DEVELOPMENT OF XENOTRANSPLANTATION MODELS FOR STURGEON GERMPLASM: ZEBRAFISH (*DANIO RERIO*) AS MODEL SURROGATES

BY

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TABLE OF CONTENTS:

ABSTRACT

Sturgeon are the most endangered vertebrate group in the world (IUCN 2016). Sturgeon are endangered due to anthropogenic factors such as overfishing, industrial activity, and harvesting for their caviar (Pikitch et al. 2005). Xenotransplantation is a model for conservation of endangered sturgeon species, but has not yet been performed between a sturgeon donor and a teleost recipient. The goal of this study was to create sterile triploid surrogates. Zebrafish (*Danio rerio*) were used as model organisms to lay the foundation for the xenotransplantation process for shortnose sturgeon (*Acipenser brevirostrum*) germplasm to blue tilapia (*Oreochromis aureus*) surrogates. Triploid induction was tested using a heat shock method. Indicators of ploidy were examined using erythrocyte imaging and larval morphology analysis. A linear discriminant analysis was run on larval morphology traits to discriminate between 14-day old heat shock and non-heat shock larvae. As well, reanimated cryopreserved sturgeon gonad tissue was dissociated and run through a Percoll gradient to determine gonad cell isolation. A t-test revealed a significant difference in survival between heat shock and non heat shock zebrafish embryos at 24 hours post fertilization ($t = 15.649$, $df = 4$, $p\text{-value} = 9.739e\text{-}05$). Survival was significantly greater for the non-heat shock group compared to the heat shock group. The linear discriminant analysis reported discrimination between the heat shock versus non heat shock zebrafish embryos with an accuracy of 80%. Sturgeon cells were dissociated and colonized the top three Percoll gradient layers. The methodology employed in this study is essential to the research on sturgeon germline chimerism. More specifically, my study contributes to the production of triploid surrogates for transplantation of sturgeon oogonial cells in order to preserve valuable genetic information that is at risk of extinction.

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LIST OF FIGURES

LIST OF TABLES

PREAMBLE

The initial study was designed for shortnose sturgeon (*Acipenser brevirostrum*) and blue tilapia (*Oreochromis aureus*). The research goal was to harvest sturgeon gonad and isolate oogonial cells for microinjection into blue tilapia sterile triploid surrogates. Due to unforeseen circumstances, such as shipping restrictions and time restraints presented by the COVID-19 pandemic, the Litvak Lab was unable to obtain blue tilapia in the time frame intended for this study. These interferences in the study design were mitigated by the use of a model organism in place of the sturgeon and tilapia. In this study, I used zebrafish (*Danio rerio*) to lay the groundwork for the research design using sturgeon, which makes the implications of my research pertinent to the Litvak Lab.

CHAPTER I: LITERATURE REVIEW

CONSERVATION OF SHORTNOSESTURGEON (ACIPENSER BREVIROSTRUM) THROUGH XENOTRANSPLANTATION OF GERMPLASM TO TRIPLOID SURROGATES USING ZEBRAFISH (DANIO RERIO) AS A MODEL ORGANISM

Introduction to Sturgeon

Sturgeon belong to the order Acipenseriformes which includes 27 different species (IUCN 2016). Sturgeon are often referred to as "living fossils", as they represent an ancient lineage, dating back to the Devonian period approximately 400 to 300 million years ago (Bemis et al. 1997; Ye et al. 2017). Sturgeon (Acipenseridae), along with paddlefish (Polydontidae), are the only two descendants of Chondrosteans and were the dominant large fishes in all major Northern American rivers (Schultz 1980; Haxton et al. 2016). Sturgeon have retained many features of their ancestors that are now extinct which distinguish them from modern teleosts (Bemis et al. 1997). Sturgeon are the most endangered vertebrate group in the world, making it imperative to implement conservation efforts (IUCN 2016). Sturgeon are sought after commercially for their flesh and caviar, which contributes to their endangered status (Pikitch et al. 2005).

Sturgeon as an endangered species

Shortnose sturgeon (*Acipenser brevirostrum*) and Atlantic sturgeon (*Acipenser oxyrhynchus*) along with many other North American fish species have suffered drastic declines in the last 30 years due to anthropogenic factors such as overfishing, destruction of resources, and water pollution (Smith and Dingley 1984; Bronzi and Rosenthal 2014). Sturgeon farming for the production of caviar has become increasingly popular as the market for such a delicacy is quite valuable (Bronzi and Rosenthal 2014). In 1967, shortnose sturgeon were listed as an endangered species by the Endangered Species Preservation Act in the United States and have been listed as threatened in Canada since 1980 (COSEWIC 2015). All species in the order Acipenseriformes are on the Red List of

the International Union for Conservation of Nature, most of which are listed as critically endangered (IUCN 2016; Haxton et al. 2016). Sturgeon have a long reproductive cycle, which has served them well historically, but recent anthropogenic factors have made this trait a disadvantage (Secor et al. 2002). Sturgeon are subject to ingesting toxins from the accumulation of toxic compounds in sediments and within the trophic web as a result of industrial activity (Billard and Lecointre 2000). Climate change is one of the greatest emerging factors affecting sturgeon endangerment as ecosystems can change state drastically through arctic ice melting, changes in salinity, and many other harmful effects (Greene and Pershing 2007).

Shortnose Sturgeon

In Latin, *Acipenser* means sturgeon and *brevirostrum* means short snout, a literal translation of their common name, shortnose (Shortnose Sturgeon Status Review Team 2010). Shortnose sturgeon (SNS) are found along the North east coast of North America (Dadswell, 1984). The only known location of shortnose sturgeon in Canada is the Saint John River, New Brunswick (COSEWIC, 2005, 2015). The lack of sturgeon in the south of North America is thought to be due to ongoing anthropogenic factors (Kynard, 1997). SNS migrate between fresh and salt water for breeding purposes which makes them an anadromous species (Dadswell 1984). The shortnose sturgeon is the smallest of the three Eastern North American species of sturgeon, including the Atlantic sturgeon (*Acipenser oxyrinchus oxyrinchus*) and the Gulf sturgeon (*Acipenser oxyrinchus desotoi*) (Dadswell 1984; The North American Sturgeon and Paddlefish Society (NASPS) 2020). Shortnose sturgeon are similar in appearance to juvenile Atlantic sturgeon, but can be distinguished by identifying features such as their larger mouth, smaller snout shape, and tail scute pattern (Dadswell 1984).

Scott and Crossman (1973) describe shortnose sturgeon as a dark fish with black to dark brown colouring that fades into yellow on the ventral side. SNS are covered in small protrusions called denticles as well as five rows of large bony scutes: two lateral, one dorsal, and two ventral rows (Scott and Crossman 1973). The scutes act as protective bony plates and run from the skull to the caudal peduncle, or stem of caudal fin

(Shortnose Sturgeon Status Review Team 2010). The scutes are used as a way to identify individuals as they form patterns on the dorsal side that are made visible by their lighter colour relative to the rest of the sturgeon body (Gilbert 1989). SNS have an elongate body which is cylindrical at the abdomen and which narrows in at the head and caudal peduncle (Shortnose Sturgeon Status Review Team 2010). The species has a heterocercal tail, meaning that the tail is asymmetric, and the upper lobe of the tail is longer than the lower lobe (Dadswell et al. 1984). On the anterior portion of the sturgeon body, there are four fleshy barbels that hang halfway between the tip of the snout and the mouth (COSEWIC 2005). Shortnose sturgeon have bones present in the skull, jaw, and pectoral girdle, but the rest of their skeleton is cartilaginous (Dadswell 1984). The largest recorded SNS was found in the Saint John River in Canada and was 143 cm long and weighed 23 kilograms (Dadswell 1984). Growth of shortnose sturgeon is not linear, with rapid growth occurring in the first year and then decreasing in subsequent years (Shortnose Sturgeon Status Review Team 2010). It is thought that SNS prioritize somatic growth over reproductive development, which is why they become increasingly larger overtime (Billard and Lecointre 2000).

Sturgeon are typically benthic feeders foraging on the small organisms present at the bottom of rivers and the ocean floor (Billard and Lecointre 2000). The barbels, located ventrally beside their mouths, aid in foraging by providing tactile sensation of the benthos (Shortnose Sturgeon Status Review Team 2010). Sturgeon feed on infaunal macroinvertebrates found in the benthic substrate such as, polychaetes, crustaceans, molluscs, and insects, which are found in muddy and sandy sediments (Shortnose Sturgeon Status Review Team 2010; Dadswell et al. 1984).

Adult shortnose sturgeon were thought to be a sexually monomorphic species (Williot 2011), but recent evidence indicates that they are in fact sexually dimorphic (Balazadeh & Litvak, 2018). Adult male SNS are typically smaller than adult female sturgeon and are only sexed during spawning by presence of running milt and eggs (COSEWIC 2005, 2015). However, Balazadeh and Litvak (2018), in a geometric study on head shape, found a number of descriptors that suggest shortnose sturgeon are sexually dimorphic. Male sturgeon have a narrowing and elongate head while female

sturgeon have a wider and shorter head (Balazadeh and Litvak 2018). They developed an algorithm that allows a non-invasive delineation of sex at over 90% accuracy.

Shortnose sturgeon explore river systems they live in, but aggregate in specific areas for longer periods of time for feeding and resting in groups (O'herron et al. 1993; Kieffer and Kynard 1993). Species in the order Acipenseriformes are found only in the northern hemisphere (Billard and Lecointre 2000). The only Canadian river known to house shortnose sturgeon is the Saint John River, New Brunswick (COSEWIC 2015). Shortnose sturgeon migrate for several reasons, spawning upriver, for feeding in the spring, and overwintering in the fall (Shortnose Sturgeon Status Review Team 2010). In late winter/early spring, SNS migrate from overwintering locations to spawning grounds in northern rivers and in southern rivers (Dadswell, 1979; Kynard, 1997). In the Kennebecasis River in Saint John, a tributary to the Saint John River, adult and subadult shortnose sturgeon were found to overwinter in large aggregates (COSEWIC 2015). The overwintering locations observed in Saint John may be considered critical habitats for shortnose sturgeon (Li et al. 2007).

