Cryopreservation of blue catfish stem cells for conserving genetic resources and xenogenic transplantation

By

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A dissertation submitted to the Graduate Faculty of Auburn University
in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

Auburn, Alabama May 02, 2020

Keywords: Cryopreservation; Selective Breeding; Xenogenesis; Germplasm; Germ cell

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Abstract

In the United States, channel catfish (*Ictalurus punctatus*) female × blue catfish (*I. furcatus*) male hybrids now account for 70% of total freshwater aquaculture production. Hybrid fry production can be improved by cryopreserving valuable male and female gamete resources from blue catfish. Our long-term goal is to develop cryopreservation protocols for spermatogonial and oogonial stem cells (SSCs and OSCs) for gene banking and xenogenesis applications, which will increase gamete availability, improve options for genetic improvement of blue catfish, and increase hybrid fry output. Stem cells frozen in germplasm repositories would help facilitate this initiative beyond traditional cryopreservation of sperm and eggs. The effects of using various permeating and nonpermeating cryoprotectants, concentrations, freezing rates, antioxidants, and antifreeze proteins (AFP) on post-thaw live cell production and viability of both blue catfish SSCs and OSCs were examined. The choice of permeating or non-permeating cryoprotectant notably impacted post-thaw SSC and OSC production and viability. By using the top-performing cryoprotectant at its target concentration for each cell type, the quantity of live cells after thawing greatly increases. The best freezing rate was -1 °C/min overall, and post-thaw production and viability decreased at rates of -2.5, -5, and -10 °C/min. Combining antioxidants and antifreeze proteins by adding hypotaurine with either AFPI 1.0 µg/mL or AFPIII 0.1 µg/mL to the cryomedia maximizes the number of live SSCs after freezing. Together, these findings show that catfish SSCs and OSCs can be successfully frozen for future cell transplantation into channel catfish or other hosts. This may become a very valuable resource for catfish genetic enhancement programs and hybrid catfish aquaculture.

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Chapter 1- General Introduction

1.1. The growing aquaculture sector

Aquaculture, the breeding of fish stocks and other aquatic animals in captivity for commercial or conservation purposes, has been implemented for more than 220 species of finfish, shellfish, crustaceans, and other organisms of economic value (Naylor et al., 2000). Captive fisheries are a growing sector of the global food economy as market demands for fresh fish and seafood continue to grow but cannot be supplemented from wild fisheries alone (Brander 2007, FAO 2018). In fact, a majority of all aquatic protein consumed now originates from commercial hatcheries instead of rivers and oceans. Global seafood consumption has reached 155 billion kg (Ritchie & Roser, 2020) and is projected to spike by another 50 billion kg in the upcoming decade (FAO, 2018). Aquaculture is becoming more prominent than ever on the global stage as new species are being established in captivity and advancements arise in how these organisms are cultured.

Hatchery environments present fish with vastly different conditions than those encountered in the wild and require them to adapt accordingly. This transition may ultimately lead to domestication (both intentionally and unintentionally) in which successive generations start exhibiting new traits that help them thrive in a captive setting (Teletchea & Fontaine, 2014). Domesticated fish stocks usually have genetic traits that are dissimilar from the wild-type stocks of the same species (i.e. differences in growth rates, disease resistance), making them more adapted to hatchery environments (Lorenzen et al., 2012). Benefits that abound from domestication and selective breeding can be taken advantage of to improve these traits even faster and in a specific direction with each new generation. Such a revelation has been responsible for increased aquaculture expansion across the world.

The genetic makeup of aquatic organisms presents several advantages over terrestrial species for genetic improvement and selection programs (Dunham, 2011). Firstly, the fecundity in most fishes is very high and time to maturation short (Murua & Saborido-Rey, 2003), meaning that genetic change can happen at a much faster rate than for low-fecundity mammals. The fertilization process can be manually controlled within hatcheries in order to choose specific broodstock, the timing of spawning, and number of fry that are produced (Dunham et al., 2008). Since the generation of most fish embryos occurs via external fertilization (sperm and egg come into contact outside the body cavity of the female), this allows for a high potential for genetic manipulation when all mating individuals can be hand selected (Dunham, 2011). Crossing specific males and females with desired genotypic or phenotypic traits strategically generates fish stocks that are superior to their wild type counterparts or even the previous generation in terms of growth, feed conversion, disease resistance, fertility rates, and other desirable traits (Dunham, 2011; Lorenzen et al., 2012), making it a powerful tool for aquaculture advancement.

1.2 Hybrid catfish industry in the United States

Catfish are a diverse group found in many regions of the world, encompassing multiple families and individual species (Lundberg et al., 2003). Similar to other aquaculture sectors, catfish aquaculture has shown significant growth worldwide (especially in Asian countries) since 2005 (Fig. 1.1). In the southern US, commercial farming of channel catfish began in the 1930s with channel catfish reared in warmwater earthen ponds. Channel catfish are well adapted to the environmental conditions of this region, and historically, they have been broadly accepted in local markets (Dunham & Smitherman, 1983). The expanding growth of the catfish industry started in the 1960s and was rapidly developing by the 1980s as more farms were

established and communication between these farms took hold.

Production yields then remained fairly constant for the next three decades (FAO, 2018). In 2007, the first major economic downturn for the industry occurred. The US catfish industry struggled with high production costs following the national recession and had to cope with increasing cost of fish feed and fuel as well as growing international competition.

Inexpensive imported catfish from countries such as Vietnam and China resulted in the production yield to decrease in 2012 (Shang, 2013; www.nass.usda.gov, 2012), and now Vietnam supplies 70% of the catfish fillets consumed in the US. However, implementations by the USDA in recent years are helping to make the US more competitive by reducing these foreign imports, causing the industry to rebound (NOAA, 2018).

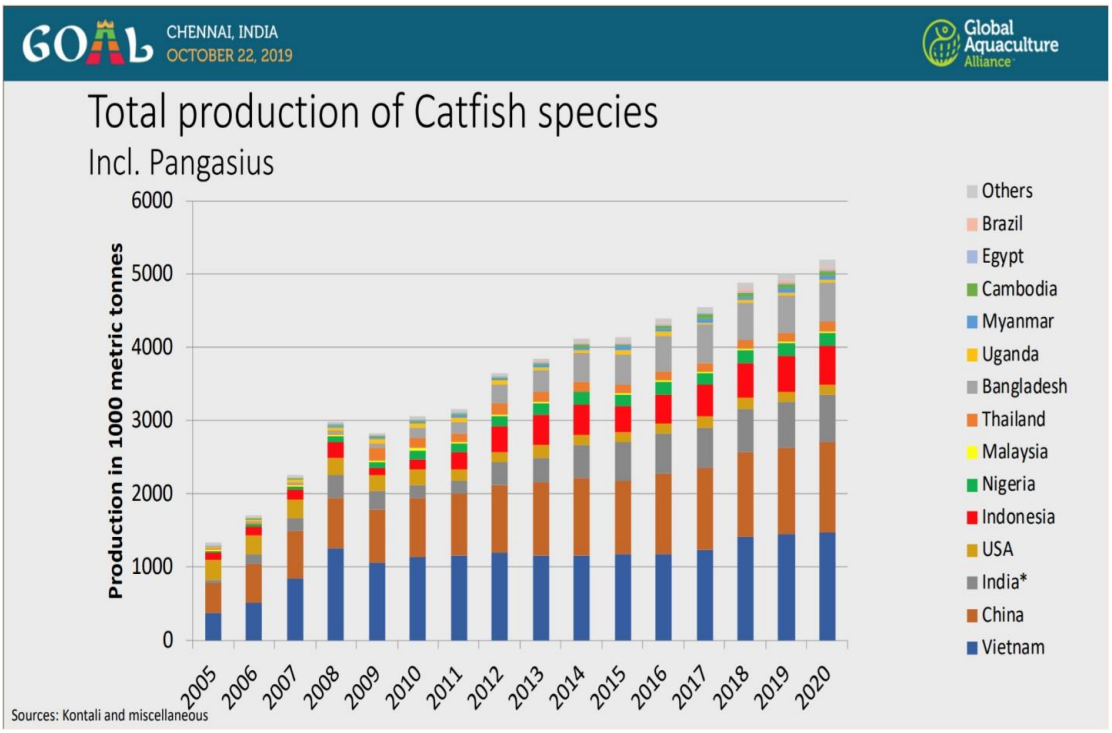


Fig. 1.1 Total production of catfish species worldwide from 2005-2020. Courtesy of Global Aquaculture Alliance 2019, https://www.undercurrentnews.com/2019/10/29/goal-survey-expects-major-farmed-finfish-species-to-smash-40m-metric-tons-in-2020/

Economic setbacks have prompted catfish farmers and researchers to assess how and where improvements can be made. Improving culturing techniques and technologies has always been a reliable method to increase production efficiency and maintain profitability, but the next major breakthrough is needed. Over the past 40 years and according to reports by the NASS, US catfish production per unit area has increased 5-fold (Fig. 1.2). Approximately, 50% of this increase is due to genetic enhancement of catfish growth and survival at Auburn University.

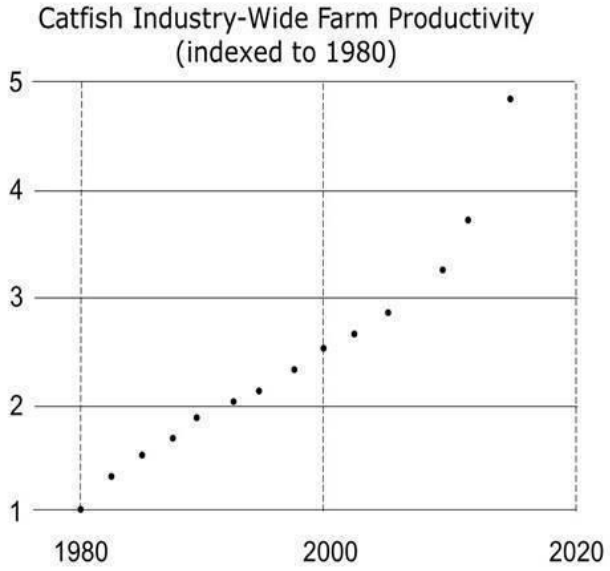


Fig. 1.2 – Production of catfish in the US from 1980-2020, showing continual increase until 2014 and then a large spike in production in the most recent years. Such large increases are partly attributable to the growth of hybrid catfish aquaculture (Craig Tucker, USDA, personal communication, data generated from annual USDA NASS reports)

Selection creates better-performing parental lines and families within a species, which can help to create an overall better hybrid (Dunham, 2011). Interspecific hybridization is a process where two distinct species are crossed with the objective of producing a hybrid superior to either parent (Dunham & Masser, 2012). Hybridization has been thoroughly examined among several species of catfishes with minimal success. Of over 40 crosses examined in the 1960s, only the channel catfish (*Ictalurus punctatus*) ♀ × blue catfish (*I.*

furcatus) ♂ hybrid (Fig. 1.3) stood out as having market potential (Giudice, 1966; Dunham and Smitherman, 1983; Dunham, 2011). This interspecific hybrid has a variety of desirable traits such as high production, increased carcass yield, better feed conversion, uniformity in size and shape, disease resistance, survival, tolerance to low oxygen levels, high dressout percentage, increased seinability, and the ability to grow under high density conditions (Dunham and Smitherman, 1983; Dunham and Argue, 1998; Dunham and Brummett, 1999; Argue et al. 2003; Dunham and Masser, 2012; Argue et al. 2014). Because this fish is so well adapted for pond culture, most commercial farms have now switched from culturing channel catfish to hybrid catfish to maximize their profit margins (Torrans and Ott, 2018). Therefore, generation of hybrid catfish is essential for the survival and growth of the catfish industry in the years to come if the U.S. is to become more competitive in world production markets (Dunham 2011).

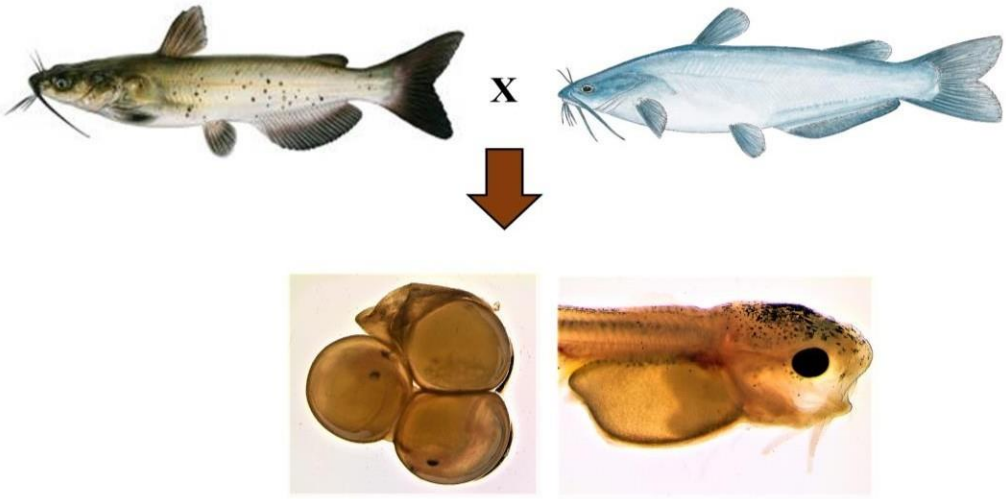


Fig. 1.3 Crossing channel catfish (*Ictalurus punctatus*) ♀ × blue catfish (*I. furcatus*) ♂ creates hybrid fry that show increased performance compared to either parent species.

1.3 Limitations of hybrid catfish production.

Previously, the main obstacle that constrained commercial production of hybrid catfish was the lack of robust, cost effective methods for acquiring reliable and sustainable quantities of fry (USDA, 2011). For large-scale production, relying on traditional pond spawning was not an option because the two parent species do not mate naturally (Hu et al., 2011). Reproductive barriers including different behavioral and psychological traits prevent hybrid fry from being produced in ponds the same way as channel catfish (Tucker et al., 1990; Hu et al., 2011). Induced spawning in pens is an option, but the results are too inconsistent for commercialization (Dunham et al. 2000, Rex Dunham, Auburn University, personal communication). Thus, artificial fertilization (or in-vitro fertilization (IVF)) must be employed in which all aspects of spawning are controlled. Technology has been developed that allows commercial-scale production of hybrid catfish embryos using artificial fertilization, causing their production capacity to increase tremendously over time (Su et al., 2013) (Fig 1.4).

Artificial reproduction is carried out by inducing ovulation in the channel catfish females by hormonal treatment followed by hand stripping of the eggs (Tave & Smitherman 1982; Dunham et al. 2000). Shortly prior to this, male blue catfish are euthanized, and the testes are harvested for sperm preparation (Dunham & Masser, 2012). IVF is then carried out by manually mixing the gametes together and incubating the hybrid catfish embryos. This process starts anew each season with the individual parent species since the F1 generation cannot successfully continue the reproduction cycle (Dunham & Argue, 2000). When weighing the costs and benefits, IVF requires much resources, specialized personnel and labor, but it remains the most effective way to produce fish that do not readily breed in captivity (www.fao.org, 1984). Improvements in IVF techniques and strategies have made it a reliable method for spawning a variety of hatchery species (Cabrita et al., 2008). Some

methodologies that have been evaluated for ictalurid catfish are hormonal administration for the females (Su et al., 2013) and sperm extraction/processing of the males (Dunham & Masser, 2012). Overall, the most limiting factor of reproduction comes from the paternal contribution. The male blue catfish must be cultured alongside channel catfish in hatcheries to provide the much-needed sperm each season. However, the blue catfish takes longer to sexually mature (at 4 to 6 years of age post-hatching) than the female channel catfish (at 2 to 4 years; Dunham et al., 1994). Another disadvantage is that the males can only be used once, making each male's sperm quite valuable. More feed resources, labor, and space must be provided to accommodate these males, so it is essential that these males provide high-quality sperm. Best-performing sperm from individual males could transcend a single fertilization event with successful gamete cryopreservation and creation of genetic repositories. Of course, the other main drawback of the current technology is the intensive labor input.

1.4 Xenogenesis technology

Another solution for tackling the paternal limitations within the hybrid catfish industry is to obtain gametes from both males and females in the form of stem cells. By extracting the non-committed germ cells from the parent individuals, they have several potential applications such as gene banking, genetic enhancement programs, cell transplantation, and xenogenesis. Xenogenesis is a method of reproduction in which successive generations differ from each other, and xenogenic organisms acquire genetic traits that are not observed in its parents of origin (Dunham, 2011; Higuchi et al., 2011).

Xenogenesis is carried out by the isolation of primordial germ cells (PGCs), spermatogonial stem cells (SSCs) or oogonial stem cells (OSCs). These cells are transplanted into a host organism so that the host matures normally but develops the ovaries or testes of the donor

fish (Shang et al., 2018). Xenogenesis is still an emerging technology and has not yet been as widely investigated for fishes as it has in terrestrial mammals (Lacerda et al. 2010), but it

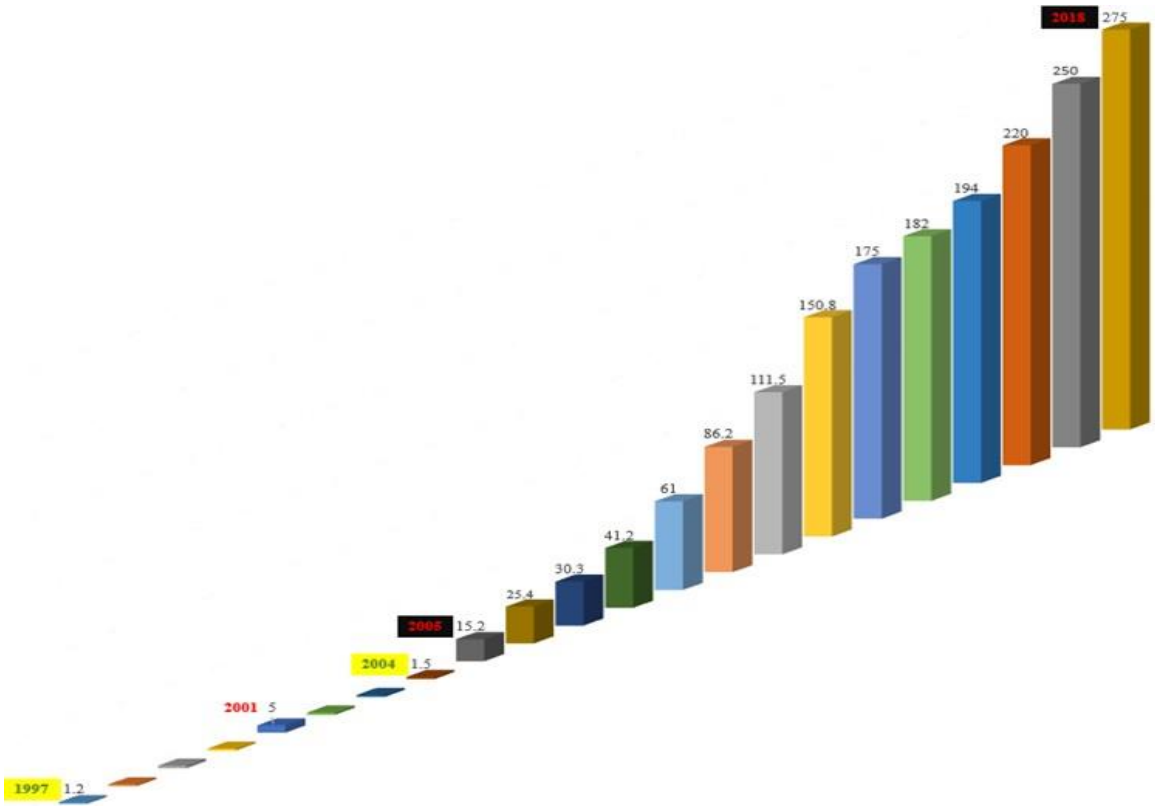


Fig. 1.4. Channel catfish (*Ictalurus punctatus*), female x blue catfish (*I. furcatus*) male hybrid embryos were commercially produced from 1997-2018 in the United States. In 1997, there were 1.2 million hybrid embryos produced, but the production of hybrid embryos remarkably increased 230 times more than the production in 1997, reaching 275 million embryos in 2018 (figure courtesy of Dr. Nagaraj Chatakondi).

offers a promising outlet for aquaculture advancement, especially in regard to development of transgenic fish (Shang et al., 2018). Stem cell transplantation is being utilized to generate gametes from fish that have longer developmental times or are otherwise inconvenient to keep in the hatchery environment within the body of a different host species (Franěk, 2019). For blue catfish, specifically, transplanting PGCs from blue catfish into channel catfish before gonadal development is beneficial because of the host's accelerated growth rates, earlier age of sexual maturity, and more convenient husbandry requirements. Blue catfish

gametes (which are more costly and time-consuming to obtain) then become more easily assessible for hybrid catfish production (Shang et al., 2018).

Transplantation of these extracted germ cells is a flexible process that can be done during different developmental stages of the host. After transplantation, cells migrate and colonize the gonads, eventually forming mature gametes from the donor species (Lacerda et al., 2013). Many different methods have proven to be successful in fishes. Takeuchi et al. (2004) accomplished xenogeneic transplantation of primordial germ cells (PGCs) isolated from rainbow trout (*Oncorhynchus mykiss*) fry into newly hatched larvae of masu salmon (*Oncorhynchus masou*), which were later able to generate functional sperm of the donor rainbow trout. Another method involves the transplantation of primordial cells into blastula by micromanipulation techniques. This involves the harvesting of primordial cells from donor embryos and transplanting these cells into blastulae for which endogenous primordial germ cell development was disabled with dead-end morpholino antisense oligonucleotides (Saito et al., 2008; Yoshizaki et al., 2019). Shang et al. (2018) attempted to transplant primordial germ cells into the blastulae of triploid channel catfish embryos to produce interspecific xenogenic catfish and obtained 33% success. Xenogenesis can also be performed by transplanting primordial germ cells into the gonads of juvenile or adult sexually mature fish (Lacerda et al., 2013). As one relevant example of this method, Perera et al. (2017) fertilized channel catfish eggs with sperm from triploid channel catfish males possessing blue catfish germ cells introduced when they were sub-adults. The authors reported a 100% success rate that hybrid progeny had the desired DNA profile from the donor fish (Perera et al. 2017). Success rate of xenogenesis techniques is variable given the fact that some fish have extremely small gonads and, as such, it is difficult to isolate the genital ridges surgically from small larvae and implant PGCs (Ye et al., 2019).

Despite its disadvantages, xenogenesis research is becoming more widely recognized

in the eyes of researchers and industry stakeholders for its potential in producing blue catfish gametes more efficiently. Overcoming the bottleneck associated with gamete availability is the next big hurdle to push hybrid aquaculture forward. On a broader scale, xenogenesis may be a more efficient method to propagate embryos for a wide variety of economically important species with applications for aquaculture, conservation, and biological research. Additionally, the benefits of germ cell transplantation can be taken further if PGCs are readily available within cryogenic gene banks (Lee et al., 2013; Franěk et al., 2019).

1.5 Cryopreservation of primordial germ cells

Cryopreservation has several benefits and has been recognized for its importance in maintaining everlasting gene banks and gamete supply for agriculture and aquaculture (reviewed by Mandawala et al., 2016). This technology simplifies broodstock management by requiring less live fish to be housed at commercial farms at a given time. It also facilitates transport of gametes from different fish farms, allowing gamete storage for genetic selection or conservation of endangered species (Cabrita et al., 2010). For aquatic animals, cryopreservation technology was first introduced to cryopreserve fish sperm. Freezing protocols have been utilized for many species with extensive studies on salmonids and cyprinids due to their high commercial value and recreational purposes (Martínez-Páramo et al., 2017). Testes tissues and sperm cells have already been successfully cryopreserved in several freshwater and marine fish species (Suquet et al., 2000; Rathore et al., 2016). There have been multiple accounts that high fertilization and hatch can be achieved using cryopreserved sperm, and thus, many hatcheries already maintain their own sperm banks. Preserving sperm from valuable males is an excellent tool for genetic enhancement.

Despite current advances in cryopreservation technology, the successful freezing of oocytes has not yet been accomplished, preventing it from being commercially applied in hatcheries (reviewed by Asturiano et al., 2017). Preserving maternal resources for the next generation remains a limitation since propagation continues to rely solely on fresh eggs. This dependence is not ideal for species with low fecundity and short reproduction windows. For researchers conducting fertility experiments in fish, it creates major restrictions if experiments can only be carried out when females are actively producing eggs. Since oocytes are so susceptible to cryo-damage and have little to no viability post-thaw (Zhang et al., 2009), cryopreserving oogonial stem cells (OSCs) from the female may overcome this issue (Yoshizaki et al., 2018). Several studies have already reported generating functional sperm from spermatogonia stem cells (SSCs) cryopreserved from whole testes in fish, and authors of these studies are quite optimistic of the future directions such research could take (Lee et al. 2013; Lee et al. 2014). By successfully freezing stem cells from both the males and females, selection can occur from both ends of the parental spectrum. Since these cells can differentiate into either functional ovaries or testes once implanted in the host organism, they can have enormous potential and versatility.

