, since they are toxic substances that can cause damage to cells.

Cryopreservation as a Method of Conservation

Cryopreservation can serve as an incredibly useful tool for the conservation of endangered or threatened species (Rivers et al. 2020). Cryopreserving sperm, eggs, their progenitor cells (such as spermatocytes and oocytes), and embryos enable germplasm cryobanks to be established, which would allow donor gametes to be created when needed and can thus protect genetic lineages (Tiersch 2008; Tsai and Lin 2012). Germplasm cryobanks reduce the probability of inbreeding and genetic drift, allowing gene pools to persist through time without being affected by catastrophic events or natural disasters (Rivers et al. 2020). Cryopreserved material can be used in germplasm xenotransplantation and assisted reproduction, to help preserve the genomes of endangered species (Martínez-Páramo et al. 2017). Cryopreserved sturgeon germplasm can be thawed and then transplanted into a sterile triploid surrogate, such as blue tilapia (*Oreochromis aureus*), which has a shorter life cycle that matures much earlier than sturgeons (Xie et al. 2019). This will enable gametes to be created more efficiently, which is critical due to the threatened nature of sturgeons.

There has been no research regarding developing cryopreservation protocols for shortnose sturgeon gonadal tissue. Currently, studies have been completed on Siberian sturgeon (Pšenička et al. 2016), Pallid sturgeon (Wayman et al. 2008) and lake sturgeon (Ciereszko et al. 2006). This is an area of research that needs more work, so that these threatened species can be protected.

Xenotransplantation

Xenotransplantation is the transplantation of living cells or tissue from one species to another. It can be used as a valuable conservation strategy, as germ cells can be transplanted from a donor into a surrogate species, which will then produce donor-derived gametes (Yoshizaki and Yazawa 2019). Spermatogonial stem cells (SSCs), oogonial stem cells (OSCs), and primordial germ cells (PGCs) can be extracted from cryopreserved tissue, and are able to be transplanted into a recipient fish and undergo gametogenesis to develop into functional sperm and eggs (Franěk et al. 2019). Germ cell transplantation eliminates the challenge of spawning the threatened species in captivity. The combination of germplasm cryopreservation and germ cell transplantation is an incredibly valuable tool for preserving and later reintroducing the genetic material of threatened species back into a population (Rivers et al. 2020). Future studies in the Litvak Lab will work on establishing xenotransplantation protocols, using cryopreserved shortnose sturgeon germplasm obtained in this study.

Non-Lethal Acquisition of Gonadal Tissue for Cryopreservation

As mentioned previously, laparoscopic surgery can be used as a minimally invasive, non-lethal method for determining sex of sturgeons. This is also an excellent method for retrieving gonadal samples for the purpose of cryopreservation. Developing protocols for the non-lethal acquisition of gonadal tissue is a critical component of the cryopreservation process, as it is inherently counter-productive to sacrifice endangered species for the purpose of enhancing conservation efforts. Laparoscopic surgery provides a method of obtaining gonad samples to be cryopreserved, without having to euthanize the fish. Differences between various laparoscopic surgery protocols include differences in the number of ports used, and whether or not insufflation is used. Insufflation is a technique that involves injecting a substance into the body cavity during laparoscopic surgery to create space between the telescope and the internal organs in order to achieve a clear view of the internal organs, and is typically done with either carbon dioxide or sterile saline (Divers et al. 2013). Saline is advantageous over carbon dioxide, as it is inexpensive, doesn't necessitate a tight seal between the operating sheath and the fish, and does not cause any significant buoyancy

changes after surgery (Divers et al. 2013). Divers et al. (2009) used 0.9% sterile saline for insufflation and a single-entry technique for evaluating gonads in free-ranging sturgeon. Munhofen et al. (2013) also used sterile saline insufflation for gender identification of juvenile Siberian sturgeon.

My Study

The aim of my study was to refine protocols for the non-lethal acquisition of gonadal material though laparoscopic surgery, as well as develop protocols for the cryopreservation of shortnose sturgeon germplasm. Specifically, my goal was to determine the freezing rate and cryoprotectant type that will yield the highest post-thaw viability. There is a lack of research surrounding the cryopreservation of ovarian tissue, yet preserving female gonadal material is critical for species who exhibit female heterogamety, such as the shortnose sturgeon. In my initial efforts to identify the sex of the experimental sturgeon, based on laparoscopic images and gonad morphology, I identified the fish as female. However, once histological analysis was completed and I was able to examine a cross sectional slice of the gonad, what I once thought were oogonia may have been spermatogonia. I cannot conclude for certain if the material I was cryopreserving was ovarian or testicular tissue but nevertheless, it is still important to continually refine germplasm cryopreservation protocols. I performed laparoscopic surgery on three shortnose sturgeon to explore the surgical techniques that provided optimal visualization and gonadal biopsy collection. One fish was sacrificed, and I cryopreserved fragments of their gonadal tissues, subjecting fragments to one of two cryoprotectants: dimethyl sulfoxide (DMSO) or ethylene glycol, and one of three freezing rates: -1^{O}C/min, -5°C/min, or -10°C/min. I assessed post-thaw viability using a trypan blue stain. Research on germplasm cryopreservation is pivotal to enhancing cryopreservation efforts. Properly cryopreserving sturgeon germplasm allows their genetic material to be added to existing germ banks, which will serve as a solid protection for their genetic linage. Advancements in cryopreservation techniques will also provide an avenue for xenotransplantation research to be studied, as it will provide sturgeon germplasm that can be transplanted into a sterile surrogate fish and proliferate into functional gametes. Optimizing germplasm cryopreservation techniques, as well as developing protocols for the non-lethal

acquisition of gonadal material, is critical for protecting shortnose sturgeon, as well as other threatened species from climate change and other anthropogenic disturbances.

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CHAPTER II

OPTIMIZING CRYOPRESERVATION PROTOCOLS TO IMPROVE POST-THAW VIABILITY OF SHORTNOSE STURGEON (*Acipenser brevirostrum*) GERMPLASM

Introduction

We are currently experiencing accelerated losses of biodiversity, and thousands of species are undergoing rapid population decline. Fish species in particular, are experiencing a more rapid population decline compared to any other vertebrate species (Li et al. 2016). Sturgeon, the common name for the ancient family Acipenseridae, are the most endangered vertebrate group in the world, with all 25 species listed on the International Union on the Conservation of Nature's Red List (IUCN, 2019). Sturgeon are primarily threatened by anthropogenic factors, such as habitat destruction and climate change, and have continually been overfished and exploited for their meat and caviar, highly valued luxury food items (Bronzi et al. 2011; Alavi et al. 2012). Sturgeon are late maturing, and have a long reproductive cycle which renders them more vulnerable to extinction (Dadswell *et al*., 1984; COSEWIC, 2015). Shortnose sturgeon (*Acipenser brevirostrum*) are a freshwater benthic species of sturgeon that inhabit the Saint John River, and are listed as a Species of Special Concern in Canada and as endangered on the United States' Endangered Species Act (COSEWIC, 2015). Thus, it is necessary that we turn to *ex-situ* conservation strategies, such as the cryopreservation of germplasm (reproductive tissue), to protect this and other sturgeon species.

Cryopreservation of germplasm is an incredibly useful conservation tool, as it allows genetic material of endangered species to be preserved indefinitely (Tsai and Lin 2012; Rivers et al. 2020). Cryopreservation involves freezing reproductive tissue at extremely low temperatures, followed by extended storage in liquid nitrogen at -196°C, so that it is viable when it is thawed (Tsai and Lin 2012). This technique has been used for a wide variety of animal and plant species as a way to store material for artificial reproduction, as well as to

preserve their genetic resources in germplasm cryobanks (Martínez-Páramo et al. 2017; Hagedorn et al. 2018). The establishment of cryobanks allows genetic material to be safeguarded from external threats or catastrophic events, and in combination with reproductive biology techniques, presents the opportunity to replenish a diminished population (Qvenild 2008; Martínez-Páramo et al. 2017). For these conservation efforts to be successful, we need to focus on improving minimally invasive techniques of acquiring gonadal tissue to be used for cryopreservation, as well as improving cryopreservation protocols.

It is imperative that we develop non-lethal methods to acquire gonadal tissue. In threatened species like sturgeons, because of their rarity, it is not advisable to terminally sample fish to obtain gonadal material for the purpose of cryopreservation. Laparoscopic surgery is a minimally invasive method of taking gonadal biopsies, that causes little harm to the fish. Post-operative mortality is low, and most often occurs as a result of infection from the procedure being performed in poor quality water (Webb et al. 2019). With increased practice and expertise, this procedure can be performed in as little as five minutes (Webb et al. 2019). Laparoscopic surgery in sturgeon has largely been used as a way to directly view and biopsy gonads for the purpose of sex identification (Divers et al. 2009; Matsche et al. 2011; Munhofen et al. 2014; Webb et al. 2019). The use of laparoscopic surgery for the purpose of retrieving germplasm samples for cryopreservation has not been well studied, but remains an important and overlooked component of the cryopreservation process.