Female sturgeon spawn approximately five years after they have reached full sexual maturity, when they are on average 10 to 12 years old (Dadswell 1979; Kynard et al. 2016). When spawning, female sturgeon deposit thousands of 3-4 mm dark coloured eggs on the riverbed or ocean floor (Dadswell 1979; Kynard 1997). Each female on average produces 11,600 eggs per kg of fish (Dadswell 1979; COSEWIC 2015). Shortnose sturgeon are long duration spawners, depositing their eggs over a period of 24-36 hours (Kynard et al., 2012). The drawn-out process of depositing eggs discreetly is hypothesized to be an advantage to the females to carefully select the specific environment in which they place them (Kynard et al., 2012). Once deposited in the water, the eggs become adhesive and remain on the bottom as they are quite dense (Dadswell et al. 1984). Sturgeon usually spawn over rocks, gravel, or other hard substrate (Dadswell, 1979; Taubert & Dadswell, 1980; Buckley & Kynard, 1985; Kynard, 1997). After the initial spawning, females will spawn approximately every three years, but spawning can occur at various rates and can even be as long as five years in between spawning events (Dadswell 1979). Male shortnose sturgeon spawn for the first time at roughly 12 years of

age, and spawn from then on approximately every 2 years (Dadswell 1979). Once released, shortnose sturgeon sperm are active for over five minutes which increases likelihood of fertilizing an egg compared to most freshwater fishes which have sperm that are active for around one minute or less (Gilroy and Litvak 2019a, 2019b).

Life stages of shortnose sturgeon

From the time of conception until about day eight post hatch, eggs are considered to be yolk-sac embryos (Shortnose Sturgeon Status Review Team 2010). As their name indicates, these larvae are dependent on the yolk sac membrane which distends ventrally and is used for nutrients (Shortnose Sturgeon Status Review Team 2010). The embryos are a dark colour and are 7-11 mm long, resembling tadpoles (Buckley and Kynard 1981; Dadswell et al. 1984). At this stage, embryos have very poorly developed facial features such as the eyes and mouth and poorly developed fins, but are capable of swimming (Richmond and Kynard 1995). The embryos are vulnerable to water flow and typically remain in dark crevices and close to rocks or coverings (Richmond and Kynard 1995; Parker and Kynard 2014).

When larval sturgeon reach about 15 mm in length (8-12 days after hatching) they absorb the yolk sac (Buckley and Kynard 1981). At this stage, sturgeon now have developed eyes, fins and a mouth with teeth, which are thought to be used specifically for larval feeding (Taubert and Dadswell 1980). The larvae begin to resemble adult shortnose sturgeon and develop the coloring pattern with dark dorsally and light ventrally (Taubert and Dadswell 1980). Sturgeon are considered juvenile once they have grown to weigh about 9 grams (Kieffer et al. 2001). At this stage, sturgeon have complete ossification and well developed fins (Shortnose Sturgeon Status Review Team 2010). The sturgeon have now absorbed their teeth used in the larval stage and have fully protruding mouths for benthic feeding (Richmond and Kynard 1995). Juvenile sturgeon ingest more than just their prey items, their feeding manner is a nonselective process which often involves eating mud, rocks and plants unintentionally (Dadswell 1979; Carlson and Simpson 1987). Juvenile sturgeon commonly feed on isopods, amphipods, and aquatic insects (Dadswell 1979; Carlson and Simpson 1987; Bain 1997). Shortnose sturgeon that have reached the length of 45 cm and develop mature gonads, they are considered to be in the

adult life stage (Dadswell et al. 1984; Shortnose Sturgeon Status Review Team 2010). The oldest documented shortnose sturgeon were identified in the Saint John River, Canada with a female aged 67 years old and a male 32 year old (Dadswell et al. 1984).

Sexual Reproduction and Gametogenesis

Fish are a diverse group of animals, and vary considerably in their reproductive strategies and capabilities (Redding and Patiño 1993). Sturgeon, along with many other groups of fish, are considered to be gonochoristic, meaning they reproduce sexually and have at least two sexes (Henne et al. 2006; Subramoniam 2017). Sturgeon have a genetic inheritance pattern with the female being the heterogametic sex (Eenennaam et al. 1999); females are ZW and the males ZZ (Ye et al. 2017). Sturgeon are oviparous, meaning that male and females deposit their gametes into the environment and allow the eggs to be fertilized and develop outside the body (Ganguly 2013). The sturgeon spawn in early spring, typically in the freshwater areas of their range (Dadswell 1984). Spawning occurs when the water temperature is between 9-15^{o}C (Dadswell 1979; Taubert and Dadswell 1980; Kynard 1997). The life cycle of sturgeon is quite long and puberty only occurs later on in life (Billard and Lecointre 2000).

Gametogenesis

Gametogenesis is the process in which gametes are formed either as spermatozoa in males or ova in females (Redding and Patiño 1993). The process starts with germ cells that undergo mitotic proliferation and meiotic division to differentiate from diploid to haploid gametes (Redding and Patiño 1993). Primordial germ cells (PGCs) are the precursors to germ cells (Lacerda et al. 2013). They originate in the embryo and upon development the cells migrate to the genital ridge to settle and form germ cells (Lacerda et al. 2013).

Spermatogenesis

In the spermatogenic epithelium, the functional unit is a spermatogenic cyst (Schulz et al. 2010). A spermatogenic cyst is comprised of Sertoli cells that surround germ cells (Schulz et al. 2010). Sertoli cells are responsible for the development and protection of the germ cells (Schulz et al. 2010). In the early stages of spermiation, the close contact of germ cells with the Sertoli cells diminishes and becomes continuous with

the spermatogenic tubules (Schulz et al. 2010). Upon the completion of this process, sperm enters the spermatic duct system (Schulz et al. 2010).

Oogenesis

Vitellogenesis is the process in which there is massive growth of the oocyte as it accumulates nutrients that are to be used in embryo development (Lubzens et al. 2010). In nonmammalian species, vitellogenin is responsible for the growth of the oocyte (Redding and Patiño 1993). Vitellogenin is produced and secreted by the liver in response to estradiol and it is modified and deposited as yolk in the oocyte (Redding and Patiño 1993). Female sturgeon are gymnovarian, meaning that the oocytes pass through the coelomic cavity and enter the ostium then leave through the oviduct (Doroshov 1985; Ganguly 2013).

Triploidy

Biology of Triploids

Polyploidy is the possession of more than two sets of chromosomes (Piferrer et al. 2009). The majority of metazoa are diploid organisms, possessing two sets of chromosomes in their somatic cells (Piferrer et al. 2009). Triploids are organisms with three sets of chromosomes in each cell (Piferrer et al. 2009). Triploid cells have an increased nuclear volume to accommodate for the extra 50% of DNA, although the organism as a whole is not larger (Benfey 1999). Most of the germ cells in triploids do not make it past the first meiotic prophase (Benfey 1999). After fertilization the polar body is expelled to maintain ploidy of the developing embryo. To create a triploid, the expulsion of the polar body is prevented and triploid embryos are created (Benfey 1999). During the first meiotic division, homologous chromosomes are unable to synapse properly which disrupts gametogenesis (Hussain et al. 1991). Due to the change in normal development shortly after fertilization, triploid organisms are genetically sterile and unable to produce viable offspring (Benfey et al. 1989).

Triploids are typically used in aquaculture to create sterile species to avoid unwanted reproduction as well as allocate growth to somatic tissue, therefore producing more meat for harvest and commercial value (Sellars 2013). Rearing fish that are sterile provide researchers multiple benefits. In the case of a malfunction in equipment or human error that results in fish being released into the natural population, if they are sterile there

is a reduction of harm of reproduction with the wild fish. Creating triploid fish can also be beneficial for commercial value, as if you create a unique genetic line of fish for sale, there is the chance that others will breed them for profit but if they are functionally sterile, this is not possible. Triploids can also be used as a surrogate species for many different experimental conservation transplantation procedures. Surrogate germline technology stems from the experiment run by Lin et al. (1992) who tested whether tissue from the embryo of one zebrafish could integrate into the germline of a surrogate zebrafish embryo. Their experiment was successful and started a new line of research of surrogacy in aquaculture (Lin et al. 1992).

Inducing and Identifying Triploids

Typically, triploids are created directly by shocking recently fertilized eggs resulting in the retention of the second polar body (Hussain et al. 1991). In figure 1, meiosis I and meiosis II are outlined with induction of triploidy through physical or chemical shock, demonstrating the disruption in development that occurs during triploid induction. Creating triploid fish is advantageous as they allocate more of the energy reserve to somatic cell development, since the germ cells are not going to be used for reproduction (Piferrer et al. 2009). By focusing the energy on somatic cells, the fish often have greater flesh quality and quantity which is an advantage in fish culture because sexually maturing fish often have lower growth rates compared to sterile fish (Hussain et al. 1991; Poontawee et al. 2007).

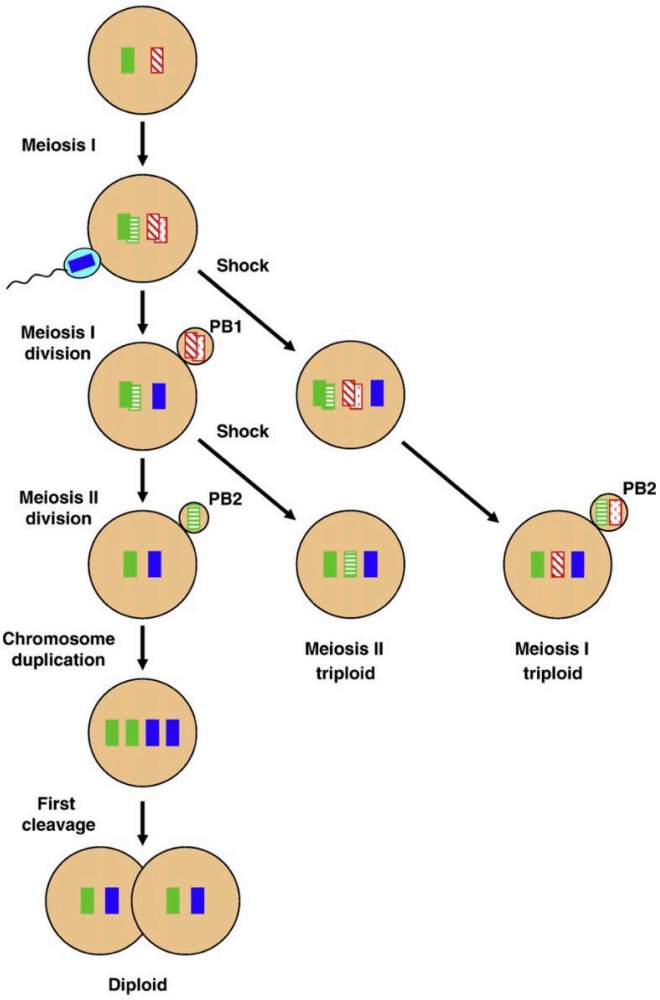

Figure 1. Piferrer et al. 2009; Ploidy manipulation with physical or chemical shock applied during meiosis I or meiosis II to suppress cell division, producing triploids by retention of the first polar body (PB1) or second (PB2) polar body. Each bar inside the cell represents one chromosome and overlapping bars indicate the sister chromatids after DNA replication during meiosis I.

