THE SHORT-TERM COLD STORAGE OF BLUE TILAPIA (OREOCHROMIS AUREUS) SPERMATOZOA IN HBSS

BY

MACKENZIE ALLEN

A thesis submitted to the

Department of Biology

Mount Allison University

In partial fulfillment of the requirements for the

Bachelor of Science degree with Honours

April 2022

TABLE OF CONTENTS

Abstract

Spermatozoa are an essential part of fertilization in sexual reproduction. Sperm are commonly collected for in vitro fertilization, quality assessment, and research purposes. Sperm cells have incredibly short lifespans which make it difficult for use in aquaculture management and in research. For that reason, it is essential to develop methods for optimal short-term storage of semen. Short-term storage of semen involves temporary cooling of sperm cells (>0 ^{o}C), which reduces the metabolic rate and thus extends the lifespan of the cell. That being said, sperm are vulnerable to desiccation as they are highly packed in seminal plasma. Diluents function to reduce the potential for dehydration and decrease sperm density while preventing activation. Extenders are added to semen to mitigate sperm cell damage when stored in cooler temperatures while supplying nutrients to the cells. This is particularly important for work on external fertilizing freshwater fish. Fortunately, there are a variety of diluents and extenders that have already been developed and tested on a variety of freshwater fish species. There has been no work currently done on Blue Tilapia (*Oreochromis aureus*) in this area. Blue Tilapia are a very important fish for aquaculture and in our lab. The goal of my honours research is to fill this gap and determine if we can successfully store Blue Tilapia semen. I collected semen samples from Blue Tilapia males, diluted it with HBSS, and left samples in the fridge for varying amounts of time. I then examined the effects of the length time in the fridge on sperm percent motility, and swimming velocity. I found that the velocity of spermatozoa of Tilapia in HBSS is not impacted by time in the fridge up to 18 hours. This work opens the door for further research on reproductive biology of Blue Tilapia and improved use for aquaculture and conservation purposes.

Acknowledgements

I would like to firstly thank my supervisor Dr. Matthew K. Litvak. I have had the pleasure of working under his supervision for the past year. I have learned many skills in my time as his honour student, and more importantly life lessons, which I will carry with me always. I would like to thank Dr. Morash for her contribution as my second reader and Dr. Hamilton for her guidance when it was most needed. I would also like to acknowledge my lab member Elise Hansen for the endless help and kindness she has shown me in aiding me complete my project. I would also like to thank my other lab members Claire, Olivia, Caleb, Kerri, and Danny for all your help in the lab and for the many laughs we have shared. I would also like to thank classmate

This project would not have been completed without the support from countless people at Mount Allison. Mount Allison has no shortage of generous and intelligent people, and I am extremely grateful to have been able to be a part of this community. Thank you.

Lastly, I would like to thank my friends and family. I struggle to understand how I ended up deserving of you all in the first place. I am humbled by your unwavering support, and not a day goes by that I don't appreciate every single one of you.

List of Figures and Tables

CHAPTER I

LITERATURE REVIEW EXPLORING OVIPAROUS FISH SPERMATOZOA AND THEIR STORAGE

Fish spermatozoa

Spermatozoa are the male gamete which is responsible for fertilizing the egg in sexual reproduction. Sperm reside within the seminal plasma; a medium which nourishes and protects it. Together, sperm and seminal plasma are called semen. Fish semen is commonly referred to as milt.

Spermatozoa resemble a tadpole and are composed of a head region, midpiece, and tail. The spermatozoon nucleus in the head region carries the paternal DNA. Mitochondria in the midpiece fuels the beating of the flagellum which results in sperm motility. Species have sperm which is suited to their environment and reproductive strategy (Haesler et al., 2011; Prakash et al., 2014). Due to this, sperm is also one of the most diverse cell types and exhibits dramatic evolutionary divergence over taxa (Ramon et al., 2014). Among fish, there are over 70 derived spermatozoa ultra-structures from their common ancestor (Pitnick et al., 2009). Fish spermatozoa morphology and biology vary widely and is suited to their habitat (Mattei, 1991). These morphological differences impact sperm's ability to carry genetic information, and just as importantly, to move.

Fish can be oviparous which are external fertilizers, and viviparous which are internal fertilizers. Sperm of external fertilizers is ejected into a foreign environment where they exist as essentially a free-living organism before fertilizing the egg. In oviparous species, which are most freshwater fish species, sperm competition and competition between males plays a large role in reproduction (Stockley et al., 1997). Sperm play a crucial role in reproduction and given the importance in oviparous fish, factors influencing sperm in the environment is well studied (Morita et al., 2003; Guasto et al., 2020). This type of reproduction also translates well in vitro settings given they occur external to the fish.

Sperm Activation

For sperm to fertilize an egg it must get to the egg. For this to occur, sperm require the ability to move. Fish sperm are activated when they come into contact with water. Sperm are activated due to a triggering signal like a change in osmotic pressure, ionic concentration, pH, and in some cases the presence of egg derived substances in the surrounding environment.

Osmotic pressure is one of the main factors in many fish which inhibits sperm activation (Stoss 1983; Billard, 1986; Cosson, 2004; Alavi and Cosson, 2006; Alavi et al., 2009). For freshwater fish, osmolality around 100-300 typically inhibits sperm motility (Alavi et al., 2009) and a hypotonic shock to sperm cells typically activates sperm motility (Billard 1978; Yang et al., 2006).

Changes in ion concentration is another main mode of sperm activation. High concentrations of potassium have been linked to the inhibition of motility in semen in many salmonids and in other fish (Stoss 1983; Billard 1986; Blake et al., 1969). The effect of Ca^{+} has also been linked to activation in trout spermatozoa and in Tilapia (Cosson et al., 1989). Different concentrations of ions alter the membrane potential opening voltage gated potassium channels which can initiate motility (Krazni et al., 2000; Takai and Morisawa, 1995).

The pH of an activation solution has a limited effect on sperm motility (Alavi and Cosson, 2005). Egg derived substances have also been loosely linked to activation of sperm (Cherr et al., 2008; Kholodnyy et al., 2020).

Sperm motility

Activation modes vary among fish and needs to be studied on a species-specific basis. There are several proposed signal transduction pathways known and thought to be conserved across many species (Dzyuba and Cosson, 2014). Spermatozoa are immotile in the testes and in plasma for most species. Some activation events occur in the external environment to activate motility.

Once a spermatozoon is activated, there is a cascade of events which cause it to become motile. External signals initiated by an activating event result in changes in the plasma membrane which cause it to become polarized. This change in polarization is due to changes in ion channels and aquaporin activity which activate the spermatozoon (Krasznai et al., 2000). The plasma membrane is tightly wrapped around the head of the spermatozoon where there is a thin layer of cytoplasm immediately interior. Through this medium, signals are transduced. Cyclic Adenosine Monophosphate (cAMP) signalling pathway regulates spermatozoa motility (Dzyuba and Cosson, 2014). Dynein proteins in the midpiece of the spermatozoon acts as a molecular motor which produce directed movement along axonemal microtubules (Dzyuba and Cosson, 2014). This axoneme activity is responsible for sperm movement. Hydrolysis of ATP catalyzed by dynein ATPase creates the chemical energy responsible for the mechanical sliding of adjacent microtubules in the axoneme causing it to move thus resulting in the beating of the flagella (Krasznai et al., 2000; Dzyuba and Cosson, 2014). The way this energy is supplied to produce ATP differs among fish species. It may include glycolysis, phospholipid catabolism, triglyceride metabolism, the Krebs cycle and oxidative phosphorylation. The mode of energy supply likely is a large contributor to the short duration of spermatozoa motility in fish sperm (Stoss, 1983; Billard and Cosson, 1992). External fertilizers rely in large part on endogenous energy for motility and survival. Fish sperm are typically only motile for short periods of time which can be explained given they have limited energy reserves (Stoss, 1983).

Quality parameters

Sperm quality, the measure of a spermatozoon's ability to fertilize an egg, is of interest to those collecting milt for in vitro fertilization or research. Given that this is hard to directly measure, researchers use quantifiable parameters to indirectly measure quality. Depending on the environmental and physical limitations, natural selection will select for certain characters of sperm that will optimize these attributes for each species. Species have different reproductive strategies and thus produce different types and quantities of sperm (Haesler et al., 2011). For this reason, there are different parameters for different species which work as a proxy for "quality". Common parameters used to assess sperm quality is sperm motility, swimming kinematics, metabolism, and biochemistry (Alavi and Cosson, 2005). Biomarkers have been used increasingly to evaluate sperm quality (Sutovsky and Lovercamp, 2012). Concentrations of sperm, or spermatocrit, has also been a mode used for measuring sperm quality. This can be measured using a haemocytometer, a counting chamber, or using centrifugation techniques to determine optical density using spectrophotometry to determine density of sperm in a given sample. However, the most common parameters used to assess sperm quality is sperm motility and swimming kinematics. In the case of oviparous fish species, high motility is a major characteristic of high-quality sperm. Descriptors of sperm motility has been established to be a key factor to determine overall sperm quality (Billard, 1978; Stoss, 1983).