Cell viability following cryopreservation is variable, and depends on a number of factors, including cooling rate, thawing rate, cryoprotectant type, and equilibration time. Cooling rate is of particular importance to the viability of the cells post-thaw. If the cooling rate is too slow, the cells are put at risk of damage by prolonged exposure to high concentrations of cryoprotectants, causing cryoprotectant toxicity (Tiersch et al. 2007). Conversely, if the rate is too fast then intracellular ice may form, which is lethal to cells (Marques et al. 2019). Thus, optimizing the cooling rate is essential to the success of cryopreservation. Most cryopreservation research has been focused on optimizing protocols for cryopreservation of sperm. Cryopreservation of female genetic material, i.e. eggs and embryos, is more difficult than the cryopreservation of sperm due to their larger size, higher

yolk content, and lower membrane permeability (Asturiano et al. 2017; Yoshizaki and Lee 2018; Mayer 2019). These factors prevent cryoprotective agents from penetrating the cell as efficiently, and put the cells more at risk for damage during the freezing process. Thus, there is a severe knowledge gap regarding the cryopreservation of oogonial cells in fish.

Sturgeon demonstrate female heterogamety; females, not the males, have two different sex chromosomes (WZ) (Van Eenennaam 1999). It is insufficient to only focus on cryopreserving sperm or sperm progenitor cells, as that will only protect the paternal genome of sturgeon (Van Eenennaam 1999; Pšenička et al. 2016; Asturiano et al. 2017). A promising alternative to cryopreserving sperm and egg cells, is the cryopreservation of gonadal tissue containing undifferentiated primordial germ cells, spermatogonia or oogonia, as these cells can retain proliferative capacities post-thaw (Anil 2013; Yoshizaki and Lee 2018; Ye et al. 2021). In conjunction with reproductive biotechnologies such as xenotransplantation, these cryopreserved undifferentiated germ cells can be transplanted into a sterile recipient fish and develop gonads that will produce sperm or eggs, depending on the sex of the surrogate (Yoshizaki and Lee 2018).

Cryopreservation of sperm has been studied in many species of sturgeon, including Pallid sturgeon (Wayman et al. 2008), Lake sturgeon (Ciereszko et al. 2006), Persian and Beluga sturgeon (Golshahi et al. 2018) but the cryopreservation of sturgeon germ cells has been far less studied. Psenicka et al. (2016) compared the viability of whole and dissociated gonadal tissue of Siberian sturgeon (*Acipenser baerii*) after freezing. They determined the optimal cryopreservation protocol for sturgeon early germ cells to be 1.5 M ethylene glycol as a cryoprotectant combined with extenders 0.5% BSA and 50 mM glucose, with a freezing rate of -1^{O}C/min. The cryopreservation of ovarian tissue has also been studied in zebrafish (*Danio rerio*), an excellent model organism for shortnose sturgeon. Anil (2013) and Tsai (2009) found the optimal cooling rate for zebrafish ovarian tissue fragments to be -4°C/min. There are few studies that stray from using cooling rates other than -1^{O}C/min, yet if higher cooling rates yield cells with similar post-thaw viabilities, this would greatly speed up the cryopreservation process, and enable quicker construction of germplasm cryobanks. To my knowledge, no one has compared different freezing rates of slow-cooling cryopreservation of shortnose sturgeon gonadal tissue.

The goal of my research was to refine laparoscopic surgery protocols for the non-lethal acquisition of gonadal material, and to optimize the freezing rate and cryoprotectant type used during the cryopreservation of shortnose sturgeon germplasm. In my first experiment, I examined two methods of laparoscopic surgery, with and without using saline insufflation, and monitored the post-operative recovery of the fish. Next, I used the slow-cooling method of cryopreservation and froze gonadal tissue fragments with one of two cryoprotectants, ethylene glycol (EG) or dimethylsulfoxide (DMSO), at three freezing rates: -1^{O}C, -5°C, and -10°C per minute. I hypothesized that slower freezing rates, and ethylene glycol as a cryoprotectant would result in higher post-thaw viability of germ cells. Membrane integrity as a measure of viability was assessed using a trypan blue stain, and the percent viability was recorded. My study has established protocols for the development of a shortnose sturgeon germplasm cryobank at Mount Allison University, which is an incredibly important step in preserving the genome of this threatened species, and will serve as a protective factor against extinction.

Materials and Methods

Experimental Animals

Three 4 year-old shortnose sturgeon (*Acipenser brevirostrum*) were maintained in a recirculating aquaculture system in the Gairdner building at Mount Allison University (Sackville, New Brunswick). All sturgeon were captured as larvae and reared in culture for their entire lives. At the start of sampling, the mean weight ± SD was 2.14 ± 0.43 kg and mean total length ± SD was 77 ± 3.61cm. Experimental fish were kept in a 5300 L tank recirculating system. The system was maintained at a temperature of 16-17°C, a pH of 7.6 and ammonia levels were kept at 0. Temperature was checked three times a week, and pH, ammonia, and nitrate levels were checked once a week. Sturgeon were maintained on a 14/10h light/dark cycle, and were fed twice a day with Optimum RAS 4 mm sinking pellets (Corey Nutrition). All experiments were conducted in accordance with the guidelines published by the Canadian Council on Animal Care and were approved by the Mount Allison Animal Care Committee (Protocol #102528).

Table 1. Weight (kg) and total length (cm) of the three experimental shortnose sturgeon

Sturgeon ID	Weight (kg)	Total Length (cm)
Fish 1	1.90	80
Fish 2	2.61	78
Fish 3	1.84	73

Sex Determination

To identify the sex of the fish, gonads were imaged using a Sonosite Edge II ultrasound with a 16-6 MHz transducer (HFL50 probe). Fish were placed into a 60 L holding tank with tricaine methane sulfonate (MS222, 100 mg/L) buffered with sodium bicarbonate. Once fish were anesthetized, they were placed on a V-trough surgical platform (Figure 1) while tank water was recirculated continually over the gills. Ultrasound images were taken between scutes 7-8 and 8-9 (count starting at the anterior end, near the pectoral fins) on the right lateral and ventral sides of the fish (Figure 1). Images were taken using the GEN and RES modes on the ultrasound, and the gain was adjusted until the clearest image was achieved. The probe was oriented parallel to the transverse plane. Once the images were taken, the fish were placed into a recovery net submerged in the large tank and closely monitored for an hour to ensure proper recovery from the anaesthetic. There were no mortalities from the procedure, all fish recovered well.

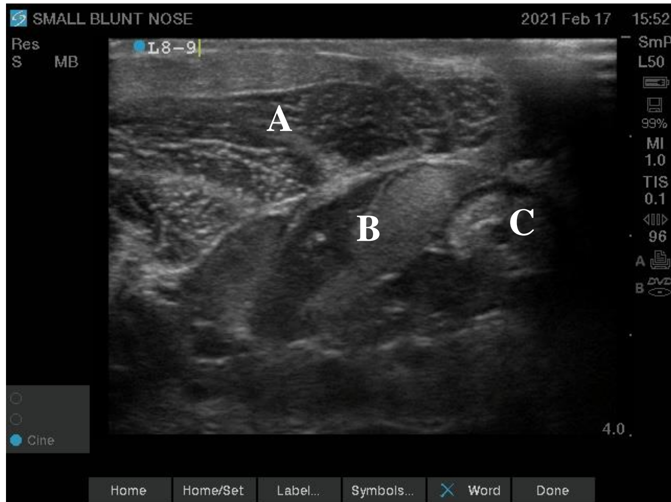

Figure 1. Ultrasound image of Fish 1. Picture was taken in RES mode with a lateral transducer. Image was taken on the left lateral side between scutes 8 and 9, with the count starting at the anterior of the fish. This image shows the muscle wall (A), the gonad (B), and the alimentary tract (C).

Experiment 1- Optimizing Protocols for the Non-Lethal Laparoscopic Collection of Gonadal Tissue

Surgical Set-up

The aim of this experiment was to further develop laparoscopic surgical techniques, to obtain gonad samples for cryopreservation while causing minimal harm to the fish. Methods established by Divers et al. (2009) were followed. Sturgeon were fasted for 24-78 hours prior to surgery. Fish were anesthetized as described above with a slightly higher concentration of anesthetic (MS 222, 150 mg/L). Once the fish were anesthetized, they were placed ventral side up on a V trough surgery platform (Figure 2). A pump continuously circulated water from a bucket containing MS222 (150 mg/L) using a plastic tube with an internal diameter of 16 mm. The tube was placed in the sturgeon's mouth to provide constant anaesthesia. The water drained passively with gravity into a bucket, to be recirculated (Figure 3). Both a 10° and 30° telescope (Medit Inc; Winnipeg, MB) were used and entered the body cavity through an operating sheath (diameter 5 mm). Biopsies were collected using flexible 4 mm biopsy forceps, and endoscopic images were captured using an Image Pro USB3 camera. The surgical instruments, telescopes, operating sheath and biopsy tool (Figure.4) were sterilized

prior to surgery by immersion in 70% isopropanol, and rinsed with distilled water immediately before use.