Induction of triploidy in fish has been done successfully on numerous species (Benfey et al., 1989; Kavumpurath & Pandian, 1990; Hussain et al., 1991; Benfey, 1999; Franěk et al., 2019; Okutsu et al., 2007). Triploid Nile tilapia (*Oreochromis niloticus L.*) were created through pressure, heat and cold shocks to determine the optimum triploid induction protocol for the species (Hussain et al. 1991). Triploid zebrafish (*Danio rerio*)

created with a heat shock at 2.5 minutes post fertilization for a duration of 4 minutes at 41ºC (Kavumpurath and Pandian 1990). The optimum heat shock protocol for zebrafish was shifted towards a slightly warmer and shorter heat shock duration of 2 minutes at 41.4 ºC at 2 minutes post fertilization and resulted in a greater percent survival and percent triploidy than at 41 ºC (Franěk et al. 2019).

Triploid induction is generally confirmed by the presence of polyploidy in an organism including direct measurements such as chromosome counting and DNA measurement (Benfey 1999). Although they are well established methods of identification they are time consuming and require advanced technology (Okumura et al. 2001). Alternatively, measurement of erythrocyte nuclear volume and counting of nucleoli are easier for researchers to perform and provide an accurate indication of ploidy (Okumura et al. 2001). There are non-lethal techniques to determine ploidy, including taking small blood samples to analyze red blood cells. Typically, triploid fish have less erythrocytes, but the size of the cells are larger and have a greater nucleolar volume (Benfey et al. 1984, 1989; Benfey, 1999).

Because of the disruption in sexual development, triploid females do not appear sexually mature which is likely due to their ability to only produce small numbers of mature post-meiotic cells at an extremely slow rate which is not observed at the normal time of sexual maturation (Benfey 1999). The oocytes of female triploid fish most likely do not reach maturity because there is no vitellogenin, which is responsible for developing the yolk of the egg (Benfey et al. 1989). The lack of vitellogenin is attributable to the lack of ovarian estrogen synthesis making the oocytes underdeveloped therefore the oogonia underdeveloped. (Benfey 1999). Triploid males do appear to reach full sexual maturity as they usually will develop fully formed gonads (Benfey et al. 1989). The mature appearance is the result of the numerous pre-meiotic spermatogonia and primary spermatocytes (Benfey et al. 1989). Since male triploid fish are able to develop testes, they also have a small amount of cells that are able to reach maturity to create spermatozoa (Benfey et al. 1989). However, these sperm are aneuploid, meaning that they have an abnormal number of chromosomes and are not the normal haploid presentation of diploid gamete which makes them sterile and unable to fertilize an egg (Griffiths et al. 2000).

Cryobanking and Transplantation in Aquaculture

Wild populations of fish are threatened and since sturgeon are very slow maturing fish, they are especially vulnerable (Dadswell 1984; Tiersch and Green 2011; COSEWIC 2015). There is a need for the preservation of genetic material to survive the genes that are threatened with extinction (Tiersch and Green 2011). Cryopreservation of tissue for a gene bank to sustain genetic diversity has proved as a useful tool in conservation biology (Tiersch & Green, 2011). Maintaining genetic diversity is essential in conserving a species as the greater the genetic diversity, the greater the opportunity for resiliency to future change (Lynch 2016). An advantageous method of maintaining genetic diversity is live gene banking, which involves rearing endangered species in captivity and genetically managing them. Cryobanking requires adequate samples representative of the natural genetic variation of endangered species which are held in a state of suspended animation through freezing with liquid nitrogen, known as cryopreservation (Mijkherjee et al. 2002). Cryogenic gene banking takes minimal space and equipment, making it a cost and time effective strategy compared to live gene banking (Tiersch and Green 2011).

Cryopreservation

Cryopreservation is the freezing of tissues to very low temperatures to preserve them (Pegg 2007). Cryopreservation of sperm is the most established technique in aquaculture (Martínez-Páramo et al. 2017). Spermatozoa are easily cryopreserved because of their small size and resistance to extremely low temperatures, therefore protocols for many different aquatic species have been produced (Martínez-Páramo et al. 2017). Though cryopreservation techniques for sperm have been established in many fish species, there is still no viable way to cryopreserve eggs or embryos (Yoshizaki and Lee 2018). Cryopreservation of embryos is difficult as they have low membrane permeability, low tolerance to chilling, low surface to volume ratio and a high yolk content (Hagedorn et al. 1997a, 1997b; Zhang and Rawson 1998; Zhang et al. 2003).

Alternately, primordial germ cells (PGCs) have been used in place of eggs and embryos to cryopreserve maternal genomic information (Yoshizaki and Lee 2018). Because of their small size relative to eggs, they are able to be temporarily frozen in liquid nitrogen and then reanimated (Yoshizaki and Lee 2018). PGCs are the first cells in

the process of germ cell development (Okutsu et al. 2006). PGCs are undifferentiated, and have the ability to develop into either spermatogonial (male) stem cells or oogonial (female) stem cells (Okutsu et al. 2006). Thus, PGCs are an ideal cell type for the focus of cryopreservation research. Once cryopreservation techniques are optimized, there will be cryobanks with viable frozen tissue which can be used in genetic studies aimed at the conservation of sturgeon (Martínez-Páramo et al. 2017).

Germ cell transplantation

PGCs of fish have previously been transplanted to embryos and became germline chimeras which after reaching sexual maturity, were able to produce donor- derived offspring, marking the successful procedure of germ cell intra-species or allogenic transplantation (Lacerda et al. 2013). The first successful inter-species or xenotransplantation of spermatogonia was done with rainbow trout (*Oncorhynchus mykiss*) donors and masu salmon (*Oncorhynchus masou*) recipients (Okutsu et al., 2007). Ye et al. (2017) were the first to perform xenotransplantation using sturgeon, Chinese (*Acipenser sinensis*) to Dabry's sturgeon (*Acipenser dabryanus*). In the experiment, Ye et al. (2017) transplanted the PGCs intraperitoneally to the Dabry's sturgeon larvae which resulted in over 70% of transplanted larvae surviving to two days post transplantation (Ye et al. 2017). The Dabry's sturgeon is easier to house in a laboratory which allows them to mature easily in captivity which is reason for selecting them as a xenotransplantation recipient species (Ye et al. 2017).

Animals can be generated via the transplantation of thawed PGCs or spermatogonia into recipients (Okutsu et al. 2007). The process is described by Yoshizaki and Lee (2018) as using primordial germ cells that have been harvested from an endangered species and transplanted into a sterile host species to produce donor-derived gametes. The goal of the model seen in Figure 2 was to be able to regenerate endangered species from the frozen genetic material which would be performed through mating the recipients (Yoshizaki and Lee 2018).

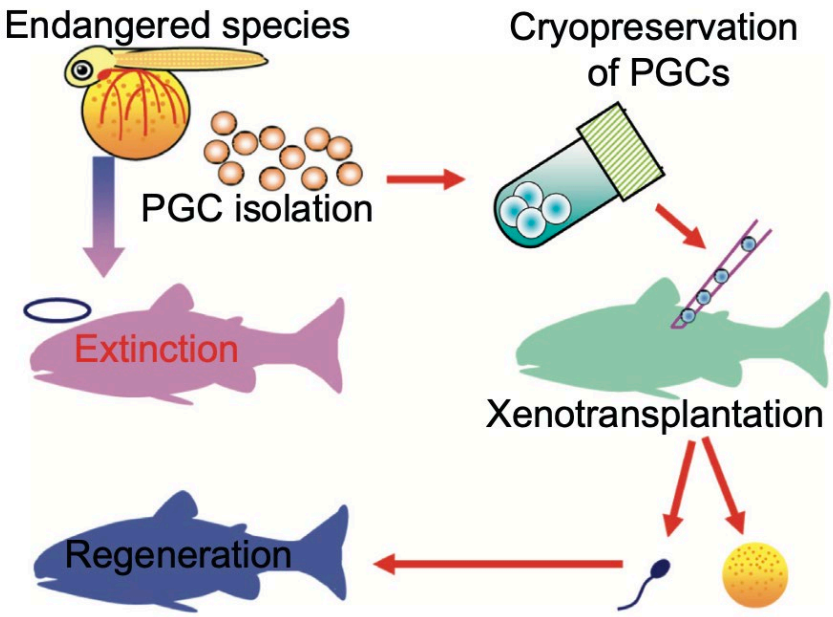

Figure 2. Yoshizaki and Lee (2018); Model for regeneration of endangered species through cryopreservation of primordial germ cells (PGCs) for transplantation to closely related species.

Zebrafish as a model organism

Zebrafish (*Danio rerio*) are a small freshwater, cyprinid teleost fish (Briggs 2002). Zebrafish are native to the flood-plains of India in shallow, slow-flowing waters (Spence et al. 2007). They are extremely popular laboratory model organisms due to their small size, availability of genetic strains, and availability for purchase at most local pet stores (Duff et al. 2019). Their short generation time make them a popular fish to use in reproductive biology as they typically reach sexual maturity 3-4 months after hatching (Spence et al. 2007). Also, laboratory husbandry procedures are well established for zebrafish due to their popularity (Briggs 2002). Zebrafish are a key player in developmental biology due to their method of mating, external fertilization, which ensures that all stages of development can be observed (Briggs 2002). External fertilization makes the organism valuable for embryo manipulation and many other possibilities (Spence et al. 2007). The zebrafish is also commonly used for the study of human diseases as it is a non-mammalian vertebrate, therefore including complexity but not being hindered by cost, latency, and animal care issues (Griffiths et al. 2000).