Nevertheless, successful cryopreservation of SSCs, OSCs, or any tissue is highly dependent on a multitude of specific factors within each protocol. “One size does not fit all”, and it is crucial to optimize cryopreservation protocols for each species and cell type (Elliot et al., 2017; Magnotti et al., 2018). Because cryopreservation inevitably puts stress on the cells and puts them at risk, reducing intracellular damage during deep freezing is the best way to ensure viable live cells survive for downstream applications (Muchlisin, 2005).

Cryopreservation often involves adding permeating and non-permeating cryoprotectants to maintain biological integrity during the freezing process. Additionally, optimizing factors such as freezing and thawing rates and additives to the cryo-media can cause significant

impacts on overall cryopreservation success (Cabrita et al., 2010). These important details for SSC and OSC protocols in fishes are sorely lacking, and thus, widescale cryopreservation of these cell types have not yet been possible. With interest in xenogenesis and germ cell transplantation technologies rapidly evolving, optimizing a cryopreservation protocol for stem cells of important species is essential.

1.6 Objectives

The objective of the following studies was to develop a cryopreservation protocol for OSCs and SSCs of blue catfish using several targeted approaches. For each cell type, different permeating and non-permeating cryoprotectants as well as their concentrations were assessed to identify which combinations yield the highest post-thaw viability for SSCs and OSCs after cryopreservation. Different freezing rates were also paired with the top- performing cryoprotectants. As a final step, antioxidants and anti-freeze proteins were applied to the best treatments from the prior analyses to evaluate their efficacy. Combining cryopreservation protocols with xenogenesis technology will broaden our understanding of long-term cell storage, germ cell transplantation, and xenogenesis outcomes. Specifically, this research will help to surmount the obstacles associated with shortages in blue catfish gametes, which may improve sustainability for the hybrid catfish industry.

References

Argue, B. J., Kuhlers, D. L., Liu, Z., & Dunham, R. A. (2014). Growth of channel catfish (Ictalurus punctatus), blue catfish (I. furcatus), and their F1, F2, F3, and F1 reciprocal backcross hybrids in earthen ponds. *Journal of Animal Science*, *92*(10), 4297–4305. doi: 10.2527/jas.2013-7549

Argue, B. J., Liu, Z., & Dunham, R. A. (2003). Dress-out and fillet yields of channel catfish, Ictalurus punctatus, blue catfish, Ictalurus furcatus, and their F1, F2 and backcross hybrids. *Aquaculture*, *228*(1-4), 81–90. doi: 10.1016/s0044-8486(03)00245-x

Asturiano, J. F., Cabrita, E., & Horváth, Á. (2017). Progress, challenges and perspectives on fish gamete cryopreservation: a mini-review. *General and comparative endocrinology*, *245*, 69-76.

Brander, K. M. (2007). Global fish production and climate change. *Proceedings of the National Academy of Sciences*, *104*(50), 19709-19714.

Cabrita, E., Robles, V., & Herráez, P. (Eds.). (2008). *Methods in reproductive aquaculture: marine and freshwater species*. CRC press.

Cabrita, E., Sarasquete, C., Martínez-Páramo, S., Robles, V., Beirão, J., Pérez-Cerezales, S., & Herráez, M. P. (2010). Cryopreservation of fish sperm: Applications and perspectives. *Journal of Applied Ichthyology*, *26*(5), 623–635. https://doi.org/10.1111/j.1439-0426.2010.01556.x

Dunham, R. A., Smitherman, R. O., & Webber, C. (1983). Relative Tolerance of Channel X Blue Hybrid and Channel Catfish to Low Oxygen Concentrations. *The Progressive Fish-Culturist*, *45*(1), 55–57. doi: 10.1577/1548-8659(1983)45[55:rtocxb]2.0.co;2

Dunham, R. A., & Argue, B. J. (1998). Seinability of Channel Catfish, Blue Catfish, and Their F1, F2, F3, and Backcross Hybrids in Earthen Ponds. *The Progressive Fish-Culturist*, *60*(3), 214–220. doi: 10.1577/1548-8640(1998)060<0214:soccbc>2.0.co;2

Dunham, R. A., & Brummett, R. E. (1999). Response of Two Generations of Selection to Increased Body Weight in Channel Catfish,Ictalurus punctatus, Compared to Hybridization with Blue Catfish, I.furcatus, Males. *Journal of Applied Aquaculture*, *9*(3), 37–45. doi: 10.1300/j028v09n03_03

Dunham, R. A., & Argue, B. J. (2000). Reproduction among Channel Catfish, Blue Catfish, and Their F1and F2Hybrids. *Transactions of the American Fisheries Society*, *129*(1), 222–231. doi: 10.1577/1548-8659(2000)129<0222:raccbc>2.0.co;2

Dunham, R. A. (2011). *Aquaculture and fisheries biotechnology genetic approaches*. Wallingford: CABI

Dunham, R. A., and Masser, M. P. 2012. Production of hybrid catfish (Vol. 436). Stoneville, Mississippi, USA: Southern Regional Aquaculture Center.

Elliott, G. D., Wang, S., & Fuller, B. J. (2017). Cryoprotectants: A review of the actions andapplications of cryoprotective solutes that modulate cell recovery from ultra-low temperatures. *Cryobiology*, *76*, 74-91.

FAO (Food and Agriculture Organization of the United Nations). 1984. The artificial propagation of warm-water finfishes: A manual for extension. URL http://www.fao.org/3/ac742e/AC742E00.htm

FAO (Food and Agriculture Organization of the United Nations). 2018. The state of world fisheries and aquaculture: meeting the sustainable development goals. URL http://www.fao.org/3/i9540en/i9540en.pdf (accessed May 2019).

Franěk, Roman, et al. "Cryopreservation and Transplantation of Common Carp Spermatogonia." *PLOS ONE*, vol. 14, no. 4, Public Library of Science, Apr. 2019, p. e0205481, https://doi.org/10.1371/journal.pone.0205481.

Giudice, J. J. (1966). Growth of a Blue X Channel Catfish Hybrid as Compared to its Parent Species. *The Progressive Fish-Culturist*, *28*(3), 142–145. doi: 10.1577/1548-8640(1966)28[142:goabxc]2.0.co;2

Green, B. W., & Engle, C. R. (2004). Growth of stocker channel catfish to large market size in single-batch culture. *Journal of the World Aquaculture Society*, *35*(1), 25–32. https://doi.org/10.1111/j.1749-7345.2004.tb01056.x

Higuchi, K., Takeuchi, Y., Miwa, M., Yamamoto, Y., Tsunemoto, K., & Yoshizaki, G. (2011). Colonization, proliferation, and survival of intraperitoneally transplanted yellowtail Seriola quinqueradiata spermatogonia in nibe croaker Nibea mitsukurii recipient. *Fisheries Science*, *77*(1), 69–77. https://doi.org/10.1007/s12562-010-03147

Hu, E., et al. "High-Throughput Cryopreservation of Spermatozoa of Blue Catfish (Ictalurus Furcatus): Establishment of an Approach for Commercial-Scale Processing." *Cryobiology*, 2010/12/19, vol. 62, no. 1, Feb. 2011, pp. 74–82, doi:10.1016/j.cryobiol.2010.12.006.

Lacerda, S. M., Batlouni, S. R., Costa, G. M., Segatelli, T. M., Quirino, B. R., Queiroz, B. M., ... & Franca, L. R. (2010). A new and fast technique to generate offspring after germ cells transplantation in adult fish: the Nile tilapia (Oreochromis niloticus) model. *PloS one*, *5*(5).

Lacerda, S. M. S. N., Costa, G. M. J., Campos-Junior, P. H. A., Segatelli, T. M., Yazawa, R., Takeuchi, Y., … França, L. R. (2013). Germ cell transplantation as a potential biotechnological approach to fish reproduction. *Fish Physiology and Biochemistry*, *39*(1), 3–11. https://doi.org/10.1007/s10695-012-9606-4

Lee, S., Iwasaki, Y., Shikina, S., & Yoshizaki, G. (2013). Generation of functional eggs and sperm from cryopreserved whole testes. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(5), 1640–1645. https://doi.org/10.1073/pnas.1218468110

Lee, Y. A., et al. "Cryopreservation of Porcine Spermatogonial Stem Cells by Slow-Freezing Testis Tissue in Trehalose1." *Journal of Animal Science*, vol. 92, no. 3, Mar. 2014, pp. 984–95, doi:10.2527/jas.2013-6843.

Lorenzen, K., Beveridge, M. C., & Mangel, M. (2012). Cultured fish: integrative biology and management of domestication and interactions with wild fish. *Biological Reviews, 87*(3), 639–660. doi: 10.1111/j.1469-185x.2011.00215.x

Lundberg, John G.; Friel, John P. (20 January 2003). "Siluriformes". Tree of Life Web Project. Retrieved 18 April 2007.

Magnotti, C., Cerqueira, V., Lee-Estevez, M., Farias, J. G., Valdebenito, I., & Figueroa, E. (2018). Cryopreservation and vitrification of fish semen: a review with special emphasis on marine species. *Reviews in Aquaculture*, *10*(1), 15-25.

Mandawala, A. A., Harvey, S. C., Roy, T. K., & Fowler, K. E. (2016). Cryopreservation of animal oocytes and embryos: Current progress and future prospects. *Theriogenology*, *86*(7), 1637-1644

Martínez-Páramo, S., Horváth, Á., Labbé, C., Zhang, T., Robles, V., Herráez, P., ... & Cabrita, E. (2017). Cryobanking of aquatic species. *Aquaculture*, *472*, 156-177.

Muchlisin, Z. A. (2005). Current status of extenders and cryoprotectants on fish spermatozoa cryopreservation. *Biodiversitas Journal of Biological Diversity*, *6*(1).

Murua, H., & Saborido-Rey, F. (2003). Female Reproductive Strategies of Marine Fish Species of the North Atlantic. *Journal of Northwest Atlantic Fishery Science, 33*, 23–31. doi: 10.2960/j.v33.a2

Naylor, R. L., Goldburg, R. J., Primavera, J. H., Kautsky, N., Beveridge, M. C., Clay, J., ... & Troell, M. (2000). Effect of aquaculture on world fish supplies. *Nature*, *405*(6790), 1017-1024.

Perera, D. A., Alsaqufi, A., Shang, M., Wade, D. C., Su, B., Elaswad, A., … Dunham, R. A.(2017). Xenogenesis-Production of Channel Catfish × Blue Catfish Hybrid Progeny by Fertilization of Channel Catfish Eggs with Sperm from Triploid Channel Catfish Males with Transplanted Blue Catfish Germ Cells. *North American Journal ofAquaculture*, *79*(1), 61–74. https://doi.org/10.1080/15222055.2016.1221008

Rathore, S. S., Chandravanshi, A., Chandravanshi, P., & Jaiswal, K. (2016). Review on cryopreservation in fisheries science: Applications and perspectives. *International Journal of Science and Research*, *5*(10), 573-579.

Ritchie, H., & Roser, M. (2020). Agricultural Production. *Our World in Data*.

Saito, T., Goto-Kazeto, R., Arai, K., & Yamaha, E. (2008). Xenogenesis in Teleost Fish Through Generation of Germ-Line Chimeras by Single Primordial Germ Cell Transplantation1. *Biology of Reproduction*, *78*(1), 159–166. https://doi.org/10.1095/biolreprod.107.060038

Shang, M. 2013. Isolation, Identification, Culture, Cryopreservation .... Retrieved from https://etd.auburn.edu/bitstream/handle/10415/3934/Mei

Shang, M., Su, B., Perera, D. A., Alsaqufi, A., Lipke, E. A., Cek, S., … Dunham, R. A. (2018). Testicular germ line cell identification, isolation, and transplantation in two North American catfish species. *Fish Physiology and Biochemistry*, *44*(2), 717–733. https://doi.org/10.1007/s10695-018-0467-3

Su, B., Perera, D. A., Zohar, Y., Abraham, E., Stubblefield, J., Fobes, M., ... & Waters, P. (2013). Relative effectiveness of carp pituitary extract, luteininzing hormone releasing hormone analog (LHRHa) injections and LHRHa implants for producing hybrid catfish fry. *Aquaculture*, *372*, 133-136.

Suquet, M., Dreanno, C., Fauvel, C., Cosson, J., & Billard, R. (2000). Cryopreservation of sperm in marine fish. *Aquaculture Research: Original Articles*, *31*(3), 231-243.

Takeuchi, Y., Yoshizaki, G. & Takeuchi, T. Surrogate broodstock produces salmonids. *Nature* **430,** 629–630 (2004). https://doi.org/10.1038/430629a

Tave, D., & Smitherman, R. O. (1982). Spawning Success of Reciprocal Hybrid Pairings Between Blue and Channel Catfishes with and Without Hormone Injection. *The Progressive Fish-Culturist*, *44*(2), 73–74. doi: 10.1577/1548-8659(1982)44[73:ssorhp]2.0.co;2

Teletchea, F., & Fontaine, P. (2014). Levels of domestication in fish: implications for the sustainable future of aquaculture. *Fish and fisheries*, *15*(2), 181-195.

Torrans, E. L., Ott, B. 2018. Effect of grading fingerling hybrid catfish (female channel catfish x male blue catfish) on growth, production, feed conversion and food fish size distribution. North American Journal of Aquaculture, 80:187-192.

Tucker, C. S., & Robinson, E. H. (1990). *Channel catfish farming handbook*. New York: Van Nostrand Reinhold.

USDA. 2011. Producing Hybrid Catfish Fry, Workshop Manual, Mississippi State University & National Warmwater & Aquaculture Center

Ye, D., Zhu, L., Zhang, Q., Xiong, F., Wang, H., Wang, X., ... & Sun, Y. (2019). Abundance of early embryonic primordial germ cells promotes zebrafish female differentiation as revealed by lifetime labeling of germline. *Marine biotechnology*, *21*(2), 217-228.

Yoshizaki, G., & Lee, S. (2018). Production of live fish derived from frozen germ cells via germ cell transplantation. *Stem cell research*, *29*, 103-110.

Yoshizaki, G., & Yazawa, R. (2019). Application of surrogate broodstock technology in aquaculture. *Fisheries Science*, *85*(3), 429–437. https://doi.org/10.1007/s12562-019-01299-y

Zhang, T., & Lubzens, E. (2009). Cryopreservation of fish oocytes. *Methods in reproductive aquaculture: marine and freshwater species. CRC Press, Boca Raton*, 251-264.

Chapter 2 - Development of a spermatogonial stem cell cryopreservation protocol for

blue catfish

Abstract

Sustainability of channel catfish (*Ictalurus punctatus*) ♀ × blue catfish (*I. furcatus*) ♂ hybrid aquaculture relies on new innovative technologies to maximize fry output. Transplanting spermatogonial stem cells (SSCs) from blue catfish into channel catfish hosts has the potential to greatly increase gamete availability, and cryopreservation would make these cells readily accessible year-round. However, a protocol for blue catfish SSCs has not yet been fully developed. The objectives of this experiment were to identify the best permeating cryoprotectants (DMSO, ethylene glycol (EG), glycerol, methanol), non- permeating cryoprotectants (lactose or trehalose with egg yolk or BSA), their optimal concentrations, and freezing rates (-1, -5, or -10°C/min) that maximize cryopreservation success. All of these factors had significant impacts on post-thaw SSC production and viability. DMSO was the most efficient permeating cryoprotectant at a concentration of 1.0 M. Optimal concentration of each cryoprotectant depended on the specific cryoprotectant due to interactions between the two factors. Of the non-permeating cryoprotectants, 0.2 M lactose with egg yolk further improved SSC production and viability beyond that of the 1.0 M DMSO control. The overall best freezing rate was consistent at -1°C/min, but similar results were obtained using -0.5°C/min. Overall, we recommend freezing SSCs with 1.0 M DMSO with 0.2 M lactose and egg yolk at a rate of -1°C/min to achieve the best cryopreservation outcomes. Continued development of cryopreservation protocols for blue catfish and other species will make SSCs available for xenogenic applications and genetic improvement programs.

2.1. Introduction

Hybridization between the channel catfish, *Ictalurus punctatus* ♀ × blue catfish, *I. furcatus*

♂ was first reported by Giudice (1966). This specific hybrid is one of the best genetic types for pond culture in the southern U.S. However, the two parent species do not spawn naturally due to reproductive isolation mechanisms (Hu et al., 2011). Thus, artificial, human- controlled reproduction remains the most practical option to create hybrid fry. Besides the extra labor and time required for artificial fertilization, another obstacle to sustain hybrid catfish production is having a reliable supply of mature blue catfish males to provide gametes when the females are ready to spawn. Fry production is limited by the quality and quantity of male gamete resources since males can only be used for a single spawn. Alternatively, channel catfish females produce an abundance of eggs and can be spawned for multiple years (Dunham and Brummett, 1999; Torrans et al., 2013). Since sperm collection requires sacrifice of the male blue catfish, it is essential to develop strategies to efficiently use sperm resources and minimize the number of males needed to be housed in commercial hatcheries.

Xenogenesis may be a more efficient method to propagate hybrid catfish embryos. Xenogenesis is a method of reproduction in which successive generations differ from each other, and xenogenic organisms possess genetic traits that are not observed in their parents of origin (Dunham, 2011; Higuchi et al., 2011). For blue catfish, successfully transplanting spermatogonial stem cells (SSCs) into channel catfish (a species with accelerated growth, earlier age of sexual maturity, and more convenient husbandry requirements) creates a xenogenic channel catfish capable of producing blue catfish sperm (Shang et al., 2018). This may cause tremendous increases in male sperm availability and allows a single male to fertilize channel catfish females for multiple years (Perera et al., 2017). By producing blue catfish gametes while taking advantage of the faster life cycle of the channel catfish, this generates enormous potential for overcoming the paternal limitations in the hybrid catfish industry. Cryopreservation of these cell types can further improve xenogenesis applicability by making them consistently available for cell transplantation and xenogenesis procedures (Lee et al.,

2013; Franěk et al., 2019). Reducing intracellular damage is the best way to ensure successful production of live SSCs for downstream xenogenesis applications. Success is highly dependent on the cryoprotectant, concentrations, and freezing rates that are utilized, in which specific protocols vary among species (Elliot et al., 2017; Magnotti et al., 2018).

Permeable cryoprotectants are substances with low molecular weight that can pass through the cell membrane, replacing the volume of intracellular water to avoid damage caused by the formation of ice crystals (Wowk, 2007). By maintaining the cell volume, they prevent cellular collapse due to excessive dehydration (Rota et al. 2006). Cryopreservation usually involves the use of permeating cryoprotectants with similar properties such as those examined in this study: dimethyl sulfoxide (DMSO), ethylene glycol (EG), glycerol, and methanol (Rana, 1995; Linhart et al., 2005; Wowk, 2007). Dimethyl sulfoxide (DMSO) is a promising cryoprotective agent that has been widely utilized by cryobiologists due to its relatively low costs and low cytotoxicity (Bhattacharya, 2018). EG and glycerol have also been universally used in the freezing of ovarian tissue and embryos of many organisms (Robertson et al., 1986; Voelkel, 1992; Chi et al., 2002). Additionally, methanol has been shown to protect fragile fish embryos by reducing their chilling sensitivity during cold storage (Zhang et al., 1993; 2003). Some permeating cryoprotectants are also paired with non-permeating substances to increase post-thaw viability. These agents, characterized by their large molecules and lower toxicity than their permeating counterparts, have been utilized to enhance cell viability without actually penetrating the plasma membrane (Berghmans et al., 2004; Corcuera et al., 2007; Meryman, 2007; Carmichael et al., 2009; Lee et al., 2014; Matthews et al., 2018). Some common substances that have been investigated are egg yolk (10%), lactose, trehalose, glucose, and honey, among others (Viveiros et al., 2000; Lee et al., 2013; Muchlisin et al., 2015).

Post-thaw viability of tissues is also highly dependent on the freezing rate with options being slow freezing or rapid vitrification (Lima & Silva, 2017). Slow cooling of tissues causes

dehydration of the cell, and ice forms in the regions where water was previously located, while quick freezing causes dangerous ice crystal formation (Iussig et al., 2019). Changes in cell volume due to freezing is a result of water efflux during the freezing phase that causes an imbalance in osmotic pressure, causing cryo-injuries to cell membranes and organelles (Dattena et al., 2004; Isachenko et al., 2005; Bart et al., 2016). Therefore, finding an optimal freezing rate that mitigates the consequences of both extremes is an essential component to a successful cryopreservation protocol.

The objectives of these experiments were to identify the best permeating and non-permeating cryoprotectants, their optimal concentrations, and the best freezing rate that yields the highest number of live SSCs from blue catfish testicular tissue after cryopreservation. Developing an effective and practical protocol for cryopreserving blue catfish stem cells will provide another reliable resource of male gametes for artificial fertilization and minimize the need to maintain as many blue catfish in ponds. Coupling cryopreservation of SSCs with xenogenesis technologies is the next innovative step to improve production of hybrid catfish. Furthermore, knowledge gained may also be applicable to other hatchery cultured species.

2.2. Materials and Methods

2.2.1 Brood stock management

All experimental procedures were performed in accordance with an Institutional Animal Care and Use Committee (IACUC) for care and use of animals in the laboratory of genetics at Auburn University, Auburn, AL, United States. Blue catfish (n = 27) were obtained from 0.04-ha earthen ponds at the EW Shell Fisheries Center at Auburn University, AL, USA. Fish were fed 5 times per week with 32% protein pelleted catfish feed at 1-2% of total fish weight per pond prior to experimentation. Fish were seined from these ponds and transferred to

260 × 72 × 50 cm flow-through raceways. At the time of gamete collection, raceways were supplied with diffused aeration so that oxygen levels were maintained above 5 ppm, pH levels between 6.8 and 7.8, and holding temperatures between 23-27 °C. Fish were then euthanized by a percussive blow to the center of the head followed by pithing. Fish were cleaned, and body weights and total length were recorded. All fish were two to three years old with a total length ranging from 35 to 44 cm and body weight from 500 to 1000 g.

2.2.2 Testes preparation

Testes preparations and extractions were performed using methods modified from Shang et al. (2018). First, a sterile scalpel was used to make an incision along the midline of the body cavity, and the paired testes were removed. Testes weight ranged from 0.33 to 0.80 g, and gonadosomatic index (gonad weight / body weight × 100) ranged from 0.04 to 0.08. Testes were placed in 15 mL tubes of anti-agent medium solution [contains Hanks' Balanced Salt Solution (HBSS21-020-CV, Mediatech, Inc., VA, USA) supplemented with 1.0 ug/mL of $NaHCO_3$ (470302-440, VWR International, PA, USA) and a combination of 100 ug/mL streptomycin and 100 unit/mL penicillin (17-602E, Lonza Group AG, MD, USA)]. The testes were removed from the 15 mL tube and soaked in 0.5% bleach solution to remove blood clots and any connective tissue. Next, testes were rehydrated again with the anti-agent medium solution followed by three washes with phosphate-buffered saline (PBS, J67802, Alfa Aesar, MA, USA) and another three washes with HBSS solution. Testes were dried and weighed using an analytical balance (Mettler Toledo AE 240). Testes were then randomly pooled from three fish (considered one replicate) and 0.05 g were distributed into 1.5 mL cryovials containing Eagle's Minimum Essential Medium (EMEM, 15-015-CV, Mediatec Inc., VA, USA) supplemented with 5% (v/v) fetal bovine serum (FBS, 0332, VWR International, PA,

USA), 2 mM L-glutamine (25-005-CI, Mediatech Inc., VA, USA), and 25 mM Hepes (0511, VWR International, PA, USA). Then, three samples per replicate were immediately processed as the control group, while the rest of the samples were transferred to new cryovials containing cryomedium composed of 55.27 mM Hepes (0511, VWR International, PA, USA), 375.48 mM NaCl (3628-05, Avantor, PA, USA), 7.28 mM KCl (BDH9258, VWR International, Pa, USA), 23.10 mM KH_2PO4 (0781, VWR, PA, USA), 3.82 mM Na_2HPO_4 (0404, VWR International, PA, USA), 3.64 mM sodium pyruvate (A1114, Alfa Aesar, MA, USA), 2.6 mM $CaCl_2 \times 2H_2O$ (470300-570, Ward's Science, ON, Canada), and 1.4 mM $MgCl_2 \times 6H_2O$ (442615, EMD Millipore Corp, MA, USA) (Lee et al. 2013).

Treatments of permeating and non-permeating cryoprotectants were also added to the cryomedia, as detailed below. Overall, testes from 9 fish were minced and three testes pools were created and used for each objective of this study.

2.2.3 Effect of permeating cryoprotectants

Using a full factorial design, pooled testes samples (0.05 g) for each replicate were distributed into 1.5 mL cryovials containing 500 µL of cryomedium supplemented with either DMSO (4948-02, Avantor, PA, USA), EG (BDH9258, VWR, PA, USA), glycerol (0854, VWR, PA, USA), or methanol (BDH1135, VWR, PA, USA) at three different concentrations: 1.0 M, 1.3 M, or 1.6 M. In total, there were twelve combinations (4 cryoprotectants × 3 concentrations) and the fresh control. Treatments were equilibrated on ice for 60 min and then frozen using a control rate freezer (PLANER Kryo 560-16) based on the following settings: start temperature at 5 ºC, hold for 5 min at 5 ºC, freeze at -1.0 ºC/min from 5 ºC to -80 ºC, and then held for 5 min at -80 ºC/min. Samples were then transferred directly to liquid nitrogen (-196ºC).