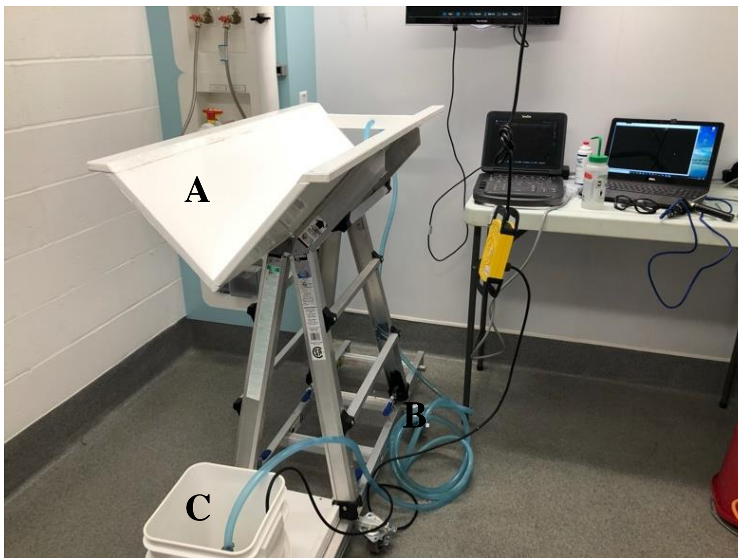

Figure 2. Surgical setup for the surgery without insufflation. The fish were placed in the V trough surgical platform (A) and the PVC tube (B) was used to recirculate water containing 150mg/L MS222 over the gills. The water returns to a holding bucket (C) through a gravity-fed return, and is pumped back up to the platform through the plastic tubing.

Figure 3. Fish on its dorsal side on the surgical platform. Water containing 150 mg/L MS 222 was pumped through the blue PVC tube into the mouth to maintain anesthesia.

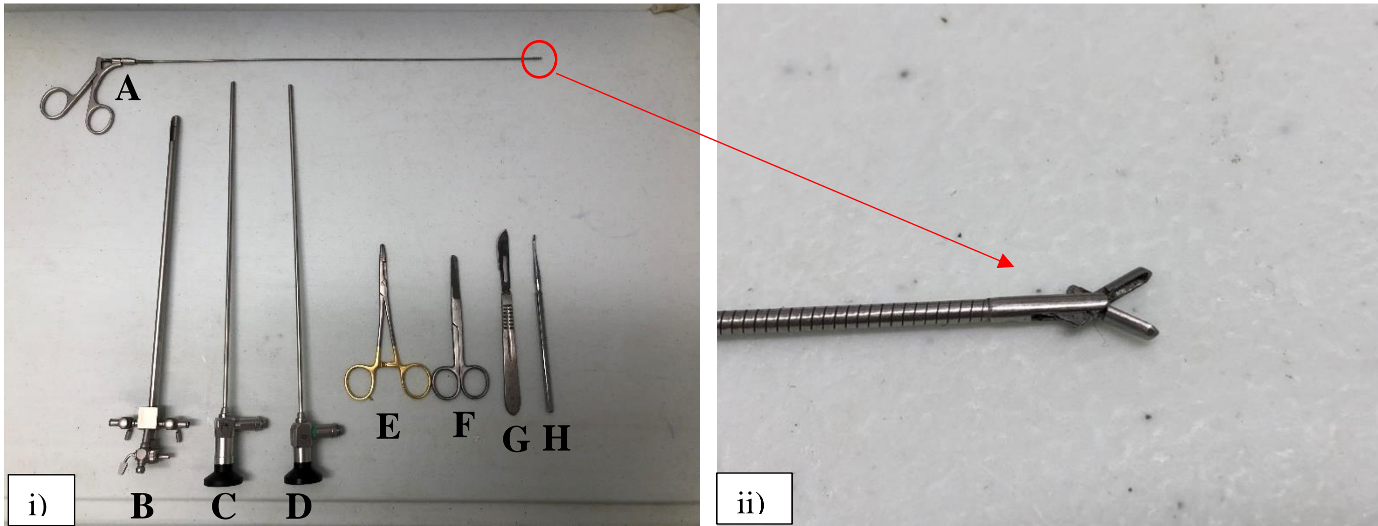

Figure 4. i) Surgical tools used during laparoscopic surgery: biopsy tool (A), operating sheath (B), 0^0 telescope (C), 30^0 telescope (D), haemostats (E), blunt scissors (F), 22 blade scalpel (G), probe (H). ii) Close up view of the 4 mm forceps on the biopsy tool.

Laparoscopic Surgery Without Insufflation

For the first two surgeries on Fish 1 and Fish 2, no insufflation was used. A 3 cm incision was made with a scalpel deep enough to penetrate to the body cavity on the right side of the ventral midline, between the 2nd and 3rd scute (count starting from posterior end near the pelvic fin). The telescope and operating sheath entered the coelem, and the gonads were scanned on both sides of the fish, until a region of desired gonad was encountered (Figure 5). Once that area was reached, the biopsy tool was inserted through the channel on the operating sheath, and samples of the gonad with an average surface area of 4.41 ± 3.10 mm^2 were taken. Samples were placed into 2.0 mL cryovials (Fisherbrand, 05-669-57), containing 0.25 mL of phosphate buffered saline (PBS) with an osmolarity of 280 mOsm kg^{-1} and kept on ice until cryopreservation. The incision was closed using two sutures of 4/0 non-absorbable nylon monofilament (Atraumatic Sutures). Each sample was imaged (Nikon CoolPix), and Image J was used to measure the surface area of each sample.

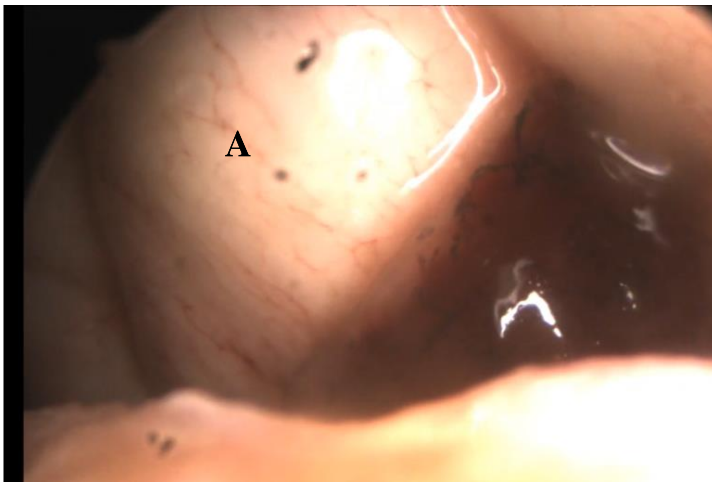

Figure 5. An endoscopic view of a region of shortnose sturgeon (*Acipenser brevirostrum*) gonad (A).

Laparoscopic Surgery Using Insufflation

Saline insufflation is often used in laparoscopic surgery to provide a clearer image. 0.9% sterile saline was used for insufflation in the third surgery performed on Fish 3. Saline was poured into half of a plastic bottle suspended from the ceiling. Flexible airline tubing with an internal diameter of 7 mm connected the saline supply to the port on the endoscope (Figure 6). A haemostat was used to clamp the plastic tubing when flow was not needed. A small incision of about 1 cm was made using a scalpel between the 2nd and 3rd scute, just deep enough to break through the first layer of skin. Blunt scissors were then used in an attempt to break through to the body cavity. This was not successful, and the operating sheath of the endoscope was used instead to try and puncture into the body cavity. The endoscope and operating sheath were inserted, with the body cavity forming a tight seal around the sheath. Saline was then permitted to flow with gravity into the operating sheath to fill and expand the body cavity. The biopsy tool was inserted through the cannula, and gonad samples were taken as described in the previous experiment. Each sample was imaged with a Nikon camera and Image J was used to measure the surface area of each sample.