Motility and swimming kinematics of sperm can be measured in a variety of different ways. Velocity, percent motile, frequency of flagellar beating are all measures to describe motility. The use of one single parameter will likely not give a good indicator or sperm quality given motility is complex (Locatello et al., 2020). The evaluation of various motility parameters may yield more accurate and precise descriptions of sperm quality. Special methods for recording sperm motility have been developed to deal with typically short duration of motility, and the tedious work that comes with calculating these values by hand (Billard and Cosson 1992). Computer assisted semen analysis (CASA) systems were developed to rapidly evaluate specific motility parameters which are difficult to examine with the human eye. CASA allows for objective comparison in swimming kinematics between sperm in different environments (Cosson et al., 1997). CASA systems can track spermatozoa simultaneously and generates copious amounts of data which can be used to study sperm quality (Kime et al., 2001). This gives us the ability to choose the highest quality sperm but also examine which physiological conditions are optimal for sperm motility. CASA tracks the head of spermatozoa over a certain period, calculating its distance traveled, speed, and multiple other parameters. In CASA systems, sperm velocity can be categorized into immotile (velocity <5 μm s^{-1}), locally motile (velocity = 5-20 μm s^{-1}) and fully motile (velocity >20 μm s^{-1}) to facilitate a quick understanding of sperm quality. There are many kinematic parameters which CASA measures. Beat cross frequency (BCF) which is the amount the sperm flagellum beats, and amplitude lateral head displacement (ALH) is the amplitude of these beats. Linearity, wobble, and straightness are other CASA parameters which are composites of velocity parameters. CASA systems divide velocity into three parameters as defined in Table 1 and illustrated in Figure 1.

Table 1. Definition of CASA velocity parameters

Data Acronym	Name	Definition
VSL	Straight-Line Velocity	Velocity as calculated by the distance between first and last points divided by elapsed time.
VAP	Average Path Velocity	Velocity as calculated by the length of the average path of the sperm divided by elapsed time.
VCL	Curvilinear Velocity	Velocity as calculated by the distance between each sperm head position divided by elapsed time.

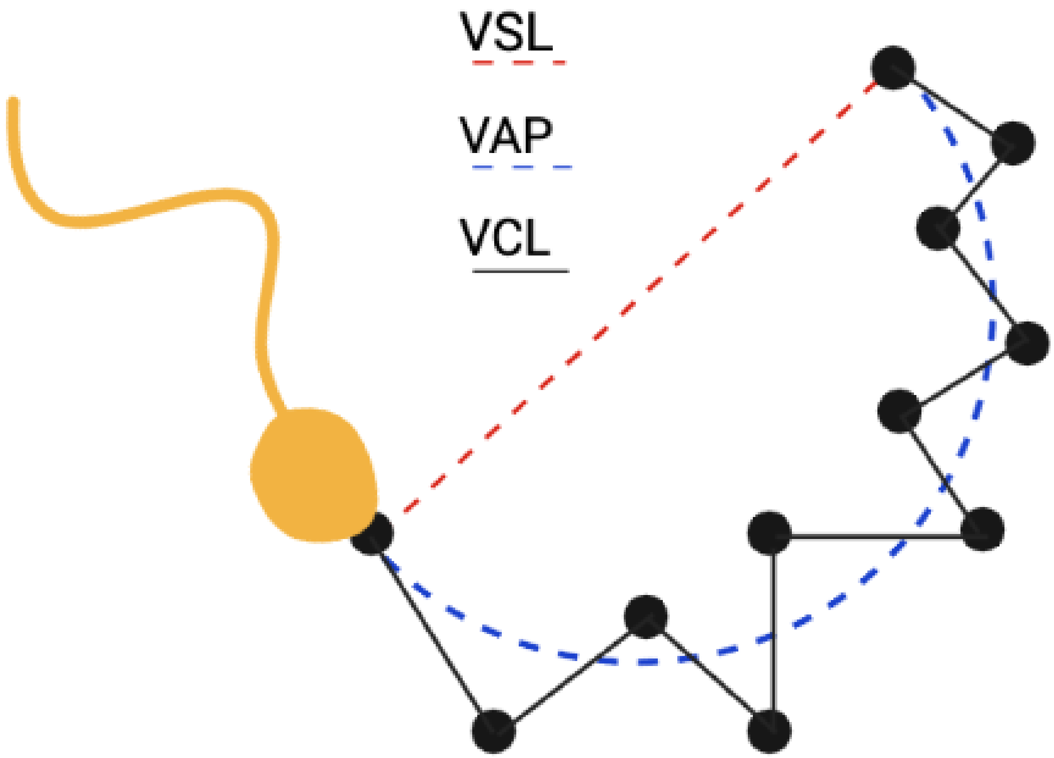

Figure 1. Illustration of spermatozoa being tracked by a CASA system. The lines represent the distance of the parameter which is divided by time. VCL = curvilinear velocity, VSL = straight line velocity, VAP = average path velocity.

Only velocity parameters were chosen for this study given velocity has been linked to ATP production. While BCF and ALH are also linked to ATP production, in fish velocity is more applicable to fertilization success in some species (Locatello et al., 2020). Although BCF and ALH are linked to velocity and have importance to evaluate, in the case of this study, I wanted to focus solely on velocity.

Influence of Temperature on sperm quality

Many environmental conditions affect the motility and duration of swimming activity of fish spermatozoa. Ion concentration, photoperiod, nutrition, stress, social interactions, age of broodstock, osmotic pressure, pH, and temperature all play a huge role. These are in addition to the influence of broodstock genetics on gamete quality (Gennotte et al., 2012).

Temperature can not only affect quality of sperm, but can also affect its lifespan (Merino et al., 2017). Temperature has direct influences on motility and velocity (Alavi and Cosson, 2005; Dyzuba et al., 2019). The influence of temperature depends on whether it is chronic or acute. Warmer temperatures increase the activity of enzymes in the mitochondria resulting in a higher production of ATP and thus energy (Perchec et al., 1995; Dyzuba et al., 2019). For sperm, this may mean higher velocity, but at the cost of motility duration as energy stores become depleted sooner.

On the other hand, temperature reductions decrease the activity of enzymes in the mitochondria. This leads to lower production of ATP and thus reduced energy production and use in the cell. Survival of diluted sperm over a length of time is inversely related to the metabolic activity (Zidni et al., 2020). With reduction in temperature, the activity of Na^+/K^+ pump declines which can result in an increase in intracellular ion concentration and a decrease in sperm survival (Zidni et al., 2020). For this reason, storing cells at lower temperatures has been utilized to extend the lifespan of sperm cells.

Lower temperatures do reduce the metabolic rate of sperm cells prolonging the lifespan and energy availability of the sperm. It also limits the production of reactive oxygen species (ROS) which can damage the cell. However, despite the benefits to lifespan of the cell, lower temperatures can lead to damaged DNA, membrane, mitochondria, and reduced overall quality (Yang et al., 2020). These damages are caused in large due to the decrease of elasticity of membranes and other important structures. Being stored in refrigerators can also cause hypoxic conditions because samples are in a sealed to reduce dehydration. These hypoxic conditions can lead to the production of ROS and cellular damage once reoxygenated.

Motility duration and velocity of spermatozoa greatly depends on the temperature. The temperature of the activation medium and of storage can both play a role in sperm motility. This is particularly important as the samples often experience rapid changes in temperature when cooled and warmed. For example, Vilela et al. (2003) found that spermatogenesis in Nile tilapia is critically sensitive to temperature changes.

Sperm storage

Short-term storage of sperm is a common practice in aquaculture and key to development of artificial reproduction protocols. It is also an essential aspect of time sensitive sperm research as spermatozoa can degrade at room temperatures quickly (Contreas et al., 2019). Short-term storage is the storage of gametes at low temperatures range from hours to weeks. Typical storage consists of semen in the absence of light around 0-4 ^{o}C. Storage on ice or in a refrigerator is a simple and inexpensive procedure.

Short-term storage of sperm can be used for transport and for species where male and female sexual maturity are asynchronous. Techniques for short-term storage of semen has been developed in several species (Jenkins-Keeran and Woods, 2002; Merino et al., 2017; Kristan et al., 2021). By storing sperm in the cold, the metabolic rate and of the sperm slows allowing it to last longer and ideally maintain quality. However, these quality parameters may decrease after being cold stored. The degree of decrease in quality depends on the fish quality, age, and spawning season (Contreas et al., 2019). Oxidative stress is usually the cause of this. For this reason, antioxidants are typically added to long term preservation of spermatozoa (Sandoval-Vargas, 2021). Other difficulties with storage are oxygen depletion and contamination. These negatives can be minimized through better storage containers and dilution and/or extender mediums.

Extenders and Diluents

The main objective of semen extenders is preventing activation, providing nutrient, and protecting sperm cells from cooler temperatures. To prevent sperm activation, extenders should have high salt content or high concentrations of cations like potassium calcium sodium or magnesium (Jenkins-Keeran and Woods, 2011). To supply nutrient, sugars or sucrose may be added to an extender.