2.2.4 Effect of non-permeating cryoprotectants

Testes samples (0.05 g) were distributed into 1.5 mL cryovials containing 500 µL of cryomedium supplemented with 1.0 M DMSO. Lactose (36218, Alfa Aesar, MA, USA) and trehalose (T0331, TCI, Tokyo, Japan (0.1M and 0.2 M) were each combined with either bovine serum albumin (BSA) 1.5% (w/v) or egg yolk 10% (v/v). Overall, there were ten treatment combinations and two control groups: fresh SSCs and those cryopreserved with 1.0 M DMSO. All samples were equilibrated and frozen, as above.

2.2.5 Effect of freezing rates

Different freezing rates (-0.5, -1.0, -5.0, -10 °C/min until -80 °C) were factorially tested using the top four cryoprotectant combinations with 1.0 M DMSO: the DMSO control, egg yolk, 0.1 M lactose with egg yolk, and 0.2 M lactose with egg yolk. To avoid bias associated with post-extraction SSC aging, the freezing order was randomized for each replicate (replicate 1: -0.5, -5.0, -1.0, -10°C/min; replicate 2: -1.0, -10, -5.0, -0.5 °C/min; and replicate 3: -10, -0.5, -5.0, -1.0 °C/min). In total, there were sixteen treatment combinations (4 cryoprotectants × 4 freezing rates) and the fresh control.

2.2.6 Thawing and spermatogonia stem cell isolation

For thawing, samples were quickly placed in a 10 °C water bath for 1 min. Testes from each

sample were collected and rehydrated by transferring them into new cryovials containing 500 µL of Dulbecco's Modified Eagle Medium (DMEM/Ham's F-12 50/50).

Fresh and thawed testes were placed on a sterile plastic Petri dish. Testes were crushed and minced into tiny pieces using a sterile scalpel (Okutsu et al. 2006). Then, 500 µL of 0.25% trypsin - EDTA (2.5 g/L trypsin 1:250 and 0.2 g/L EDTA) was added to the homogenized testes. Any intact portions of the testes were transferred to 50 mL autoclaved glass flasks containing plastic stir bars. The flasks were incubated on ice for 30 min followed by 45 min of mechanical stirring to dissociate the SSCs from the tissue and allow more digestion time. Next, 500 µL of DMEM was added to each flask to neutralize trypsin digestion. The cell suspensions were filtered twice by using two different sizes of nylon mesh (70 µm and 40 µm) to remove undigested tissues followed by centrifuging at 2.9 RCF for 20 min. The supernatant was discarded, and the pellet was rehydrated with 300 µL DMEM solution.

2.2.7 SSC production and cell viability

First, 10 uL of the cell suspension was gently pipetted to disperse the pellet and then mixed with 10 uL of trypan blue at a ratio 1:1 to differentiate live cells and dead cells. Only SSCs were counted, excluding any other cell types that could be present in the cell suspension (i.e. spermatids, sperm, spermatogonia B). Then 10 uL of the sample was pipetted onto a Neubauer hemocytometer. A total of five squares were counted for stained cells, and the average of these counts was calculated. This process was done three times, accounting for the dilution factor. The dilution factor was multiplied by the average SSC count and the volume of the hemocytometer grid to obtain the final post-thaw spermatogonia A (hereafter referred to as SSC) production for each replicate.

The viability of SSCs was checked using a live/dead double staining kit (QIA76, Sigma-

Aldrich) following manufacturer's instructions. Live cells were counted using an Axio Imager.A2 microscope (Carl Zeiss Microscopy, White Plains, NY) with a 40× objective. The viability was then calculated using the following formula, taking the proportion of the total SSCs collected from the cryopreserved testes divided by the total number of SSCs from the fresh testes.

Viability recovery index (%) = (*Number of live cryopreserved SSCs* / *Number of live fresh SSCs*) × 100.

2.2.8 Statistical analyses

All data were analyzed using SAS statistical analysis software (v.9.4; SAS Institute Inc., Cary, NC, USA). Residuals were tested for normality (Shapiro-Wilk test; PROC UNIVARIATE) and homogeneity of variance (plot of residuals vs. predicted values; PROC GPLOT). Data were arcsin square root or $\log_{10}$ transformed to meet assumptions of normality and homoscedasticity when necessary. Post-hoc testing was done using Tukey's HSD test with values considered statistically significant at alpha = 0.05. All reported values are stated as LSMs ± standard error (SEM).

Firstly, one-way ANOVA was used to compare post-thaw SSC production and viability from the permeating cryoprotectant treatments and the fresh control. Moreover, for viability the cryoprotectant × concentration interaction term was significant ($P < 0.0001$), as such, the two-way ANOVA was decomposed into a series of one-way ANOVAs to determine the effect of concentration at each level of cryoprotectant. Secondly, to analyze the effects nonpermeating cryoprotectant treatments (n = 12) and controls (fresh SSCs and SSCs cryopreserved with 1.0 DMSO), one-way ANOVA was again implemented. Lastly, one-way ANOVAs were also used to compare treatments of the four top-performing cryoprotectants

with the different freezing rates (16 total combinations) and the fresh control. Again, for viability, the interaction term between cryoprotectant and freezing rate was significant (P = 0.0004). Thus, the two-way ANOVA model was analyzed as separate one-way ANOVAs to determine the effect of freezing rate within each cryoprotectant.

2.3. Results

2.3.1 Permeating cryoprotectants

As expected, the fresh control yielded significantly higher SSC production than the cryopreservation treatments (Fig. 1A, Fig. 4A), which all showed varying degrees of success. Implementing permeating cryoprotectants at various concentrations significantly impacted the amount SSCs after cryopreservation ($P < 0.0001$; Fig. 1A), and the optimal concentration depended on the cryoprotectant. 1.0 M was the best concentration for DMSO, but 1.6 M was better for EG. 1.0 M DMSO yielded the most SSCs ($3.88 \times 10^5 \pm 1.13 \times 10^4$), which was significantly higher than the other cryopreserved treatments. For DMSO, SSC counts dropped considerably at 1.3 M and 1.6 M. EG had the second highest amount of SSCs at 1.6 M ($2.70 \times 10^5 \pm 1.13 \times 10^4$) and no live SSCs at 1.0 M (Fig. 1A), again demonstrating the impacts of concentration. SSC count was low for glycerol at all concentrations. When comparing the results visually to that of 1.0 DMSO, it is clear that methanol was an ineffective cryoprotectant since no live SSCs were detected (Fig. 1A, Fig. 4B,D). SSC viability was highest when SSCs were frozen with 1.0 M DMSO (48.0 ± 1%). Viability was lower for EG at 1.6 M (33.2 ± 0.9%), but this difference was not statistically significant. Methanol was ineffective at all concentrations, yielding no post-thaw viability (Fig 1B, $P < 0.0001$).

2.3.2 Non-permeating cryoprotectants

For most combinations tested, post-thaw SSC production was not improved beyond that of the 1.0 M DMSO control. However, cells frozen with 1.0 M DMSO supplemented with 0.2 M lactose and egg yolk did yield the greatest quantity of live SSCs ($5.8 \times 10^5 \pm 1.80 \times 10^4$) ($P < 0.0001$; Fig. 2.2A, Fig. 2.4C). The next top performing treatments were 1.0 M DMSO with egg yolk and 1.0 M DMSO with 0.1 M lactose and egg yolk, which had similar post- thaw SSC production as the 1.0 M DMSO control (Fig. 2.2A). Overall, treatments with trehalose resulted in low SSC counts, the highest being $1.47 \times 10^5 \pm 1.80 \times 10^4$. Like for SSC production, highest SSC viability was also observed when the cells were frozen with 1.0 M DMSO supplemented with 0.2 M lactose and egg yolk (Fig. 2.2B). All other treatments showed significantly less post-thaw viability (Fig. 2.2B).

2.3.3 Freezing rates

For most combinations tested, post-thaw SSC production was not improved beyond that of the 1.0 M DMSO control. However, cells frozen with 1.0 M DMSO supplemented with 0.2 M lactose and egg yolk did yield the greatest quantity of live SSCs ($5.8 \times 10^5 \pm 1.80 \times 10^4$) ($P < 0.0001$; Fig. 2A, Fig. 4C). Of the cryopreservation treatments, the highest-performing combination was DMSO with 0.2 M lactose at a freezing temperature of -1 ^oC/min, which gave $6.74\times 10^5 \pm 6.82 \times 10^4$ SSCs. This amount was not significantly lower than the fresh control, indicating high post-thaw success. The three worst-performing combinations had a freezing rate of -10 ^oC/min, which were DMSO, DMSO with egg yolk, and DMSO with 0.1 M lactose. These treatments yielded SSC counts as low as $5.2 \times 10^4 \pm 6.82 \times 10^4$. As for freezing rates, -10°C/min had the lowest average SSCs among treatments (1.41×10^5) and -1°C/min the highest (4.88×10^5).

For viability, treatments with -5 ^{o}C/min and -10 ^{o}C/min freezing rates had consistently lower viability for all cryoprotectants except 1.0 M DMSO with egg yolk, in which -0.5, -5, and -10 ^{o}C/min were all statistically the same (Fig. 2.3B). Additionally, the cryoprotectant affected the viability within each freezing temperature for -0.5, -1, and -10 ^{o}C/min ($P < 0.05$), but no such effect was observed at -5 ^{o}C/min ($P = 0.0763$). Overall, the best-performing combinations contained either 0.1 or 0.2 M lactose at freezing rates of -0.5 and -1 ^{o}C/min.

2.4. Discussion

Transplanting SSCs from a donor species into an interspecific host to produce male gametes has gained ground as an innovative approach for aquaculture, but xenogenesis technology remains relatively new (Lacerda et al., 2010). Prior to this study, a protocol for blue catfish SSC cryopreservation had not yet been evaluated. Like with any protocol, the adequate conditions for SSC survival must be provided, and subtle differences in methodology may play a critical role in its success (Magnotti et al., 2018). In the present study, such effects were investigated by assessing various cryoprotective agents, concentrations of these agents, and freezing rates on post-thaw viability of SSCs. There were significant impacts on post-thaw SSC production and viability for all three of these factors, indicating that specific combinations of cryoprotectants and freezing rates can greatly influence cryopreservation success.

The cryoprotectants described above were tested at three concentrations: 1.0 M, 1.3 M, and 1.6 M, which were chosen to represent a range of low to high concentrations and reflect what have been used in previous cryopreservation studies (Wowk, 2007; Bozkurt et al., 2019). In our case, the lowest concentration of DMSO (1.0 M) was the best cryoprotectant after evaluation under various conditions, producing greater numbers of live, viable cells. However, higher concentrations of 1.3 M and 1.6 M were more suitable for EG. The other two

cryoprotectants, glycerol and methanol, yielded low SSC production and viability at all concentrations, making them poor candidates as cryoprotectants for SSCs of this species. Previous studies on cryopreservation of testes tissues have also shown that the cryoprotectants such as those used in this study affect cryopreservation success. For example, Marinović et al. (2017) isolated testes tissues from tench, *Tinca tinca*, and goldfish, *Carassius auratus*, and effects of DMSO, methanol, and EG at three concentrations (1 M, 2 M and 3 M) on post-thaw cell viability were assessed. In both species DMSO and EG generally yielded higher cryo-survival of early-stage germ cells, and methanol was consistently the worst-performing cryoprotectant, similar to the results here presented. However, regardless of the cryoprotectant, 3 M generally yielded the highest post-thaw viability and 1 M the lowest. These results contrast with our own findings, in which post-thaw production and viability tended to be lower as concentration increased for DMSO. In another study, Lee et al. (2013) sought to demonstrate the efficacy of 1.3 M DMSO, glycerol, and EG for the cryopreservation of whole male testes from rainbow trout. Of these, DMSO was the most effective cryoprotectant compared to glycerol and EG.

All of these findings together point to the fact that cells are sensitive to cryoprotectants as well as their concentration, and a balance must be maintained to optimize most-thaw cell recovery. This is because when the concentration is too low, internal machinery within the cells cannot withstand the damaging effects of deep freezing. Moreover, when the concentration exceeds the optimal, cells become vulnerable to cryoprotectant toxicity, also compromising their post-thaw viability (Best, 2015). Further interactions among other components of the cryomedium may also impact cellular responses to specific cryoprotectants in unknown ways and are worth further exploration.

In the same study mentioned previously, Lee et al. (2013) found that the subsequent use of the non-permeating agent trehalose with 1.3 M DMSO yielded a higher viability recovery

index than DMSO alone for rainbow trout. Furthermore, freezing of whole testes from Manchurian trout, *Brachymystax lenok,* was accomplished by using 1.3 M methanol supplemented with 0.2 M trehalose and 10% egg yolk (Lee & Yoshizaki, 2016). In our study, most of the non-permeating cryoprotectant treatments did not increase SSC production or viability beyond that of the 1.0 M DMSO control. Previous studies have shown to have higher post-thaw viability with trehalose, but we saw no improvements in any of the trehalose treatments. However, addition of 0.2 M lactose and egg yolk did cause a significant increase, indicating that success can be improved when these substances are added during cryopreservation. These results are evidence that variability exists between species, cell types, and even methodologies that cause results to differ on a case-by-case basis.

Effects of freezing rate are not always held consistent across the broad range of possible cryoprotectant combinations because of the interaction that exists between the two factors (as indicated by the ANOVA model for viability). For whole fish testes, the optimum freezing temperature is typically -1 °C/min (Lee et al., 2013; Franěk et al., 2019). Similar results have been obtained from several studies in other fish species rainbow trout, starry goby, *Asterropteryx semipunctata*, common carp, *Cyprinus carpio*, and brycon, *Brycon orthotaenia* (Melo and Godinho, 2006; Lee et al., 2013; Lee & Iwasaki, 2013; Hagedorn et al, 2018; Lima and Silva, 2017). Corresponding to our own findings, 1°C/min was the best freezing rate for blue catfish SSCs at all four top-performing cryoprotectant combinations. However, the rate of -0.5°C/min did not yield significantly lower results, so it, too, can be used and still retain high post-thaw viability. More extreme temperatures (-5 and -10°C/min) had the opposite effect, greatly reducing the number of viable SSCs, likely due to intracellular damage caused by rapid ice crystal formation. Therefore, it would not be practical to use these freezing rates for cryopreservation.

Many aspects regarding the proper freezing of SSCs in fishes are still unknown despite

the obvious benefits of having these cells available in gene banks. Overall, we emphasize the need to optimize a cryopreservation protocol for each species and cell type that takes into account all underlying and interacting factors. Future work may yet identify the impacts of other specific factors and methodologies that would improve cryopreservation success. Moreover, understanding what makes a suitable donor male would improve standardization and consistency of germ cell transplantation procedures to come. High quantity and quality of SSCs is essential for xenogenesis (Shang et al., 2018) but is not held consistent across individual males, which may alter cell vulnerability to cryopreservation.

Research should continue to link physical, biochemical, and molecular markers to SSC quality to identify fish whose SSCs would best tolerate cryopreservation. Some molecular markers have already been assessed in other fishes and mammals (described by Shang et al., 2018), which provide promising future directions to tackle individual variability. On the other end of the spectrum, similar biomarkers can also be used to identify receptive channel catfish hosts. As a final consideration, oocytes, unlike sperm are highly sensitive to cryodamage and, thus, have not been cryopreserved successfully. Because of the lack of maternally derived gametes in long term storage, developing protocols for female oogonia stem cells would also provide valuable resources for gene banks.

2.5. Conclusions

Cryopreservation of immature germ cells (i.e. spermatogonia) was completed successfully in blue catfish, offering an alternative to the traditional freezing of eggs and sperm. From these results, it is recommended that 1.0 M DMSO paired with the non-

permeating cryoprotectants 0.2 M lactose and egg yolk be added to the cryomedia and that cells be frozen at a rate of either -0.5 or -1 °C/min. With this proposed protocol, SSCs can stored long-term for genetic enhancement programs and xenogenesis procedures. This has potential to maximize efficiency of hybrid catfish production by increasing availability of blue catfish gametes and reducing the need to keep as many live blue catfish on-hand in commercial hatcheries. Continued development of SSC cryopreservation protocols will strengthen our knowledge of other mechanisms determining cryopreservation outcomes for blue catfish that may also be applied to other aquaculture species.

Acknowledgements

This project was supported by the Agriculture and Food Research Initiative Competitive Grant no. 2018-67015-27614 from the USDA National Institute of Food and Agriculture.

References

Bart, Amrit N., et al. *Transactions of the American Fisheries Society Cryopreservation of Blue Catfish Spermatozoa and Subsequent Fertilization of Channel Catfish Eggs*. Vol. 8487, no. March, 2016, doi:10.1577/1548-8659(1998)127<0819.

Berghmans, Stéphane, et al. "Zebrafish Sperm Cryopreservation." *Methods in Cell Biology*, vol. 2004, no. 77, 2004, pp. 645–58, doi:10.1016/s0091-679x(04)77034-x.

Best, B. P. (2015). Cryoprotectant toxicity: facts, issues, and questions. *Rejuvenation research*, *18*(5), 422-436.

Bhattacharya, S. (2018). Cryoprotectants and Their Usage in Cryopreservation Process. *BIOTECHNOLOGY IN BIOMEDICAL AND BIOLOGICAL SCIENCES*, 7.

Bozkurt, Y. (2019). Introductory Chapter: Cryopreservation Biotechnology in Aquatic Science. *Biological Research in Aquatic Science*. doi: 10.5772/intechopen.85901

Carmichael, Carrie, et al. "Cryopreservation and in Vitro Fertilization at the Zebrafish International Resource Center." *Methods in Molecular Biology (Clifton, N.J.)*, vol. 546, 2009, pp. 45–65, doi:10.1007/978-1-60327-977-2_4.

Chi, H.-J. (2002). Cryopreservation of human embryos using ethylene glycol in controlled slow freezing. *Human Reproduction*, *17*(8), 2146–2151. doi: 10.1093/humrep/17.8.2146

Corcuera, B., Marigorta, P., Sagüés, A., Saiz-Cidoncha, F., & Pérez-Gutiérrez, J. (2007). Effect of lactose and glycerol on the motility, normal apical ridge, chromatin condensation and chromatin stability of frozen boar spermatozoa. *Theriogenology*, *67*(6), 1150–1157. doi: 10.1016/j.theriogenology.2007.01.002

Dattena, M., et al. "Comparison of Different Vitrification Protocols on Viability after Transfer of Ovine Blastocysts in Vitro Produced and in Vivo Derived." *Theriogenology*, vol. 62, no. 3, 2004, pp. 481–93, doi:https://doi.org/10.1016/j.theriogenology.2003.10.010.

Dunham, R. A., & Brummett, R. E. (1999). Response of Two Generations of Selection to Increased Body Weight in Channel Catfish,Ictalurus punctatus, Compared to Hybridization with Blue Catfish, I.furcatus, Males. *Journal of Applied Aquaculture*, *9*(3), 37–45. doi: 10.1300/j028v09n03_03

Dunham, R. A. (2011). *Aquaculture and fisheries biotechnology genetic approaches*. Wallingford: CABI

Elliott, G. D., Wang, S., & Fuller, B. J. (2017). Cryoprotectants: A review of the actions and applications of cryoprotective solutes that modulate cell recovery from ultra-low temperatures. *Cryobiology*, *76*, 74-91.

Franěk, Roman, et al. "Cryopreservation and Transplantation of Common Carp Spermatogonia." *PLOS ONE*, vol. 14, no. 4, Public Library of Science, Apr. 2019, p. e0205481, https://doi.org/10.1371/journal.pone.0205481.

Giudice, J. J. (1966). Growth of a Blue X Channel Catfish Hybrid as Compared to its Parent Species. *The Progressive Fish-Culturist*, *28*(3), 142–145. doi: 10.1577/1548-8640(1966)28[142:goabxc]2.0.co;2

Hagedorn, Mary M., et al. "Cryopreservation of Fish Spermatogonial Cells: The Future of Natural History Collections." *Scientific Reports*, vol. 8, no. 1, 2018, pp. 1–11, doi:10.1038/s41598-018-24269-3.

Higuchi, K., Takeuchi, Y., Miwa, M., Yamamoto, Y., Tsunemoto, K., & Yoshizaki, G. (2011).

Colonization, proliferation, and survival of intraperitoneally transplanted yellowtail Seriola quinqueradiata spermatogonia in nibe croaker Nibea mitsukurii recipient. *Fisheries Science*, *77*(1), 69–77. https://doi.org/10.1007/s12562-010-0314-7

Hu, E., et al. "High-Throughput Cryopreservation of Spermatozoa of Blue Catfish (Ictalurus Furcatus): Establishment of an Approach for Commercial-Scale Processing." *Cryobiology*, 2010/12/19, vol. 62, no. 1, Feb. 2011, pp. 74–82, doi:10.1016/j.cryobiol.2010.12.006.

Iussig, B., Maggiulli, R., Fabozzi, G., Bertelle, S., Vaiarelli, A., Cimadomo, D., ... & Rienzi, L. (2019). A brief history of oocyte cryopreservation: arguments and facts. *Acta obstetricia et gynecologica Scandinavica*, *98*(5), 550-558.

Isachenko, Vladimir, et al. "Clean Technique for Cryoprotectant-Free Vitrification of Human Spermatozoa." *Reproductive BioMedicine Online*, vol. 10, no. 3, Reproductive Healthcare Ltd, Duck End Farm, Dry Drayton, Cambridge CB23 8DB, UK, 2005, pp. 350–54, doi:10.1016/S1472-6483(10)61795-6.

Lacerda, S. M., Batlouni, S. R., Costa, G. M., Segatelli, T. M., Quirino, B. R., Queiroz, B. M., ... & Franca, L. R. (2010). A new and fast technique to generate offspring after germ cells transplantation in adult fish: the Nile tilapia (Oreochromis niloticus) model. *PloS one*, *5*(5).

Lacerda, S. M. S. N., Costa, G. M. J., Campos-Junior, P. H. A., Segatelli, T. M., Yazawa, R., Takeuchi, Y., … França, L. R. (2013). Germ cell transplantation as a potential biotechnological approach to fish reproduction. *Fish Physiology and Biochemistry*, *39*(1), 3–11. https://doi.org/10.1007/s10695-012-9606-4

Lee, S., Iwasaki, Y., Shikina, S., & Yoshizaki, G. (2013). Generation of functional eggs and sperm from cryopreserved whole testes. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(5), 1640–1645. https://doi.org/10.1073/pnas.1218468110

Lee, Y. A., et al. "Cryopreservation of Porcine Spermatogonial Stem Cells by Slow-Freezing Testis Tissue in Trehalose1." *Journal of Animal Science*, vol. 92, no. 3, Mar. 2014, pp. 984–95, doi:10.2527/jas.2013-6843.

Lima, D. B. C., & Silva, L. D. M. da. (2017). Cryopreservation of testicular tissue: an alternative to maintain the reproductive capacity in different animal species. *Ciência Rural*, *47*(11). https://doi.org/10.1590/0103-8478cr20170135

Linhart, O., Rodina, M., Flajshans, M., Gela, D., & Kocour, M. (2005). Cryopreservation of European catfish Silurus glanis sperm: Sperm motility, viability, and hatching success of embryos. *Cryobiology*, *51*(3), 250–261. https://doi.org/https://doi.org/10.1016/j.cryobiol.2005.07.005

Magnotti, C., Cerqueira, V., Lee-Estevez, M., Farias, J. G., Valdebenito, I., & Figueroa, E. (2018). Cryopreservation and vitrification of fish semen: a review with special emphasis on marine species. *Reviews in Aquaculture*, *10*(1), 15-25.

Mandawala, A. A., Harvey, S. C., Roy, T. K., & Fowler, K. E. (2016). Cryopreservation of animal oocytes and embryos: Current progress and future prospects. *Theriogenology*, *86*(7), 1637-1644.

Marinović, Z., Lujić, J., Kása, E., Bernáth, G., Urbányi, B., & Horváth, Á. (2017). Cryosurvival of isolated testicular cells and testicular tissue of tench Tinca tinca and goldfish Carassius auratus following slow-rate freezing. *General and comparative endocrinology*, *245*, 77-83.

Matthews, Jennifer L., et al. "Changes to Extender, Cryoprotective Medium, and In Vitro Fertilization Improve Zebrafish Sperm Cryopreservation." *Zebrafish*, vol. 15, no. 3, Mary Ann Liebert, Inc., publishers, Jan. 2018, pp. 279–90, doi:10.1089/zeb.2017.1521.

Melo, F. C. S. A., and H. P. Godinho. "A Protocol for Cryopreservation of Spermatozoa of the Fish Brycon Orthotaenia." *Animal Reproduction*, vol. 3, no. 3, 2006, pp. 380–85.