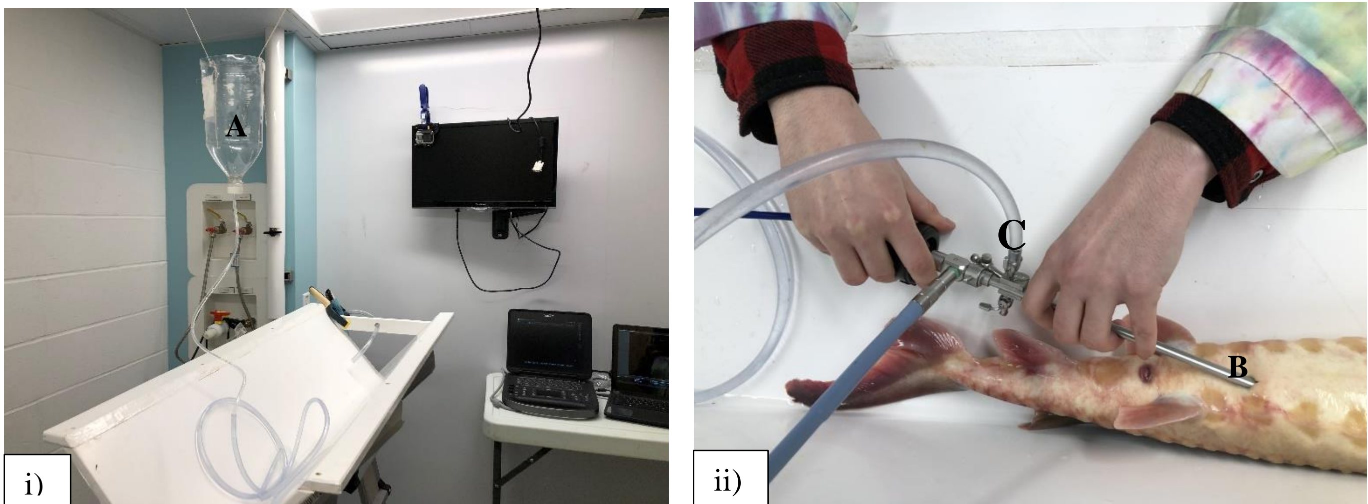

Figure 6. i) Surgical set up for the insufflation surgery. The plastic bottle (A) was filled with 0.9% sterile saline, which flowed through the plastic tubing into the side port in the endoscope. ii) The plastic tube containing sterile saline entered the operating sheath (B) through the side entry port (C).

Experiment 2- Optimizing Protocols for the Cryopreservation of Shortnose Sturgeon Germplasm

The aim of this experiment was to determine the optimal cooling rate and cryoprotectant that would contribute to the highest viability of gonadal tissue post-thaw.

Preparation of the Cryoprotectant Solution

A cryoprotectant solution for Siberian sturgeon ovarian tissue was optimized by Psenicka et al. (2016) as 1.5M cryoprotectant, and the extenders 0.5% Bovine Serum Albumin (BSA) and 50 mM glucose (D-glucose; Fisher Scientific, 186122A). The two cryoprotectants used in this study were 1.5M dimethyl sulfoxide (DMSO; Fisher Scientific, 195679) and 1.5M ethylene glycol (EG; Fisher Scientific, 193115).

Cryopreservation of Biopsy Fragments

Each biopsy sample was kept in 0.25 mL of PBS and then combined with 0.75 mL of one of the two cryoprotectant solutions (N=15, DMSO; N=15, EG). Samples were then put in a PLANER Kryo-360 cryogenic freezer (Planer Limited, United Kingdom) and exposed to one of three freezing rates. All profiles started with a 10-minute hold at 10°C for the samples to equilibrate with the cryoprotectant solution. The samples were cooled at a rate of either -1^{O}C/min (Fish 2; N=10), -5°C/min (Fish 3; N= 10) or -10°C/min (Fish 1; N= 10) down to -80°C. After cooling, the samples were immediately placed in liquid nitrogen.

Cryopreservation of Gonadal Material

One sturgeon (Fish 3) was sacrificed, which enabled larger sections of gonad to be used. Gonads were dissected from the fish and washed in PBS. Gonads were cut with scissors into pieces weighing ~0.1 g, the weight was recorded, and the pieces were imaged. The samples frozen at a rate of -1^{O}C/min were cooled first, followed by the -5°C/min group, and the -10°C/min was frozen last. The same cryopreservation protocols were followed as for the cryopreservation of biopsy fragments, except using a larger sample size (Figure 7).

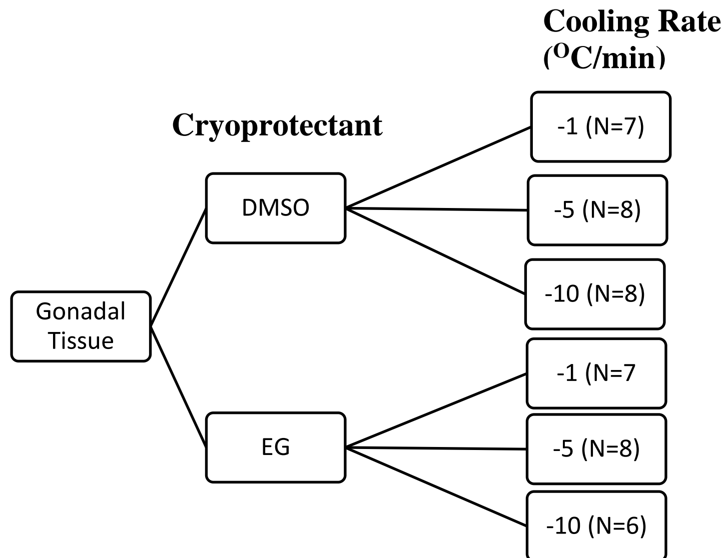

Figure 7. Experimental design for the cryopreservation of shortnose sturgeon (*Acipenser brevirostrum*) gonadal tissue fragments. DMSO is dimethyl sulfoxide and EG is ethylene glycol. Gonad from Fish 3 was dissected into pieces weighing ~1 g each, and exposed to one of two cryoprotectant solutions (N=23, DMSO; N=21, EG).

Image Analysis

The gonadal biopsies were too small to weigh with the balance we had on hand, so they were instead imaged and analyzed with Image J. The surface area of one side of the biopsy was measured. The pieces of the whole gonad were able to be weighed as well as imaged. Using the weight and surface area estimate of the whole gonad pieces, a regression was performed to try and extrapolate the weight of the biopsy pieces.

Thawing and gonad dissociation

Cryopreserved gonadal fragments were removed from liquid nitrogen, and placed in a 37°C water bath for one minute. Each gonadal fragment was rinsed with PBS, submerged in PBS, and minced with scissors.

Testing for Cell Membrane Viability

Following thawing, 100 µL of the dissociated cell solution was placed in a 2mL cryotube along with 50 µL of 0.4% Trypan Blue stain (Gibco, 2167528). The solution was

gently mixed by pipetting up and down, and left to incubate for 1.5 minutes. Approximately 15 µL of the cell suspension was pipetted into each notch of a hemocytometer (Neubauer) and observed using a compound microscope (Olympus BX40). Cells were counted in the large corner squares (that each contain 16 smaller squares). Cells that touched the border were only counted on the top and left sides, and ignored if they touched the bottom and right borders (Strober 2015). Cells were designated as either alive (unstained, membrane intact) or dead (stained blue, compromised membrane) (Figure 8). Cell membrane viability was calculated by using the equation:

$$\text{Percent viable cells} = \frac{\text{Number of viable cells}}{\text{Total number of cells}} \times 100\%$$

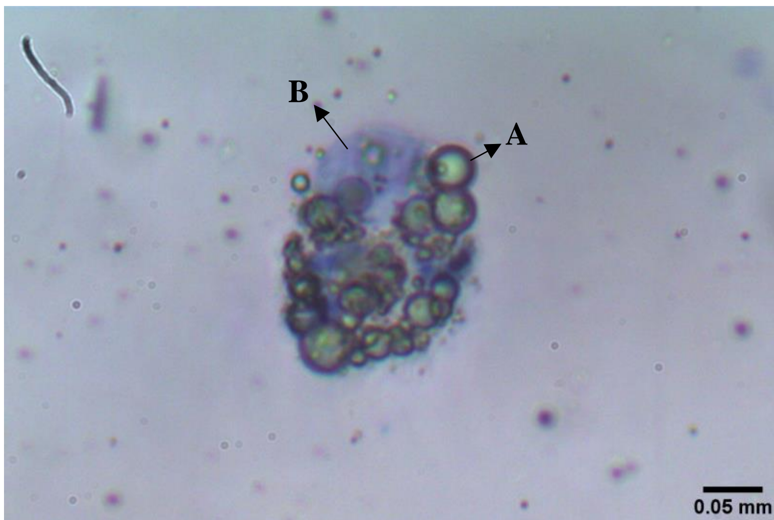

Figure 8. Cryopreserved shortnose sturgeon germ cells stained with trypan blue post-thaw. Cells were exposed to DMSO as a cryoprotectant and cooled at a rate of -1^{O}C/min. Membrane intact cells remain unstained (A), whereas cells with compromised membranes are stained blue (B). Image taken on Olympus BX40 microscope at 40x objective.

Histology

Fresh tissue was dehydrated in a graded series of ethanol and embedded in paraffin. Gonadal tissue was sliced into 7 µm sections using a microtome (Leica RM2135) and stained with trypan blue. Pictures were taken using the Infinity 1 (Lumenera equipped with Infinity Capture Software) microscope camera (Figure S1).

Statistical Analysis

A linear regression was used to assess the relationship between tissue fragment weight and surface area. Viability data are presented as mean ± standard deviation (SD). Statistical analyses were performed using R version 4.0.4. A 2-way ANOVA was applied to assess effects of cooling rate and cryoprotectant and their interaction on post-thaw viability. Normality was assessed using a Shapiro-Wilk test, and homogeneity of variance was assessed using Levene's test. When an interaction was detected (experiment 2: cooling rate x CPA), the data were split by CPA, and two one-way ANOVAs were performed. Post-hoc testing was conducted using Tukey's HSD. An alpha of 0.05 was used to assess significance.