Extenders can be made by hand by adding appropriate reagents to create a solution with the appropriate pH and osmolality. Some extenders have been developed for specific species but can be used across many taxa. Common extenders are Mounib's sucrose-based diluent, egg yolk, and Hanks balanced salt solution (HBSS) (Contreras et al., 2019; Garcia et al., 2016). HBSS is a salt solution with added glucose made for handling cells and retaining their viability (Hanks, 1975). High osmolality and potassium relative to the surroundings makes it an optimal extender or diluent as it resembles the environment of the testis.

Blue Tilapia (*Oreochromis aureus*)

Tilapia (several closely related cichlid species) is a popular fish used in integrated aquaculture agriculture (aquaponics). Blue Tilapia (*Oreochromis aureus*) are a smaller more cold tolerant Tilapia. They are natively found in the tropical and subtropical Africa and in the middle east and typically co-occur with Nile tilapia. They are an incredible invasive species having populations now worldwide (Canonico et al., 2005).

Tilapia are separated into three genera, *Tilapia* the substrate spawners, *Sarotherdon* paternal mouthbrooders, and *Oreochromis* the maternal mouthbrooders. *Oreochromis* Fry and adult fish are omnivorous, and this genus resides in freshwater exclusively. *Oreochromis* are the most popular species to be used in aquaculture, and it is no coincidence these hardy highly reproductive fish are popular in the most densely populated areas of the world. Their small size and hardiness make them ideal for aquaculture as they are easy to rear and cost effective. While Tilapia is second to carps in total fish farming, yields have projected to increase in the near future (Gupta et al., 2004).

Reproduction

Tilapias mature and can spawn naturally in captivity. The most appropriate broodstock for aquaculture are around 1 year old fish between 150-200g (Rana, 1988; Little and Hulata, 2000). Optimal temperatures for spawning are between 25-30^{o}C and no spawning occurs under 20^{o}C (El-sayed, 2019). pH may range between 7-9 (Rothbart and Pruginin, 1975). Given their range is mostly in equatorial, subtropical, and Mediterranean locations, populations tend to breed year-round (Coward and Bromage, 2000). Sudden environmental changes like cold shocks or density changes can improve intensity and spawning synchrony (Rothbart and Prugini 1975; Coward and Bromage, 2000). Another key attribute of this species in aquaculture production and work on reproduction biology.

Eggs

The outer surface of the egg is the chorion (El-sayed, 2019). When in water, eggs absorb water, and the chorion becomes turgid, and the egg hardens preventing polyspermy. Eggs then become ellipsoid or ovoid shape. Like all other teleost's, Tilapia eggs have a micropyle which is a tunnel and the only entry point for sperm in fertilization. The micropyle is located at animal pole along the line of the major axis (Muir and Roberts 2012).

Sperm

Blue Tilapia sperm have a tubular shape with a spherical head shape (Chao et al., 1987). They have the best motility around approximately 4 minutes and are brownish white in appearance (Chao et al., 1987). In Teleost's, spermatogenesis develops in cysts in the seminiferous tubules formed by Sertoli cells with primary spermatogonia. Testis size and sperm production generally increases into adulthood (Vilela et al., 2003).

Breeding behaviour

Tilapia breeding occurs around the lek, which is an arena that males create and defend (Rana, 1988). Males create the lek by manipulating substratum with their mouths creating a cleared location which is their lek. It is within the lek that males attract, court, and spawn with females. Breeding is a highly visual and density dependent interaction. During breeding pigmentation of Tilapia becomes intensified, with male pigmentation being more intense than their female counterparts (Rothbart and Prugini 1975). The genital papilla will descend and become engorged and turn red. When breeding, males choose their mating partner and drive away the rest of females. The process of spawning lasts up to 2 h (Rothbart and Prugini 1975). Post spawn, females will attempt to avoid other fish, and will not eat. Once the previous female vacates nest, males will mate with another female. Males can mate throughout entire year if kept in optimal conditions. Once females' complete incubation and nursing, they can reproduce again as well.

While older bigger fish reproduce less, the number and size of eggs they produce is larger. These eggs are more likely to survive as larger eggs makes them more tolerant to starvation (Rana 1988). Older fish are typically more experienced and will incubate eggs more successfully than younger fish (Rana 1988). Females and males can easily be stripped by applying pressure to the sides of the abdomen along the gonads. By expelling eggs in milt and gently mixing them, eggs can be artificially fertilized and can then be artificially incubated in plastic containers or McDonald hatching jars. Round bottom jars are preferred over conical jars offering better survival (Rana 1988). Eggs will hatch around 50 h after spawning (Rothbart and Prugini 1975).

Fish conservation

Fish are the most diverse vertebrate group which make up almost 50% of all known vertebrate species (Nelson 2016). Unfortunately, natural fish populations have decreased in large due to habitat loss and overfishing both thanks to human activity (Henrique de Siqueira-Silva et al., 2017). As of 2016, the IUCN (2016) reported over 50% of fish species are either extinct, extinct in the wild, critically endangered, endangered, vulnerable, or near threatened.

It is vital that natural fish populations replenish, and aquaculture practices become more efficient to feed our growing global population. Being able to understand and assess the reproductive strategies of different species, especially of those readily available to us like Tilapia, informs researchers how to better conserve endangered species and improve production of commercially valuable species.

Aquaculture

With the increasing importance of aquaculture and harvesting from wild stocks, there is a growing importance of artificial reproduction procedures. Being able to assess for high quality sperm and increase the yield and identify the best fish for broodstock. Identification and understanding the biology of sperm is also essential for cryopreservation of valuable genetic strains. While sperm is produced in larger quantities than eggs, this does not mean its produced in unlimited quantities or that the quality does not matter. Differences in ejaculate quantity, morphology, motility, and duration of motility affects fertilization success and shapes reproductive strategies and behaviour (Gilroy and Litvak, 2019).

Understanding the motility parameters of blue tilapia sperm gives insight into the reproductive strategies employed by these fish. Further experimentation would be required to discover which kinematic parameters of sperm would yield an optimized fertilization rate.

Cryopreservation

Development of short-term storage protocols are important to the development of cryopreservation for germplasm banking. Cryopreservation is the long-term cooling of genetic material cryogenically to be stored for an indefinite amount of time. This is considered long-term storage of genetic material, compared to that of short-term as described above. Choosing optimal sperm and understanding the mechanisms and which conditions lead to optimal fertilization is crucial for the reanimation process. Cryopreservation has many benefits. It can be used in aquaculture and conservation to improve broodstock management in hatcheries and preserve important genetic strains. It can solve synchrony problems in terms of gamete production and maturation or organisms with low sperm production. Cryopreservation can also assist in maintaining original wild genotypes which could be recovered in the future (akin to a genetic backup plan). During cryopreservation there are many steps which involve cooling freezing thawing, osmotic changes dehydration, and rehydration. Cells, including sperm, can be sensitive to these changes (Cabrita et al., 2014). Understanding the factors influencing sperm quality in short-term settings helps researcher choose suitable cryoprotectants and optimize methods for the cryopreservation of different species spermatozoa.

Cryopreservation of eggs and embryos is very difficult and research in this area has not been successful. For this reason, the focus has been on sperm to be used as the genetic material for cryopreservation and germbank cryobank development in fish. Protocols for many species sperm exist and more are being investigated currently. My hope is that work on short-term storage of fish sperm, like the research presented here, will help speed the development of sperm cryopreservation protocols of fishes for conservation and aquaculture development.

REFERENCES

Alavi, S. M., Rodina, M., Viveiros, A. T. M., Cosson, J., Gela, D., Boryshpolets, S., & Linhart, O. (2009). Effects of osmolality on sperm morphology, motility and flagellar wave parameters in Northern pike (*Esox lucius L.). Theriogenology*, *72*(1), 32–43.

Alavi, S., & Cosson, J. (2005). Sperm motility in fishes. I. Effects of temperature and pH: A review. *Cell Biology International*, *29*(2), 101–110.

Alavi, S., & Cosson, J. (2006). Sperm motility in fishes. (II) Effects of ions and osmolality: A review. *Cell Biology International*, *30*(1), 1–14.

Billard, R. (1978). Changes in structure and fertilizing ability of marine and freshwater fish spermatozoa diluted in media of various salinities. *Aquaculture*, *14*(3), 187–198.

Billard, R. (1986). Spermatogenesis and spermatology of some teleost fish species. *Reproduction Nutrition Développement*, *26*(4), 877–920.

Billard, R., & Cosson, M. P. (1992). Some problems related to the assessment of sperm motility in freshwater fish. *Journal of Experimental Zoology*, *261*(2), 122–131.

Blake Grant, F., Pang, P. K. T., & Griffith, R. W. (1969). The twenty-four-hour seminal hydration response in goldfish (Carassius auratus)—I. sodium, potassium, calcium, magnesium, chloride and osmolality of serum and seminal fluid. *Comparative Biochemistry and Physiology*, *30*(2), 273–280.

Cabrita, E., Martínez-Páramo, S., Gavaia, P. J., Riesco, M. F., Valcarce, D. G., Sarasquete, C., Herráez, M. P., & Robles, V. (2014). Factors enhancing fish sperm quality and emerging tools for sperm analysis. *Aquaculture*, *432*, 389–401.