Meryman, Harold T. "Cryopreservation of Living Cells: Principles and Practice." *Transfusion*, vol. 47, no. 5, John Wiley & Sons, Ltd (10.1111), May 2007, pp. 935–45, doi:10.1111/j.1537-2995.2007.01212.x.

Muchlisin, Zainal Abidin, et al. "Exploration of Natural Cryoprotectants for Cryopreservation of African Catfish, Clarias Gariepinus, Burchell 1822 (Pisces: Clariidae) Spermatozoa." *Czech Journal of Animal Science*, vol. 60, no. 2, 2015, pp. 10–15, doi:10.17221/7906-CJAS.

Okutsu, T., Yano, A., Nagasawa, K., Shikina, S., Kobayashi, T., Takeuchi, Y., & Yoshizaki, G. (2006). Manipulation of fish germ cell: visualization, cryopreservation and transplantation. *Journal of Reproduction and Development*, *52*(6), 685-693.

Perera, D. A., Alsaqufi, A., Shang, M., Wade, D. C., Su, B., Elaswad, A., … Dunham, R. A. (2017). Xenogenesis-Production of Channel Catfish × Blue Catfish Hybrid Progeny by Fertilization of Channel Catfish Eggs with Sperm from Triploid Channel Catfish Males with Transplanted Blue Catfish Germ Cells. *North American Journal of Aquaculture*, *79*(1), 61–74. https://doi.org/10.1080/15222055.2016.1221008

Rana, K. J. (1995). Cryopreservation of Fish Spermatozoa BT - Cryopreservation and Freeze-Drying Protocols. In J. G. Day & M. W. Pennington (Eds.) (pp. 151–165). Totowa, NJ: Humana Press. https://doi.org/10.1385/0-89603-296-5:151

Robertson, S. M., Lawrence, A. L., Neill, W. H., Arnold, C. R., & McCarty, G. (1988). Toxicity of the cryoprotectants glycerol, dimethyl sulfoxide, ethylene glycol, methanol, sucrose, and sea salt solutions to the embryos of red drum. *The Progressive Fish-Culturist*, *50*(3), 148-154.

Rota, A., Milani, C., Cabianca, G., & Martini, M. (2006). Comparison between glycerol and ethylene glycol for dog semen cryopreservation. *Theriogenology*, *65*(9), 1848–1858. doi: 10.1016/j.theriogenology.2005.10.015

Shang, M., Su, B., Perera, D. A., Alsaqufi, A., Lipke, E. A., Cek, S., … Dunham, R. A. (2018). Testicular germ line cell identification, isolation, and transplantation in two North

American catfish species. *Fish Physiology and Biochemistry*, *44*(2), 717–733. https://doi.org/10.1007/s10695-018-0467-3

Torrans, L., Ott, B., Jones, R. "Shorty," & Jones, R. (2013). Channel Catfish Hatchery Production Efficiency Using a Vertical-Lift Incubator (the See-Saw) at Various Egg Loading Rates. *North American Journal of Aquaculture*, *75*(2), 235–243. https://doi.org/10.1080/15222055.2013.763879

Viveiros, A. T. M., et al. "Sperm Cryopreservation of African Catfish, Clarias Gariepinus: Cryoprotectants, Freezing Rates and Sperm:Egg Dilution Ratio." *Theriogenology*, vol. 54, no. 9, 2000, pp. 1395–408, doi:https://doi.org/10.1016/S0093-691X(00)00462-3.

Voelkel, S. A. (1992). *U.S. Patent No. 5,160,312*. Washington, DC: U.S. Patent and Trademark Office.

Wowk, B. (2007). How cryoprotectants work. *Cryonics*, *28*(3), 3-7.

Ye, D., Zhu, L., Zhang, Q., Xiong, F., Wang, H., Wang, X., … Sun, Y. (2019). Abundance of Early Embryonic Primordial Germ Cells Promotes Zebrafish Female Differentiation as Revealed by Lifetime Labeling of Germline. *Marine Biotechnology*, *21*(2), 217–228. https://doi.org/10.1007/s10126-019-09874-1

Yoshizaki, G., Takashiba, K., Shimamori, S., Fujinuma, K., Shikina, S., Okutsu, T., … Hayashi, M. (2016). Production of germ cell-deficient salmonids bydead endgene knockdown, and their use as recipients for germ cell transplantation. *Molecular Reproduction and Development*, *83*(4), 298–311. doi: 10.1002/mrd.22625

Zhang, T., Rawson, D. M., & Morris, G. J. (1993). Cryopreservation of pre-hatch embryos of zebrafish (Brachydanio rerio). *Aquatic Living Resources*, *6*(2), 145-153.Zhang T, Rawson DM. Studies on chilling sensitivity of zebrafish (Brachydanio rerio) embryos. Cryobiology. 1995; 32:239–246.

Zhang T, Liu XH, Rawson DM. Effects of methanol and developmental arrest on chilling injury in zebrafish (Danio rerio) embryos. Theriogenology. 2003; 59:1545–1556. [PubMed: 12559459]

Figure captions

Fig. 2.1. The effects of permeating cryoprotectants treatments on post-thaw spermatogonia A (SSC) production for blue catfish, *Ictalurus furcatus* (A). The effects of concentration are given within each cryoprotectant on the post-thaw viability recovery index (B). Bars represent LSMs + SEM. Letters indicate significant differences, determined by Tukey's HSD ($P < 0.05$).

Fig. 2.2. The effects of nonpermeating cryoprotectant treatments paired with 1.0 M DMSO on post-thaw spermatogonia A (SSC) production (A) and post-thaw viability index (B) for blue catfish, *Ictalurus furcatus* (A). Bars represent LSMs + SEM. Letters indicate significant differences, determined by Tukey's HSD ($P < 0.05$).

Fig. 2.3. Cryoprotectant treatments that yielded the highest post-thaw SSC viability were combined with four different freezing rates to assess the impacts for blue catfish, *Ictalurus furcatus* SSCs. All combinations were compared for spermatogonia A (SSC) production (A). The effects of concentration within each cryoprotectant are shown for the post-thaw viability recovery index (B). Bars represent LSMs + SEM. Letters indicate significant differences, determined by Tukey's HSD ($P < 0.05$).

Fig. 2.4. Images of blue catfish, *Ictalurus furcatus*, SSCs were obtained by fluorescent microscopy with a 40× objective. In the first column, images were taken with Trypan Blue stain. In the following columns, SSCs are represented in green and dead SSCs in red followed by a merged view. Shown by row is a series of cryoprotectant treatments as follows: fresh control (A), 1.0 M DMSO (B), 1.0 M DMSO with 0.2 M lactose and egg yolk (C) and 1.3 M methanol (D).

Fig. 2.1

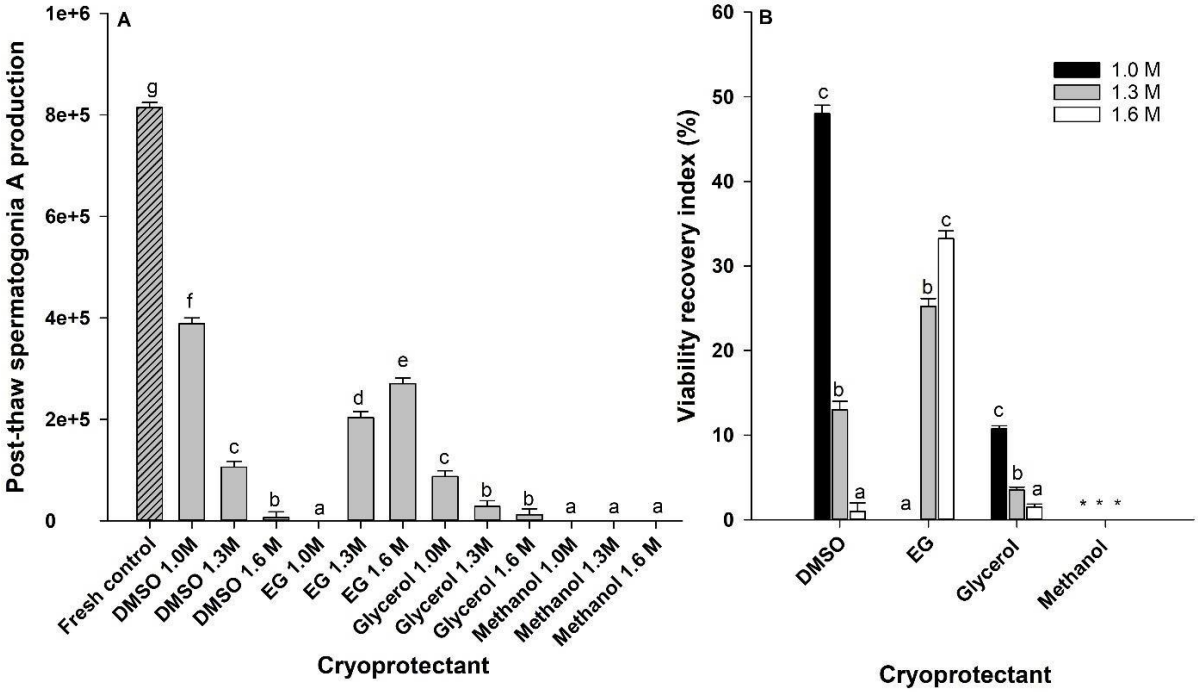

A
Post-thaw spermatogonia A production
1e+6
8e+5
6e+5
4e+5
2e+5
0
g
f
c
b
a
d
e
c
b
b
a
a
a
Fresh control
DMSO 1.0M
DMSO 1.3M
DMSO 1.6 M
EG 1.0M
EG 1.3M
EG 1.6 M
Glycerol 1.0M
Glycerol 1.3M
Glycerol 1.6 M
Methanol 1.0M
Methanol 1.3M
Methanol 1.6 M
Cryoprotectant
B
Viability recovery index (%)
60
50
40
30
20
10
0
1.0 M
1.3 M
1.6 M
c
b
a
a
b
c
c
b
a
* * *
DMSO
EG
Glycerol
Methanol
Cryoprotectant

Fig. 2.2

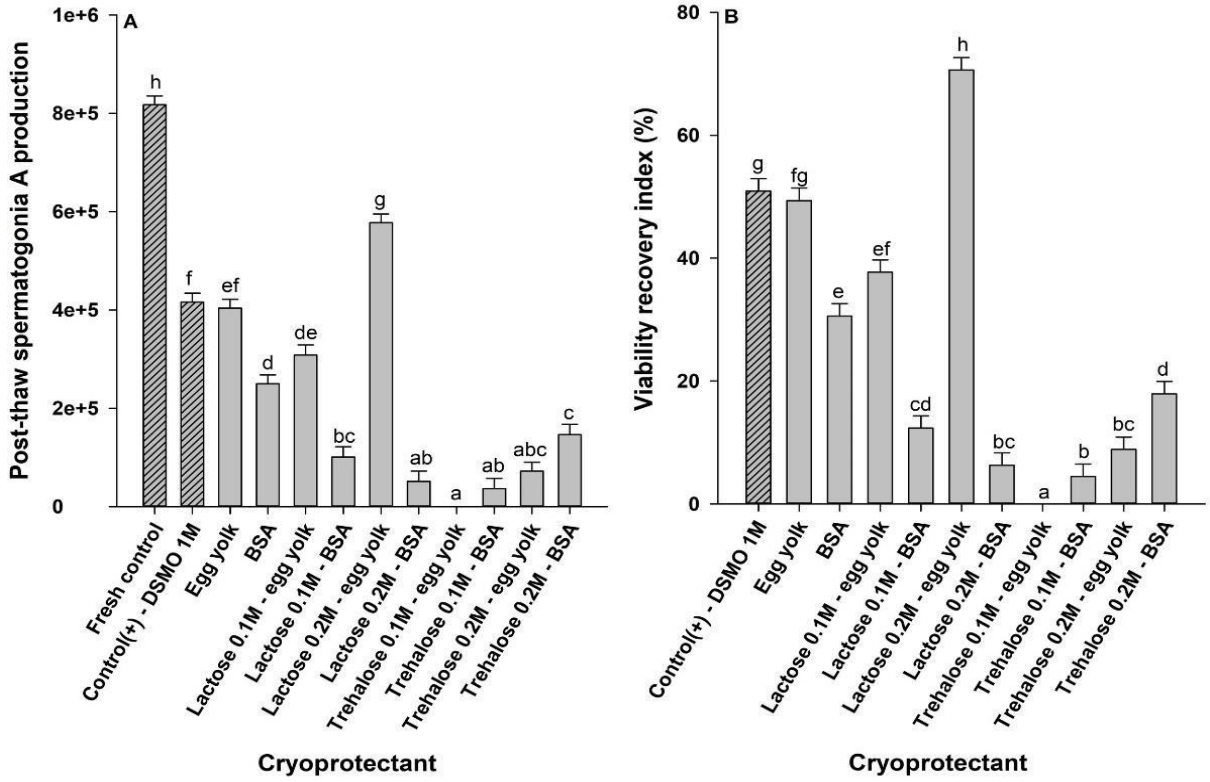

A
Post-thaw spermatogonia A production
1e+6
8e+5
6e+5
4e+5
2e+5
0
h
f
ef
d
de
bc
g
ab
a
ab
abc
c
Fresh control
Control(+) - DSMO 1M
Egg yolk
BSA
Lactose 0.1M - egg yolk
Lactose 0.1M - BSA
Lactose 0.2M - egg yolk
Lactose 0.2M - BSA
Trehalose 0.1M - egg yolk
Trehalose 0.1M - BSA
Trehalose 0.2M - egg yolk
Trehalose 0.2M - BSA
Cryoprotectant
B
Viability recovery index (%)
80
60
40
20
0
g
fg
e
ef
cd
h
bc
a
b
bc
d
Control(+) - DSMO 1M
Egg yolk
BSA
Lactose 0.1M - egg yolk
Lactose 0.1M - BSA
Lactose 0.2M - egg yolk
Lactose 0.2M - BSA
Trehalose 0.1M - egg yolk
Trehalose 0.1M - BSA
Trehalose 0.2M - egg yolk
Trehalose 0.2M - BSA
Cryoprotectant

Fig. 2.3

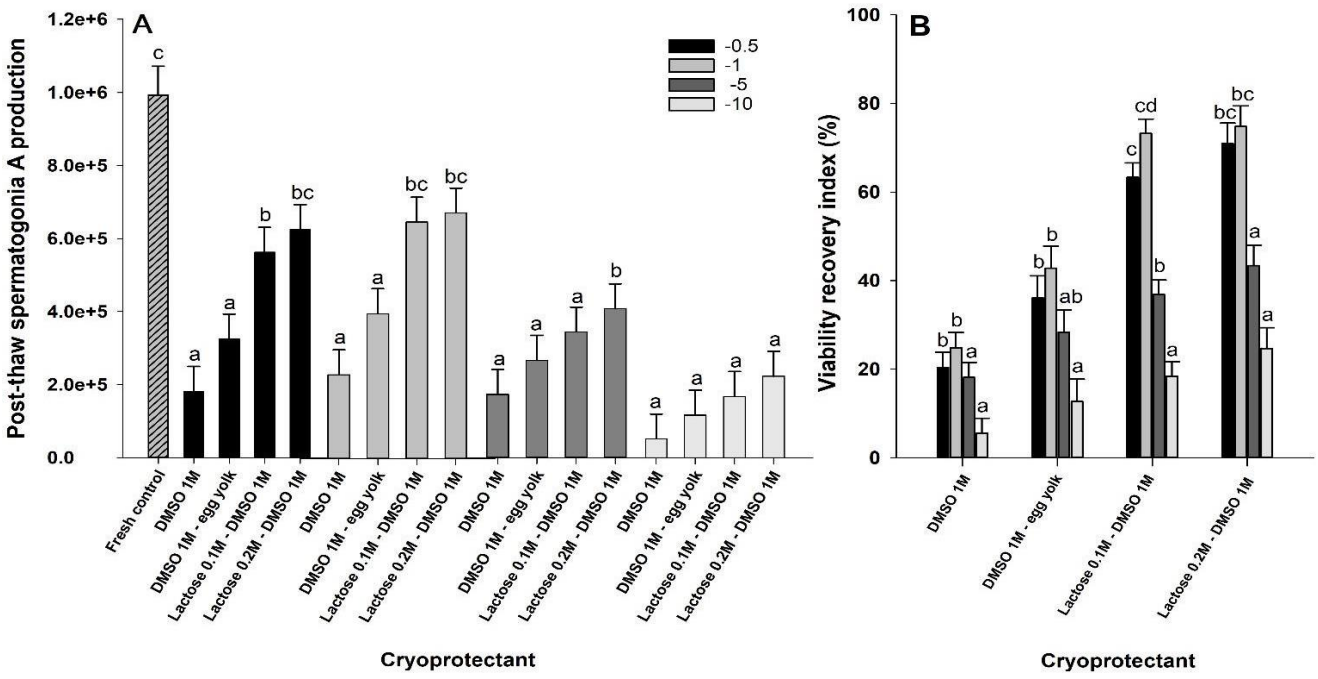
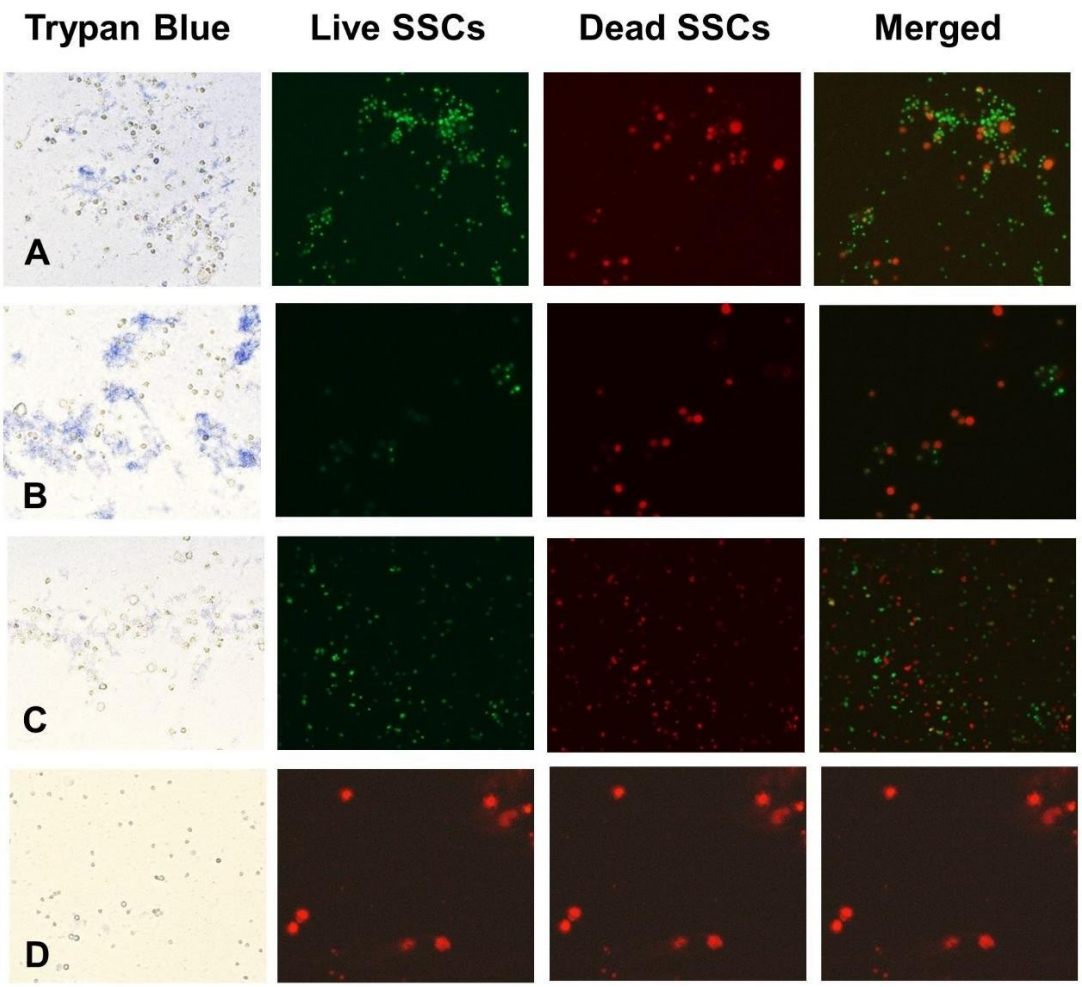

A
Post-thaw spermatogonia A production
1.2e+6
1.0e+6
8.0e+5
6.0e+5
4.0e+5
2.0e+5
0.0
-0.5
-1
-5
-10
Fresh control
DMSO 1M
DMSO 1M - egg yolk
Lactose 0.1M - DMSO 1M
Lactose 0.2M - DMSO 1M
Cryoprotectant
B
Viability recovery index (%)
100
80
60
40
20
0
DMSO 1M
DMSO 1M - egg yolk
Lactose 0.1M - DMSO 1M
Lactose 0.2M - DMSO 1M
Cryoprotectant

Fig. 2.4



Chapter 3 - Development of an oogonial stem cell cryopreservation protocol for blue catfish

Abstract

Production of hybrid catfish *(Ictalurus punctatus*) ♀ × blue catfish (*I. furcatus*) ♂ is limited by the availability of blue catfish gametes. Xenogenesis and transplantation of primordial germ cells (PGCs) are powerful tools for generating blue catfish eggs and sperm within faster growing channel catfish hosts, which has potential to greatly increase hybrid fry outputs. Cryopreservation of oogonial stem cells (OSCs) stores the female contribution for gene banking and makes them accessible for xenogenesis applications, but an optimized protocol has not yet been developed for blue catfish. This study is the first of its kind that assessed several combinations of permeating cryoprotectants (DMSO, ethylene glycol (EG), glycerol, and methanol at 1.0 M, 1.3 M, or 1.6 M), non-permeating cryoprotectants (0.1 M lactose, 0.2 M lactose, or 0.1 M trehalose with 10% egg yolk or 1.5% BSA), and freezing rates (-1.0, -2.5, -5.0 °C/min) on OSC cryopreservation success. All three factors impacted post-thaw OSC production and viability, and there were also specific interactions, such that effects of concentration and freezing rate varied among cryoprotectant treatments. Overall, OSCs best retained their post-thaw viability using our proposed specifications: a cryoprotectant mixture of 1.0 M DMSO with 0.2 M lactose and 10% egg yolk and freezing at a rate of -1°C/min. Continuing to develop consistent cryopreservation protocols for blue catfish and other species will make OSCs available for genetic enhancement programs and emerging xenogenesis technologies.

3.1 Introduction

Producing the next generation of channel catfish (*Ictalurus punctatus*) ♀ × blue catfish (*I. furcatus*) ♂ hybrids within commercial hatcheries relies heavily on the availability of gamete resources from both parent species (Migaud et al., 2013). Therefore, it is a priority for catfish farmers to produce fish with enough high-quality sperm and eggs to meet growing market demands. Due to the lack of natural hybridization between channel catfish and blue catfish, hybrids are produced by *in vitro* fertilization (IVF), in which all aspects of reproduction are controlled (Dunham, 2000). Although an effective and consistent method, IVF is accompanied by its own complications (Dunham et al., 2000). Blue catfish males fulfill the paternal role for the hybrid catfish offspring, and commercial hatcheries must maintain healthy stocks of this species to produce the annual cohort of fry. Maintaining blue catfish is necessary but also a limitation, as blue catfish males take longer to mature than channel catfish (Dunham et al., 1994), requiring more resources and space before they can be utilized as broodfish. This dilemma has made it necessary to take new innovative approaches to obtain, store, and process gametes from blue catfish in order to improve hybrid catfish production and sustainability.

One solution for indefinite long-term storage of gamete resources for use in future gene banking and IVF is cryopreservation (Cabrita et al., 2015). From an aquaculture perspective, storing gametes from both male and female origins would aid genetic enhancement programs, facilitate gamete transport between hatcheries, and ensure that sperm is available when females are induced to spawn (Hu et al., 2011; Cabrita et al., 2015). Testes tissues and sperm cells have already been successfully cryopreserved in several freshwater and marine fish species (Suquet et al., 2000; Rathore et al., 2016). Despite current advances in cryopreservation technology, the successful freezing of oocytes has had its shortcomings,

preventing it from being applied commercially in hatcheries (reviewed by Asturiano et al.,

2017). Eggs exhibit different properties such as their larger size, yolk and liquid content, which make them more vulnerable to freezing and post-thaw damage (Zhang et al., 2009; Franěk et al., 2019a). Thus, cryopreservation of early-stage female germ cells [oogonial stem cells (OSCs)] from ovarian tissues is an ideal alternative because they are small in size and have less lipid/yolk material, making them less sensitive to chilling (Yoshizaki et al., 2018). Multiple accounts have confirmed that these cells are capable of surviving cryopreservation (Lee et al., 2016; Lujić et al., 2017). Recent research has developed protocols for cryopreservation of spermatogonial stem cells (SSCs) (Perera et al., 2017; Shang et al., 2018; Chapter 2), but freezing protocols for OSCs are still largely exploratory with many unknown factors (Franěk et al., 2019a).