Results

Experimental Animals

Once surgery protocols had been developed on frozen fish, we performed laparoscopic surgery on three live sturgeon to optimize our methodology. The surgeries on Fish 1 and Fish 2 were performed without insufflation, and the surgery on Fish 3 was performed using 0.9% sterile saline insufflation. Ten gonadal biopsies were taken from each fish, although not analyzed post-cryopreservation due to uncertainty regarding tissue sample type. The third sturgeon was euthanized two weeks after its laparoscopic surgery, and its gonad was dissected into 126 samples each weighing ~0.1 g. Of these, 44 samples were thawed, manually dissociated, and assessed for viability.

Optimization of Laparoscopic Surgery Protocols

Each surgery took ~20 minutes to perform, from initial incision to suturing. The first two surgeries on live fish (Fish 1 and 2) were performed without insufflation, and an incision site of 2-3 cm. 0° and 30° endoscopes were both used at different points during each surgery. I found that using the 0° and 30° is at the preference of the surgeon; personal preference for angle of view shown on the monitor as you explore the coelomic cavity and take biopsies. This method using a larger incision site enabled smooth movement of the endoscope in the body cavity, making it easier for the surgeon to manipulate the endoscope to scan different internal organs. Conversely, visualization was poor at times, and the biopsy forceps were not always visible in the endoscope camera. Additionally, when threading the biopsy forceps through the channel in the endoscope, the forceps approached the gonads at an angle $<45^{O}$, close to parallel to the gonads, that made it difficult to obtain gonadal samples. Due to difficulties in retrieving gonadal tissue with the biopsy tool, on Fish 2 the endoscope was removed and tissue samples were collected directly from the initial incision site. This eliminated the poor visibility and inadequate angle problems that we were facing while using the endoscope to collect biopsies. The third surgery on Fish 3 was performed using saline insufflation and a much smaller initial incision of ~5 mm. The insufflation surgery improved visibility as a result of distension of the coelomic cavity, but the small incision made it more difficult to maneuver the endoscope. This method also presented challenges in retrieving gonadal samples, as the biopsy tool would often approach the gonads at an inadequate angle which made it harder to physically collect a sample of the gonad. I found the optimal protocol for the acquisition of gonadal material to not use the endoscope, but to take a biopsy of the gonad directly from the initial incision site. All fish survived the laparoscopic surgery procedures, had a strong recovery and no post-operative complications were observed.

Relationship Between Gonad Fragment Weight and Surface Area

To decrease handling time during gonadal biopsies, we assessed if there is a correlation between weight and 2-D area of gonad pieces. Biopsy samples had a mean surface area ± SD of 4.41 ± 3.10 mm^2. There is a weak relationship between surface area and weight for tissue fragments (Figure 9; linear regression, R^2=0.18, F(df=1, 117)=25.38 p=1.73e-06, SE of slope=13.56).

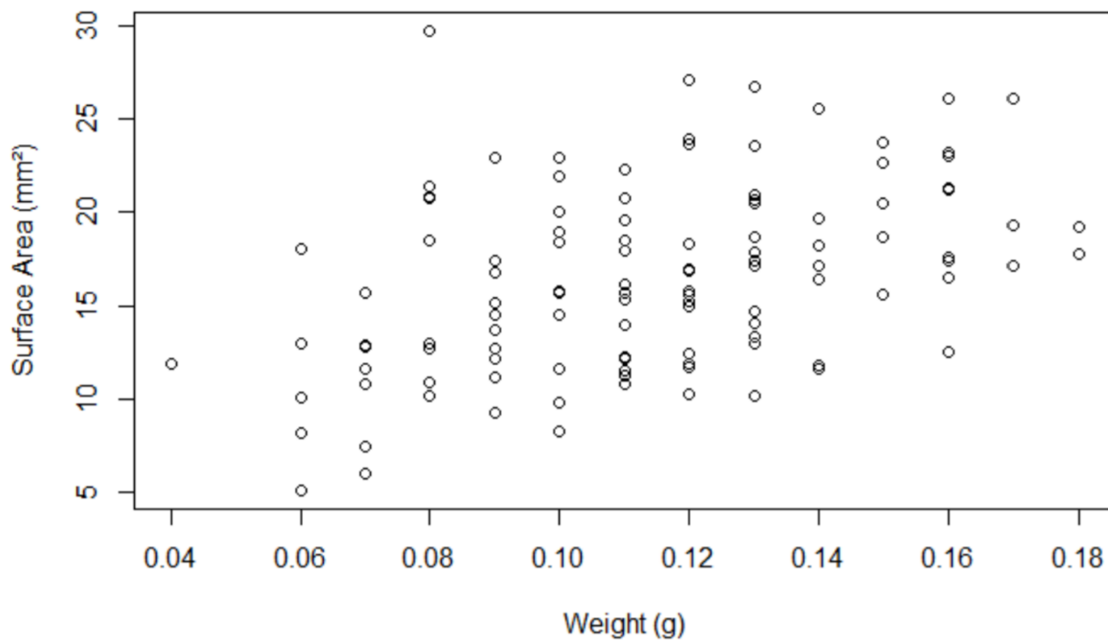

Figure 9. Linear relationship between surface area (mm^2) and weight (g) of gonad samples collected from the three laparoscopic surgeries on laboratory reared shortnose sturgeon (*Acipenser brevirostrum*) (R^2=0.18).

Effect of Cooling Rate on Post-Thaw Viability

Normality was assessed using the Shapiro-Wilk test (p=0.3866) and homogeneity of variances was assessed with Levene's test (p=0.1358). I found a significant interaction between cooling rate and CPA (2W ANOVA, F(df=2, 38)=5.918, p=0.00579), which indicates that the effect of cooling rate on post-thaw viability varied with cryoprotectant type. Due to this interaction, I decided to split up the data and run two one-way ANOVAs, examining the effect of cooling rate on viability at each CPA. With EG as a CPA, cooling rate had a significant effect on post-thaw viability at all cooling rates (1W ANOVA, F(df=2, 20)=3.646, p=1.3e-06). A cooling rate of -5°C/min yielded the highest post-thaw viability of 61.5 ± 7.5%, and a cooling rate of -10°C/min promoted the lowest post-thaw viability of 22.6 ± 3.5% (Figure 10).

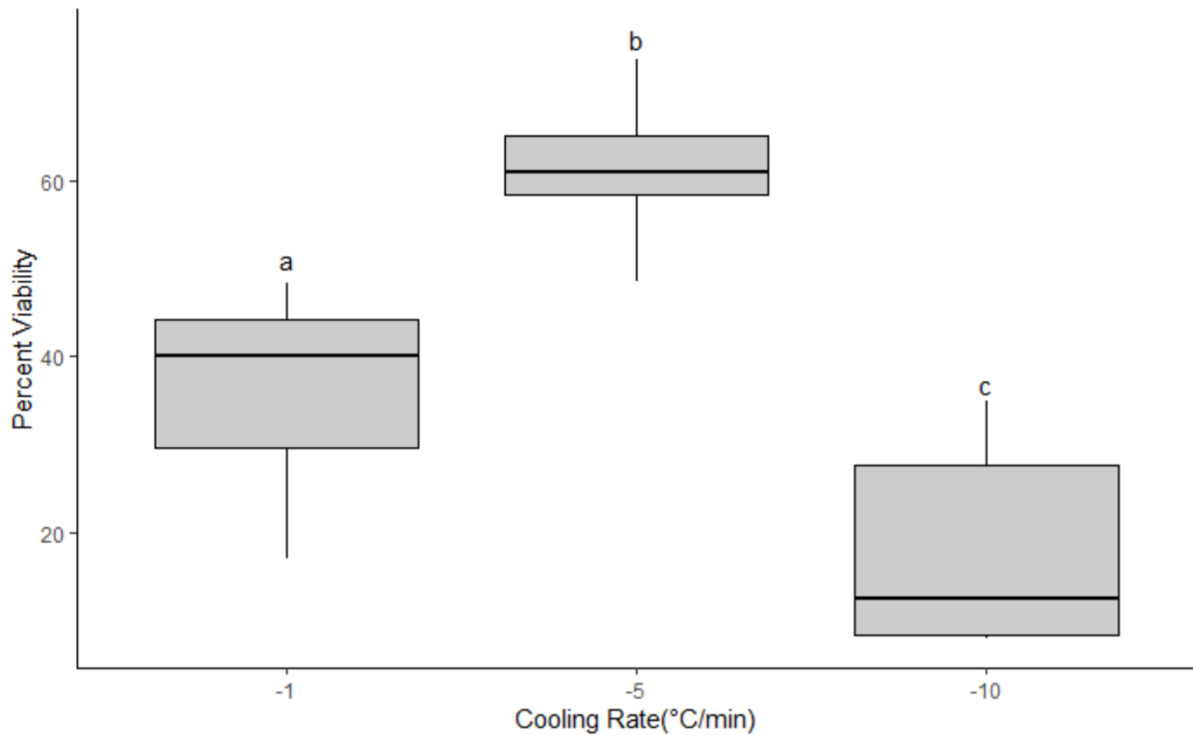

Figure 10. Post-thaw viability of shortnose sturgeon germ cells following exposure to three cooling rates (-1, -5 and -10°C/min). Cells were cryopreserved using 1.5 M ethylene glycol (EG) as a cryoprotectant, combined with 0.5% BSA, 50 mM glucose and PBS. Samples were rapidly thawed, manually dissociated, and assessed for viability using a trypan blue stain. Letters denote statistical significance ($P<0.05$).