Microinjection to zebrafish embryos and larvae

The use of microinjection techniques for intracellular/extracellular injection and perfusion has become a popular procedure in numerous areas of experimental biology research as it allows researchers to inject genetic information from one individual into another (Schubert et al. 2014;Warner Instruments 2021). Microinjection is also favoured over other methods as it allows for quantitative reliability, with specific volumes and rates controlled by the microinjector there is less room for error. Previous work in the Litvak Lab has optimized time at microinjection to embryos for increased survival rate of zebrafish (Burbridge 2019). Microinjection of embryos is favoured due to the lack of immune system at this stage of development, which promotes integration of foreign material that would be rejected in an adult fish (Yoshizaki and Lee 2018). Zebrafish are popular subject for use in embryology as they develop optically transparent embryos that are relatively large compared to the size of the adult fish, allowing manipulation of the embryo and observation of all developmental stages (Kimmel et al. 1995).

Ovarian germ tissue taken from three-month-old zebrafish was dissociated and injected into two-week-old zebrafish larvae with and successfully colonized the gonads and differentiated into sperm in the male recipients (Wong et al. 2011). Ovarian germ cells are present in the ovary of adult zebrafish and can function as germline stem cells, which can subsequently proliferate and function as donor-derived female and male gametes depending on the sex of the recipient fish (Wong et al. 2011). In addition to the transplantation of PGCs and spermatogonia the development of a novel technique using oogonial cells using zebrafish advances the field of fish germ cell transplantation, especially for surrogate brood stock technology (Takeuchi, 2003; Okutsu et al., 2006; Yoshizaki et al., 2010; Wong et al., 2011).

My Study

Originally, the purpose of my study was to determine an optimal protocol for the xenotransplantation of germplasm from shortnose sturgeon (*Acipenser brevirostrum*) into sterile blue tilapia (*Oreochromis aureus*) recipients. However, because of our inability due to the pandemic to get access to blue tilapia, the target surrogate, I decided to work on Zebrafish as a model for development of these protocols for sturgeons. I decided upon zebrafish as the protocols will be similar for tilapia and I did not want to waste valuable

research time nor lab resources, as the research program is on a time restraint. To do this, I performed four separate experiments. The goal of my first study was to confirm a successful and effective method of creating triploid zebrafish (Franěk et al. 2019). The second experiment was done in attempt to identify triploid zebrafish larvae through erythrocyte analysis (Benfey et al. 1984; Kavumpurath and Pandian 1990; Pase et al. 2009; Bianchini and Wright 2013; Kim et al. 2017; van de Pol et al. 2020). The third experiment investigated external methods of ploidy confirmation by examining possible discrimination between larval morphology in zebrafish. The final question I aimed to address is whether it is possible to dissociate and isolate shortnose sturgeon oogonial cells using a Percoll gradient (Wong et al. 2011). Ultimately, my research will further our understanding of the first steps of xenotransplantation of germplasm in fish. The advancement of xenotransplantation techniques is essential to the continuation of research on this critically endangered sturgeon species and could play a large role in future conservation efforts. More specifically, the creation of a successful protocol for triploid induction and isolation of oogonial cells will advance the research for xenotransplantation of sturgeon germplasm to a teleost recipient.

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CHAPTER II: OPTIMIZING TRIPLOID ZEBRAFISH (*DANIO RERIO*) SURROGATE PROTOCOLS IN PREPARATION FOR XENOTRANSPLANTATION OF STURGEON GERMPLASM TO TRIPLOID TELEOST RECIPIENTS

INTRODUCTION

Xenotransplantation, or xenogeneic transplantation, is the transplantation of tissue, cells, or other biological material between species (Government of Canada 2006). Xenotransplantation provides the ability for researchers to create novel transgenic lines and has potential applications in the preservation of endangered species (Lacerda et al. 2013). Cryopreservation, or the freezing of tissues to very low temperatures by submersion in liquid nitrogen, can be used in conjunction with xenotransplantation (Pegg 2007). Cryopreservation of embryos has not yet been successfully performed as embryos have low membrane permeability, low tolerance to chilling, low surface to volume ratio and a high yolk content, which are all traits that make them unsuitable for cryopreservation (Hagedorn et al. 1997a, 1997b; Zhang and Rawson 1998; Zhang et al. 2003). As an alternative, primordial germ cells (PGCs) can be cryopreserved as they are much smaller and are tolerant to freezing temperatures (Yoshizaki and Lee 2018). Thawed cryopreserved material, such as PGCs, can be used to generate fish via transplantation into recipients (Okutsu et al. 2007). The process is done using primordial germ cells that have been harvested from a donor species, potentially cryopreserved cells that have been reanimated, and transplanted into a sterile host species to produce donor-derived gametes (Yoshizaki and Lee 2018). Germ cell transplantation has made significant contributions to the fields of experimental zoology as well as conservation biology (Lacerda et al. 2013). Using this method for augmentation of endangered species populations is favoured as it requires a small biopsy of gonadal tissue from the donor species, therefore not harvesting entire organisms and not require large amounts of capture of populations at risk (Yoshizaki and Lee 2018). Triploids, organisms possessing 3 sets of chromosomes, are often used in aquaculture as recipient surrogates in xenotransplantation experiments (Benfey 1999). Triploids are functionally sterile due to an interruption in development which makes them essential recipients of transplantation

as they allocate resources to donor materials, rather than their own reproduction (Benfey 1999).

Zebrafish (*Danio rerio*) are a small tropical fish that are endemic to India and other small bodies of water in Asia (Spence et al. 2007). They are invaluable to medical and developmental research as they are easy to house, have a fast reproductive turnover rate, and are external fertilizers (Briggs 2002). Zebrafish embryos from the moment they are spawned are completely transparent which serves as a major advantage for developmental biologists as it allows the observation of all developmental stages straight through to adulthood (Spence et al. 2007). Because of the small size and resilience of the embryos, they are easily handled and manipulated by researchers. Burbidge (2019) assessed yolk swapping in zebrafish embryos, highlighting the resilience of embryos and the ease of micromanipulation without damaging development. Sommerfeld (2016) studied the impact that egg size has on growth, development and reproductive performance post yolk-sac manipulation, optimizing egg size reduction. Previous studies in the Litvak Lab show the use of zebrafish as a laboratory organism and the further need for research on specifics of zebrafish embryology, development, and micromanipulation for use as model organisms. The work done in the Litvak Lab has demonstrated the ability of zebrafish embryos to undergo micromanipulation and injection of foreign substances (Duff et al. 2019). These factors support the use of zebrafish as a model for xenotransplantation of germplasm to sterile embryo surrogates. In this chapter, I will focus on triploidization of zebrafish.

To that end, the goal of my study was to create triploid zebrafish embryos using an optimized heat shock protocol as well as determine success of triploid induction (Franěk et al. 2019). There are no known morphological markers that distinguish zebrafish diploid larvae from triploid larvae, other than size and number of erythrocytes (Benfey 1999). Standardization of a set of geometric morphometric markers in zebrafish larvae would significantly simplify the ability to identify production of triploid zebrafish and reduce sacrifice of larvae for blood cell analysis in attempt to determine triploidy. The final goal of my study was to reanimate develop a selection protocol for collection cryopreserved shortnose sturgeon (*Acipenser brevirostrum*) gonad and dissociated and identify gonadal cells. My study investigates the use of a protocol for zebrafish as a

model organism to lay the groundwork for other species. Xenotransplantation between species such as shortnose sturgeon and a teleost such as blue tilapia (*Oreochromis aureus*) is the next step to broadening our knowledge of reproductive biology in these species and creating conservation protocols for endangered sturgeon species.

MATERIALS AND METHODS

Fish Husbandry

Zebrafish (*Danio rerio*) were bought at a local PetSmart. All fish were given a chloramine T disinfection bath of 18mg/L for 4 hours upon arrival and quarantined from the rest of the fish for 14 days before introduction (Noga 2010). All fish were kept in a zebrafish recirculation system (Pentair 18 tanks: 12 x 3.0 L tank part #: PCT3-D; 6 x 10.0 L tank part #: 10-7-A) with a 14:10 light:dark photoperiod using a light timer. Dawn was simulated using string lights connected to a timer starting half an hour before the overhead lights to allow the fish to adjust to the light. The recirculation system was maintained at a temperature of 27 ºC +/- 1 ºC and pH was kept stable between 6-7, with ammonia levels at 0. Fish were fed twice a day with Tetra Tetra-Min tropical food flakes or freeze-dried bloodworms (Omega One) and systems were cleaned regularly.

Experiment 1 – Heat Shock

The evening before heat shock, three male and three female zebrafish were each placed in their own individual breeding tank. Fish were sexed by using geometric morphometrics such as size of abdomen and length of caudal peduncle (Duff et al. 2019). Each tank contained glass marbles lining the bottom surface and a plastic sex plant to stimulate spawning. The fish were organized by sex, having male tanks beside female tanks so that each fish was able to see the opposite sex through the tank wall to ensure visual breeding cues were available. The fish were removed just after the simulated dawn from their individual tanks and placed in tanks together for breeding. The fish were placed in a double layer tank with the first tank having perforation at the bottom to allow eggs to sink through for fast collection. Fish were observed to identify breeding cues such as tail chasing and release of eggs (Hutter et al. 2010). Once fertilization was observed, the fish were quickly transferred by removing the top tank and the eggs were passed through a stainless-steel sieve. Half of the eggs were placed aside as a control group and the other half were kept for subsequent triploid induction.

A previous trialed method was performed to determine whether observation of fertilization would be sufficient or if manual stripping would be required. The manual stripping method was trialed by anaesthetizing zebrafish using a non-lethal dose of MS-222 (0.168g/L) and using a blunt probe to massage the eggs and sperm out of the fish.

Sperm and eggs were combined in a petri dish and 1 ml of 27 ºC +/- 1 ºC zebrafish recirculation system water was added to induce fertilization, marking time zero of fertilization. The petri dish was gently mixed to distribute sperm. After 1 minute, the petri dish was filled with 15 ml of zebrafish water and was gently mixed. This method followed the same heat shock method outlined below, starting at 2 minutes post fertilization. The eggs from both the heat shock and control groups of the manual stripping method did not survive, indicating lack of fertilization and no embryo production. Therefore, the heat shock method of observation of fertilization was used for this study.