Gene banks comprised of OSCs provide the necessary materials for preserving the female genotype for the next generation. OSCs can be transplanted into a host organism of different species to produce xenogens – organisms in which the two consecutive generations differ from each other (Dunham 2011; Higuchi et al. 2011). For blue catfish, transplanting OSCs from blue catfish into channel catfish before they have developed their own gonads is beneficial because of the host's accelerated growth rates, earlier age of sexual maturity, and more convenient husbandry requirements. Once the hosts mature, they can then produce blue catfish gametes that continue the life cycle for the blue catfish parent and hybrid offspring. Cryopreservation of these cell types can further improve xenogenesis applicability by making them consistently available for cell transplantation.

Successful cryopreservation depends heavily on the substances added to the cryopreserved cells that mitigate the harmful effects of deep freezing. The loss of volume the cells encounter during such drastic drops in temperature can lead to ice crystal formation and irreparable intracellular damage (Chao & Liao, 2001; Wowk, 2007). Maintaining the cell volume during freezing protects the cell from these damaging effects, which is why

cryoprotectants are often necessary to ensure high post-thaw viability (Muchlisin, 2005). Permeable cryoprotectants are substances with low molecular weight that can pass through the cell membrane and protect its organelles during cryopreservation (Wowk, 2007). Some common, widely-used permeating cryoprotectants for gamete and tissue cryopreservation are dimethyl sulfoxide (DMSO), ethylene glycol (EG), glycerol, propylene glycol, or methanol, all of which have unique chemical properties and structure (Rana, 1995; Linhart et al., 2005; Wowk, 2007). Of those listed, DMSO has been widely utilized by cryobiologists due to its relatively low cost and cytotoxicity (Bhattacharya, 2018). EG and glycerol have also been universally used in the freezing of ovarian tissue and embryos of many organisms for research and agricultural use (Voelkel, 1992; Chi et al., 2002). Methanol has also been investigated, and results show that it has potential for reducing chilling sensitivity of fragile fish embryos (Zhang et al., 1998; 2003; Bart & Kyaw, 2003). Such reports justify the testing of these chemicals for OSC cryopreservation. Permeating cryoprotectants are sometimes paired with non-permeating substances that add an additional protective shield to enhance post-thaw viability. These agents, characterized by their large molecules and lower toxicity than their permeating counterparts, protect the cell membrane without actually entering the cell (Meryman, 2007; Lee et al., 2014; Matthews et al., 2018). Some common substances that have been used either on their own or coupled with permeating cryoprotectants for cell cryopreservation are 10% egg yolk, lactose, 0.5% bovine serum albumin (BSA), trehalose, glucose, and skim milk (Freshney, 1994; Viveiros et al., 2000; Lee et al., 2013; Yoshizaki et al., 2011; Muchlisin et al., 2015).

Post-thaw viability of tissues is also highly dependent on the freezing rate (Muchlisin, 2005), making it a very important input factor for any cryopreservation protocol. Slow cooling of tissues causes dehydration of the cell with ice forming in the regions where water was previously located, while quick freezing causes dangerous ice crystal formation (Iussig et al., 2019). Therefore, finding a balance between the two extremes that minimizes

stress on the cells is an important factor to consider. Studies on cryopreserving germ cells universally report using a standard slow freezing rate of -1°C/min, which has been successful for species including zebrafish (Danio rerio) (Bono-Mestre et al., 2009), Siberian sturgeon (*Acipenser baerii*) **(**Pšenička et al., 2016), rainbow trout (*Oncorhynchus mykiss*) (Lee et al., 2016; Yoshizaki et al., 2018), Nile tilapia (*Oreochromis niloticus*) (Lacerda et al., 2010), honmoroko (*Gnathopogon caerulescens*) (Higaki et al., 2018) and common carp (*Cyprinus carpio*) (Franěk et al., 2019b). Rapid freezing rates via vitrification have also been examined with high success rates (Bono-Mestre et al., 2009; Higaki et al., 2018)

Cryopreservation success is highly dependent on a multitude of factors, and protocols must be optimized for each cell type and species (Magnotti et al., 2018). Many aspects regarding the proper freezing of OSCs in fish are still unknown despite the obvious benefits of having these cells available in gene banks. To answer these questions, the objectives of this study were to test various permeating/non-permeating cryoprotectants and freezing rates that produce the highest count of live OSCs and give the highest post-thaw viability. By developing a cryopreservation protocol for OSCs from female blue catfish, it allows for the female genetic contribution for reproduction to be stored in gene banks and also offers an alternative to the freezing of sperm for gamete preservation. Moreover, transplantation of stem cells by xenogenesis may help overcome the obstacles associated with shortages of blue catfish gametes and subsequent hybrid catfish production.

3.1. Materials and Methods

3.1.1 Experimental fish

All experimental procedures were performed in accordance with an Institutional

Animal Care and Use Committee (IACUC) protocol for care and use of animals at the Fish Genetics Unit at Auburn University, Auburn, AL, United States. Female blue catfish (n = 15) were obtained from 0.04-ha earthen ponds at the EW Shell Fisheries Center at Auburn University, AL, USA. Fish were fed 5 times per week with 32% protein pelleted catfish feed at 1-2% of total fish weight per pond prior to experimentation. Fish were collected and transferred to 260 × 72 × 50 cm flow-through holding tanks. The tanks were supplied with diffused aeration and oxygen level maintained above 5 ppm, and pH levels were between 6.8-7.8. Holding temperatures were between 26-30°C. Body weights and total length were recorded from each individual. All fish were two to three years old, with body weights ranging 340 to 700 g and total length between 29 to 36 cm.

3.1.2 Ovary extraction and preparation

After euthanasia, the surface of each fish was sterilized with 70% ethanol. Ovary preparations and extractions were performed using methods modified from Shang et al. (2018) for testes. Ovaries were dissected, weight was taken, and gonad somatic index (gonad weight divided by body weight × 100) was calculated. Ovary weights ranged from 0.35 to 0.75 g and gonad somatic index from 0.06 to 0.13. Ovaries were placed in 15 mL tubes of anti-agent medium solution [contains Hanks' Balanced Salt Solution (HBSS, 21-020-CV, Mediatech, Inc., VA, USA) supplemented with 1.0 ug/mL of $NaHCO_3$ (470302-440, VWR International, PA, USA), and a combination of 100 ug/mL streptomycin and 100 unit/mL penicillin (17-602E, Lonza Group AG, MD, USA)]. Ovaries were removed and soaked in 0.5% bleach solution to remove blood clots and any connective tissue. Next, ovaries were rehydrated again with the anti-agent medium solution followed by three washes with phosphate-buffered saline (PBS, J67802, Alfa Aesar, MA, USA) and another three washes with HBSS solution. Ovaries

were dried and weighed using an analytical balance (Mettler Toledo AE 240). For each part of the study, ovaries were randomly pooled from five fish with 3 replicates per treatment, and 0.05 g was distributed into 1.5 mL cryovials containing eagle's minimum essential medium (EMEM, 15-015-CV, Mediatec Inc., VA, USA), 5% (v/v) fetal bovine serum (FBS, 0332, VWR International, PA, USA), 2 mM L-glutamine (25-005-CI, Mediatech Inc., VA, USA), and 25 mM Hepes (0511, VWR International, PA, USA). Then, three replicates were immediately processed as the control group (See Section 3.2.6). The samples for cryopreservation were transferred to new cryovials containing 500 uL cryomedium composed of 55.27 mM Hepes (0511, VWR International, PA, USA), 375.48 mM NaCl (3628-05, Avantor, PA, USA), 7.28 mM KCl (BDH9258, VWR International, Pa, USA), 23.10 mM KH_2PO_4 (0781, VWR, PA, USA), 3.82 mM Na_2HPO_4 (0404, VWR International, PA, USA), 3.64 mM sodium pyruvate (A1114, Alfa Aesar, MA, USA), 2.6 mM $CaCl_2 \times 2H_2O$ (470300-570, Ward's Science, ON, Canada), and 1.4 mM $MgCl_2 \times 6H_2O$ (442615, EMD Millipore Corp, MA, USA); Lee et al. 2013). Only OSCs were counted, excluding any other cell types that could be present in the cell suspension (i.e. oocytes, eggs and somatic cells).

3.1.3 Permeating cryoprotectants

Samples were supplemented with either DMSO (4948-02, Avantor, PA, USA), EG (BDH1125, VWR, PA, USA), glycerol (0854, VWR, PA, USA), or methanol (BDH1135, VWR, PA, USA) at three different concentrations: 1.0 M, 1.3 M, or 1.6 M. In total, there were 12 combinations (4 cryoprotectants × 3 concentrations × 3 replicates) and 3 fresh controls. Treatments were equilibrated on ice for 60 min and then frozen using a control rate freezer (PLANER Kryo 560-16) based on the following settings: start temperature at 5 ^{o}C, hold for 5 min at 5 ^{o}C, freeze at -1.0 ^{o}C/min from 5 ^{o}C to -80 ^{o}C, and then held for 5 min at -80 ^{o}C/min.

Samples were then transferred directly to liquid nitrogen (-196 ^{o}C).

3.1.4 Non-permeating cryoprotectants

OSCs were cryopreserved in 1.0 M DMSO and paired with three non-permeating cryoprotectants: 0.1 M lactose, 0.2 M lactose, or 0.1 M trehalose. These cryoprotectants were factorially tested with 10% egg yolk or 1.5% BSA. The fresh control was OSCs that did not undergo cryopreservation, and additional controls consisted of OSCs cryopreserved with 1.0 M DMSO and either egg yolk or BSA. All samples were processed as previously described.

3.1.5 Freezing rates

Different freezing rates (-1.0, -2.5, -5.0 °C/min until -80 °C) were combined with the top three cryoprotectant combinations: 1.0 M DMSO with 0.1 M trehalose and egg yolk, 0.1 M lactose and egg yolk; and 0.2 M lactose and egg yolk. All samples were processed as previously described.

3.1.6 Oogonia stem cell thawing and isolation

For thawing, samples were quickly placed in a 10 °C water bath for 1 min. Samples were rehydrated by transferring them into new cryovials containing 500 µL of Dulbecco's modified eagle medium (DMEM/Ham's F-12 50/50). Fresh and thawed ovaries were placed on a sterile plastic Petri dish, minced into small pieces, and 500 µL of 0.25% trypsin-EDTA (2.5 g/L trypsin 1:250 and 0.2 g/L EDTA) was added. Any intact portions of the ovaries were transferred to 50 mL autoclaved glass flasks containing plastic stir bars. The flasks were incubated on ice for 30 min followed by 45 min of mechanical stirring. Next, 500 µL of DMEM

was added to each flask to neutralize trypsin digestion. The cell suspensions were filtered twice by using two different sizes of nylon mesh (70 μm and 40 μm) to remove undigested tissues followed by centrifuging at 2.9 RCF for 20 min. The supernatant was discarded, and the pellet was rehydrated with 300 μL DMEM solution.

3.1.7 OSC production and viability

First, 10 uL of the cell suspension was mixed with 10 uL of trypan blue at a ratio 1:1 to differentiate live and dead cells. The sample was incubated for ~5 s, then 10 uL was pipetted onto a Neubauer hemocytometer. A total of five squares (1 mm^2) was counted for live stained cells, and the average of these counts was calculated. This process was done three times. The dilution factor was then factored in to obtain the final OSC for each replicate.

Viability of OSCs was assessed using a live/dead double staining kit (QIA76, Sigma-Aldrich) following manufacturer's instructions. Live cells were counted using an Axio Imager.A2 (Carl Zeiss Microscopy, White Plains, NY) with a 40x objective. The viability of OSCs was then assessed by taking the proportion of the total OSCs collected from the cryopreserved ovaries divided by the total number of OSCs obtained from the fresh ovaries.

Viability recovery index (%) = (*Number of live cryopreserved cells* /*Number of live fresh cells*) × 100

3.1.8 Statistical analyses

All data were analyzed using SAS statistical analysis software (v.9.4; SAS Institute Inc., Cary, NC, USA). Residuals were tested for normality (Shapiro-Wilk test; PROC UNIVARIATE) and homogeneity of variance (plot of residuals vs. predicted values; PROC

GPLOT). Data were arcsin square root (for percentage data) or $\log_{10}$ transformed to meet assumptions of normality and homoscedasticity when necessary. Post-hoc testing was done with Tukey's HSD with values considered statistically significant at alpha = 0.05.

First, one-way ANOVA was used compare post-thaw OSC production from the 12 permeating cryoprotectant/concentration combinations and the fresh control. For viability, the same combinations were analyzed by a two-way ANOVA. The cryoprotectant × concentration interaction term was significant ($P < 0.0001$), as such, the two-way ANOVA was decomposed into a series of one-way ANOVAs to determine the impacts of concentration within each cryoprotectant. Second, to analyze the effects of adding nonpermeating cryoprotectants, one-way ANOVA was used to analyze all treatment combinations on post-thaw OSC production. For viability, only the fresh control and 1.0 M DMSO with 10% egg yolk were used, and data were analyzed with the same process as post-thaw production. Lastly, to determine the effect of freezing rate intensity on post-thaw OSC production, one-way ANOVA was again implemented. Moreover, viability recovery index was analyzed using a two-way ANOVA with input factors of cryoprotectant, freezing rate, and cryoprotectant × freezing rate. There was an interaction between treatment and freezing rate ($P < 0.0001$), thus, the impacts of freezing rate were analyzed separately within each cryoprotectant using one-way ANOVA models.

3.3. Results

3.3.1. Effects of permeating cryoprotectants

The use of permeating cryoprotectants at various concentrations resulted in differences in the amount of live post-thaw oogonial stem cells after cryopreservation ($P < 0.0001$; Fig. 3.1A). As expected, the fresh control produced the highest amount of live OSCs ($4.40 \times 10^5 \pm$

1.44×10^4) ($P < 0.0001$). Within each treatment, optimal concentration varied depending on the cryoprotectant. Here, 1.0 M DMSO yielded the most OSCs ($2.47 \times 10^5 \pm 4.40 \times 10^4$), which outperformed DMSO at 1.3 M and 1.6 M. EG had the second highest amount of OSCs at 1.0 M ($1.47 \times 10^5 \pm 4.40 \times 10^4$) and 1.3 M ($9.33 \times 10^4 \pm 4.40 \times 10^4$). OSC production was similarly low for glycerol and methanol at all concentrations, ranging from only 0 cells to 2×10^4. Methanol at 1.6 M was ineffective as a cryoprotectant, for which no live OSCs were observed. For DMSO and EG, concentrations between 1.0 and 1.6 M significantly impacted OSC viability ($P = 0.0001$ for DMSO and 0.0067 for EG, respectively; Fig. 3.1B). Viability was lowest for glycerol and methanol, ranging from only 0 to 3%, and there were no differences between concentrations ($P = 0.359$) for glycerol and ($P = 0.259$) for methanol, respectively. OSCs frozen with 1.0 M DMSO gave the highest post-thaw viability (37.0 ± 1.6%), which dropped to 21% at 1.3 M and to only 5% at 1.6 M. Viability was similar to the second best treatment (1.3 M DMSO) for 1.0 M EG (22 ± 1.6%), and 1.3 M (14.0 ± 1.6%).

3.3.2. Effects of non-permeating cryoprotectants

After testing various combinations of nonpermeating cryoprotectants with 1.0 M DMSO, there were significant changes in the amount of live post-thaw oogonial stem cells between treatments ($P < 0.0001$; Fig. 3.2A). As expected, the fresh control produced the highest amount of live OSCs ($8.53 \times 10^5 \pm 2.98 \times 10^4$). The other two positive controls, 1.0 M DMSO supplemented with egg yolk or BSA, yielded much fewer OSCs (2.33×10^5 and $2.40 \times 10^5 \pm 2.98 \times 10^4$, respectively) and were similar to several other treatments. Lactose at 0.2 M with egg yolk was the top performer, producing $5.80 \times 10^5 \pm 2.98 \times 10^4$ OSCs. Lactose at 0.1 M with egg yolk achieved the next highest amount of OSCs, but the two other treatments with lactose had low cell numbers. The remaining combinations with trehalose all gave similar

results to the egg yolk and BSA controls with 2.33×10^5 cells or less. Viability was also highly impacted by treatment combination ($P < 0.0001$; Fig. 3.2B). Lactose at 0.2 M with egg yolk was also the best treatment with a viability of 67.7 ± 3.6%. There was a difference of 24% between this treatment and the second highest, 0.1 M lactose with egg yolk. Viability was similar for all other treatments to that of the positive controls, ranging from 15.3 to 29.7%.

3.3.3. Effects of freezing rates

Post-thaw OSC production was largely dependent on the treatment × temperature combination ($P < 0.0001$; Fig. 3.3A). The three cryoprotectants utilized were 0.1 M lactose, 0.2 M lactose, and 0.1 M trehalose paired with 1.0 M DMSO, for which 0.2 M lactose consistently yielded the highest number of post-thaw cells at freezing rates of -1, -2.5, and -5 °C/min (values of 4.93×10^5, 2.97×10^5, and $1.33 \times 10^5 \pm 1.85 \times 10^4$, respectively). The best treatment was 0.2 M lactose at a freezing rate of -1 °C/min. Average number of live OSCs produced decreased at the more rapid freezing rates. As for OSC viability, the effect of freezing rate was also significant within each cryoprotectant [0.1 M lactose ($P = 0.0004$); 0.2 M lactose ($P < 0.0001$); and 0.1 M trehalose ($P = 0.001$)]. Viability was highest at -1°C/min within each cryoprotectant, and notable decreases were observed at -2.5 and -5°C/min for 0.1 M lactose and 0.2 M lactose. The treatment with 0.1 M trehalose did not show a significant decrease at -5 °C/min, and viability was similar to that of -1 °C/min. Live post-thaw OSCs were visually distinguished from images obtained by fluorescent microscopy (Fig. 3.3C). It can be observed that the profile for the number of live/dead OSCs (indicated in green/red) varied among cryoprotectants and freezing rates.

3.4. Discussion

Cryo-storage of the female gamete in fishes has so far achieved minimal success due to the sensitivity of eggs to freezing damage that leads to very little or no viability post-thaw (Zhang et al., 2009). Therefore, freezing of OSCs offers a method for preserving the female contribution for the next generation (Yamaha et al., 2007). Additionally, cryopreserved OSCs from blue catfish can be used for advancing xenogenesis technologies and faster production of gametes, which may greatly benefit the catfish industry in the near future. This study developed a protocol for cryopreservation of blue catfish OSCs by assessing which cryoprotectants (both permeating and non-permeating), concentrations, and freezing rates yielded the most viable OSCs after thawing. As expected, all three of these components were proven to be important factors in overall cryopreservation success of OSCs.

DMSO, EG, glycerol, and methanol have been widely utilized as permeating cryoprotectants across a variety of cell types (Wowk, 2007; Bhattacharya, 2018). Given the current shortage of data available on OSCs, it was hypothesized that the same cryoprotectants would be suitable for OSCs. Of the four cryoprotectants tested in our study, DMSO resulted in the overall highest post-thaw OSC production and viability. Previously, Franěk et al. (2019) used DMSO, methanol, and propylene glycol to freeze ovaries from the common carp with concentrations ranging from 1.0 to 3.0 M. From these treatments, DMSO produced the highest post-thaw viability from cryopreserved ovarian tissue. In another case, spermatogonia stem cells were extracted from testes of rainbow trout, in which DMSO was responsible for higher cell survival than propylene glycol or glycerol (Lee et al., 2013). In contrast, propylene glycol was reported to be the best for the same species and cell type (Yoshizaki et al., 2011). Additionally, Pšenička et al. (2016) found that EG was the best cryoprotectant for SSCs and OSCs of Siberian sturgeon and that DMSO caused higher levels of cellular damage. The conclusion the authors drew from this inconsistency is that cryopreservation protocols are highly specific, and differences may occur between fish of different phylogenetic origins (i.e.

chondrostean or teleostean).

The optimal concentration of a cryoprotectant varies among studies, likely due to variability among species and other factors. An example from the same studies mentioned above shows that both Pšenička et al. (2016) and Franěk et al. (2019) concluded that 1.5 M DMSO and EG were the most successful concentrations. The latter study determined their conclusion after testing a series of low-to-high concentrations, while Pšenička et al. (2016) justified their use of 1.3 M from previous protocols. Furthermore, different optimal concentrations have also been drawn from studies on the same species and cell type. For example, Lee et al. (2013) found a lesser amount of 1.3 M to be optimal for spermatogonia, but Yoshizaki et al. (2011) reported 1.8 M for the same species. However, the difference between the two studies was a difference in cryoprotectant, in which 1.3 M was optimal for DMSO and 1.8 M for propylene glycol. Altogether, it can be concluded that the optimal concentration is not held universal across all possible cryoprotectants. Subtle differences in cytotoxicity of each substance are probable factors that influence a cell's sensitivity to each concentration (Zhang et al., 2007). Thus, it is important to analyze each cryoprotectant at multiple concentrations when developing new cryopreservation protocols. For blue catfish

OSCs, general trends were that post-thaw OSC production and viability were highest at 1.0 M and decreased at 1.3 M and 1.6 M. It is possible that OSCs from blue catfish are more sensitive to cytotoxicity than the other species analyzed, which is why concentrations >1.3 M reduced post-thaw viability. More research on cryoprotectant and concentrations for blue catfish or other ictalurid catfishes would be needed to solidify such conclusions.

Pairing permeating cryoprotectants with non-permeating cryoprotectants has been shown to enhance cellular post-thaw viability. For blue catfish, 0.2 M lactose with egg yolk was the best of the combinations tested that outperformed the egg yolk and BSA controls. To

our knowledge, this is the first account confirming lactose as a superior cryoprotectant for OSCs in a fish species. However, besides the best lactose treatment, most combinations of lactose and trehalose gave similar results, and there were no apparent differences between egg yolk and BSA within each treatment. Significant effects in the ANOVA models were likely driven by the large gaps between the fresh control, the best, and the low-performing treatments. Overall, OSC cryopreservation success was not highly dependent on specific treatments.

Despite the common use of trehalose for tissue cryopreservation, our results did not support trehalose as a suitable non-permeating cryoprotectant in contrast to results by Lee et al. (2016) for SSCs of Manchurian trout (*Brachymystax lenok*) and Franěk et al. (2019a) for OSCs of common carp. Similar to our study, Franěk et al. (2019a) used DMSO as the permeating cryoprotectant and added glucose, trehalose, or sucrose. Of those, there were no significant differences between glucose and trehalose. However, because lactose was not tested in their protocol, it cannot be concluded that trehalose would exceed lactose for post- thaw viability. As for the other case, Lee et al. (2016) used methanol instead of DMSO, which could have caused interactions between permeating cryoprotectants and non-permeating additives. No such inferences can be made from our data since only DMSO was used. DMSO was already confirmed as the best permeating cryoprotectant from the first part of this study, so assessing other permeating cryoprotectants would not have optimized our cryopreservation protocol any further. Overall, non-permeating cryoprotectants that are deemed successful for other species and cell types must be validated on a case-by-case basis. For our species of interest and others, further research should assess more cryoprotectant combinations to improve consistency between protocols and reduce among-study variability. For blue catfish OSCs, it can be concluded that post-thaw viability can be best improved by adding 0.2 M lactose with egg yolk to the cryomedium.

In the current study, -1°C/min was the best freezing rate for cryopreserving OSCs, and more rapid freezing rates (-2.5 and -5°C/min) reduced overall post-thaw OSC production. The significant interaction between freezing rate and cryoprotectant on post-thaw viability also showed that the effects of temperature depend on the cryoprotectant. However, it can be concluded that, overall, -1°C/min was consistently more successful when paired with our predetermined top-performing cryoprotectant combinations. This confirms what has previously been established for germ cell and tissue cryopreservation for several other fish species **(**Lacerda et al., 2010; Lee et al., 2016; Pšenička et al., 2016; Franěk et al., 2019b). Due to its efficacy and growing popularity, further research should also focus on developing a vitrification procedure for ovarian tissue as an alternative to slow freezing (Franěk et al., 2019a). This technique prevents the negative effects of crystallization by using a rapid freezing rate and high levels of cryoprotectants (Fahy et al., 1984). Although still in its preliminary stages, successful vitrification of ovarian tissue has been reported in zebrafish (*Danio rerio*) (Marques et al., 2015), showing that vitrification might be a promising technology that may increase OSC post-thaw success.

Germ cell cryopreservation is still relatively new and exploratory for use in aquaculture. Continuing to develop more consistent protocols for blue catfish and related species may point to aspects of methodology (i.e. freezing of whole ovaries or individual cells; thawing rates) that could increase cryopreservation success. Moreover, variability in oogonia/gamete quantity and quality arises from environmental and genetic factors (Fitzpatrick, 2012) that may affect cryo-freezing potential of the OSCs. If donor females can be ranked based on physical, biochemical, or molecular markers, it would help reduce variability caused by individual fish and standardize cryopreservation operations. Effects of individual variation relative to these factors can then be assessed by cell transplantation of cells and determining possible effects on fertilization and hatch success of the resulting hybrid catfish

progeny.