When DMSO was used as a cryoprotectant, cooling rate had a significant effect on post-thaw viability (1W ANOVA, $F(df=2, 18)=31.59$, $p=0.0447$). A cooling rate of -1^{O}C/min had significantly higher post-thaw viability compared to a cooling rate of -10°C/min ($p=0.0445$). A cooling rate of -10°C/min had the lowest viability of 18 ± 12.3 %, and a cooling rate of -1^{O}C/min had the highest viability of 62.3% ± 8.9%. There were no significant differences in post-thaw viability between the -1^{O}C/min and -5°C/min cooling rates ($p=0.7515$), or between the -5°C/min and -10°C/min cooling rates (Figure 11; $p=0.1571$).

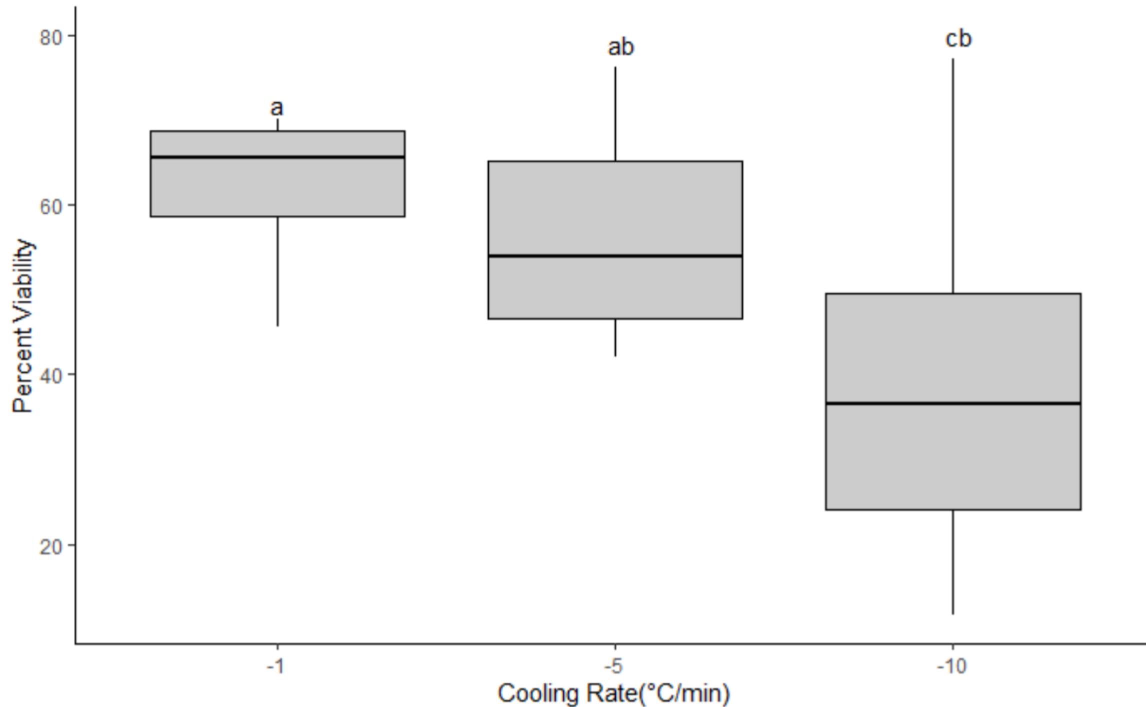

Figure 11. Post-thaw viability of shortnose sturgeon germ cells following exposure to three cooling rates (-1, -5 and -10°C/min). Cells were cryopreserved using 1.5 M dimethyl sulfoxide (DMSO) as a cryoprotectant, combined with 0.5% BSA, 50 mM glucose and PBS. Samples were rapidly thawed, manually dissociated, and assessed for viability using a trypan blue stain. Letters denote statistical significance ($P<0.05$).

Discussion

Cryopreservation of germ cells is a promising strategy for the conservation of threatened species; yet, reliable methods for the cryopreservation of female genetic material has yet to be developed. Preserving female genetic material is of particular importance for sturgeon, as they exhibit female heterogamety, so only focusing efforts on cryopreserving male genetic material would only protect the paternal genome, resulting in a loss of genetic information (Van Eenennaam 1999; Pšenička et al. 2016). However, I was unable to confidently identify the sex of the experimental sturgeon, leaving me unable to confirm the cryopreserved tissue as ovarian tissue. Thus, I focused my research on developing laparoscopic surgery protocols for the collection of gonadal tissue, and on optimizing protocols for the cryopreservation of shortnose sturgeon germ cells. In experiment 1, I

performed 14 surgeries on dead and 3 surgeries on live fish in order to practice laparoscopic surgery protocols. In experiment 2, I exposed gonadal tissue to either EG or DMSO at one of three freezing rates. I hypothesized that EG and a rate of -1^{O}C/min would yield the highest post-thaw viability. Ultimately, I determined that gonad collection by celiotomy was the most effective, though using endoscopes for assessment of gonadal maturity appear to be a promising avenue for future studies. In addition, I found that EG yielded the highest viability at a freezing rate of -5°C/min and DMSO yielded highest viabilities at -1^{O}C/min and -5°C/min (no significant difference). This research is pivotal to the timely construction of cryobanks to protect shortnose sturgeon from extinction.

Acquisition of Gonadal Material

Due to the threatened status of sturgeons, it is imperative that we develop protocols for the acquisition of gonadal material that do not result in fish mortality. My first surgical protocol (performed on Fish 1 and Fish 2) using no insufflation and a larger incision (2-3cm) was deemed ineffective. With this method, I ran into two major challenges. The first, was that there were times when the biopsy forceps were not visible in the endoscope camera. This resulted in having to "go in blind" and not be able to visualize the organ/tissue I was taking a biopsy of. This is problematic, as it may result in cryopreservation of a tissue type that is not gonadal tissue, or it could cause surgical complications for the fish. Gonads are closely associated with a layer of fat (Webb et al. 2019), which can make it difficult to distinguish those two tissue types from one another, particularly if the biopsy forceps are not visible. The second problem faced was a poor angle at which the biopsy forceps approached the gonads, which made it difficult to grab a gonad sample. Due to poor ergonomics surrounding the insertion of the biopsy tool through the channel in the operating sheath of the endoscope, the biopsy cups would often seem to slide against the surface of the gonads, running almost parallel to them. To retrieve a biopsy of adequate size, I found that the angle the biopsy tool approaching the gonad at is very important. The endoscope needs to be at least 50 degrees from the body wall of the fish, but approaching perpendicular is the optimal sampling angle. In the future, it would be beneficial to use a larger pair of biopsy forceps to collect the gonadal tissue. The biopsy forceps that we used to collect samples were 4 mm long, which

led to the collection miniscule samples of tissue that were difficult to process due to its small size. Divers et al. (2013) used biopsy forcep cups nearly 4 cm in length to biopsy the liver of free-ranging river sturgeon, so in the future it would be beneficial to attempt this surgery with larger biopsy tools.

The surgery using saline insufflation increased visibility, due to increased space between the endoscope and the body wall but decreased the maneuverability of the endoscope, as a result of the tight seal required between the body wall and the endoscope. Insufflation is a technique that is widely used for laparoscopic surgery, and air, carbon dioxide and saline are all used as agents of insufflation in fish (Divers et al. 2009; Matsche et al. 2011; Webb et al. 2019). I found that the increased visibility that insufflation provided did not outweigh the technical difficulties that came with maneuvering the endoscope, and ultimately it made the biopsy process more challenging. I think that moving forward, insufflation should be used as a tool for increased visualization when it is necessary to have a clear view of the internal organs, such as when assessing gonadal maturity rather than as a tool for biopsy sampling.

I found that the optimal method of retrieving gonadal samples was by celiotomy, which involves a making a larger incision (~2-3 cm) on the ventral side that penetrates directly to the body cavity, and directly sampling the gonad from the initial incision site (Webb et al. 2019). This method has been used on sturgeon to collect gonad biopsy samples for the purpose of sex identification, and has been shown to inflict minimal trauma onto the fish (Falahatkar et al. 2013; Eenennaam 2018). Celiotomy eliminates the two main problems that we were facing with the other surgeries, as this it allows you to visualize the gonad without using cameras and, ensuring that you are sampling the correct tissue. Additionally, this method allows the biopsy tool to go straight down so that it is nearly perpendicular with the gonad, a better angle for taking biopsy samples. The endoscope was helpful for viewing the internal organs and scanning the gonads, but actually hindered the biopsy sampling process. The endoscope is a tool more suited for assessing gonadal maturity and sex determination, rather than taking gonadal biopsies. Ultimately, all laparoscopic surgeries were successful. All fish recovered well, incision sites healed, and no postoperative damage or distress was observed indicating that this is a safe procedure to perform on sturgeon.