At 2 minutes post-fertilization, the fertilized eggs were submerged in 41.4 ºC embryo medium in 1-inch diameter glass vials for a duration of 2 minutes (Westerfield 1995; Franěk et al. 2019). The glass vials each containing 50 ml of embryo medium were pre-heated to 41.4 ºC to ensure that time of heat shock was controlled for upon submersion. Temperature was controlled using a general-purpose water bath (VWR model WB20: 120/50-60/12.0). After the 2 minutes, they were removed and placed into individual vials of 28.5 ºC embryo medium. All control and heat shock embryos were reared at the same temperature in the same water bath, each in their own glass vial. String lights were mounted above the water bath on a timer to match the photoperiod in the zebrafish recirculation system. Heat shock embryo survival was determined by observation at 3-hour intervals post-fertilization.

Experiment 2 – Triploid Identification

Once the larvae hatched (48 to 72 hpf), they were sacrificed by removing their tails and bloodletting on a clean microscope slide (Pase et al. 2009; Bianchini and Wright 2013; Kim et al. 2017). A drop of phosphate buffered saline solution (PBS) was administered to each slide before use under the microscope. Red blood cells were observed using an Olympus BX40 light microscope. Images were taken of collections of red blood cells using the ZEN 3.0 ZEN-pro system for the Zeiss SteREO Discovery V.20 microscope (magnification 320.0 x).

Experiment 3 – Larval Morphology Analysis

Fourteen-day old larvae from both the heat shock and non-heat shock groups were imaged using the ZEN 3.0 ZEN-pro system for the Zeiss SteREO Discovery V.20

microscope. Larvae were individually placed in 2% methyl cellulose (Fisher Chemical M352-500) for imaging purposes, and each was imaged from lateral and dorsal positions (Duff 2018). The body area, fin fold area, and dorsal view of the head area (excluding the eyes) were measured (mm^2) using the active region graphics function on the ZEN 3.0 ZEN-pro system (Figure 3). Body area was calculated by taking the full body area and subtracting the swim bladder. Fin fold area was calculated by taking the total area of the fish and subtracting the full body area (with swim bladder) and subtracting the total head area (with eyes). Head area excluding eyes was done by taking the area of the head and subtracting the summed area from both eyes.

Figure 3. 14-day old zebrafish (Danio rerio) larvae imaged using ZEN 3.0 ZEN-pro system for the Zeiss SteREO Discovery V.20 microscope (magnification 2.3 x). A) Lateral view of zebrafish larvae used to calculate body area (True body area = body area with swim bladder – swim bladder area). B) Lateral view of zebrafish larvae used to calculate fin fold area (Fin fold area = total area - body area with swim bladder – head area with eyes). C) Dorsal view of zebrafish larvae used to calculate head area excluding the eyes (True head area = area of head including the eyes - area of the eyes).

Experiment 4 - Dissociation of Sturgeon Gonad

Shortnose sturgeon (*Acipenser brevirostrum*) gonads were harvested and cryopreserved in liquid nitrogen. The gonads were frozen using two different cooling rates, -1 °C per minute and -10 °C per minute, both cryoprotected in dimethyl sulfoxide (DMSO). Gonad tissue was then thawed for one minute in a 37 °C water bath and finely minced using scissors. The cell suspension was then washed and resuspended with PBS. The gonad suspension was dyed using a 2:1, gonad:trypan blue staining protocol and loaded onto a discontinuous Percoll (GE Healthcare) gradient in a conical test tube with 20%, 25%, 30%, 35%, 40%, 50%, and 60% fractions (Wong et al. 2011). A Percoll gradient is typically used to isolate viable human spermatogonia for artificial insemination, and was used in this method to isolate the gonad germ cells (Arcidiacono et al. 2008). The conical test tube was centrifuged in the Thermo Scientific Sorvall ST16R centrifuge for 30 minutes at 4 ºC at 800 X g. After centrifuging, the cell suspension from the 20%, 30%, 40% Percoll fractions were sampled and imaged using the Olympus BX40 light microscope (magnification 40.0 x). The diameter of each imaged cell was measured using Image J Version 1.51 (100; 2015). Viable cells were identified using the trypan blue stain indication. The cells that retained a blue outline were considered viable. The cells that were a full blue tint were considered dead/non-viable.

Statistical Analyses

All statistical analyses were conducted using R Studio Version 1.2.5042. Two separate t-tests were done to compare survival proportions for heat shock zebrafish embryos versus non-heat shock embryos from experiment 1. The first t-test was the survival from both groups at 3 hours post fertilization (hpf) and the second t-test was the survival from both groups at 24 hpf. Assumption of normality was tested using Shapiro-Wilk test and homogeneity of variance was assessed using a Bartlett's test.

A linear discriminant function analysis was conducted on the three morphological measurements taken on the heat shock and non-heat shock larvae at 14 days post fertilization (dpf). The analysis was done on the surviving 20 embryos, 15 from the control group and 5 from the triploid group. The linear discriminant function analysis (LDA) was done to maximize the morphological differences between the heat shock and the control embryos, using the three measurements as predictor variables of group.

Essentially, the LDA uses body area, fin fold area, and head area to predict whether the embryos can be classified as heat shock or control. The discriminant function for LDA using the two groups, heat shock and control, and the three measurements, body area, fin fold area, and head area is seen below.

$$DF = a\,(x - \bar{x}) + b\,(y - \bar{y}) + c\,(z - \bar{z})$$

The equation describes a, b, and c as the slopes of the discriminant function, and x, y, and z as the three variables measured (Davis 2002; Maindonald and Braun 2010). Therefore, each sample has a value using this equation that is called a score. One discriminant function (DF) is calculated in this analysis as there are two groups to be distinguished between, heat shock and control. This is due to the DF creating axes by taking the number of groups minus one, therefore one for this analysis.

Jackknife validated sampling was done to reduce bias and assess the accuracy of the prediction. The jackknife LDA removes one observation and formulates the DF using the remaining data. This analysis produces a percent of accuracy of the prediction of the jackknife sampling which can be compared to the original LDA to determine overestimation in the model. Original LDA prediction uses resubstitution by taking all the samples to develop the discriminant function (Davis 2002; Maindonald and Braun 2010). Both jackknife and the original LDA prediction are compared using output tables describing the accuracy of the discrimination on the diagonal of the table.

Cell diameter and number of cells observed in from the top three Percoll gradient layers (20%, 30%, 40%) were visualized to determine any possible trends. Samples were also taken from the layer on top of the 20% Percoll gradient. Due to the low number of samples collected, no statistical analyses were run on this data.

RESULTS

Experiment 1 – Heat Shock Survival

There was no significant difference in survival at 3 hpf between the two groups (Figure 4). Survival for control embryos was significantly higher than for heat shock embryos at 24 hpf (t = 15.649, df = 4, p-$value$ = 9.739e-05) (Figure 5).

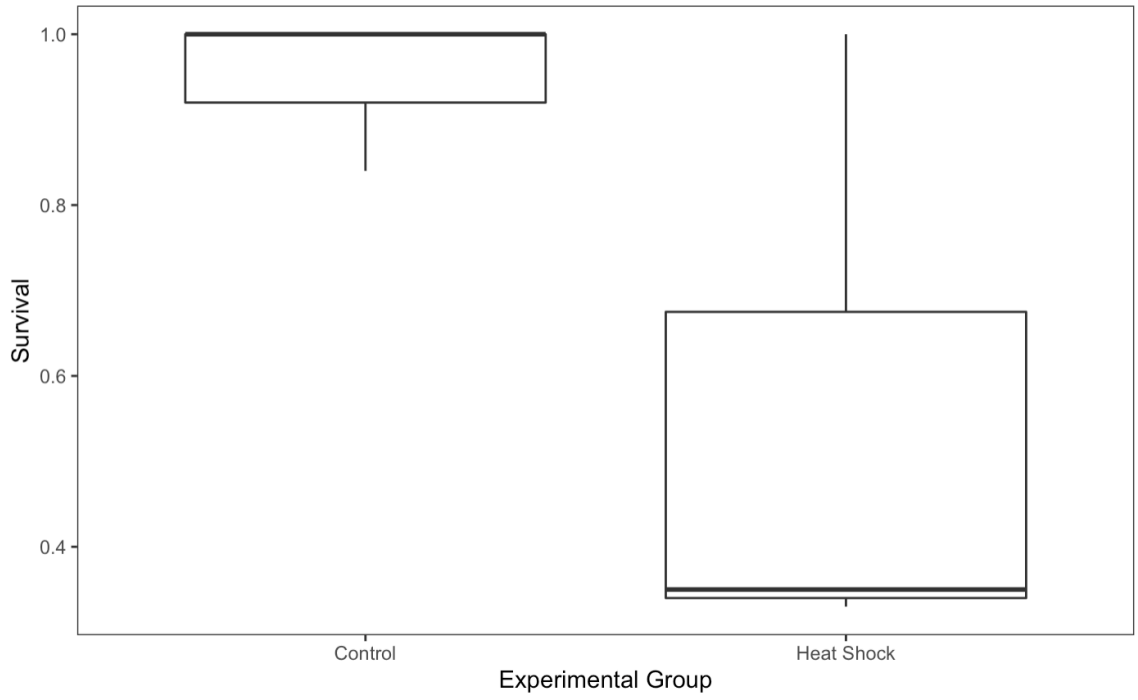

Figure 4. Survival of Heat Shock zebrafish (*Danio rerio*) embryos and Control (non-heat shock) zebrafish embryos at 3 hours post fertilization (hpf).