3.5. Conclusions

This study is the first of its kind that optimized a cryopreservation protocol for blue catfish OSCs after a thorough assessment of several cryoprotectants and freezing rates. OSCs retained their viability post-thaw using our proposed specifications: cryoprotectant mixture of DMSO 1 M with 0.2 M lactose and egg yolk frozen at a rate of -1°C/min. The hybrid catfish industry can benefit from cryopreserved OSCs by using them for genetic enhancement programs, germ cell transplantation, and emerging xenogenesis technologies.

Acknowledgements

This project was supported by the Agriculture and Food Research Initiative Competitive Grant no. 2018-67015-27614 from the USDA National Institute of Food and Agriculture.

References

Ahammad, M. M., Bhattacharyya, D., & Jana, B. B. (1998). Effect of different concentrations of cryoprotectant and extender on the hatching of Indian major carp embryos (Labeo

rohita, Catla catla, and Cirrhinus mrigala) stored at low temperature. *Cryobiology*, *37*(4), 318-324.

Asturiano, J. F., Cabrita, E., & Horváth, Á. (2017). Progress, challenges and perspectives on fish gamete cryopreservation: a mini-review. *General and comparative endocrinology*, *245*, 69-76.

Bart, A. N., & Kyaw, H. A. (2003). Survival of zebrafish, Brachydanio rerio (Hamilton-Buchanan), embryo after immersion in methanol and exposure to ultrasound with implications to cryopreservation. *Aquaculture Research*, *34*(8), 609-615.

Bhattacharya, S. (2018). Cryoprotectants and Their Usage in Cryopreservation Process. *BIOTECHNOLOGY IN BIOMEDICAL AND BIOLOGICAL SCIENCES*, 7.

Bono-Mestre, C., Cardona-Costa, J., & García-Ximénez, F. (2009). Effects on cell viability of three zebrafish testicular cell or tissue cryopreservation methods. *Cryoletters*, *30*(2), 148-152.

Cabrita, E., Labbé, C., Horváth, Á., Herráez, P., Robles, V., Asturiano, J. F., … Martínez-Páramo, S. (2015). Cryobanking in aquatic species: Applications and perspectives in fish germ cells. *Cryobiology*, *71*(3), 556. doi: 10.1016/j.cryobiol.2015.10.082

Chao, N. H., & Liao, I. C. (2001). Cryopreservation of finfish and shellfish gametes and embryos. In *Reproductive Biotechnology in Finfish Aquaculture* (pp. 161-189). Elsevier.

Chi, H. J., Koo, J. J., Kim, M. Y., Joo, J. Y., Chang, S. S., & Chung, K. S. (2002). Cryopreservation of human embryos using ethylene glycol in controlled slow freezing. *Human Reproduction*, *17*(8), 2146-2151.

Dunham, R. A., Hyde, C., Masser, M., Plumb, J. A., Smitherman, R. O., Perez, R., & Ramboux, A. C. (1994). Comparison of culture traits of channel catfish, Ictalurus punctatus, and blue catfish I. furcatus. *Journal of Applied Aquaculture*, *3*(3-4), 257-268.

Dunham, R. A., Lambert, D. M., Argue, B. J., Ligeon, C., Yant, D. R., & Liu, Z. (2000). Comparison of manual stripping and pen spawning for production of channel catfish× blue catfish hybrids and aquarium spawning of channel catfish. *North American Journal of Aquaculture*, *62*(4), 260-265.

Dunham, R. A. 2011. Aquaculture and Fisheries Biotechnology: Genetic Approaches. Cambridge, MA.

Fahy, G. M., MacFarlane, D. R., Angell, C. A., & Meryman, H. T. (1984). Vitrification as an approach to cryopreservation. *Cryobiology*, *21*(4), 407-426.

Fitzpatrick, M. S. (2012). Effects of stress on fish reproduction, gamete quality, and progeny. *Reproductive Biotechnology in Finfish Aquaculture*, 3.

Freshney, R. I. (1983). Instability, variation, and preservation. *Culture of Animal Cells*, *3*, 253-266.

Franěk, R., Tichopád, T., Steinbach, C., Xie, X., Lujić, J., Marinović, Z., ... & Pšenička, M. (2019a). Preservation of female genetic resources of common carp through oogonial stem cell manipulation. *Cryobiology*, *87*, 78-85.

Franěk, R., Marinović, Z., Lujić, J., Urbányi, B., Fučíková, M., Kašpar, V., ... & Horváth, Á. (2019b). Cryopreservation and transplantation of common carp spermatogonia. *PloS one*, *14*(4).

Higaki, S., Todo, T., Teshima, R., Tooyama, I., Fujioka, Y., Sakai, N., & Takada, T. (2018). Cryopreservation of male and female gonial cells by vitrification in the critically endangered cyprinid honmoroko Gnathopogon caerulescens. *Fish physiology and biochemistry*, *44*(2), 503-513.

Higuchi, K., Takeuchi, Y., Miwa, M., Yamamoto, Y., Tsunemoto, K., & Yoshizaki, G. (2011). Colonization, proliferation, and survival of intraperitoneally transplanted yellowtail Seriola quinqueradiata spermatogonia in nibe croaker Nibea mitsukurii recipient. *Fisheries Science*, *77*(1), 69–77. https://doi.org/10.1007/s12562-010-0314-7

Hu, E., Yang, H., Tiersch, T. R. 2011. High-throughput cryopreservation of spermatozoa of blue catfish (*Ictalurus furcatus*): establishment of an approach for commercial-scale processing. Cryobiology, 62(1):74-82.

Iussig, B., Maggiulli, R., Fabozzi, G., Bertelle, S., Vaiarelli, A., Cimadomo, D., ... & Rienzi, L. (2019). A brief history of oocyte cryopreservation: arguments and facts. *Acta obstetricia et gynecologica Scandinavica*, *98*(5), 550-558.

Lacerda, S. M., Batlouni, S. R., Costa, G. M., Segatelli, T. M., Quirino, B. R., Queiroz, B. M., ... & Franca, L. R. (2010). A new and fast technique to generate offspring after germ cells transplantation in adult fish: the Nile tilapia (Oreochromis niloticus) model. *PloS one*, *5*(5).

Lee, S., Iwasaki, Y., Shikina, S., & Yoshizaki, G. (2013). Generation of functional eggs and sperm from cryopreserved whole testes. *Proceedings of the National Academy of Sciences*, *110*(5), 1640-1645.

Lee, Y. A., Kim, Y. H., Ha, S. J., Kim, K. J., Kim, B. J., Kim, B. G., ... & Ryu, B. Y. (2014). Cryopreservation of porcine spermatogonial stem cells by slow-freezing testis tissue in trehalose. *Journal of Animal Science*, *92*(3), 984-995.

Lee, S., N. Katayama, G. Yoshizaki, Generation of juvenile rainbow trout derived from cryopreserved whole ovaries by intraperitoneal transplantation of ovarian germ cells, Biochem. Biophys. Res. Commun. (2016) 6–11, https://doi.org/10.1016/j.bbrc.2016.08.156.

Linhart, O., Rodina, M., Flajshans, M., Gela, D., & Kocour, M. (2005). Cryopreservation of European catfish Silurus glanis sperm: sperm motility, viability, and hatching success of embryos. *Cryobiology*, *51*(3), 250-261.

Lujić, J., Marinović, Z., Bajec, S. S., Djurdjevič, I., Kása, E., Urbányi, B., & Horváth, Á. (2017). First successful vitrification of salmonid ovarian tissue. *Cryobiology*, *76*, 154-157.

Magnotti, C., Cerqueira, V., Lee-Estevez, M., Farias, J. G., Valdebenito, I., & Figueroa, E. (2018). Cryopreservation and vitrification of fish semen: a review with special emphasis on marine species. *Reviews in Aquaculture*, *10*(1), 15-25.

Marques, L. S., Bos-Mikich, A., Godoy, L. C., Silva, L. A., Maschio, D., Zhang, T., & Streit Jr, D. P. (2015). Viability of zebrafish (Danio rerio) ovarian follicles after vitrification in a metal container. *Cryobiology*, *71*(3), 367-373.

Matthews, J. L., Murphy, J. M., Carmichael, C., Yang, H., Tiersch, T., Westerfield, M., & Varga, Z. M. (2018). Changes to extender, cryoprotective medium, and in vitro fertilization improve zebrafish sperm cryopreservation. *Zebrafish*, *15*(3), 279-290.

Meryman, H. T. (2007). Cryopreservation of living cells: principles and practice. *Transfusion*, *47*(5), 935-945.

Migaud, H., Bell, G., Cabrita, E., McAndrew, B., Davie, A., Bobe, J., ... & Carrillo, M. (2013). Gamete quality and broodstock management in temperate fish. *Reviews in Aquaculture*, *5*, S194-S223.

Muchlisin, Z. A. (2005). Current status of extenders and cryoprotectants on fish spermatozoa cryopreservation. *Biodiversitas Journal of Biological Diversity*, *6*(1).

ZA, Muchlisin., WN, N., & MN, S. A. (2015). Exploration of natural cryoprotectants for cryopreservation of African catfish, Clarias gariepinus, Burchell 1822 (Pisces: Clariidae) spermatozoa. *Chezch Journal of Animal Sciences*, *60*(1), 10-15.

Perera, D. A., Alsaqufi, A., Shang, M., Wade, D. C., Su, B., Elaswad, A., ... & Lipke, E. A. (2017). Xenogenesis-production of channel catfish× blue catfish hybrid progeny by fertilization of channel catfish eggs with sperm from triploid channel catfish males with transplanted blue catfish germ cells. *North American Journal of Aquaculture*, *79*(1), 61-74.

Pšenička, M., Saito, T., Rodina, M., & Dzyuba, B. (2016). Cryopreservation of early stage Siberian sturgeon Acipenser baerii germ cells, comparison of whole tissue and dissociated cells. *Cryobiology*, *72*(2), 119-122.

Rana, K. J. (1995). Cryopreservation of aquatic gametes and embryos: recent advances and applications.

Rathore, S. S., Chandravanshi, A., Chandravanshi, P., & Jaiswal, K. (2016). Review on cryopreservation in fisheries science: Applications and perspectives. *International Journal of Science and Research*, *5*(10), 573-579.

Suquet, M., Dreanno, C., Fauvel, C., Cosson, J., & Billard, R. (2000). Cryopreservation of sperm in marine fish. *Aquaculture Research: Original Articles*, *31*(3), 231-243.

Viveiros, A. T. M., So, N., & Komen, J. (2000). Sperm cryopreservation of African catfish, Clarias gariepinus: cryoprotectants, freezing rates and sperm: egg dilution ratio. *Theriogenology*, *54*(9), 1395-1408.

Voelkel, S. A., & Hu, Y. X. (1992). Use of ethylene glycol as a cryoprotectant for bovine embryos allowing direct transfer of frozen-thawed embryos to recipient females. *Theriogenology*, *37*(3), 687-697.

Wowk, B. (2007). How cryoprotectants work. *Cryonics*, *28*(3), 3-7.

Yamaha E., Saito T., Goto-Kazeto R., Arai K. (2007): Developmental biotechnology for aquaculture, with special reference to surrogate production in teleost fishes. Journal of Sea Research, 58, 8–22.

Yoshizaki, G., & Lee, S. (2018). Production of live fish derived from frozen germ cells via germ cell transplantation. *Stem cell research*, *29*, 103-110.

Zhang, T., & Rawson, D. M. (1998). Permeability of dechorionated one-cell and six-somite stage zebrafish (Brachydanio rerio) embryos to water and methanol. *Cryobiology*, *37*(1), 13-21.

Zhang T, Rawson DM, Pekarsky I, Blais I, Lubzens E. Low temperature preservation of fish gonad cells and oocytes. In: Babin PJ, Cerda` J, Lubzens E. (eds) The fish Oocyte: from basic studies to biotechnological applications. Online: Springer: 2007, p. 411-436.

Zhang, T., & Lubzens, E. (2009). Cryopreservation of fish oocytes. *Methods in reproductive aquaculture: marine and freshwater species. CRC Press, Boca Raton*, 251-264.

Figure Captions

Fig. 1. Oogonial stem cells (OSCs) from blue catfish (*Ictalurus furcatus*) were cryopreserved with a series of permeating cryoprotectants (DMSO, ethylene glycol (EG), glycerol, and methanol) at three concentrations (1.0 M, 1.3 M, and 1.6 M). Means of OSC production for each treatment were compared by one-way ANOVA (A). Due to a significant interaction in the two-way ANOVA model, viability recovery index was analyzed with separate one-way ANOVA within each cryoprotectant (B). LSMs + SEM are represented, and ascending letters are different by Tukey's HSD.

Fig. 2. Oogonial stem cells (OSCs) from blue catfish (*Ictalurus furcatus*) were cryopreserved in 1.0 M DMSO and paired with lactose or trehalose (at 1.0 or 2.0 M). Each treatment was tested with 10% egg yolk or 1.5% BSA. Means of OSC production and viability recovery index for each treatment were compared by one-way ANOVAs. LSMs + SEM are shown for the post-thaw OSC production (A) and viability recovery index (B), in which different letters indicate significance between treatments by Tukey's HSD.

Fig. 3. Treatments that yielded the best results from the first two parts of this study were subjected to three different freezing rates (-1, -2.5, and -5°C/min). Means of OSC production and viability recovery index for each treatment were compared by one-way ANOVAs (A). Due to a significant interaction in the two-way ANOVA model, freezing rates were analyzed within each cryoprotectant for the viability recovery index (B). Bars show the LSMs + SEM, and different letters indicate significance in treatments by Tukey's HSD. For each treatment, OSCs were visually distinguished using images obtained by fluorescent microscopy (C). Green and red fluorescence indicates live and dead OSCs.

Fig. 3.1.

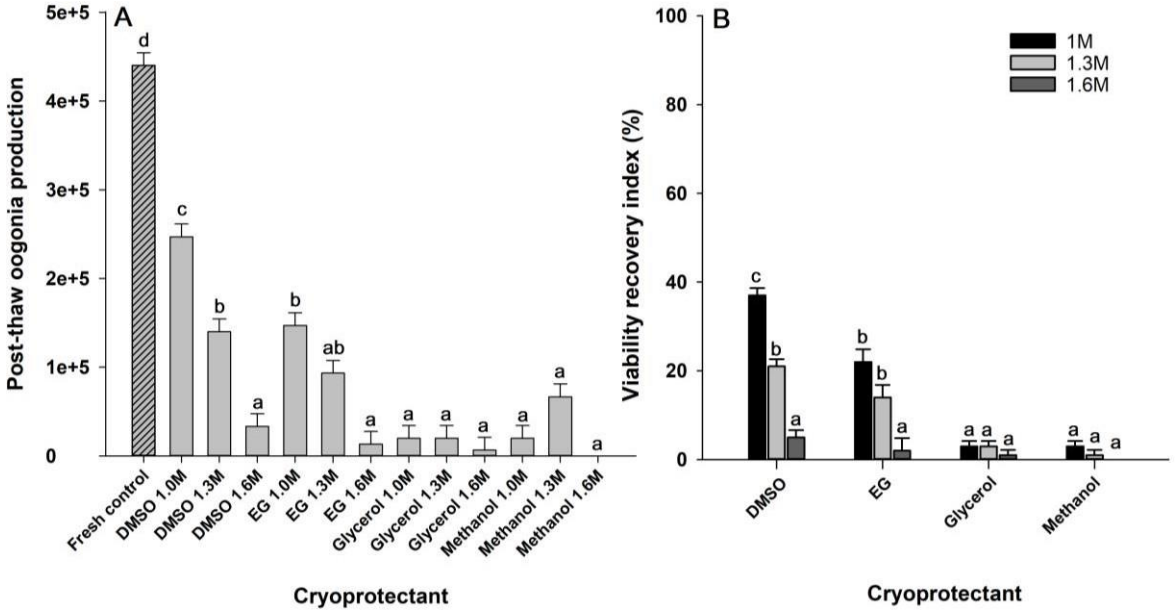

A
Post-thaw oogonia production
5e+5
4e+5
3e+5
2e+5
1e+5
0
d
c
b
a
b
ab
a
a
a
a
a
a
Fresh control
DMSO 1.0M
DMSO 1.3M
DMSO 1.6M
EG 1.0M
EG 1.3M
EG 1.6M
Glycerol 1.0M
Glycerol 1.3M
Glycerol 1.6M
Methanol 1.0M
Methanol 1.3M
Methanol 1.6M
Cryoprotectant
B
Viability recovery index (%)
100
80
60
40
20
0
1M
1.3M
1.6M
c
b
a
b
b
a
a a a
a a a
DMSO
EG
Glycerol
Methanol
Cryoprotectant

Fig. 3.2.

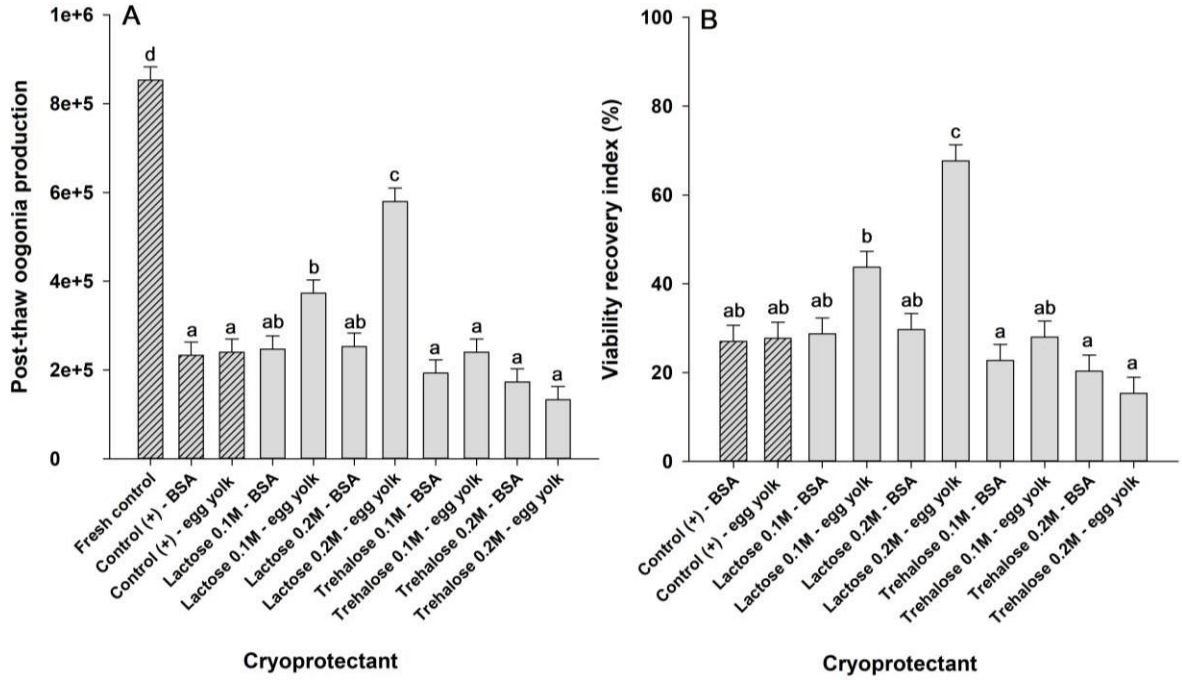
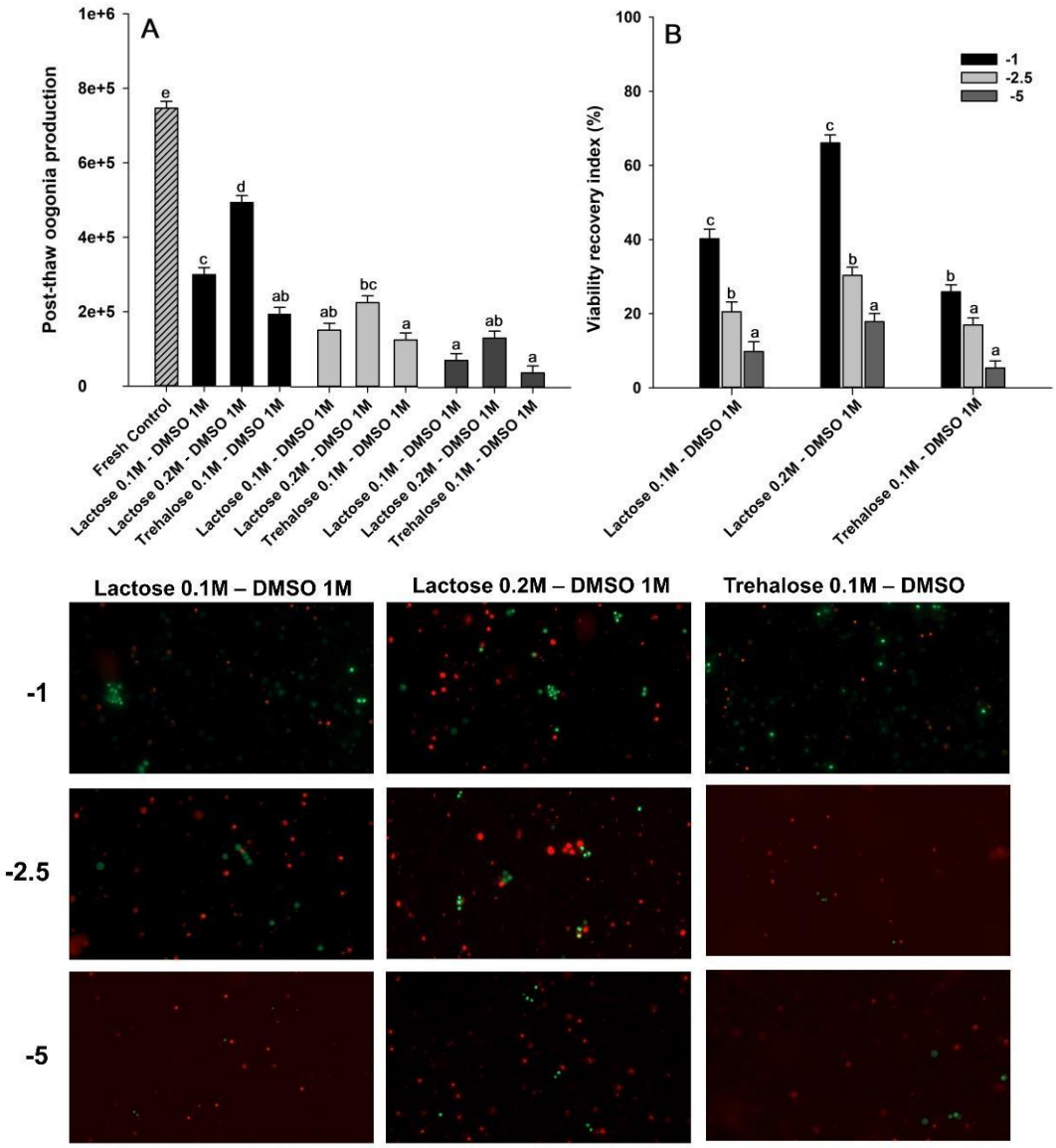

A
Post-thaw oogonia production
1e+6
8e+5
6e+5
4e+5
2e+5
0
d
a
a
ab
b
ab
c
a
a
a
a
Fresh control
Control (+) - BSA
Control (+) - egg yolk
Lactose 0.1M - BSA
Lactose 0.1M - egg yolk
Lactose 0.2M - BSA
Lactose 0.2M - egg yolk
Trehalose 0.1M - BSA
Trehalose 0.1M - egg yolk
Trehalose 0.2M - BSA
Trehalose 0.2M - egg yolk
Cryoprotectant
B
Viability recovery index (%)
100
80
60
40
20
0
ab
ab
ab
b
ab
c
a
ab
a
a
Control (+) - BSA
Control (+) - egg yolk
Lactose 0.1M - BSA
Lactose 0.1M - egg yolk
Lactose 0.2M - BSA
Lactose 0.2M - egg yolk
Trehalose 0.1M - BSA
Trehalose 0.1M - egg yolk
Trehalose 0.2M - BSA
Trehalose 0.2M - egg yolk
Cryoprotectant

Fig. 3.3.