Tissue Fragment Weight and Surface Area Relationship

All of the gonad tissue fragments were weighed and imaged prior to cryopreservation, and their weight and estimated surface area was recorded. This was done to see if there was a correlation between surface area and weight, as biopsy tissue samples are too small to weigh on our lab balance, but can easily be imaged. I did not find a strong relationship between surface area and weight, but there is a trend of increasing surface area with increasing weight of the tissue fragments. The weak relationship is likely due to surface area being an inadequate index of size for the tissue fragment. The fragments were three-dimensional, so a better estimate of size would have been volume, where the surface area was multiplied by the height of the fragment. A regression of volume and weight would likely have stronger relationship with an increased R^2 value. Despite a rather weak relationship, it is beneficial to have an idea of the correlation between surface area and weight of gonadal tissue fragments, particularly when germplasm sampling in the field, where a balance is not available.

Cryopreservation: cryoprotectant type

Although not statistically significant, Figures 1 and 2 suggest that DMSO yields higher post-thaw viabilities across the three cooling rates tested compared to EG. However, I found a significant interaction between CPA and freezing rate, which indicates that CPAs function differently at different cooling rates. The results of my study are similar to what was found by Psenicka et al. (2016), who found that a CPA of EG yielded highest viability of cryopreserved Siberian sturgeon (*Acipenser baerii)* germ cells, with the next highest viability using DMSO. Franek et al. (2019) found that DMSO promoted the highest viability of common carp (*Cyprinus carpio*) spermatogonia. DMSO was also seen to promote the highest post-thaw viability of rainbow trout (*Oncorhynchus mykiss*) ovaries. Both CPAs used in this study are widely used for germ cell cryopreservation across fish species, and there is not a clear consensus on which one consistently promotes higher viability. However, a study by Plachinta et al. (2004) showed that EG more toxic than DMSO to zebrafish (*Danio rerio)* oocytes. Here, toxicity was not measured directly but my results could indicate that moving forward, it is preferable to use DMSO as a CPA over EG, as there is less potential for toxic

effects on shortnose sturgeon oogonial cells. However, more research is needed to distinguish which of these two CPAs are optimal.

Cryopreservation: freezing rate

Freezing rate is critical to the success of cryopreservation, as a rate that is too fast or too slow creates the potential of intracellular ice formation, which is lethal to cells (Marques et al. 2019). The results of my experiment suggest that the optimal cooling rate for the cryopreservation of shortnose sturgeon gonadal tissue is either -1^{O}C/min or -5°C/min. In both the DMSO and EG groups, tissue that cooled at a rate of -1^{O}C/min had significantly higher post-thaw compared to tissue cooled at a rate of -10°C/min. These results agree with Pšenička et al. (2016), Lee et al. (2016), and Franěk et al. (2019) who all saw high post-thaw viability (65-90%) with a cooling rate of -1 ^{O}C/min. However, this difference in viability between cooling rates could be due to the order in which I conducted my trials, as I started with the -1^{O}C/min group, followed by the -5°C/min group, and ended with the -10°C/min group. Thus, tissue in the -10°C/min group was sitting on ice for 4 more hours than the -1^{O}C/min group, which was frozen immediately following its removal from the fish. While it is unavoidable to have tissue sitting on ice for some amount of time when comparing freezing rates, due to our lab only having one Kryo 360 freezer, further studies randomizing the order in which samples are frozen are needed. These studies will confirm that tissue cooled at a rate of -10°C/min yielded the lowest viability of cells post-thaw due to the rapid cooling rate, and not as a result of the tissue degrading in the time between it was harvested, and put in the Kryo 360. It would be interesting to conduct future studies examining the effect that the time the tissue spends on ice prior to cryopreservation, has on post-thaw viability, and could be included in analyses as a covariate. This would be particularly beneficial for germplasm sampling in the field, as it would inform researchers how long they have to transport the tissue from the fish to the freezer, before viability is sacrificed.

A cooling rate of -5°C/min yielded the highest viability when the tissue was exposed to EG, and there was no significant difference in viability between cooling rates of -1^{O}C/min and -5°C/min for tissue exposed to DMSO. Cooling rates are not well explored in the cryopreservation research, as most studies aim to optimize other facets of the

cryopreservation process, such as cryoprotectant type or equilibration time, and use an "industry standard" slow cooling rate of -1^{O}C/min (Pšenička et al. 2016; Lee et al. 2016; Hagedorn et al. 2018; Franěk et al. 2019). Studies that investigate freezing rate typically compare viability between slow cooling (typically at a rate of -1^{O}C/min) and vitrification (immediate immersion in liquid nitrogen), rather than examining differences between slow-cooling rates themselves (Marques et al. 2019). My results suggest that we can freeze shortnose sturgeon gonadal tissue at a rate that is five times faster than what is typically done in the literature, without sacrificing viability. This is extremely beneficial, as it reduces challenges associated with having limited space and resources to freeze material with. Our lab only has one freezer, so increasing the freezing rate enables the freezing of more samples in a 24-hour time period. Ultimately, freezing tissue at a faster rate gives us the opportunity to construct cryobanks more rapidly, which is critical due to the declining populations of threatened species, like shortnose sturgeon.

Area of Concern- Contamination

When analyzing the gonadal tissue, after reanimation, under a compound microscope, it was apparent that all of the samples had rod-shaped specimens present; I presume these to be bacteria. It is unclear what the origin of the contamination was and at what point in the tissue collection process it occurred. Nevertheless, this informs us that moving forward is it extremely important to create and maintain a sterile field, and to ensure all surgical equipment is thoroughly sterilized between surgeries.

Viability Assessment

The trypan blue method of viability testing is known as a Tier 1 assay, and assesses cell membrane integrity as an indicator of viability (Baust et al. 2017). Trypan blue is able to enter cells with compromised membranes, and stain them blue, while cells with intact membranes are impermeable to the dye. However, having a cell with an intact membrane is not always an indicator of a living cell. It has been show that cryopreservation can induce alterations in gene expression post-thaw (Kaity et al. 2008). It would be valuable in future cryopreservation research to investigate more metrics of cell viability, such as Tier 2 assays

that measure cell death indicators and metabolic activity (Baust et al. 2017). This would provide a more comprehensive and accurate measure of post-thaw viability. For example, the fluorescent JC-1 dye that can be used to measure mitochondrial activity, thus providing important information about the status of the cell's metabolic function (Baust et al. 2017). Another method of testing viability that could be explored is an ATP (adenosine 5'-triphosphate) assay, in which viability is assessed based on the levels of ATP present in the cells, as ATP is key indicator of normal development and reproductive abilities (Anil 2013). Trypan blue is an excellent tool for a quick assessment of viability based on membrane integrity, to help narrow down cryopreservation protocols. However, once the CPA solution is optimized, using these additional measures of viability will increase the accuracy of post-thaw viability results and will ultimately improve the quality of isolated cells that will eventually be transplanted into another species, increasing the likelihood that they will survive and proliferate. It would also be beneficial to test for viability multiple times during the cryopreservation process, as opposed to only immediately after thawing. Additionally, future studies should test viability of tissue before freezing and after exposure to CPA (after the equilibration time). Testing viability of fresh, unfrozen tissue would serve as a useful reference to compare the viability of cryopreserved tissue to, and testing viability after CPA exposure would serve as an indicator of how toxic the CPAs are to the cell. Exploring different methods of viability testing and testing viability at multiple times throughout the cryopreservation process would give a more holistic view of the way in which cryopreservation effects cell viability.

Developing germplasm cryobanks is an important *ex-situ* conservation strategy for threatened species, such as shortnose sturgeon. There exist protocols for the cryopreservation of sperm, but there is a lack of research on the cryopreservation of female genetic material, such as oogonial cells, eggs and embryos, mainly largely due to structural challenges that make them more vulnerable to damage during the freezing process, such as a larger size and higher lipid content (Martínez-Páramo et al. 2017; Rivers et al. 2020). In addition, there is lack of focus on developing methods to acquire gonadal material for cryopreservation that does not involve fish mortality. To the best of my knowledge, I am the first to examine the effect of different slow-cooling cryopreservation rates of shortnose sturgeon gonadal cells. I

found that celiotomy was the optimal method for retrieval of gonadal tissue, and that a freezing rate of -5 ^{O}C/min was optimal for a CPA of EG, and a freezing rate of -1 ^{O}C/min or -5 ^{O}C/min was optimal when using DMSO as a CPA. Optimizing minimally invasive gonadal acquisition methods and oogonial cell cryopreservation protocols are paramount to the conservation of shortnose sturgeon, as well as other threatened species.