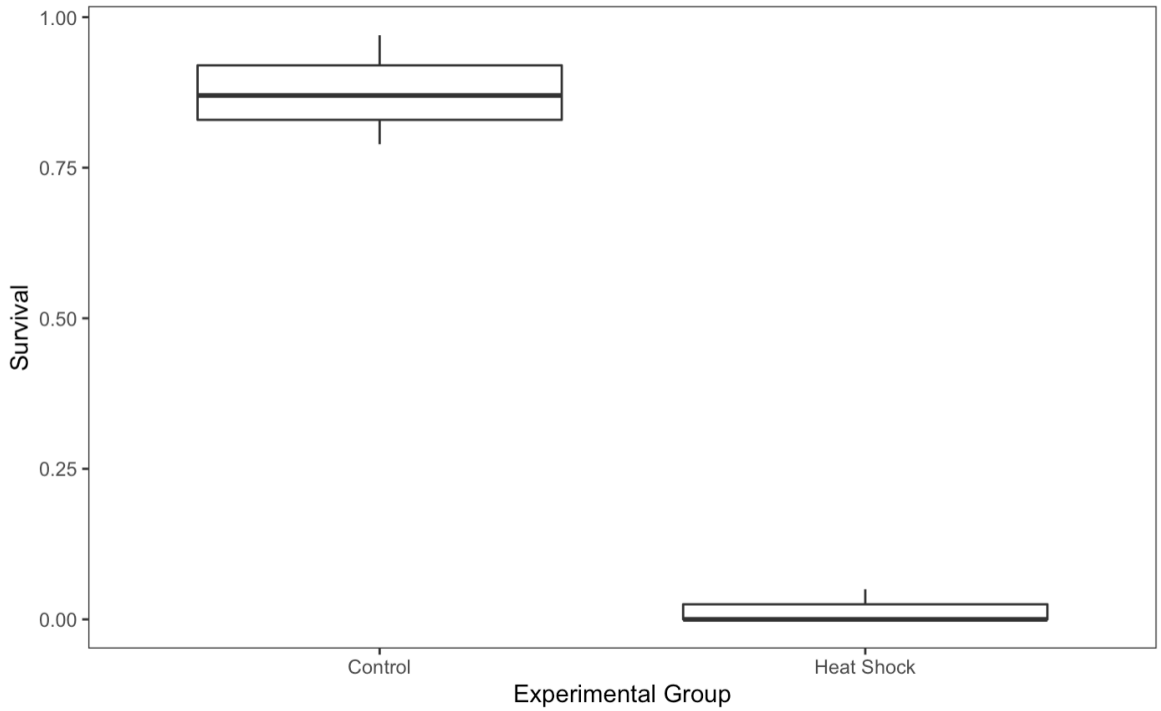

Figure 5. Survival of Heat Shock zebrafish (*Danio rerio*) embryos and Control (non-heat shock) zebrafish embryos at 24 hours post fertilization (hpf). Survival for control embryos was significantly higher than for heat shock embryos at 24 hpf (t = 15.649, df = 4, p-*value* = 9.739e-05).

Experiment 2 – Triploid Identification

Triploid identification using erythrocyte method was unsuccessful, due to lack of survival to hatching stage and red blood cell (rbc) imaging protocol. Red blood cell identification in 14-day old larvae was achieved without replication and seen in Figure 6.

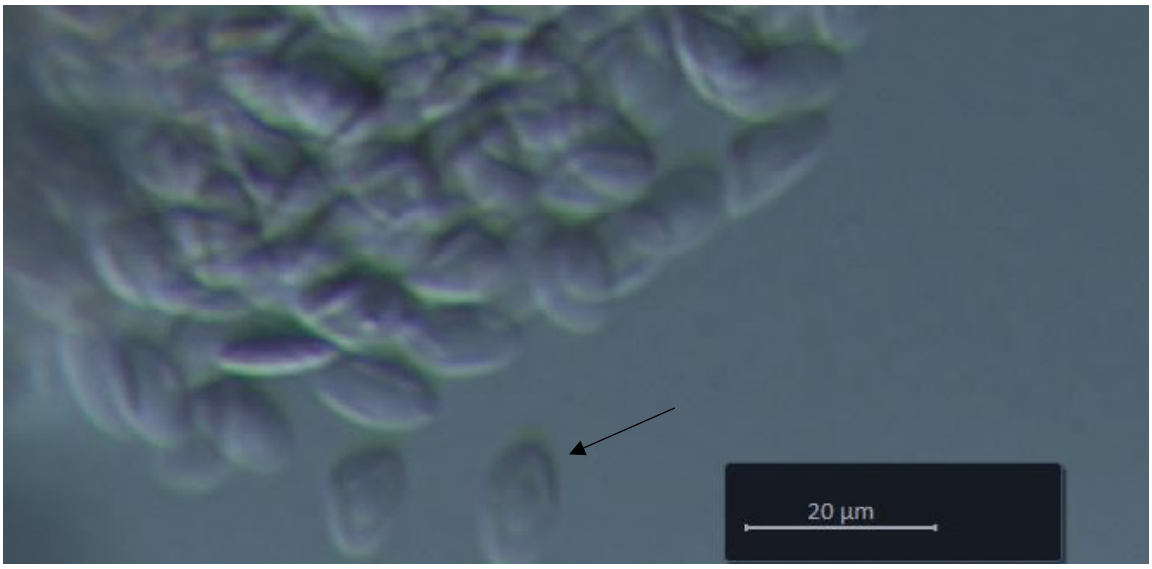

Figure 6. Red blood cells leaving tail laceration in zebrafish (*Danio rerio*). Image captured using ZEN 3.0 for ZEN-pro on the Zeiss SteREO Discovery V.20 microscope (magnification 320.0 x). Single red blood cell indicated with arrow.

Experiment 3 – Larval Morphology Analysis

Zebrafish were classified by body area (mm^2), fin fold area (mm^2) and head area (mm^2). The linear discriminant function analysis yielded 90% accuracy from 15 control (non-heat shock) zebrafish larvae and 5 heat shock larvae 14 dpf (Table 1).

Table 1. Linear discriminant function analysis between control and heat shock zebrafish embryos 14 dpf by body area (mm^2), fin fold area (mm^2) and head area (mm^2) (accuracy = 0.90). Columns specify original data and rows specify LDA predictions. Accurate discrimination of the model is organized by diagonal (top left to bottom right).

Experimental Group	**Control**	**Heat Shock**
Control	14	1
Heat Shock	1	4

Table 1 shows an almost complete discrimination between control and heat shock embryos, with only 2 individuals straying from the prediction. Table 1 indicates that 90% of samples that belong to each group were discriminated by the LDA as belonging to said group. Jackknifed validation was done to identify over-representation in the LDA and produce a more accurate prediction (Table 2).

Table 2. Jackknife prediction of linear discriminant function analysis between control and heat shock zebrafish embryos 14 dpf by body area (mm^2), fin fold area (mm^2) and head area (mm^2) (accuracy = 0.80). Columns specify original data and rows specify Jackknife predictions. Accurate discrimination of the model is organized by diagonal (top left to bottom right).

Experimental Group	**Control**	**Heat Shock**
Control	12	3
Heat Shock	1	4

Table 2 shows a more accurate representation of discrimination by the LDA. A total of 4 individuals straying from the prediction, 80% of individuals accurately grouped based off of the DFA model. Results indicate the difference of 10% between the resubstitution (original LDA model) and the jackknifed validation.

Greatest observed difference between group means was for fin fold area, with a mean difference of 0.3088 mm ($\bar{x}_{control}$ = 0.4199 ± 0.2513, $\bar{x}_{heatshock}$ = 0.7287 ± 0.1519). Figure 7 represents the discrimination between the three morphological measurements taken from the heat shock versus the control larvae.

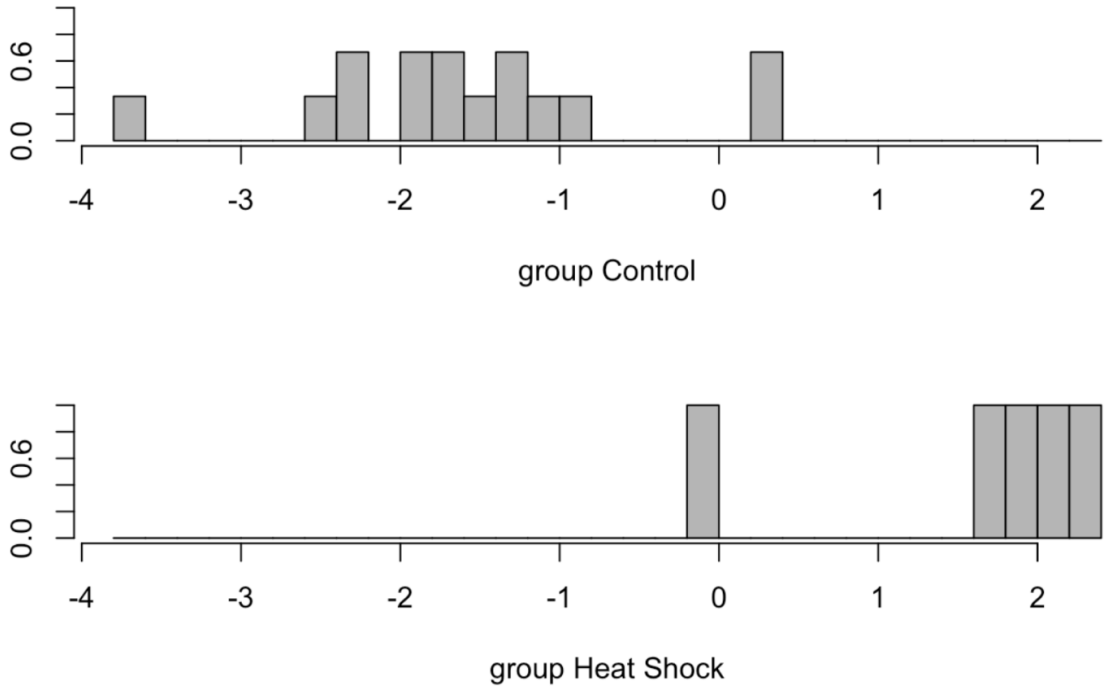

Figure 7. Stacked histogram of DFA scores of measurements taken on 20 zebrafish (*Danio rerio*) larvae 14 days post fertilization. Histogram represents body area (mm^2), fin fold area (mm^2), and head area without eyes (mm^2) for Control and Heat Shock groups. Figure displaying jackknife validation fit for reduced error.

Experiment 4 - Dissociation of Sturgeon Gonad

Dissociation of sturgeon gonad samples was done to isolate gonad precursor cells in a Percoll gradient. Samples from the top layer adhering to the Percoll gradient, and from the 20%, 30%, and 40% Percoll gradient fractions were observed. Shortnose sturgeon (*Acipenser brevirostrum*) gonadal cells were identified most confidently from the top layer and the 20% Percoll fraction in the -10 ºC (in DMSO) per minute group and in the -1 ºC (in DMSO) per minute group (Figure 8). No cells were observed in the 30%

Percoll layer in the -1 ºC per minute group. No cells were observed in the 40% Percoll layer in either of the cooling rate groups.