Canonico, G. C., Arthington, A., McCrary, J. K., & Thieme, M. L. (2005). The effects of introduced tilapias on native biodiversity. *Aquatic Conservation: Marine and Freshwater Ecosystems*, *15*(5), 463–483.

Chao, N.-H., Chao, W.-C., Liu, K.-C., & Liao, I.-C. (1987). The properties of tilapia sperm and its cryopreservation*. *Journal of Fish Biology*, *30*(2), 107–118.

Cherr, G. N., Morisawa, M., Vines, C. A., Yoshida, K., Smith, E. H., Matsubara, T., Pillai, M. C., Griffin, F. J., & Yanagimachi, R. (2008). Two egg-derived molecules in sperm motility initiation and fertilization in the Pacific herring (*Clupea pallasi*). *The International Journal of Developmental Biology*, *52*(5–6), 743–752.

Contreras, P., Dumorné, K., Ulloa-Rodríguez, P., Merino, O., Figueroa, E., Farías, J. G., Valdebenito, I., & Risopatrón, J. (2019a). Effects of short-term storage on sperm function in fish semen: A review. *Reviews in Aquaculture*, raq.12387.

Cosson, J. (2004). The Ionic and Osmotic Factors Controlling Motility of Fish Spermatozoa. *Aquaculture International*, *12*(1), 69–85.

Cosson, M. P., Billard, R., & Letellier, L. (1989). Rise of internal Ca2+ accompanies the initiation of trout sperm motility. *Cell Motility and the Cytoskeleton*, *14*(3), 424–434.

Cosson, M. P., Cosson, J., Andrè, F., & Billard, R. (1995). cAMP/ATP relationship in the activation of trout sperm motility: their interaction in membrane-deprived models and in live spermatozoa. Cell motility and the cytoskeleton, 31(2), 159-176.

Coward, K., Bromage, N. Reproductive physiology of female tilapia broodstock. *Reviews in Fish Biology and Fisheries 10***,** 1–25 (2000).

Dzyuba, B., Legendre, M., Baroiller, J. F., & Cosson, J. (2019). Sperm motility of the Nile tilapia (*Oreochromis niloticus*): Effects of temperature on the swimming characteristics. *Animal Reproduction Science*, *202*, 65–72.

Dzyuba, V., & Cosson, J. (2014). Motility of fish spermatozoa: From external signaling to flagella response. *Reproductive Biology*, *14*(3), 165–175.

El-Sayed, A. F. M. (2019). *Tilapia Culture: Second Edition*. Elsevier Science.

Garcia, R. R. F., Vasconcelos, A. C. N., Povh, J. A., Oberst, E. R., Eloy, L. R., & Streit Junior, D. P. (2016). Different extenders solutions for tambaqui semen cooling. *Pesquisa Agropecuária Brasileira*, *51*(6), 780–784.

Gennotte, V., Françôis, E., Rougeot, C., Ponthier, J., Deleuze, S., & Mélard, C. (2012). Sperm quality analysis in XX, XY and YY males of the Nile tilapia (*Oreochromis niloticus*). *Theriogenology*, *78*(1), 210–217.

Gilroy, C. E., & Litvak, M. K. (2019a). Relationship between seminal plasma composition and spermatozoa swimming speed and motility in wild and captive shortnose sturgeon (*Acipenser brevirostrum*). *Aquaculture*, *505*, 217–224.

Gilroy, C. E., & Litvak, M. K. (2019b). Swimming kinematics and temperature effects on spermatozoa from wild and captive shortnose sturgeon (Acipenser brevirostrum). *Animal Reproduction Science*, *204*, 171–182.

Gupta, M. V., & Acosta, B. O. (2004). A review of global tilapia farming practices.

Guasto, J. S., Estrada, J. B., Menolascina, F., Burton, L. J., Patel, M., Franck, C., Hosoi, A. E., Zimmer, R. K., & Stocker, R. (2020). Flagellar kinematics reveals the role of environment in shaping sperm motility. *Journal of The Royal Society Interface*, *17*(170), 20200525.

Haesler, M. P., Lindeyer, C. M., Otti, O., Bonfils, D., Heg, D., & Taborsky, M. (2011). Female mouthbrooders in control of pre- and postmating sexual selection. *Behavioral Ecology*, *22*(5), 1033–1041.

Hanks, J. H. (1975). Hanks' balanced salt solution and pH control. *Tissue Culture Association Manual*, *1*(1), 3–4.

Henrique de Siqueira-Silva, D. H., Saito, T., dos Santos-Silva, A. P., da Silva Costa, R., Psenicka, M., & Yasui, G. S. (2018). Biotechnology applied to fish reproduction: Tools for conservation. *Fish Physiology and Biochemistry*, *44*(6), 1469–1485.

International Union for Conservation of Nature and Natural Resources (IUCN). (2016). IUCN Red List of Threatened Species. http://www.iucnredlist.org/

Jenkins-Keeran, K., & Woods, L. C. (2002). An Evaluation of Extenders for the Short-Term Storage of Striped Bass Milt. *North American Journal of Aquaculture*, *64*(4), 248–256.

Kholodnyy, V., Gadêlha, H., Cosson, J., & Boryshpolets, S. (2020). How do freshwater fish sperm find the egg? The physicochemical factors guiding the gamete encounters of externally fertilizing freshwater fish. *Reviews in Aquaculture*, *12*(2), 1165–1192.

Kime, D. E., Van Look, K. J. W., McAllister, B. G., Huyskens, G., Rurangwa, E., & Ollevier, F. (2001). Computer-assisted sperm analysis (CASA) as a tool for monitoring sperm quality in fish. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *130*(4), 425–433.

Krasznai, Z., Márián, T., Izumi, H., Damjanovich, S., Balkay, L., Trón, L., & Morisawa, M. (2000). Membrane hyperpolarization removes inactivation of Ca^{2+} channels, leading to Ca^{2+} influx and subsequent initiation of sperm motility in the common carp. *Proceedings of the National Academy of Sciences*, *97*(5), 2052–2057.

Kristan, J., Dadras, H., & Policar, T. (2021). Optimization of short-term storage of pikeperch semen: An applicable approach. *Animal*, *15*(1), 100001.

Little, D. C., & Hulata, G. (2000). Strategies for tilapia seed production. In M. C. M. Beveridge & B. J. McAndrew (Eds.), *Tilapias: Biology and Exploitation* (pp. 267–326). Springer Netherlands.

Locatello, L., Rigoni, G., Soriano, M. E., Rasotto, M. B., & Poli, F. (2020). Going beyond conventional parameters to unveil sperm quality in fish: The use of fibre optic technology to assess mitochondrial respiratory performance. *Biology Open*, bio.053306.

Mattei, X. (1991). Spermatozoon ultrastructure and its systematic implications in fishes. *Canadian Journal of Zoology*, *69*(12), 3038–3055.

Merino, O., Figueroa, E., Cheuquemán, C., Valdebenito, I., Isachenko, V., Isachenko, E., Sánchez, R., Farías, J., & Risopatrón, J. (2017). Short-term storage of salmonids semen in a sodium alginate-based extender. *Andrologia*, *49*(5), e12661.

Morita, M., Takemura, A., & Okuno, M. (2003). Requirement of Ca2+ on activation of sperm motility in euryhaline tilapia *Oreochromis mossambicus*. *Journal of Experimental Biology*, *206*(5), 913–921.

Muir, J. F., & Roberts, R. J. (Eds.). (2012). Recent Advances in Aquaculture: Volume 3.

Nelson, J. S., Grande, T., & Wilson, M. V. H. (2016). *Fishes of the world* (Fifth edition). John Wiley & Sons.

Perchec, G., Jeulin, C., Cosson, J., Andre, F., & Billard, R. (1995). Relationship between sperm ATP content and motility of carp spermatozoa. *Journal of Cell Science*, *108*(2), 747–753.

Pitnick, S., Hosken, D. J., & Birkhead, T. R. (2009). Sperm morphological diversity. *In Sperm Biology* (pp. 69–149). Elsevier.

Prakash, S., Prithiviraj, E., Suresh, S., Lakshmi, N. V., Ganesh, M. K., Anuradha, M., Ganesh, L., & Dinesh, P. (2014). Morphological diversity of sperm: A mini review. *Iranian Journal of Reproductive Medicine*, *12*(4), 239–242.

Ramón, M., Jiménez-Rabadán, P., García-Álvarez, O., Maroto-Morales, A., Soler, A., Fernández-Santos, M., Pérez-Guzmán, M., & Garde, J. (2014). Understanding Sperm Heterogeneity: Biological and Practical Implications. *Reproduction in Domestic Animals*, *49*, 30–36.

Rana, K. (1988). Reproductive Biology and the Hatchery Rearing of Tilapia Eggs and Fry. In J. F. Muir & R. J. Roberts (Eds.), *Recent Advances in Aquaculture* (pp. 343–406). Springer Netherlands.

Rothbard, S., & Pruginin, Y. (1975). Induced spawning and artificial incubation of Tilapia. *Aquaculture*, *5*(4), 315–321.