Future Direction

My honours project has laid the groundwork for a shortnose sturgeon germplasm cryobank at Mount Allison University. My research has profound implications for the germplasm cryopreservation process, as I found that viability is not sacrificed when tissue is frozen at a faster cooling rate of -5 ^{O}C/min, which will increase the speed at which cryobanks can be contructed. The next steps for the Litvak Lab would be to use the cryopreserved shortnose sturgeon ovarian tissue, isolate the oogonial cells, and transplant those into a sterile triploid fish, such as blue tilapia (*Oreochromis aureus*), and be able to proliferate into functional, donor-derived sperm and eggs (Yoshizaki and Yazawa 2019). Successful isolation and transplantation of shortnose sturgeon germ cells, would enable sturgeon gametes to be produced more rapidly, which would be able to contribute to population recovery. Overall, establishing germ cell transplantation protocols would be a major step forward in the conservation of shortnose sturgeon.

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Appendix

Histological Images

I have included some histological images of shortnose sturgeon gonadal tissue, as well as histological images of Siberian sturgeon ovarian and testicular tissue. Initially, upon observation of the gonad, I thought that the sturgeon I retrieved gonadal samples from was a female, and that I was cryopreserving ovarian tissue. However, upon histological analysis and comparison with histological images from the literature, it seems that our tissue more closely resembles images showing testicular tissue (Figure S1-S2). I was unable to come to a confident conclusion regarding the sex of the fish, and if I had more time, I would have completed geometric morphometric analysis to assist my efforts in trying to distinguish the sex.

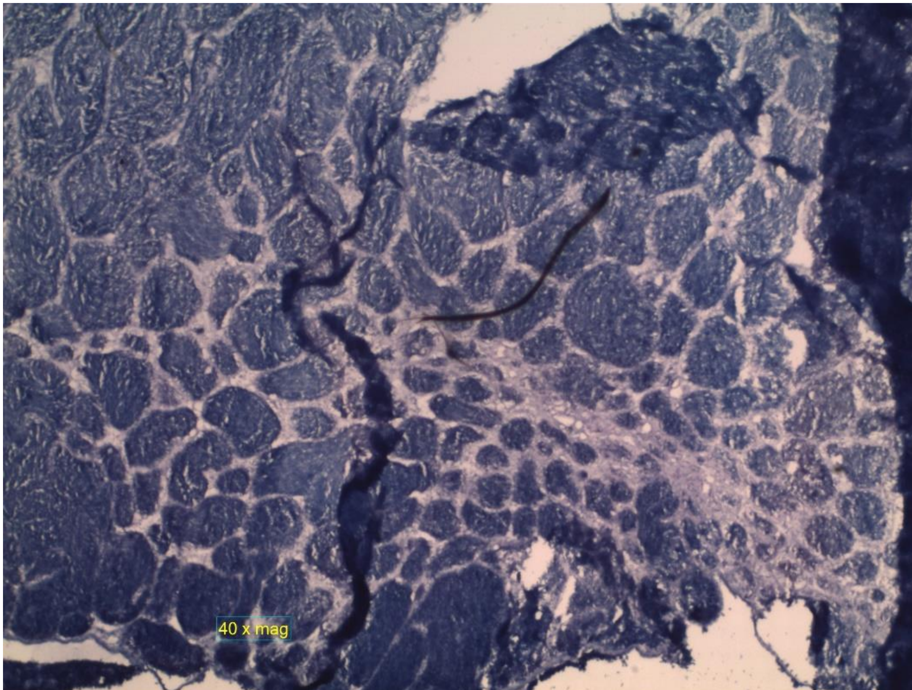

Figure S2. Histological section of 4-year-old shortnose sturgeon (*Acipenser brevirostrum*) gonadal tissue, stained with trypan blue.

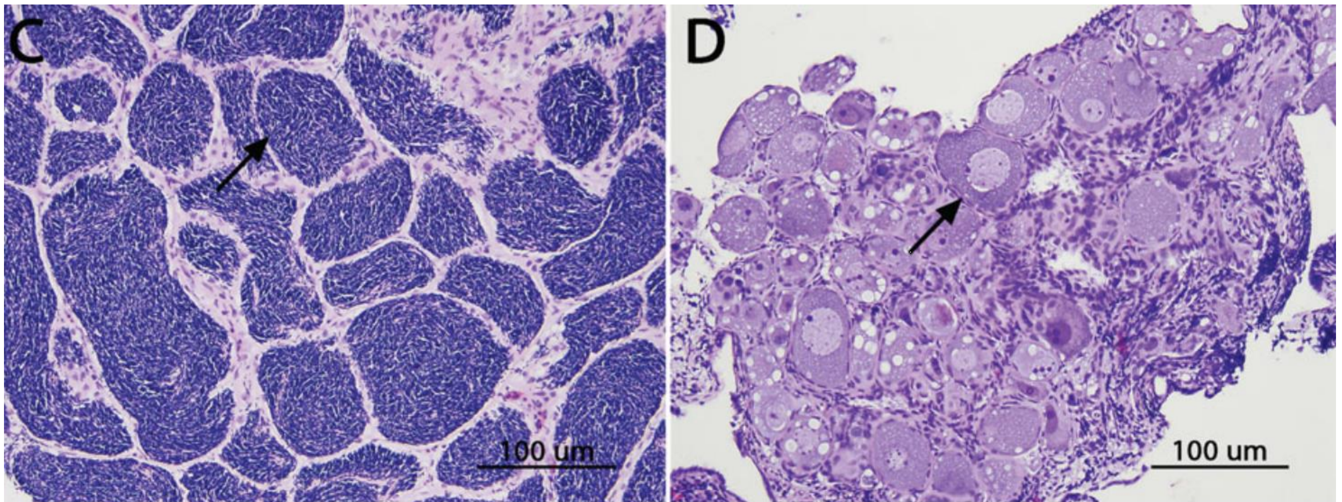

Figure S2. Figure from Munhofen et al. (2014) showing a histological section of a gonadal biopsy of a 3-year-old male Siberian sturgeon (*Acipenser baerii*) (C) and a histological section of a gonadal biopsy of a 3-year-old female Siberian sturgeon (D).

CHAPTER III: CONCLUSION

Review of Goals and Main Findings

Ultimately, my goal was to advance techniques to contribute to the development of germplasm cryobanks, to safeguard the genetic material of threatened species. There are two main components to the cryopreservation process: retrieving gonadal material, and the freezing of gonadal tissue. The first component is often overlooked, as fish are usually euthanatized and whole gonads are removed. However, since cryopreservation of germplasm is used as a conservation strategy, it is critical that we develop non-lethal ways of collecting gonadal material, as it is unfavorable to sacrifice threatened species. For the second component, there is a wide array of factors that must be optimized during the cryopreservation process in order to yield a high viability of cells post-thaw. There is a substantial lack of research comparing different slow-cooling rates, as many studies use a standard freezing rate of -1^{O}C/min. However, this freezing rate is inefficient, as it takes close to 2 hours to complete the cryopreservation process, accounting for equilibration time and the time it takes for the Kryo 360 freezer to heat/cool to specified temperatures. Thus, my research goals were twofold: to improve laparoscopic surgery protocols as a method of obtaining gonadal tissue for cryopreservation, and to optimize the cooling rate and cryoprotectant used for the cryopreservation of shortnose sturgeon gonadal tissue.

I found the optimal method for retrieving tissue was through celiotomy, where an incision is made and tissue is removed from directly there. I determined that using an endoscope may be valuable in studies of assessing gonadal maturity, but hindered the process of taking a gonadal biopsy. My research suggests that when using ethylene glycol as a cryoprotectant, the optimal cooling rate is -5°C/min and when using DMSO as a cryoprotectant, both cooling rates of -1^{O}C/min and -5°C/min are optimal, as they show no significant differences in viability. This suggest that we can freeze germplasm five times

more efficiently than what is typically done in the literature and still maintain high post-thaw viability, which enables more rapid construction of cryobanks.

Closing Remarks

We are currently in the midst of a massive biodiversity crisis, and it has reached a point where some researchers have even denoted this rapid loss of diversity in the Anthropocene as the "6th mass extinction". Vertebrate populations have decreased by 60% since 1970 and species losses are at 100 times the rate of biologically normal extinctions (Turvey and Crees, 2019). To fight against this frightening loss of species diversity, cryopreservation of germ cells is a promising *ex-situ* conservation strategy. Cryopreservation allows genetic material of threatened species to be stored in cryobanks indefinitely, and can be used in combination with reproductive biology techniques such as germ cell transplantation, as a way to reintroduce genetic information into a population. Thus, it is crucial that we focus our attention on refining cryopreservation protocols. The implications of my work on optimizing laparoscopic surgery techniques and germ cell cryopreservation protocols extend beyond shortnose sturgeon, and will contribute to the *ex-situ* conservation efforts of threatened species worldwide.

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