A
Post-thaw oogonia production
1e+6
8e+5
6e+5
4e+5
2e+5
0
e
c
d
ab
ab
bc
a
a
ab
a
Fresh Control
Lactose 0.1M - DMSO 1M
Lactose 0.2M - DMSO 1M
Trehalose 0.1M - DMSO 1M
Lactose 0.1M - DMSO 1M
Lactose 0.2M - DMSO 1M
Trehalose 0.1M - DMSO 1M
Lactose 0.1M - DMSO 1M
Lactose 0.2M - DMSO 1M
Trehalose 0.1M - DMSO 1M
B
Viability recovery index (%)
100
80
60
40
20
0
-1
-2.5
-5
c
b
a
c
b
a
b
a
a
Lactose 0.1M - DMSO 1M
Lactose 0.2M - DMSO 1M
Trehalose 0.1M - DMSO 1M
Lactose 0.1M – DMSO 1M
Lactose 0.2M – DMSO 1M
Trehalose 0.1M – DMSO
-1
-2.5
-5

Chapter 4 - Effects of Antioxidants and Antifreeze Protein on Cryopreservation of Blue Catfish (*Ictalurus furcatus)* Spermatogonial Stem Cells

Abstract

Long-term storage of spermatogonia stem cells (SSCs) adds a valuable resource to gene banks, and may improve genetic enhancement programs and xenogenesis technologies for aquaculture research. Successful cryopreservation of SSCs depends on many factors, typically involving the use of permeating and non-permeating cryoprotective agents. Hybrid catfish are produced by crossing channel catfish, *Ictalurus punctatus* ♀ with blue catfish, *I. furcatus* ♂. Of the two parent species, gametes from blue catfish males are quite valuable and in limited supply, making it necessary to improve current cryopreservation protocols. The objectives of this experiment were to analyze the individual and combined effects of antioxidants (catalase, hypotaurine, and ascorbic acid) and antifreeze proteins (AFP I and AFP III) on post-thaw viability of SSCs from blue catfish testicular tissue after cryopreservation. Overall, the addition of individual antioxidants or antifreeze proteins did not improve post-thaw SSC production or viability, but there were significant differences between the individual treatments. In the combined analysis, treatments with the antioxidant hypotaurine gave higher post-thaw SSC production and viability than catalase across the range of AFPs. Some treatments yielded higher post-thaw SSC recovery than the control, indicating that certain combinations of antioxidants and AFPs can be beneficial for cryopreservation success. These results confirm that adding hypotaurine with either AFPI 1.0 µg/mL or AFPIII 0.1 µg/mL to the cryomedia will maximize the number of live SSCs after freezing. Overall, these results also build knowledge of SSC cryopreservation and the underlying factors that must be optimized within each protocol.

4.1 Introduction

Long-term storage of gametes has been recognized for its importance in maintaining everlasting gene banks and a continuous gamete supply for terrestrial animals as well as for aquatic organisms (reviewed by Mandawala et al., 2016). For fish, cryopreservation technology was first introduced to cryopreserve spermatozoa, and protocols have been optimized for a variety of both marine and freshwater cultured species (Suquet et al., 2000; Rathore et al., 2016). Thus, many hatcheries already maintain their own sperm banks and recognize the enormous potential cryopreserved gametes have for industrial advancement (Labbé et al., 2013). Additional resources for generating gametes are in the form of spermatogonia stem cells (SSCs) cryopreserved from whole testes in fish (Lee et al., 2013; Lee et al., 2014). These cells exhibit sexual plasticity, possessing the ability to develop into either spermatozoa or oocytes once they mature (Molyneaux & Wylie, 2004). Aquaculture research has recently been focusing on SSC and OSC extraction and long-term storage because they add a valuable resource to gene banks and may improve genetic enhancement programs and xenogenesis technologies (Lacerda et al., 2013; Shang et al., 2018), especially for species that experience reproductive roadblocks in captivity.

Hybrid catfish, the cross between channel catfish, *Ictalurus punctatus* ♀ × blue catfish, *I. furcatus* ♂ are often cultured and economically valuable for U.S. aquaculture (Torrans and Ott, 2018), but due to the lack of natural hybridization between the two parent species, hybrids must be produced by *in vitro* fertilization (IVF) (Bart and Dunham, 1996; Argue et al., 2003). Maintaining blue catfish males in commercial hatcheries is a limitation since they take longer to mature than channel catfish and require more feed and space resources (Dunham et al., 1994). Furthermore, each male can only be used once due to lethal methods of testes extraction

(Argue et al., 2003; Hu et al., 2011), making each male's sperm quite valuable and in limited supply. This paternal dilemma has made it necessary to take new innovative approaches to obtain and store gametes from blue catfish in order to improve hybrid catfish production and sustainability. In response to this need, research has turned to SSC transplantation into channel catfish hosts, with results showing variable levels ofsuccess (Perera et al., 2017; Shang et al., 2018). SSCs can be transplanted into a host organism of different species to produce xenogens, defined as organisms in which comprised of elements not typical to its species (Dunham, 2011; Higuchi et al., 2011). In this process, the host matures, but develops the gametes of the donor, which is greatly beneficial if the host species possesses accelerated growth rates, earlier age of sexual maturity, and/or more convenient husbandry requirements. All of these benefits can be taken advantage of if blue catfish gametes can be produced within channel catfish hosts (Shang et al., 2018). Xenogenesis represents one method among others that SSCs can be used for aquacultural gain, and these technologies would further improve if SSCs from specific males could be stored and retain their post-thaw viability.

Successful cryopreservation of SSCs or any tissue type is highly dependent on a multitude of specific factors within each protocol that must be optimized on a case-by-case basis (Elliot et al., 2017; Magnotti et al., 2018). Because cryopreservation inevitably puts stress on the cells, reducing intracellular damage and oxidative stress during deep freezing is the best way to ensure viability of live cells for downstream applications (Muchlisin, 2005; Kim, 2017). This typically involves the use of permeating and non-permeating cryoprotective agents to prevent cryo-injuries (Hezayehei et al., 2018). Of these different additive agents, antioxidants are beneficial in slowing or even reversing the effects of oxidative stress cells endure during cryopreservation (Cabrita et al., 2011). Reactive oxygen species (ROS) usually occur in low concentrations and are natural products of cellular metabolism (Muzzalupo, 2013). However,

accumulation of ROS affects mechanisms related to cellular divisions, apoptosis, and cellular signaling (Halliwell, 2000; Len et al., 2019), and they damage proteins, lipids, and DNA if cells are unable to detoxify them (Temple et al., 2005; Holliwell, 2006; Len et al., 2019).

There are several antioxidants that have reduced cryodamage (reviewed by Len et al., 2019), and the top-performing treatments are based on the cell type and integrity prior to cryopreservation (Cabrita et al., 2011; Len et al., 2019). Substances such as α-tocoferol, ascorbic acid, taurine, and hypotaurine as well as specific vitamins have been tested as potential additives to cryomedia for fish sperm (Cabrita et al., 2011; Martínez-Páramo et al., 2012). The most efficient antioxidant for cryopreservation of male gametes varies among studies and may even be species dependent. For example, vitamins C and E reduced DNA damage of spermatozoa from gilt-head seabream, *Sparus aurata*, but contradictorily, these antioxidants increased DNA damage in European seabass, *Dicentrarchus labrax* (Cabrita et al., 2011).

Post-thaw sperm viability has been improved with antioxidants, but sperm are still more vulnerable to cryo-injuries than other male-derived cell types (i.e. spermatogonia types A or B, spermatocytes) because they have less cytoplasm, increasing their susceptibility for ROS accumulation (Celino et al., 2011). Given this advantage, SSCs may have better post- thaw viability than sperm cells of the same species with or without the antioxidant additives. Impacts on SSCs have been studied for a few select mammal species. In mice, Aliakbari et al. (2016, 2017) added catalase and α-tocopherol to the cryomedia, and survival of the SSCs was significantly improved compared to the control treatment. Similar results were found by Ha et al. (2016) with addition of 14 mM hypotaurine. In sheep, *Ovis aries*, vitamins C and E increased viability of SSCs (Shabani et al., 2017), similar to the results found for gilt-head seabream as described previously. Currently, data on antioxidant effects for aquatic

organisms are lacking, and it is not certain that SSCs from fish would respond the same way as SSCs of mammalian origin.

Antifreeze proteins (AFPs) have developed in multiple taxa, including fish, as a highly specialized biological adaptation to subzero temperatures (Hassoun & Emir, 2017; Robles et al., 2019). AFPs have allowed fish to survive in environments that would otherwise be uninhabitable (Devries, 1982). They operate at the molecular level and interact with the cellular membranes (Hassoun & Emir, 2017). One of the unique features that make these compounds relevant for cryopreservation is that they inhibit crystallization during deep freezing (Harding et al., 2003; Fuller, 2004; Irshad et al., 2020). There are many known types, but common AFPs studied across fish species are AFPI, II, and III (Robles et al., 2019). Using AFPs in cryopreservation protocols has already been attempted for sperm, oocytes, and also embryos of various organisms (as reviewed by Robles et al., 2019), but not SSCs. The authors noted that AFPs can be beneficial if the optimal type and concentration are used, but that their success is highly dependent on other methodological factors. A few notable examples on fish concluded that cryopreserving embryos of gilthead seabream and turbot, *Scophthalmus maximus*, significantly increased hatch success when AFPI and AFP III were added, respectively (Robles et al., 2006; 2007). It is currently unknown if the same positive effects would be observed for SSCs or even if combining AFPs with antioxidants could optimize current cryopreservation procedures.

The objectives of these experiments were to analyze a subset of known antioxidants (catalase, hypotaurine, and ascorbic acid) and AFPs (AFPI and AFP III) and their impacts on post-thaw viability of SSCs from blue catfish testicular tissue after cryopreservation. The role of these input factors on the cryopreservation success of blue catfish SSCs has not yet been established. By optimizing the cryopreservation protocol for SSCs, these cells can be used as an alternative to storing spermatozoa and can be used in emerging xenogenesis technologies.

From a broader perspective, these results can build on the overall knowledge of how cryoprotective additives influence cryopreservation outcomes for stem cells in aquatic organisms.

4.2. Materials and Methods

4.2.1 Experimental fish

All experimental procedures were performed in accordance with an Institutional Animal Care and Use Committee (IACUC) protocol for care and use of animals. Male blue catfish (n = 12) were obtained from 0.04-ha earthen ponds at the EW Shell Fisheries Center at Auburn University, AL, USA. Fish were fed 5 times per week with 28-32% protein pelleted catfish feed at 1% of total fish weight per pond prior to experimentation. Fish were collected and transferred to 260 × 72 × 50 cm flow-through holding tanks. The tanks were supplied with diffused aeration, and oxygen levels were maintained above 5 ppm with pH between 6.8 and 7.8. Temperatures at testicular harvest were between 26-30 °C. Body weights and total length were recorded from each individual. All fish were two years old, with body weights ranging 400 to 800 g and total length between 33 to 38 cm.

4.2.2 Testes extraction and preparation

After euthanasia, the surface of each fish was sterilized with 70% ethanol. Preparations and extractions were performed using methods modified from Shang et al. (2018). Testes were dissected, and weight and gonadosomatic index (gonad weight divided by body weight × 100) were recorded. Testes weights ranged from 0.4 to 0.8 g and gonad somatic index from 0.09 to

0.15. Testes were placed in 15 mL tubes of anti-agent medium solution [contains Hanks' Balanced Salt Solution (HBSS, 21-020-CV, Mediatech, Inc., VA, USA) supplemented with 1.0 ug/mL of ($NaHCO_3$, 470302-440, VWR International, PA, USA) and a combination of 100 ug/mL streptomycin and 100 unit/mL penicillin (17-602E, Lonza Group AG, MD, USA)]. Testes were removed, soaked in 0.5% bleach solution to remove blood clots and any connective tissue, and were rehydrated with the anti-agent medium solution followed by three washes with phosphate-buffered saline (PBS, J67802, Alfa Aesar, MA, USA) and another three washes with HBSS solution. Testes were dried and weighed using an analytical balance (Mettler Toledo AE 240). For each part of the study, testes were randomly pooled from five fish with 3 replicates per treatment, and 0.05 g was distributed into 1.5 mL cryovials containing Eagle's Minimum Essential Medium (EMEM, 15-015-CV, Mediatec Inc., VA, USA), 5% (v/v) fetal bovine serum (FBS, 0332, VWR International, PA, USA), 2 mM L-glutamine (25-005-CI, Mediatech Inc., VA, USA), and 25 mM Hepes (0511, VWR International, PA, USA). Then, three replicates were immediately processed as the fresh control group. The samples for cryopreservation were transferred to new cryovials containing 500 uL cryomedium composed of 55.27 mM Hepes (0511, VWR International, PA, USA), 375.48 mM NaCl (3628-05, Avantor, PA, USA), 7.28 mM KCl (BDH9258, VWR, PA, USA), 23.10 mM KH_2PO_4 (0781, VWR, PA, USA), 3.82 mM Na_2HPO_4 (0404, VWR, PA, USA), 3.64 mM sodium pyruvate (A1114, Alfa Aesar, MA, USA), 2.6 mM $CaCl_2 \times 2H_2O$ (470300-570, Ward's Science, ON, Canada) and 1.4 mM $MgCl_2 \times 6H_2O$ (442615, EMD Millipore Corp, MA, USA) (Lee et al. 2013).

4.2.3 Antioxidants, antifreeze proteins, and combined effects

Samples were independently tested with a total of fifteen antioxidant or AFP treatments. One treatment was added to the cryomedia of each sample prior to freezing. The antioxidants tested were catalase (50, 100, and 200 IU/mL (C1328, Spectrum, CA, USA), hypotaurine (3.5, 7, and 14 mM/mL) (159711, MP Biomedicals LLC, OH, USA), and ascorbic acid (0.1, 0.5 and 1 mM/mL) (BDH9242, VWR International, Pa, USA). AFPI and AFPIII were each tested at 0.1, 1, and 10 µg/mL (A/F Protein Inc., MA, USA). Secondly, to assess the combined effects of antioxidants and AFPs on cryopreservation, the two best concentrations of hypotaurine (3.5 mM and 7 mM/mL) and catalase (50 IU, 100 IU/mL) from the first experiment were factorially tested with the three AFP treatments (AFPI 0.1 µg/mL, AFPI 1.0 µg/mL, and AFPIII 0.1 µg/mL). Due to the poor performance of ascorbic acid, this antioxidant was discarded from the combined analysis.

Treatments were equilibrated on ice for 60 min. Upon removal, a cryoprotectant mixture of 1.0 M DMSO (4948-02, Avantor, PA, USA), 0.2 M lactose (36218, Alfa Aesar, MA, USA), and 10% egg yolk was added to each 1.5 mL sample. They were frozen using a control rate freezer (PLANER Kryo 560-16) based on the following settings: start temperature at 5 ºC, hold for 5 min at 5 ºC, freeze at -1.0 ºC/min from 5 ºC to -80 ºC, and then held for 5 min at -80 ºC/min. Samples were then transferred directly to liquid nitrogen (-196ºC).

4.2.4 Thawing and isolation of SSCs

For thawing, samples were quickly placed in a 10°C water bath for 1 min. Samples were rehydrated by transferring them into new cryovials containing 500 µL of Dulbecco's Modified Eagle Medium (DMEM/Ham's F-12 50/50) (10-090-CV, VWR International, Pa, USA). Fresh and thawed testes were placed on a sterile plastic Petri dish, minced into small pieces, and 500 µL of 0.25% trypsin - EDTA (2.5 g/L trypsin 1:250 and 0.2 g/L EDTA) (25200-

072, Gibco, NY, USA) was added. Any intact pieces were transferred to 50 mL autoclaved glass flasks containing plastic stir bars. The flasks were incubated on ice for 30 min followed by 45 min of mechanical stirring. Next, 500 µL of DMEM was added to each flask to neutralize trypsin digestion. The cell suspensions were filtered twice by using two different sizes of nylon mesh (70 µm and 40 µm) to remove undigested tissues followed by centrifuging at 2.9 RCF for 20 min. The supernatant was discarded, and the pellet was rehydrated with 300 µL DMEM solution.

4.2.5 SSC production and viability recovery index

The cell suspension (10 uL) was mixed with 10 uL of trypan blue at a ratio 1:1 to differentiate between live and dead cells. The sample was incubated for ~5 s, then 10 uL was pipetted onto a Neubauer hemocytometer. Only SSCs from each cell suspension were counted. A total of five squares (1 mm^2) was counted for live and dead stained cells, and the average of these counts was calculated. This process was done three times. The dilution factor was then factored in to obtain the final spermatogonia A (hereafter referred to as SSC) production count for each replicate.

Viability of SSCs was assessed using a live/dead double staining kit (QIA76, Sigma-Aldrich) following manufacturer's instructions. Live cells were counted using an Axio Imager.A2 (Carl Zeiss Microscopy, White Plains, NY) with a 40× objective. The viability of SSCs was then assessed by taking the proportion of the total SSCs collected from the cryopreserved testes divided by the total number of SSCs obtained from the fresh testes:

Viability recovery index (%) = (*Number of live cryopreserved cells* /*Number of live fresh cells*) × 100.

4.2.6 Statistical analyses

All data were analyzed using SAS statistical analysis software (v.9.4; SAS Institute Inc., Cary, NC, USA). Residuals were tested for normality (Shapiro-Wilk test; PROC UNIVARIATE) and homogeneity of variance (plot of residuals vs. predicted values; PROC GPLOT). Data were arcsin square root or $\log_{10}$ transformed to meet assumptions of normality and homoscedasticity when necessary. Treatment means were contrasted using the least-squares means (LSM) method, and post-hoc testing was done with Tukey's HSD. Alpha was set at 0.05 for main effects.

Separate one-way ANOVAs were used to compare post-thaw SSC production and viability from the independent antioxidant and AFP treatments (n = 15) and then for the combined antioxidant + AFP treatments (n = 18). Fresh cells without cryopreservation were used as a control for SSC production. Samples cryopreserved with 1.0 M DMSO + 0.2 M lactose and 10% egg yolk (no added antioxidants or AFPs) were used as an additional control group for both SSC production and viability.

4.3. Results

4.3.1 Independent effects of antioxidants and AFPs

The process of cryopreservation caused a notable decrease of the SSC live cells from freezing to post-thaw from 9.6×10^5 to 6.3×10^5 (Fig. 4.1A). Overall, the addition of individual antioxidants or antifreeze proteins did not improve post-thaw SSC production or the viability recovery index to that of the control (Fig. 4.1A,B), but there were significant differences between the individual treatments ($P < 0.0001$). When comparing antioxidants to

antifreeze proteins, most of the treatments yielded similar results. Of the antioxidant treatments, catalase 50 IU/mL and 100 IU/mL and all hypotaurine treatments were all statistically the same as the control, with production and viability ranging from 4.9×10^5 to 5.7×10^5 and 51.3 to 60.0%, respectively. Of the antifreeze proteins assessed, only AFPI 1 µg/mL gave the same post-thaw production and viability as the control. Within each antioxidant or AFP, there were some significant decreases at the highest concentration for catalase and ascorbic acid, but not for hypotaurine, AFPI or AFPIII. Overall, ascorbic acid at all concentrations was ineffective, resulting in the lowest SSC production and viability at 1 mM/mL.

4.3.2 Combined effects of antioxidants and AFPs

A total of sixteen treatments were compared among each other and to the control groups for SSC production and viability recovery index (Fig. 4.2). Differences between the best and worst antioxidant/AFP combinations were highly significant for both variables ($P < 0.0001$). Images obtained by fluorescent microscopy show the differences in viability percentages between treatments of antioxidants, AFPs, and both additives combined (Fig. 4.2; top panel). Overall, treatments with the antioxidant hypotaurine gave higher post-thaw SSC production and viability than catalase across the range of AFPs (6.12×10^5 compared to 4.53×10^5 for SSC production; 85.47% compared to 71.75% for viability). From these results, it was not evident AFPI was consistently better or worse than AFPIII.

For SSC yield after cryopreservation, the three treatments that gave higher SSC numbers than the control all contained hypotaurine, the highest being hypotaurine 7 mM/mL + AFPI 1.0 µg/mL with 8.53×10^5 cells (Fig. 4.2A). Hypotaurine 3.5 mM/mL + AFPI 0.1 µg/mL and hypotaurine 7 mM/mL + AFPIII 0.1 µg/mL were both statistically the same as the

best treatment with values ranging from 8.00×10^5 to 8.27×10^5. The worst performing treatment was catalase 50 IU/mL + AFPIII 0.1 µg/mL, yielding only 1.87×10^5 cells. The other treatments gave similar intermediate results within this range. For viability, several treatments outperformed the control group (Fig. 4.2B). The best treatment was hypotaurine 7 mM/mL + AFPIII 0.1 µg/mL with viability of 94.23%. Five of the other treatments that gave similarly high results, ranging from 83.27 to 93.43%. With only 74.5% viability for the control, the post-thaw viability was significantly improved by six of the total antioxidant/AFP combinations. Similar to the results for SSC production, the worst treatment was catalase 50 IU/mL + AFPIII 0.1 µg/mL with only 46.9% viability.

4.4. Discussion

Antioxidants and AFPs have been shown to be reliable cryoprotective agents for certain cell types in terrestrial mammals as well as some aquatic organisms (Len et al., 2019; Robles et al., 2019). Currently, there is little information available as to whether or not these additives would improve cryopreservation success of SSCs, especially for aquatic freshwater teleosts that make up the backbone of the global aquaculture industry (FAO, 2017). In response to this need, we assessed the impacts of a subset of antioxidants (catalase, hypotaurine, and ascorbic acid) and AFPs (AFPI and AFP III) at different concentrations on post-thaw viability of blue catfish SSCs. These substances were originally chosen because of their known efficacy as cryoprotectants for various cell types (gametes, embryos), including stem cells.

In this study, there were significant differences in post-thaw SSC production and viability among antioxidant and AFP combinations, but overall, no treatments outperformed the control group (without any antioxidants or AFPs added) when added to the cryopreservation protocol individually. The most interesting result was that there were

improvements when certain antioxidants and AFPs were combined, specifically some of the hypotaurine treatments, indicating that the interactions between these two factors can significantly influence cryopreservation success. Given the novelty of this research, comparisons cannot be made to other teleosts. To our knowledge, this is the first study of its kind to assess antioxidants and AFPs as potential additives to SSC cryopreservation protocols of a popular aquaculture species. We, therefore, compare our results to the current available data that exists for SSCs studies in mammals and for sperm cryopreservation in other fish species and taxonomic groups.

Improvements in cell viability and recovery have been observed for sperm cryopreservation in other teleost species (Karanova et al., 2002; Cabrita et al., 2011; Martínez-Páramo et al., 2012; Zilli et al., 2014). For a few species of mammals, the antioxidants catalase and hypotaurine were successful in improving post-thaw viability of SSCs (Aliakbari et al. 2016; 2017; Shabani et al., 2017), but positive responses by sperm are more evident across the available literature. This may be due to sperm cells having less cytoplasm than undifferentiated stem cells (Bucak et al., 2010). Many antioxidants originate in the cytoplasm and therefore, sperm may have lower amounts of them to combat lipid peroxidation and ROS buildup within the cellular membrane (Bucak et al., 2010). Sperm could then be expected to yield a greater response than SSCs to added antioxidants, aligning with our own findings.

Given the lack of prior data, it was not evident beforehand how SSCs would respond to AFPs in this study. AFPs do not interact with ROS or membrane lipids like antioxidants do but, perform different functions in preventing ice crystal formation (Hassoun & Emir, 2017). In multiple cases, cryopreservation success of teleost sperm has been improved with AFPs in regard to sperm survival, functionality, and intracellular composition (Robles et al., 2019). One example in gilthead seabream showed that AFPs affected the fatty acid profiles in the

sperm plasma membrane and increased cryo-resistance (Beirão et al., 2012). In the same study, improvements in post-thaw kinetic parameters of sperm were also recorded with 1 µg/mL of AFPIII. These parameters are often used as assessments of sperm quality after freezing, given that damage to the organelles and to the DNA are linked to decreased swimming performance (Suquet et al., 1998; Cabrita et al., 2001). Such examples shown in traditional gamete cryopreservation highlight the diversity of mechanisms altered during cryopreservation, and there is no reason to believe that the ability of cryoprotective agents to cross the plasma membrane and interact with the cell's interior would not also occur in SSCs. Unlike mature sperm cells, SSCs are not assessed by kinematics but may still suffer genetic and structural damage, justifying why SSC cryopreservation may be expected to improve with cryoprotective agents. However, in this study, post-thaw viability was nearly identical to the control with or without AFPs. Only when both antioxidants and AFPs were combined did a few treatments show increases in SSC production and viability. Interactions between these factors (or other factors within the protocol) are quite novel and have not yet been identified in other SSC protocols, making them interesting for future study.

Specific effects of cryoprotective agents such as those used in this study may also be highly variable because of species-specific and other methodological factors that are yet to be standardized across cryopreservation protocols (Robles et al., 2019). For example, SSCs in this study were frozen using a mixture of permeating and nonpermeating cryoprotectants (1.0 M DMSO, 0.2 M lactose, and 10% egg yolk), which improved post-thaw cell viability in prior experimental trials by our research group. With these substances already present in the cryomedia acting as cryoprotectants, antioxidants or AFPs that have the same function may not be necessary to maximize post-thaw cell recovery. Using antioxidants or AFPs in place of the previously established cryoprotectants may or may not create a more positive response but was not tested in this study since the objective was to build from our previously optimized

protocols.

Determining the exact mechanisms driving these results is challenging when the physiological, molecular, and biochemical properties of SSCs are still poorly understood (Lacerda et al., 2013). Additionally, SSCs may be highly different from their developed sperm counterparts and from other cell types. Historically SSCs, especially those in fishes, have been difficult to access, limiting detailed studies on their cryopreservation. More research in other species and other taxa could provide a reliable foundation for technical advancement in this area and may confirm these results.