Sandoval-Vargas, L., Silva Jiménez, M., Risopatrón González, J., Villalobos, E. F., Cabrita, E., & Valdebenito Isler, I. (2021). Oxidative stress and use of antioxidants in fish semen cryopreservation. *Reviews in Aquaculture*, *13*(1), 365–387.

Stockley, P., Gage, M. J. G., Parker, G. A., & Møller, A. P. (1997). Sperm Competition in Fishes: The Evolution of Testis Size and Ejaculate Characteristics. *The American Naturalist*, *149*(5), 933–954.

Stoss, J. (1983). 6 Fish Gamete Preservation and Spermatozoan Physiology. In *Fish Physiology* (Vol. 9, pp. 305–350). Elsevier.

Sutovsky, P., & Lovercamp, K. (2012). Molecular markers of sperm quality. In M. Lucy, J. Pate, M. Smith, & T. Spencer (Eds.), *Reproduction in Domestic Ruminants VII* (pp. 247–256). Nottingham University Press.

Takai, H., & Morisawa, M. (1995). Change in intracellular K+ concentration caused by external osmolality change regulates sperm motility of marine and freshwater teleosts. *Journal of Cell Science*, *108*(3), 1175–1181.

Vilela, D. A. R., Silva, S. G. B., Peixoto, M. T. D., Godinho, H. P., & França, L. R. (2003). Spermatogenesis in teleost: Insights from the Nile tilapia (Oreochromis niloticus) model. *Fish Physiology and Biochemistry*, *28*(1–4), 187–190.

Yang, H., Hazlewood, L., Walter, R. B., & Tiersch, T. R. (2006). Effect of osmotic immobilization on refrigerated storage and cryopreservation of sperm from a viviparous fish, the green swordtail Xiphophorus helleri. *Cryobiology*, *52*(2), 209–218.

Yang, S., Huang, W., Chen, H., Huang, M., Liufu, Y., & Meng, Z. (2020). Effect of chilled storage on sperm quality of basa catfish (*Pangasius bocourti*). *Fish Physiology and Biochemistry*, *46*(6), 2133–2141.

Zidni, I., Lee, Y. H., Park, J. Y., Lee, H. B., Hur, J. W., & Lim, H. K. (2020). Effects of Cryoprotective Medium Composition, Dilution Ratio, and Freezing Rates on Spotted Halibut (*Verasper variegatus*) Sperm Cryopreservation. *Animals*, *10*(11), 2153.

CHAPTER II

Introduction

Spermatozoa are the male gamete responsible for moving to and fertilizing an egg in sexual reproduction. Sperm are composed of a head region, midpiece, and tail region. The head region contains the nucleus which is the source of the paternal DNA. The midpiece below the head contains mitochondria which causes the flagellum to beat making the sperm motile. Sperm originate in the testes and are contained in a medium called the seminal plasma which nourishes and inhibits motility of sperm. The seminal plasma and sperm together are called semen, and in the case of fish, semen is often referred to as milt. Fish are for the most oviparous, which means they reproduce externally. These fish expel their gametes into the water column where fertilization occurs

Sperm must undergo an activation event to become motile. Activation is species specific and can be caused by multiple triggers. These triggers for sperm activation in oviparous fish can involve an osmotic shock, changes in specific ion concentrations, changes in pH, and egg derived substances. In freshwater fish, one of the greatest and most common factors which activate sperm is change in osmolality (Yang et al., 2006). Freshwater fish are hypertonic to their environment. Seminal plasma has an osmolality of typically around 100-300 (Yang et al., 2006; Alavi et al., 2009). When ejected into the water, sperm are shocked by a shift from an isotonic to a hypotonic environment (Yang et al., 2006). Osmotic pressure of the seminal plasma is one of the main motility inhibitors in cyprinids and other types of fish (Cosson, 2004; Kholodnyy et al., 2020). Decreases in specific ion concentrations are another major mode of activation. High levels of potassium and calcium have been linked to inhibiting sperm motility (Grant et al., 1969; Morita et al., 2003; Alavi and Cosson, 2006). pH has been loosely linked to activation of spermatozoa (Alavi and Cosson, 2005). Egg derived substances like high weight glycoproteins have also shown to aid in the activation of sperm of some species (Kholodnyy et al., 2020).

Once activated, there is a cascade of reactions which initiates motility in sperm. The activation event triggers a depolarization or a hyperpolarization of the cell (Krasznai et al., 2000). This polarization results in an increase of internal calcium concentration (Cosson et al, 1989; Kholodnyy et al., 2020). This then activates the production of ATP in the mitochondria residing in the midpiece, fueling the dynein proteins which function as a mechanical motor in the microtubules of the axoneme that allows the flagella to beat back and forth (Krasznai et al., 2000). Sperm motility is maintained until the mitochondria stops suppling energy to the axoneme. Fish sperm motility longevity is variable but is often characterized for short duration of motility under 1 minute (Billard and Cosson, 1992). Sperm of many fish do not survive outside of the testis for very long (Stoss, 1983). For this reason, when collecting sperm, it is vital to store sperm at lower temperatures to slow ageing of the cells. By lowering the temperature, enzyme activity decreases, thereby reducing the respiration of the cell. This results in energy stores being conserved resulting in a longer lifespan of cells (Polge et al., 1949; Merino et al., 2016). Short-term storage, which is the temporary storage of sperm at temperatures above 0, are used when sperm are required shortly after collection.

Sperm is collected and handled for a variety of reasons. For research purposes, collected sperm can be used to understand underlying biological and ecological reproductive strategies of different organisms. It can also be collected to understand how environmental factors affect sperm quality and thus reproduction. Collecting sperm is also essential in breeding programs. Breeding programs are used in aquaculture and for conservation purposes. They can be used to deal with sexually asynchronous species and optimizing reproduction (Beirao et al., 2019). Collecting sperm of high quality is of great importance to researchers and others alike. Correct storage of sperm following collection is essential to maintain this quality. High quality sperm are those that readily able to fertilize an egg. Given this is difficult to measure and not always possible to measure directly, researchers use alternative quantifiable measures to indirectly measure quality. Spermatocrit density, biochemistry of seminal plasma and the metabolism of spermatozoa are common parameters used to assess sperm quality. Given the importance of sperm motility in fertilization, percent motile and other kinematic parameters of motile sperm are common parameters to assess sperm quality (Jenkins-Keeran and Woods, 2011). Computer assisted sperm analysis (CASA) technology has been developed to track sperm in the place of tracking it by hand. CASA software and hardware (phase contrast microscope and camera) allows scientists to track positional coordinates the heads of individual sperm. This coordinate and time data allows the researcher to evaluate motility and a variety of kinematic parameters. Kinematics are the description of how objects move, and there are many different descriptors of kinematics including different metrics of velocity, distance, and head movement.

It is essential to dilute the sperm so that the computer can track individual spermatozoa. If it is too dense you would not be able to follow individual sperm with the CASA system. Sperm are also diluted to eliminate dehydration of the sample over time. Dilution is particularly difficult in the case of fish given sperm are activated by water. Diluents are modelled to simulate the seminal plasma. By creating an environment like the seminal plasma, diluents can be made. Diluents differ from extenders which ideally extend the life of the cell, as they do not contain added nutrient. Extenders are typically made to be species specific and are composed of salts and sugars (Kristan et al., 2021). HBSS has been used as an extender and diluent (Yang et al., 2006; Garcia et al., 2016). Its high cation content and being generally isosmotic to many fish, make it an ideal diluent and short-term extender (depending on whether it has added sugars).

Tilapias are a group of cichlids and one of the most popularly consumed fish worldwide. They are native to subtropical Africa and the middle east (El-sayed, 2019). They have now expanded their reach worldwide both in aquaculture and natural settings. Tilapia were introduced to freshwater bodies in the United States and several other countries in the 1960s (El-sayed, 2019). Genus *Oreochromis* is the most popular genus of Tilapia used in aquaculture. They are popular due to their ability to survive and thrive under a wide variety of conditions. Their size and along with their ability to reproduce repeatedly year-round makes them ideal for aquaculture. Blue Tilapia (*Oreochromis aureus*) are the smallest and most cold tolerant of their genus. Commonly co-occurring with Nile tilapia, they reside in freshwater.

Tilapia reach sexual maturity around 4 months old yet are ideal for reproduction at around 1 year old and 150-200g (Rana, 1988; Little and Hulata, 2000). Optimal temperatures for spawning are around 25-30 ^{o}C in neutral to slightly basic water (pH 7-9) (Rothbart and Pruginin, 1975). They breed year-round yet changes in temperature or density changes can illicit more intense reproduction and spawning synchrony (Rothbart and Pruginin, 1975; Rana, 1988). Tilapia eggs, like all teleost eggs, have a micropyle which is a tunnel wherein sperm may penetrate. Tilapia sperm are tubular in shape and have a spherical head (Little and Hulata, 2000). This species does not currently have a standard protocol for dilution nor for short-term extenders.

I pose the question: Can I store Blue Tilapia semen the fridge? If so, how long can I store Blue Tilapia semen short-term when diluted with HBSS before the quality of the sperm is compromised? I hypothesize that I will be able to store Tilapia sperm and that the short-term storage of semen diluted with HBSS will affect the motility and swimming kinematics. I predict that there will be a decrease in motility and velocity as time stored in the fridge increases.