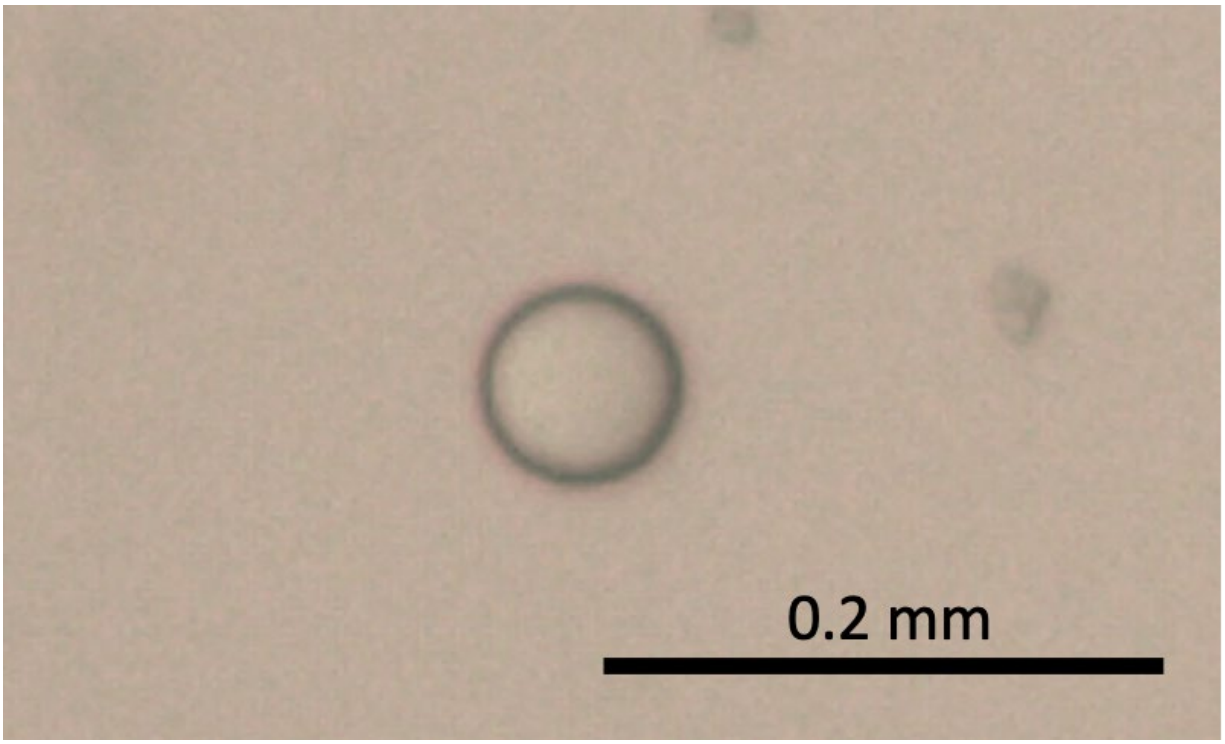

Figure 8. Shortnose sturgeon (*Acipenser brevirostrum*) gonadal cell dyed with trypan blue and captured on Olympus BX40 light microscope (magnification 40.0 x).

Cell diameter (mm) was measured for each sturgeon cell imaged. Cell diameter was only recorded for top layer, 20%, and 30% Percoll gradient fractions as there were no cells observed in the 40% Percoll gradient fraction. An overall total of 10 cells were observed across both cooling rate sample groups. There was a 7:3 ratio of cells observed between the two cooling rates (-10 ºC/min : -1 ºC/min). Average cell diameter from all 10 cells measured was 0.1195714 ± 0.05379264 mm (Figure 9).

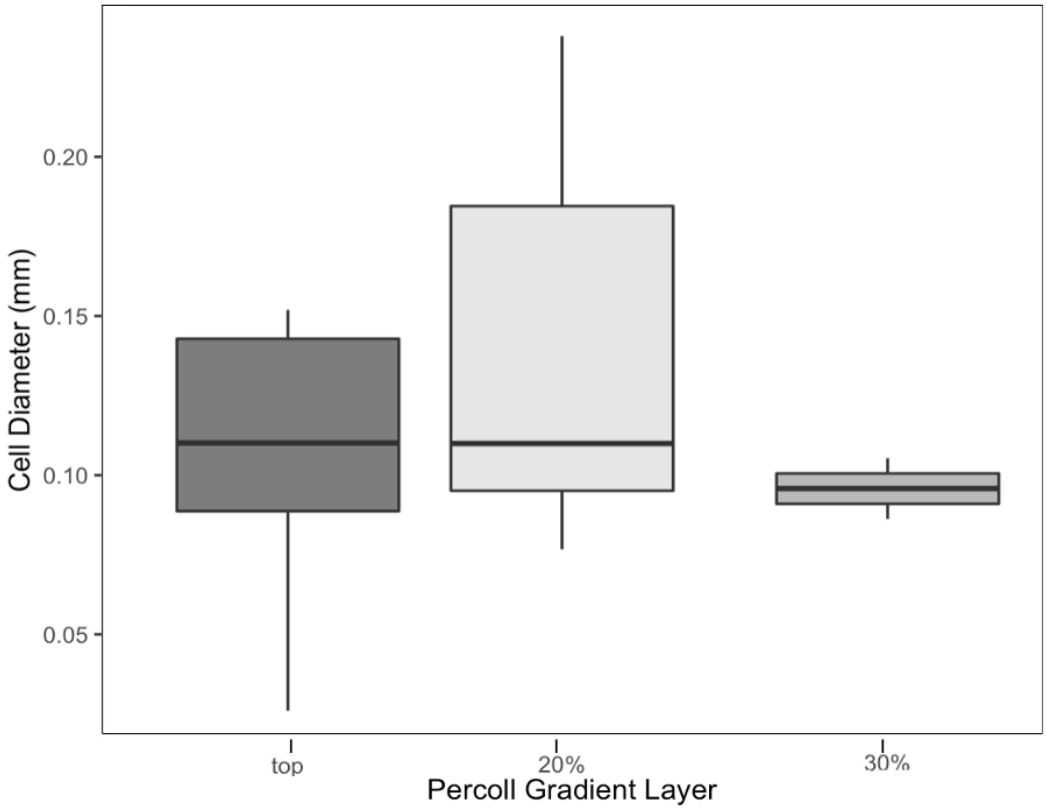

Figure 9. Shortnose sturgeon (*Acipenser brevirostrum*) gonad cell diameters (mm). Cell samples taken from the top, 20%, and 30% Percoll gradient layers from -1 ºC per minute and -10 ºC per minute, both cryoprotected in dimethyl sulfoxide (DMSO). Cell diameter measured using Image J Version 1.51 (100; 2015).

DISCUSSION

Xenotransplantation of gonadal material is a novel technique for conservation of threatened species and has been performed in multiple fish species to date. The process must be modified specifically for different species and for use in different laboratories. The creation of triploid fish for use as microinjection recipients is an essential step in the xenotransplantation model for conservation of endangered sturgeon species. The focus of my study was to create triploid zebrafish (*Danio rerio*) and identify non-lethal methods of ploidy detection. The study was designed to mimic the xenotransplantation model that has not yet been successful for sturgeon in transplanting gonadal material into a teleost recipient. In experiment 1 and 2, I have replicated the protocol for triploid induction using zebrafish. In experiment 3, I identified potential morphological indicators of ploidy in 2-week-old zebrafish larvae. Unfortunately, I was not able to do erythrocyte analysis in order to identify ploidy due to time restraints. Using shortnose sturgeon (*Acipenser brevirostrum*) gonad, I was able to isolate gonad precursor cells using a Percoll gradient. My results have initiated the xenotransplantation model for use in subsequent research in the Litvak Lab.

In experiment 1, I found an overall lower survival rate for the heat shocked zebrafish embryos compared to the non-heat shocked control embryos. Low survival of heat shock embryos was anticipated as previous studies have also documented the decreased survival following a heat shock (Hussain et al. 1991; Benfey 1999; Razak et al. 1999; Byamungu et al. 2001; Pradeep et al. 2012; Franěk et al. 2019). In the most recent literature using zebrafish, heat shock at 2 minutes post fertilization at 41.4 ºC lasting 2 min was identified as the optimal protocol for triploid induction, therefore was chosen for my study (Franěk et al. 2019). My heat shock trials yielded a much lower percent survival at 24 hpf, with the average survival rate of less than 2% with an attempted identical method. Error in my methodology resulting in low embryo survival post heat shock may have been due to the sensitivity of the heat shock to exact timing. Since heat shock must be done within minutes of fertilization of the eggs, there is the potential for possible human error in transferring the eggs in and out of the heat shock water bath. All potential sources of error were avoided but because of the need for extreme accuracy with timing,

a second or two difference in the duration or initiation of the heat shock could yield significantly different results.

Another factor that may have impacted the survival of the embryos is not related to the heat shock, but instead the accuracy of the fertilization. There was trial and error in different fertilization methods, such as manual stripping with manual fertilization, but the method that resulted in the greatest experimental control was the observation of fertilization. The manual stripping method required too much precision and time to be done in an efficient fashion and was harmful to the fish. Additionally, the manual stripping method appeared to not yield viable sperm, as the sperm was pipetted over the eggs to induce fertilization, and no fertilization was identified after performing this method. Eggs that appeared to be fertilized, and mimicked the size, colour, and overall morphology of a fertilized egg were questioned to have been successfully fertilized, as both the heat shock and the control embryos for these trials using the manual stripping method did not survive past the 24-hour stage of development. The similarity between developing embryos at 3 hours post fertilization and the so called "pseudo fertilized" eggs at 3 hours post fertilization was remarkable. Unfortunately, I was unable to find evidence of this phenomenon in other studies, therefore I determined it to be important knowledge to have when rearing zebrafish embryos. Distinguishing between fertilized and unfertilized eggs is important for future studies. In future research, observation of fertilization, rather than manual stripping, is the preferred method to determine time at fertilization.