4.5. Conclusions

This is the first research report that examined the effects of antioxidants, AFPs, and combinations of the two factors on post-thaw viability of blue catfish SSCs. From these results, we recommend that adding the antioxidant hypotaurine with either AFPI 1.0 µg/mL or AFPIII 0.1 µg/mL to the cryomedia will maximize the number of live SSCs after freezing. Although individual antioxidant and AFP treatments did not improve cryopreservation success, specific combinations of cryoprotective agents may be beneficial. Overall, having a reliable source of viable SSCs in long-term storage can enhance genetic enhancement programs for broodstock selection and germ cell transplantation technologies between channel catfish and blue catfish. Combining xenogenesis and cryopreservation will facilitate

hybrid fry production. Overall, these results also build knowledge of SSC cryopreservation and the underlying factors that must be optimized within each protocol.

Acknowledgements

This project was supported by the Agriculture and Food Research Initiative

Competitive Grant no. 2018-67015-27614 from the USDA National Institute of Food and Agriculture.

References

Aliakbari, F., Gilani, M. A. S., Amidi, F., Baazm, M., Korouji, M., Izadyar, F., ... & Abbasi, M. (2016). Improving the efficacy of cryopreservation of spermatogonia stem cells by antioxidant supplements. *Cellular Reprogramming (Formerly" Cloning and Stem Cells")*, *18*(2), 87-95.

Argue, B. J., Liu, Z., & Dunham, R. A. (2003). Dress-out and fillet yields of channel catfish, Ictalurus punctatus, blue catfish, Ictalurus furcatus, and their F1, F2 and backcross hybrids. *Aquaculture*, *228*(1-4), 81–90. doi: 10.1016/s0044-8486(03)00245-x

Bart, A. N., & Dunham, R. A. (1996). Effects of sperm concentration and egg number on fertilization efficiency with channel catfish (Ictalurus punctatus) eggs and blue catfish (I. furcatus) spermatozoa. *Theriogenology*, *45*(3), 673-682.

Beirão, J., Zilli, L., Vilella, S., Cabrita, E., Fernández-Díez, C., Schiavone, R., & Herráez, M. P. (2012). Fatty acid composition of the head membrane and flagella affects S parus aurata sperm quality. *Journal of Applied Ichthyology*, *28*(6), 1017-1019.

Bucak, M. N., Tuncer, P. B., Sarıözkan, S., Başpınar, N., Taşpınar, M., Çoyan, K., ... & Ilgaz, S. (2010). Effects of antioxidants on post-thawed bovine sperm and oxidative stress parameters: antioxidants protect DNA integrity against cryodamage. *Cryobiology*, *61*(3), 248-253.

Cabrita, E., Anel, L., & Herraez, M. P. (2001). Effect of external cryoprotectants as membrane stabilizers on cryopreserved rainbow trout sperm. *Theriogenology*, *56*(4), 623-635.

Cabrita, E., Ma, S., Diogo, P., Martínez-Páramo, S., Sarasquete, C., & Dinis, M. T. (2011). The influence of certain amino acids and vitamins on post-thaw fish sperm motility, viability and DNA fragmentation. *Animal reproduction science*, *125*(1-4), 189-195.

Celino, F. T., Yamaguchi, S., Miura, C., Ohta, T., Tozawa, Y., Iwai, T., & Miura, T. (2011). Tolerance of spermatogonia to oxidative stress is due to high levels of Zn and Cu/Zn superoxide dismutase. *Plos one*, *6*(2).

Devries, A. L. (1982). Biological antifreeze agents in coldwater fishes. *Comparative Biochemistry and Physiology Part A: Physiology*, *73*(4), 627–640. doi: 10.1016/0300-9629(82)90270-5

Dunham, R. A., Hyde, C., Masser, M., Plumb, J. A., Smitherman, R. O., Perez, R., & Ramboux, A. C. (1994). Comparison of culture traits of channel catfish, Ictalurus punctatus, and blue catfish I. furcatus. *Journal of Applied Aquaculture*, *3*(3-4), 257-268.

Dunham, R. A. (2011). *Aquaculture and fisheries biotechnology genetic approaches*. Wallingford: CABI

Elliott, G. D., Wang, S., & Fuller, B. J. (2017). Cryoprotectants: A review of the actions and applications of cryoprotective solutes that modulate cell recovery from ultra-low temperatures. *Cryobiology*, *76*, 74-91.

FAO (Food and Agriculture Organization of the United Nations). 2017. Top 10 species groups in global aquaculture 2017. http://www.fao.org/3/ca5224en/CA5224EN.pdf (accessed April 2020).

Fuller, B.J. Cryoprotectants: The essential antifreezes to protect life in the frozen state. Cryo Lett. **2004**, 25, 375–388.

Ha, S. J., Kim, B. G., Lee, Y. A., Kim, Y. H., Kim, B. J., Jung, S. E., ... & Ryu, B. Y. (2016). Effect of antioxidants and apoptosis inhibitors on cryopreservation of murine germ cells enriched for spermatogonial stem cells. *PloS one*, *11*(8).

Halliwell, B. (2000) The antioxidant paradox. Lancet 355, 1179–1180, https://doi.org/10.1016/S0140-6736(00)02075-4

Halliwell, B. (2006) Reactive species and antioxidants. Redox Biology is a fundamental theme of aerobic life. Plant Physiol. 141, 312–322, https://doi.org/10.1104/pp.106.077073 19

Harding, M.M.; Anderberg, P.I.; Haymet, A.D.J. "Antifreeze" glycoproteins from polar fish. Eur. J. Biochem. **2003**, 270, 1381–1392.

Hassoun, A., & Emir Çoban, Ö. (2017). Essential oils for antimicrobial and antioxidant applications in fish and other seafood products. *Trends in Food Science & Technology*, *68*, 26–36. https://doi.org/10.1016/j.tifs.2017.07.016

Hezavehei, M., Sharafi, M., Kouchesfahani, H. M., Henkel, R., Agarwal, A., Esmaeili, V., & Shahverdi, A. (2018). Sperm cryopreservation: A review on current molecular cryobiology and advanced approaches. *Reproductive BioMedicine Online*, *37*(3), 327–339. https://doi.org/10.1016/j.rbmo.2018.05.012

Higuchi, K., Takeuchi, Y., Miwa, M., Yamamoto, Y., Tsunemoto, K., & Yoshizaki, G. (2011). Colonization, proliferation, and survival of intraperitoneally transplanted yellowtail Seriola quinqueradiata spermatogonia in nibe croaker Nibea mitsukurii recipient. *Fisheries Science*, *77*(1), 69–77. https://doi.org/10.1007/s12562-010-03147

Hu, E., Yang, H., Tiersch, T. R. 2011. High-throughput cryopreservation of spermatozoa of blue catfish (*Ictalurus furcatus*): establishment of an approach for commercial-scale processing. Cryobiology, 62(1):74-82.

Irshad, M., Ali Subhani, M., Ali, S., & Hussain, A. (2020). Biological Importance of Essential Oils. *Essential Oils - Oils of Nature*. https://doi.org/10.5772/intechopen.87198

Karanova, M.V.; Pronina, N.D.; Tsvetkova, L.I. The effect of antifreeze glycoproteins on survival and quality of fish spermatozoa under the conditions of long-term storage at +4 degree C. Izv. Akad. Nauk. Seriia Biol. 2002, 88–92.

Kim, S. K., Youm, H. W., Lee, J. R., & Suh, C. S. (2017). Chapter 4 Role of Antioxidants and Antifreeze Proteins in Cryopreservation/Vitrification. *Methods in Molecular Biology*, 45–63. https://doi.org/10.1007/978-1-4939-6828-2_4

Labbé, C., Robles, V., & Herraez, M. P. (2013). Cryopreservation of gametes for aquaculture and alternative cell sources for genome preservation. In *Advances in aquaculture hatchery technology* (pp. 76-116). Woodhead Publishing.

Lacerda, S. M. S. N., Costa, G. M. J., Campos-Junior, P. H. A., Segatelli, T. M., Yazawa, R., Takeuchi, Y., … França, L. R. (2013). Germ cell transplantation as a potential biotechnological approach to fish reproduction. *Fish Physiology and Biochemistry*, *39*(1), 3–11. https://doi.org/10.1007/s10695-012-9606-4

Lee, S., Iwasaki, Y., Shikina, S., & Yoshizaki, G. (2013). Generation of functional eggs and sperm from cryopreserved whole testes. *Proceedings of the National Academy of Sciences*, *110*(5), 1640-1645.

Lee, Y. A., Kim, Y. H., Ha, S. J., Kim, K. J., Kim, B. J., Kim, B. G., ... & Ryu, B. Y. (2014). Cryopreservation of porcine spermatogonial stem cells by slow-freezing testis tissue in trehalose. *Journal of Animal Science*, *92*(3), 984-995.

Len, J. S., Koh, W. S. D., & Tan, S. X. (2019). The roles of reactive oxygen species and antioxidants in cryopreservation. *Bioscience reports*, *39*(8).

Magnotti, C., Cerqueira, V., Lee-Estevez, M., Farias, J. G., Valdebenito, I., & Figueroa, E. (2016). Cryopreservation and vitrification of fish semen: a review with special emphasis on marine species. *Reviews in Aquaculture*, *10*(1), 15–25. https://doi.org/10.1111/raq.12145

Mandawala, A. A., Harvey, S. C., Roy, T. K., & Fowler, K. E. (2016). Cryopreservation of animal oocytes and embryos: Current progress and future prospects. *Theriogenology*, *86*(7), 1637-1644.

Martínez-Páramo, S., Diogo, P., Dinis, M. T., Herráez, M. P., Sarasquete, C., & Cabrita, E. (2012). Incorporation of ascorbic acid and α-tocopherol to the extender media to enhance antioxidant system of cryopreserved sea bass sperm. Theriogenology, 77(6), 1129-1136.

Molyneaux, K., & Wylie, C. (2004). Primordial germ cell migration. *International Journal of Developmental Biology*, *48*(5-6), 537-543.

Muzzalupo, I. (2013). Food Industry. In *Google Books*. BoD – Books on Demand.

Muchlisin, Zainal Abidin, et al. "Exploration of Natural Cryoprotectants for Cryopreservation of African Catfish, Clarias Gariepinus, Burchell 1822 (Pisces: Clariidae) Spermatozoa." *Czech Journal of Animal Science*, vol. 60, no. 2, 2015, pp. 10–15, doi:10.17221/7906-CJAS.

Perera, D. A., Alsaqufi, A., Shang, M., Wade, D. C., Su, B., Elaswad, A., … Dunham, R. A.

(2017). Xenogenesis-Production of Channel Catfish × Blue Catfish Hybrid Progeny by Fertilization of Channel Catfish Eggs with Sperm from Triploid Channel Catfish Males with Transplanted Blue Catfish Germ Cells. *North American Journal of Aquaculture*, *79*(1), 61–74. https://doi.org/10.1080/15222055.2016.1221008

Rathore, S. S., Chandravanshi, A., Chandravanshi, P., & Jaiswal, K. (2016). Review on cryopreservation in fisheries science: Applications and perspectives. *International Journal of Science and Research*, *5*(10), 573-579.

Robles, V., Cabrita, E., Anel, L., & Herráez, M. P. (2006). Microinjection of the antifreeze protein type III (AFPIII) in turbot (Scophthalmus maximus) embryos: toxicity and protein distribution. *Aquaculture*, *261*(4), 1299-1306.

Robles, V., Barbosa, V., Herráez, M. P., Martínez-Páramo, S., & Cancela, M. L. (2007). The antifreeze protein type I (AFP I) increases seabream (Sparus aurata) embryos tolerance to low temperatures. *Theriogenology*, *68*(2), 284-289.

Robles, V., G. Valcarce, D., & F. Riesco, M. (2019). The Use of Antifreeze Proteins in the Cryopreservation of Gametes and Embryos. *Biomolecules*, *9*(5), 181. https://doi.org/10.3390/biom9050181

Shabani, H., Zandi, M., OFOGI, H., Sanjabi, M. R., & Pajooh, K. H. (2017). The effect of combining vitamin E and C on the viability improvement of transfected ovine spermatogonial stem cells after cryopreservation and thawing. *Turkish Journal of Veterinary and Animal Sciences*, *41*(5), 648-655.

Shang, M., Su, B., Perera, D. A., Alsaqufi, A., Lipke, E. A., Cek, S., … Dunham, R. A. (2018). Testicular germ line cell identification, isolation, and transplantation in two North American catfish species. *Fish Physiology and Biochemistry*, *44*(2), 717–733. https://doi.org/10.1007/s10695-018-0467-3

Suquet, M., Dreanno, C., Petton, B., Normant, Y., Omnes, M. H., & Billard, R. (1998). Long-term effects of the cryopreservation of turbot (Psetta maxima) spermatozoa. *Aquatic Living Resources*, *11*(1), 45-48.

Suquet, M., Dreanno, C., Fauvel, C., Cosson, J., & Billard, R. (2000). Cryopreservation of sperm in marine fish. *Aquaculture Research: Original Articles*, *31*(3), 231-243.

Temple, M.D., Perrone, G.G. and Dawes, I.W. (2005) Complex cellular responses to reactive oxygen species. Trends Cell Biol. 15, 319–326, https://doi.org/10.1016/j.tcb.2005.04.003 18

Torrans, E. L., Ott, B. 2018. Effect of grading fingerling hybrid catfish (female channel catfish x male blue catfish) on growth, production, feed conversion and food fish size distribution. North American Journal of Aquaculture, 80:187-192.

Zilli, L.; Beirão, J.; Schiavone, R.; Herraez, M.P.; Gnoni, A.; Vilella, S. Comparative Proteome Analysis of Cryopreserved Flagella and Head Plasma Membrane Proteins from Sea Bream Spermatozoa: Effect of Antifreeze Proteins. PLoS ONE 2014, 9, e99992.

Figure Captions

Fig. 4.1. One-way ANOVAs were used to determine individual effects of antioxidant and AFP treatments on post-thaw spermatogonia A production (A) and viability recovery index (B) for blue catfish, *Ictalurus furcatus*, SSCs after cryopreservation. Light and dark gray bars represent LSMs + standard error for antioxidant and AFP treatments, respectively. Letters signify significant differences as determined by Tukey's HSD ($P < 0.05$).

Fig. 4.2. The top panel shows images and viability percentages of blue catfish, *Ictalurus furcatus*, SSCs obtained by fluorescent microscopy with a 40× objective. Live SSCs fluoresce green and dead SSCs appear red, showing the differences in viability between hypotaurine 7 mM, AFPI 1 µg, and both additives combined. In the bottom panel, one-way ANOVAs compared the antioxidants hypotaurine and catalase with and without AFP treatments on post-thaw spermatogonia A production (A) and viability recovery index (B) for blue catfish, *Ictalurus furcatus*, SSCs after cryopreservation. Light and dark gray bars represent LSMs + standard error for the hypotaurine and catalase treatment groups, respectively. Letters signify significant differences as determined by Tukey's HSD ($P < 0.05$).

Fig. 4.1.

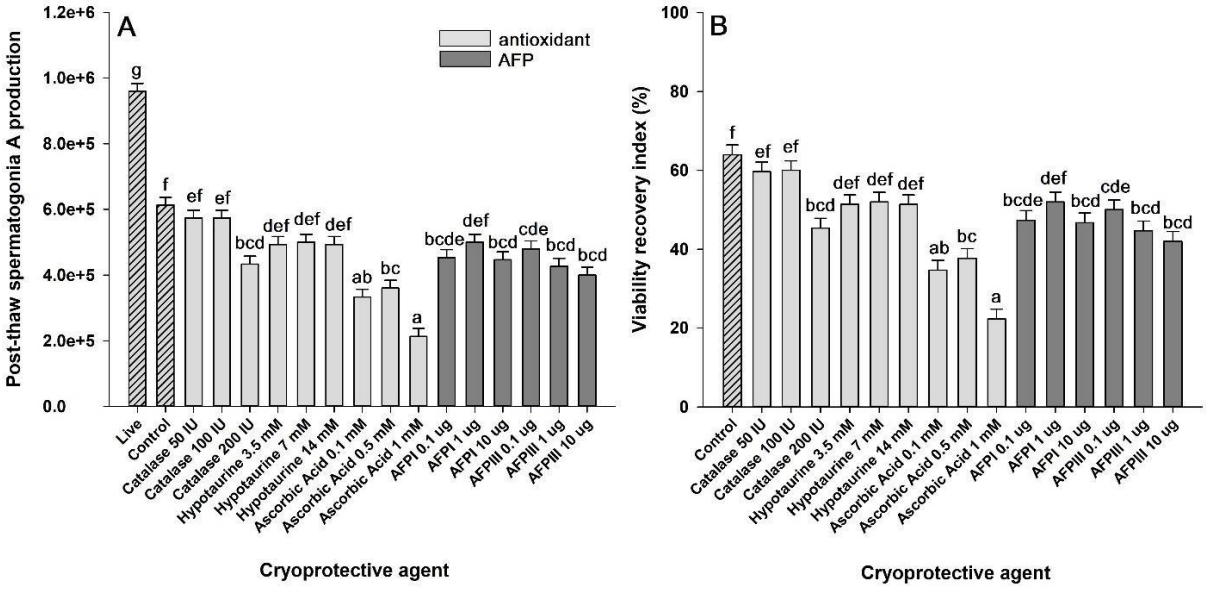
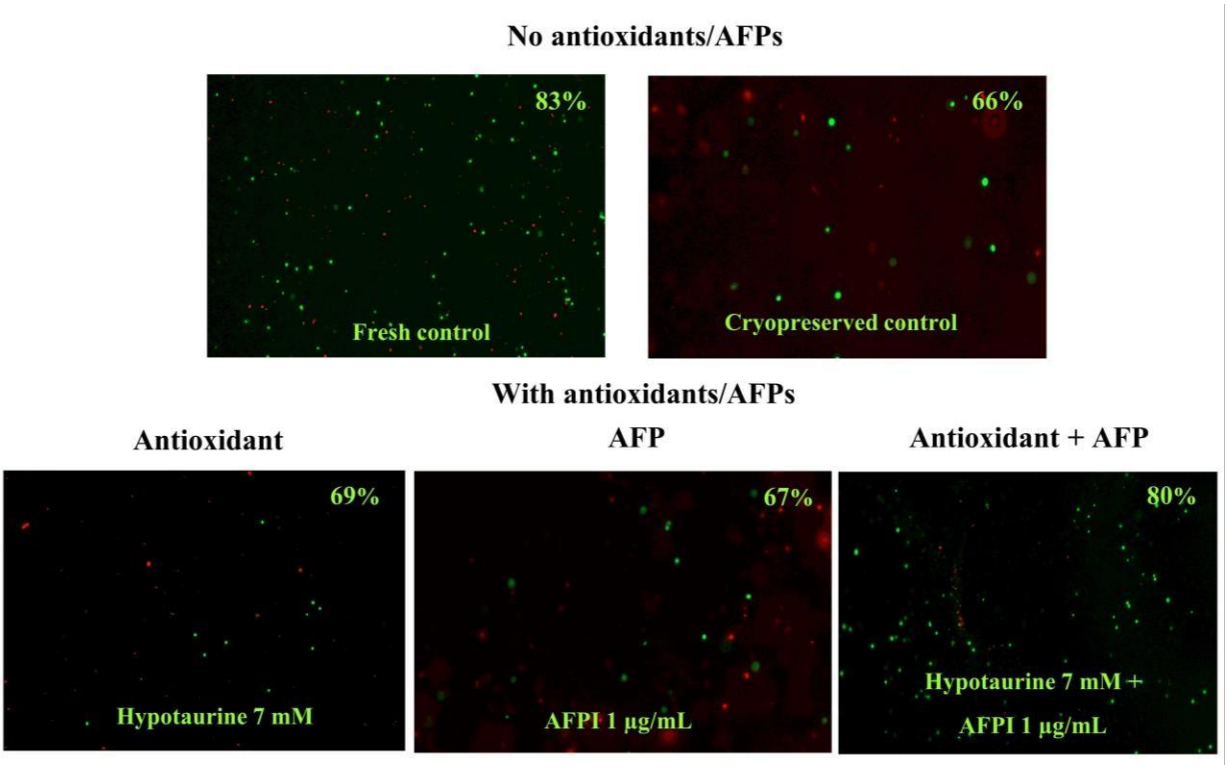
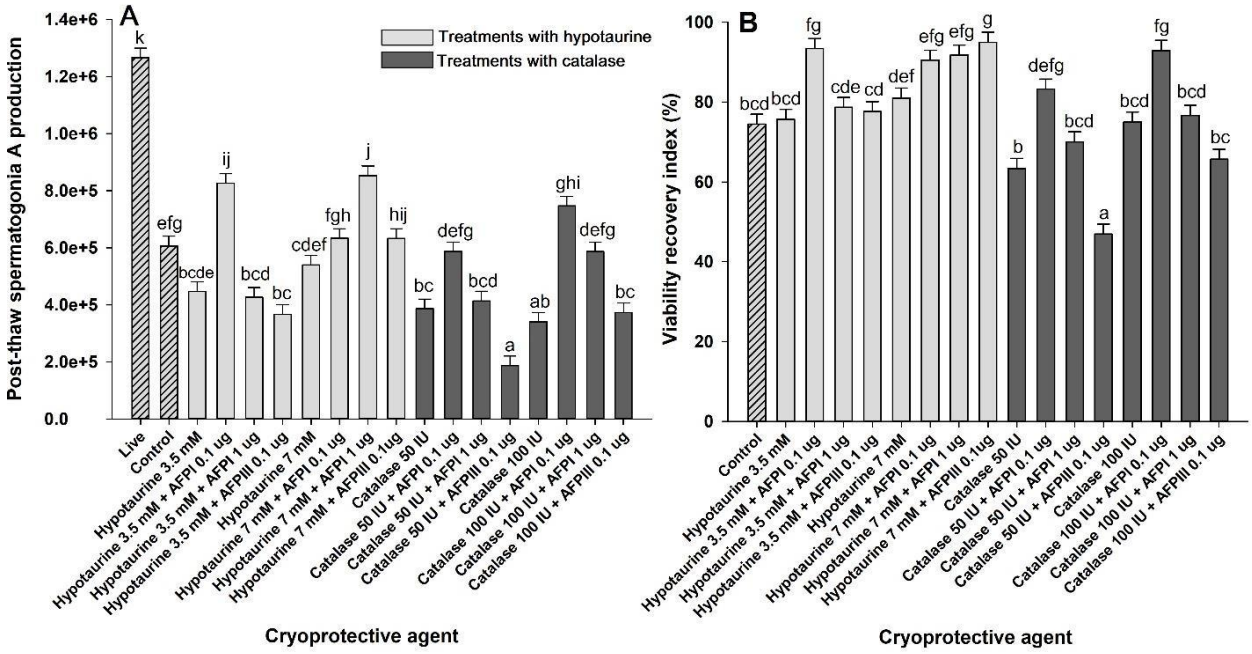


Fig. 4.2.


No antioxidants/AFPs
83%
Fresh control
66%
Cryopreserved control
With antioxidants/AFPs
Antioxidant
AFP
Antioxidant + AFP
69%
Hypotaurine 7 mM
67%
AFPI 1 µg/mL
80%
Hypotaurine 7 mM +
AFPI 1 µg/mL


A
Post-thaw spermatogonia A production
1.4e+6
1.2e+6
1.0e+6
8.0e+5
6.0e+5
4.0e+5
2.0e+5
0.0
Treatments with hypotaurine
Treatments with catalase
k
efg
bcde
ij
bcd
bc
cdef
fgh
j
hij
bc
defg
bcd
a
ab
ghi
defg
bc
Live
Control
Hypotaurine 3.5 mM
Hypotaurine 3.5 mM + AFPI 0.1 ug
Hypotaurine 3.5 mM + AFPI 1 ug
Hypotaurine 3.5 mM + AFPIII 0.1 ug
Hypotaurine 7 mM
Hypotaurine 7 mM + AFPI 0.1 ug
Hypotaurine 7 mM + AFPI 1 ug
Hypotaurine 7 mM + AFPIII 0.1ug
Catalase 50 IU
Catalase 50 IU + AFPI 0.1 ug
Catalase 50 IU + AFPI 1 ug
Catalase 50 IU + AFPIII 0.1 ug
Catalase 100 IU
Catalase 100 IU + AFPI 0.1 ug
Catalase 100 IU + AFPI 1 ug
Catalase 100 IU + AFPIII 0.1 ug
Cryoprotective agent
B
Viability recovery index (%)
100
80
60
40
20
0
bcd
bcd
fg
cde
cd
def
efg
efg
g
b
defg
bcd
a
bcd
fg
bcd
bc
Control
Hypotaurine 3.5 mM
Hypotaurine 3.5 mM + AFPI 0.1 ug
Hypotaurine 3.5 mM + AFPI 1 ug
Hypotaurine 3.5 mM + AFPIII 0.1 ug
Hypotaurine 7 mM
Hypotaurine 7 mM + AFPI 0.1 ug
Hypotaurine 7 mM + AFPI 1 ug
Hypotaurine 7 mM + AFPIII 0.1ug
Catalase 50 IU
Catalase 50 IU + AFPI 0.1 ug
Catalase 50 IU + AFPI 1 ug
Catalase 50 IU + AFPIII 0.1 ug
Catalase 100 IU
Catalase 100 IU + AFPI 0.1 ug
Catalase 100 IU + AFPI 1 ug
Catalase 100 IU + AFPIII 0.1 ug
Cryoprotective agent