Methods

Fish Care

Blue Tilapia (*Oreochromis aureus*) were purchased from a private breeder in November 2020. They were kept in a recirculating custom four tank system in the Litvak lab at Mount Allison University. This system was backwashed three times weekly. Fish were kept at approximately 25.5 ^{o}C and around 7.2 pH. Fish where fed until satiated using #3 Corey floating fish feed. 6 Male fish ranging in weight from 376.29 to 607.89 with a mean weight of 480.88 +/- 35.63 were used in this experiment. 2 fish were sampled February 25th, February 28th, and March 3d. They were kept in a tank with three additional fish to reduce aggression. Fish were tagged with Biomark Mini HPT8 PIT tags using a MK165 Implanter with a 16-gage needle. Tags were read upon sampling using an HPR Lite Handheld PIT Tag Reader. The fish care was approved by the Mount Allison Animal Care Committee.

Milt extraction

Fish where anaesthetized with MS222 (Fisher scientific). Fish where not fed for 12 hours prior to anaesthetization. Anesthetic was mixed in a 10L bucket at a concentration of 150mg/l and mixed in a 1:1 ratio with $CaCO_3$. Fish were placed in the anaesthetic until they lost the ability to keep themselves upright. They were then placed in a custom trough as seen in Figure 1. The sedated fish were placed between the sponges with their ventral side facing up. Using the index finger and the thumb, gentle pressure was applied to the sides of the fish just below the stomach where the testes are located. A stroking motion was used starting from the base of the pelvic fins down to the anal fin. This process was repeated until milt was observed. The vent region was blotted dry with a Kim wipe and a capillary tube was placed near the urogenital aperture. Milt was collected in these tubes which were subsequently emptied into microcentrifuge tubes. This collection process would take under a minute. Following collection, fish were placed in a separate tank until equilibrium was regained and were then placed back in their original tank.

Figure 2. Milt extraction set up. Custom trough with sponges attached for holding fish upright.

Extender Dilutions

HBSS with calcium, magnesium, and phenol red (Corning) was used as an extender and diluent in this experiment. Milt was diluted by adding 20X HBSS. 20 ul of this mixture was then mixed with 20ul HBSS in four separate 0.6 microcentrifuge tubes. These tubes where used for each fridge time treatment. Tubes were kept in the back of a fridge kept at approximately 6 ^{o}C. Samples were divided into 0,1,9, and 18 hours in the fridge. Samples that were not used initially were left in the fridge for 2 weeks (384 hours), and some samples were noted to still be viable. These samples were then evaluated and included in this experiment.

Motility

Samples taken from the fridge were set out in room temperature for approximately 15 minutes. 1μl of the sample solution was pipetted onto a Hamilton-Thorne two-chambered 20μm specific microscope slide (2X-CEL, Hamilton-Thorne, Massachusetts, United States of America). Immediately following this, 4 μl of 1% BSA solution was pipetted onto the slide to activate the sperm and a cover slip was placed on top. Spermatozoa activity was evaluated using an Olympus CX-41 compound microscope equipped with a 10x negative phase contrast objective and digital video camera (JAI CM-040GE, JAI Inc. Miyazaki, Japan). The camera was attached to a Dell Latitude E5540 laptop via an Ethernet cable. Images were analyzed in real-time using the CEROS II Hamilton-Thorne computer assisted semen analysis (CASA) software. The system recorded images at 60 frames/s. Once this was completed and the capillary action ceased, random areas of the slide were sampled around times 60, 180, 360, and 900 seconds. Recordings were captured continuously aiming for approximately 40-60 spermatozoa per frame. Recordings at each sampling time ceased once at least 200 spermatozoa was recognized.

Spermatozoa kinematics

Motile sperm (any spermatozoa with a VCL>0) were combined into a separate dataset. The following was only conducted on this motile sperm dataset. The mean values for each parameter for each sample was calculated, and the mean of the replicates of each fish was calculated.

CASA calculates a variety of parameters, many of which are composites of velocity while others like ALH and BCF which are innately linked to velocity (Locatello et al., 2020). Given velocity can be a crucial aspect for successful fertilization in some species, and for simplicities sake, only velocity parameters were considered (Locatello et al., 2020). Velocity parameters include curvilinear velocity (VCL), average path velocity (VAP), and straight-line velocity (VSL). VCL is the curvilinear velocity (μm/s) which is sum of the incremental distances moved in each frame along the sampled path divided by the time taken for the sperm to cover the track. VAP or average path velocity (μm/s) is the smoothed sperm head positions in a running average along the path travelled divided by the time taken for the sperm to cover the track. VSL is the straight-line velocity (μm/s) which is the smoothed straight line between the first and last position of a track. Given that these velocity parameters are innately correlated which can lead to Type 1 error if analyzed separately, a principal component analysis (PCA) was conducted to develop a single swimming kinematic descriptor to reduce the number of dependent variables. Only correlation matrices with eigen values of >1 were interpreted.

Percent motility

Percent motility was calculated for each sample by creating an additional data column wherein if the VCL>0, there would be a 1 and if VCL=0 there was a 0. These values where used to compute percent motility for all replicates. The mean of all the replicates for a given fish was calculated.

Statistical analyses

Data was collected in Excel and analyzed using RStudio (R Core Team, 2021). Packages psych, car, mass, dplyr, corrplot, doBy, multcomp, ggfortify, effectsize, tidyversy, ggubr, nlme, DescTools, ez and rstatix were used. A two-way split plot repeated measures design was used to evaluate the effect of fridge time on the sperm velocity principal component and percent motility. Fish was considered the subject, time in fridge as the between subjects' factor, and time post activation as the within subject's factor. Following this analysis, the data was divided up by time post activation to see if there was an effect of time in fridge at certain times post activation.

Results

Fish were anaesthetized in approximately 7 minutes after exposure to 150 mg/ml of buffered MS222. Fridge temperature was consistently 6 ^{o}C and samples were all kept in the back in the same location.

Principal component analysis

The correlation matrix showed that VCL, VAP and VSL parameters were highly correlated (all greater than 88). Table 3. in the appendix shows the means of each velocity parameter of each treatment group. The principal component analysis yielded one significant component with an eigenvalue of 2.86 which explained 95% of the variance. This principal component was extracted and represented sperm velocity performance.

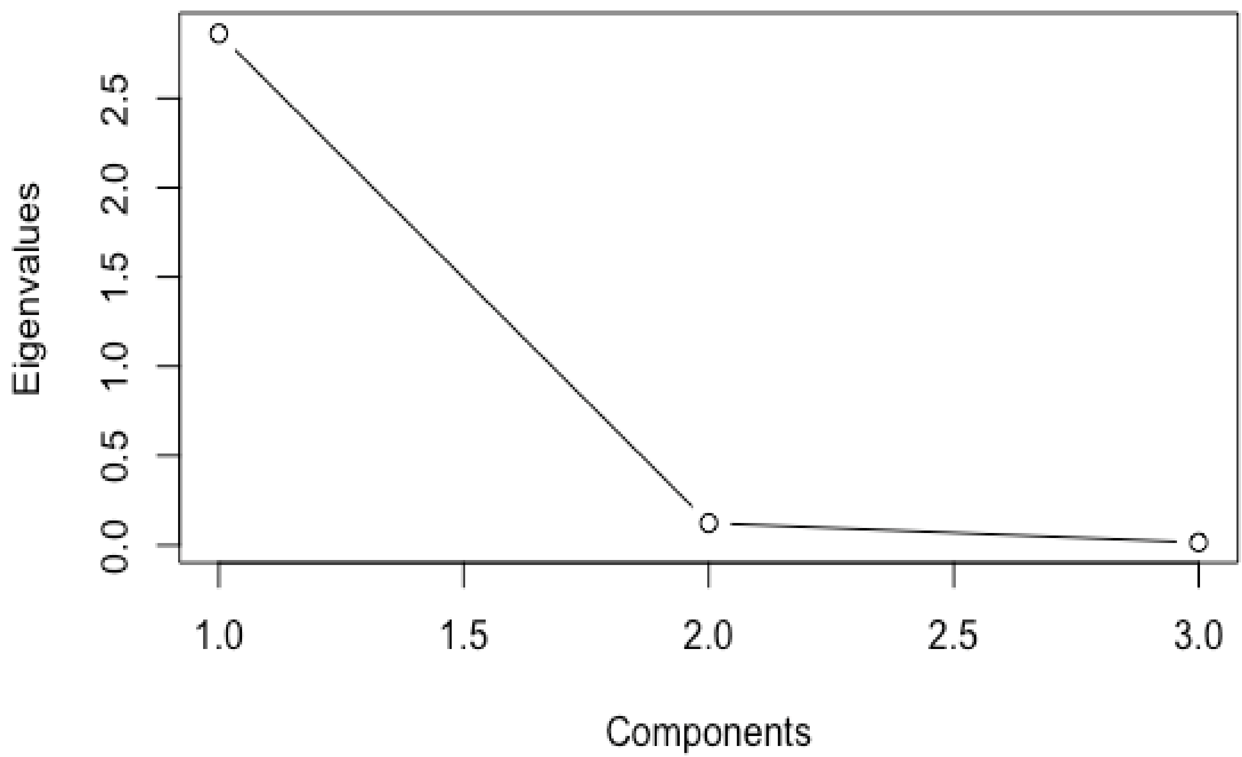

Figure 3. Scree plot showing the eigen values of components. Eigen values of >1 is deemed significant.