Triploid identification using erythrocyte numbers and dimensions was unsuccessful due to a lack of heat shock samples and human error in imaging methodology. Due to the fine focus and no stage for movement on the Zeiss SteREO Discovery V.20 microscope, it was very difficult to manipulate the microscope slide containing the red blood cells. Therefore, any small movements changed the image drastically. Thus, experiment 2 yielded only one viable image of red blood cells leaving a fresh tail wound (Figure 4) where cell boundaries and distinct features were identified. Erythrocyte nuclear volume as well as diameter and abundance have been identified as key markers of ploidy level (Benfey et al. 1984; Kavumpurath and Pandian 1990; Pase et al. 2009; Bianchini and Wright 2013; Kim et al. 2017; van de Pol et al. 2020). Due to the

replicated nature and relative success of this method by other researchers, it should be possible to reproduce in the Litvak Lab. Therefore, in future studies inducing and identifying triploids, this method should be applied as a starting point for triploid detection, and subsequent methods of identification should follow. Because of the difficulty in red blood cell imaging, a microscope stage must be added to the Zeiss SteREO Discovery V.20 microscope.

I compared larval morphological parameters to determine if there was discrimination between heat shock and non-heat shock embryos at 14 days post fertilization, that would indicate potential ploidy level. To the best of my knowledge, this was the first study to focus on body area, fin fold area, and head area as the main markers for ploidy identification. While I was not able to determine ploidy, I did find a high degree of discrimination between the heat shock and non-heat shock groups for the three morphological parameters that I tested. From the linear discriminant function analysis that I ran on body area, fin fold area, and head area, there was close to full discrimination between heat shock larvae and non-heat shock larvae. The model was accurate at 80% when analyzed using a jackknifed validation sampling method. These results indicate that the discriminant function created from the linear equation is accurate at discriminating between the heat shock and the control larvae for 80% of the individuals in the sample (n = 20). In future analysis, if these results are replicable, this would be an incredible feat to detection methods of ploidy in zebrafish and potentially other species of fish. Using external body measurements instead of erythrocyte measurements is valuable to fish health as erythrocyte analysis requires invasive techniques and sometimes total fish sacrifice. To determine if morphometric analysis is successful at detecting ploidy, the experiment will need to be replicated with greater numbers and have an established method of ploidy detection to compare the results to, such as erythrocyte analysis. Establishing a protocol for non-invasive ploidy detection for zebrafish and other fish is essential to triploid induction and identification research.

Experiment 4, the dissociation and isolation of sturgeon gonad and gonadal cells, is critical to the xenotransplantation model, as it was the only experiment in this study focusing directly on sturgeon. Cells from the sturgeon were able to be isolated and identified from the reanimated gonad samples. Due to the difficulty of sexing sturgeon, it

was not 100% confirmed that the sturgeon gonad samples were from a female fish. After a histological analysis, the results were more closely related to the literature on male shortnose sturgeon (Hartlen 2021). Use of geometric morphometrics as well as external and internal indicators of sex were also used, but due to time restraints were not fully applied to determine sex (Balazadeh and Litvak 2018). Regardless, the implication from my experiment is the future use of a protocol that isolated sturgeon cells which is essential to replicate in order to move on to the microinjection stage of the xenotransplantation model. I found that the cells colonized the top three layers of the 7-layer Percoll gradient after being centrifuged. This is a very important finding as it tells me that cells are able to be isolated using this method, and it may be applied to isolate female sturgeon gonad cells, or oogonial cells. My results from running the Percoll gradient cell isolation method are similar to the results from Wong et al. (2011), where zebrafish oogonial cells, otherwise known as ovarian germ cells, were observed in the 25%–30% and 30%– 35% Percoll fractions. When using 5 female zebrafish ovaries, an average of 755 ± 108 ovarian germ cells were recovered from each female and the majority of the cells were obtained from 30%–35% (364 ± 44 cells) and 25%–30% (247 ± 48) (Wong et al. 2011). My methodology was adapted from this work for use with sturgeon gonadal material, and it is interesting that an almost identical method was somewhat successful for a species that is not closely related to zebrafish. Therefore, this method is pertinent to the isolation of ovarian germ cells in many fish species, not only zebrafish and shortnose sturgeon, but may need adjustments depending on fish size and taxonomy.

Sturgeon are the most endangered vertebrate group in the world (IUCN 2016). It is critical to gather information on physical, chemical, and biological parameters needed to rear sturgeon in facilities in order to augment natural populations (COSEWIC 2015). Along with these abiotic and biotic factors, researchers have developed many protocols for conservation strategies such as cryopreservation, transplantation, genetic analyses, tagging and marking, and many more modern approaches. Conservation of shortnose sturgeon and investigation of cryopreservation and xenotransplantation strategies has been proposed. Currently, the Litvak Lab is developing optimal sturgeon oogonial cell dissociation measures along with methods of cryopreserving oogonial cells for use in later

transplantation (Wade 2020; Hartlen 2021). The goal of my study was to create triploid zebrafish embryos using an optimized heat shock protocol as well as determine success of triploid induction. My goal was also to explore larval morphology as a source of ploidy determination. My final research goal was to dissociate shortnose sturgeon gonad and isolate gonadal cells using a Percoll gradient. From this study, I was able to create heat shock larvae that survived past the 2-week stage of development. I was not able to determine ploidy of the larvae though the larval morphology analysis did show a discrimination between the heat shock and the non-heat shock larvae. From the gonad dissociation and Percoll Gradient, I was able to isolate shortnose sturgeon cells from the tissue sampled. Because of the uncertainty of sex of the sturgeon, I could not identify whether or not the cells were oogonial cells. Overall, my study is important in laying the groundwork for future triploid induction through heat shock in the Litvak Lab. Future projects focus on blue tilapia (*Oreochromis aureus*) triploid surrogates for transplantation of shortnose sturgeon oogonial cells. My results indicate that this method can be performed in the Litvak Lab and that there are measures to identify new ways of non-invasive detection of ploidy in larval fish. Without the creation and verification of viable triploid surrogates, the xenotransplantation model cannot progress. Therefore, my study is pertinent to advancing this work in preparation for microinjection to triploid embryos. The Percoll gradient can be applied to connect the xenotransplantation model by isolating the cells to be microinjected into the triploid embryos. This entire process is essential to the creation of germline chimeras using sturgeon germ cells and tilapia surrogates. The innovation of a technique for preservation of sturgeon germplasm through production of gonads in a host teleost fish is essential in advancing the research on xenotransplantation for endangered sturgeon, as this has not yet been done. Researchers need to invest in working with endangered species to create protocols for augmenting populations and protecting those that still exist. Due to the large negative impact that anthropogenic factors have posed on sturgeon biodiversity, we need to become aware of our actions and to work towards saving the irreplaceable and ancient lineage representatives that we still have on our planet.

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APPENDIX

Representative images

Due to the repeated use of imaging for analysis of different zebrafish embryo and larval traits, I have included multiple representative images for insight into experiments 1, 2 and 3. In experiment 1, I calculated percent survival for the heat shock embryos versus the non-heat shock embryos. Determining embryo survival was done by observation of decomposition or embryo death. Embryos that appeared cloudy or yellow in colour were determined dead (Figure A1).

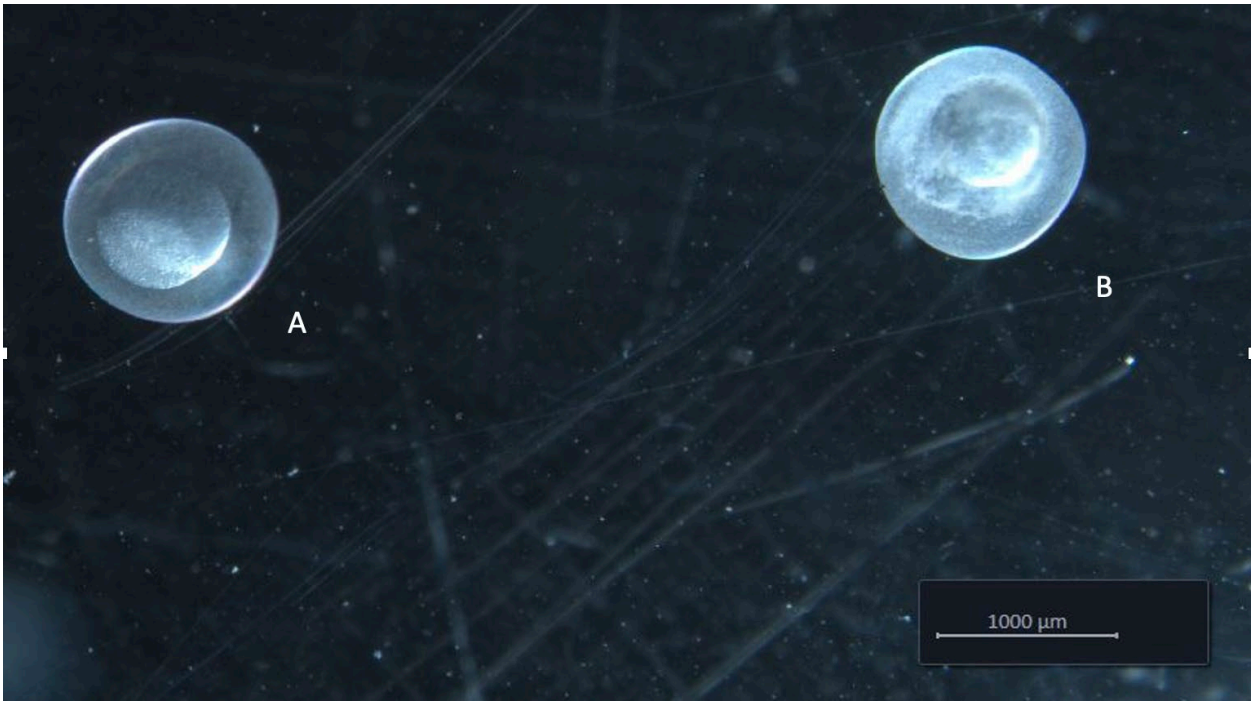

Figure A1. Zebrafish embryos 3 hours post fertilization larvae imaged using ZEN 3.0 ZEN-pro system for the Zeiss SteREO Discovery V.20 microscope (magnification 2.3 x). A) Live embryo. B) Dead/non-viable embryo.