Velocity performance

The normality and homogeneity of variance was tested on the residuals of the ANOVA. The velocity performance principal component had univariate normality (Shapiro-Wilk test, p= 0.3415). It also displayed homogeneity of variance (Levene's test, p = 0.4167). The effect of the interaction of fridge and time as well as the effect of time did not pass the assumption of sphericity (Mauchly's Test for Sphericity, p=3.81e-3). The Greenhouse-Geisser corrections outputted by the ez package in R was used.

The two-way split plot repeated measures ANOVA yielded no significant interaction between fridge treatment and time post activation on velocity performance (F-value: 1.1735, p-value: 0.7). There was no statistically significant effect of time in fridge on velocity performance (F-value: 0.746, p-value: 0.573), and there was an effect of time post activation on velocity performance (F-value: 22.673, p-value: 2.56e-6).

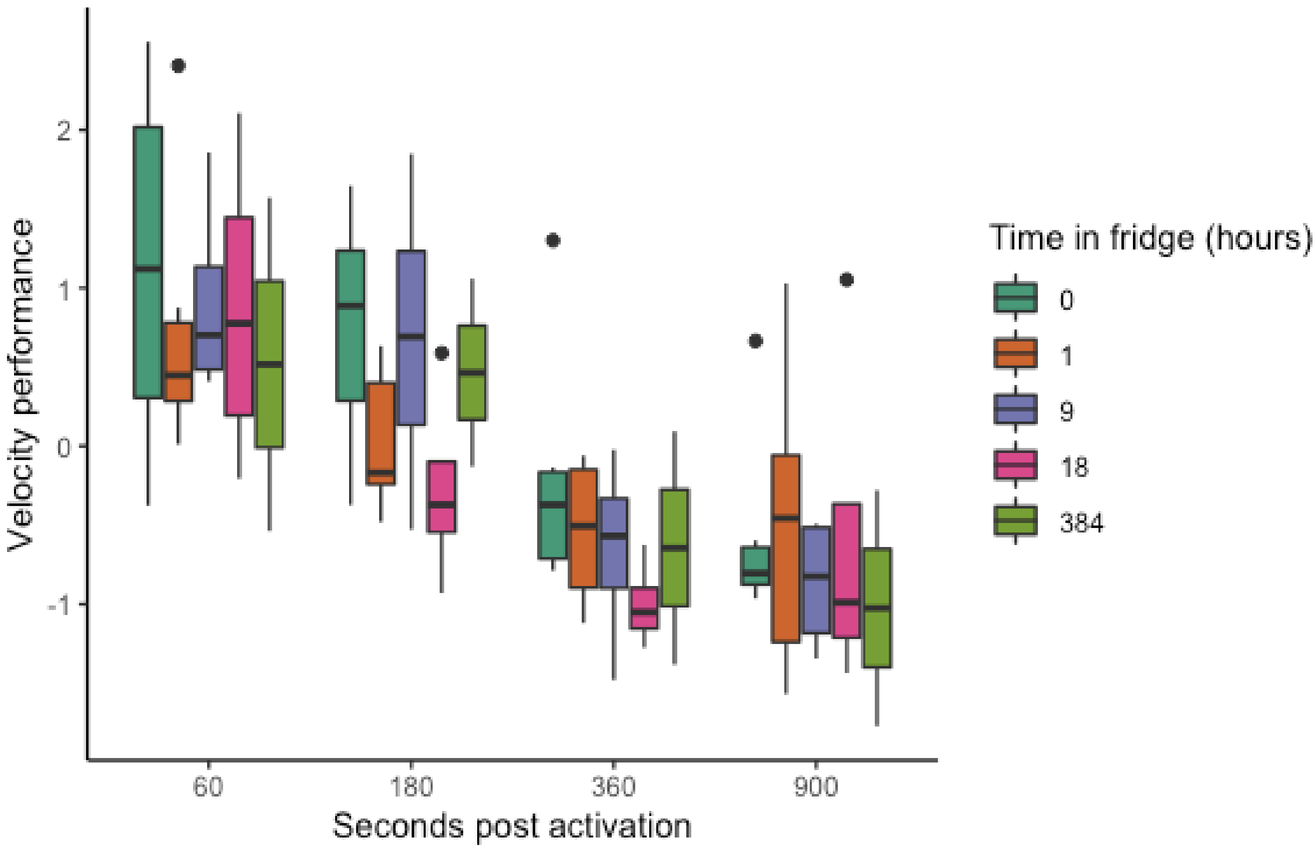

Figure 4. Mean velocity performance of spermatozoa stored at varying time spent in the fridge (hours) over seconds post activation represented by boxplots. Whiskers represent the range of data for each treatment and the middle line represents the mean. Outliers are denoted as dots.

Percent motility

The normality and homogeneity of variance was tested on the residuals of the ANOVA. The motility data was found to have homogeneity of variance (Levene's test, $p = 0.2146$), but was found to not be normal (Shapiro-Wilk test, $p= 0.2146$). Given one of the requirements for the ANOVA was not met, motility data was transformed using log10 transformation which then yielded normal and homogenous variance (Shapiro-Wilk test, $p= 0.1799$; Levene's test, $p=0.732$). After creating the model, the assumption of sphericity was satisfied for both time and the interaction between fridge treatment and time post activation (Mauchly's Test for Sphericity, $p= 0.2580$).

The two-way split plot repeated measures ANOVA yielded no significant interaction between fridge treatment and time post activation on percent motility (F-value: 1.1735, p-value:

3.26e-1). There was almost a statistically significant effect of fridge on percent motility (F-value: 56.896, p-value: 9.04e-2), and there was an effect of time post activation on percent motility (F-value: 2.401, p-value: 2.72e-16). Table 4. in the appendix shows the mean percent motility and variance for each treatment group

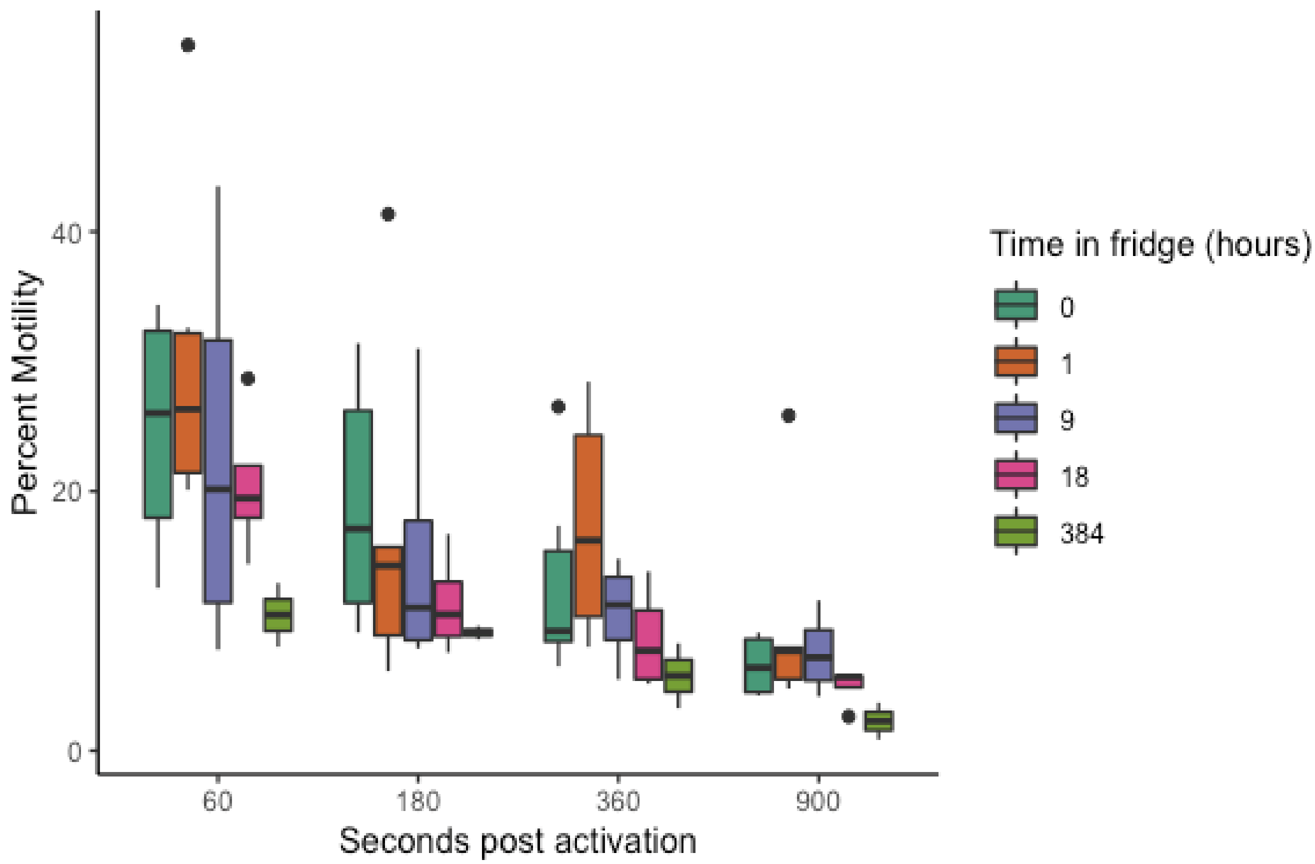

Figure 5. Mean percent motility of spermatozoa stored at varying time spent in the fridge (hours) over seconds post activation represented by boxplots. Whiskers represent the range of data for each treatment and the middle line represents the mean. Outliers are denoted as dots.

Given that there was almost a significant effect of fridge on percent motility, the data was subset by time post activation to see if there may be an effect of fridge within different levels of time post activation. Four one-way ANOVAs were carried out and their results are outlined in Table 2.

Table 2. One-way ANOVA results for the effect of fridge on percent motility at different times post activation.

One-way ANOVA time group (seconds)	Treatment	Df	F value	P-value
60	Fridge	4	1.957	0.147
180		4	0.685	0.612
360		4	2.11	0.124
900		4	3.414	0.0319*

No significant effect of fridge on time was observed at 60-, 180-, and 360-seconds post activation. At 900 seconds, there was a significant effect of fridge treatment on percent motility. Post hoc testing (Dunnet's test) comparing the fridge treatments against the 0 hours in fridge control group showed that there was a significant decrease in motility after 384 hours (2 weeks).

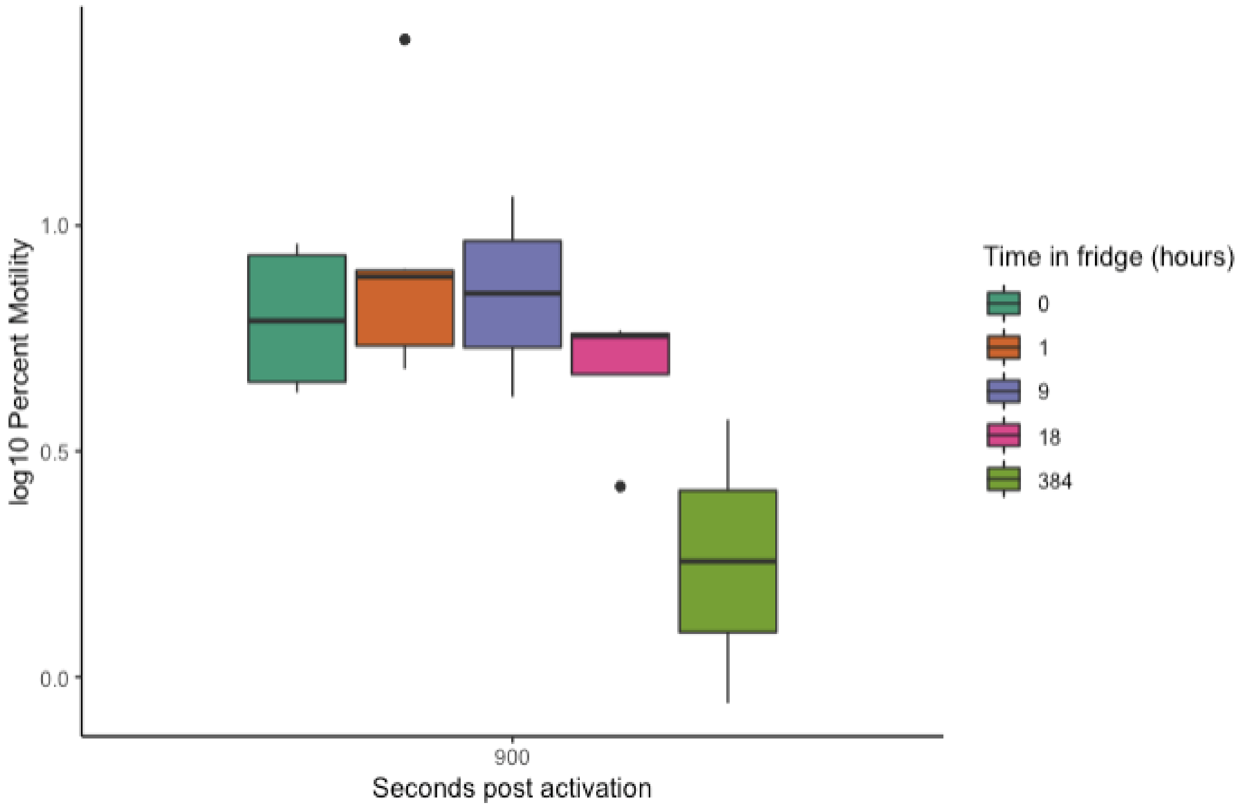

Figure 6. The log10 transformed velocity performance of spermatozoa stored at different lengths of time in the fridge at 900 seconds post activation represented by boxplots. Whiskers represent the range of data for each treatment and the middle line represents the mean. Outliers are denoted as dots.

Discussion

Scientist often use short-term cold storage of semen for their analyses but rarely factor the effect of storage on sperm. Studies like mine should be done for all semen work to account for the potentially confounding effect of storage on sperm quality. HBSS was used primarily as a diluent in our lab and in this work, I sought to understand its efficacy as an extender. Understanding the changes to sperm quality following storage is essential for optimal storage and use of milt. By being able to store Blue Tilapia semen, this work allows for further research on their reproductive biology by providing confidence future results not being confounded by storage time. Determining whether HBSS is effective as an extender and diluent informs improvements *in vitro* fertilization techniques and research on Blue Tilapia. Diluted samples were left in a fridge for 0, 1, 9, 18, and 348 hours. The first four values were chosen to represent the potential time frame in which samples may have been held in the lab for research and *in vitro* fertilization purposes. The main findings of this study were that time in the fridge did not influence the velocity performance, and that it may have affected the percent of motile sperm.

While the p-values of the velocity performance analysis were very clearly not significant in terms of the effects, the percent motility analysis was not as clear. The p-value for the main effects of fridge on the initial ANOVA model for percent motility was very close to meeting the critical 0.05 alpha level. When considering the boxplots and looking for trends in data, certain tendencies emerge. The two-week sample group looks as though it is substantially lower than the other treatment groups between all times post activation. Given the lack of literature of specifically what constitutes “good sperm” in Blue Tilapia, there are no grounds to establish what a biologically significant reduction in percent motility to determine the power of this analysis. However, in future studies a larger sample size should be considered.

When divided up by time, I found a significant decrease in sperm motility at the two-week mark 9 minutes post activation. The literature in some regard agrees with these findings. It has been noted that following 24 hours in the fridge, milt quality often declines (Harvey and Kelley, 1984). Duration of motility can have implications on fertilization success for certain species depending on their reproductive strategy (Prakash et al., 2014). Decreases in motility could have implications in fertilization. For example, fertilization typically occurs within 30 seconds post activation and requires a minimum of 34 038 sperm for fertilization in winter flounder (Butts et al., 2012). Further research is required to determine the effects for shorter duration of motility on Tilapia fertilization. We would need to understand if most fertilization of eggs occurs within 360 seconds in tilapia, then there would be less of a concern of how fridge time may affect fertilization success. However, if these decreases in the longevity of sperm motility do matter in terms of fertilization, there may be more sperm needed to fertilize the egg. While time in fridge does not seem to affect percent motile early on once activated, sperm may not remain motile for as long as if stored for longer durations. Depending on the reason for collection, understanding the implications of extended storage is essential. This is important when considering the use of HBSS as an extender in Blue Tilapia.

Previous work does not altogether agree that there is no effect of storage length on kinematic performance (Dietritch et al., 2021). Dietritch et al. (2021) found that storage of carp semen in a buffer with added penicillin resulted in reduced velocity and that multiple structures within spermatozoa were damaged in the process. When considering these comparisons, it should be noted that they are between different species and used vastly different methods for extenders and diluents. The differences observed may be an example of sperm diversity. The damage caused by cold storage and the efficacy of a given extender is unique to different species. Although, this difference in outcome could simply show that the Dietritch et al. (2021) method was not ideal for their species and my method was suitable for my species. Again, comparisons of this nature are difficult.

The lack of literature on the common velocity and percent motility of Blue Tilapia sperm makes it difficult for me to comment on the initial quality of the collected sperm or compare my results. It has been noted throughout literature however that selecting good broodstock and high-quality sperm is essential for good storage of sperm (Rurangwa et al., 2004). Common cut-offs for what are considered good quality sperm in anecdotal tenses is anywhere from 60-80% motility. Nile Tilapia, a closely related species, have been reported to have an initial motility of 60-80% (Dzyuba et al., 2002). The highest motility I sampled in Blue Tilapia was around 24%. I am concerned about the quality of broodstock selected for this experiment. This quality may have been reduced by the repeated collection of milt from this group of fish. While Tilapia continuously produce sperm, this does not mean they have endless supplies of sperm. There could be negative effects on their sperm by frequently stripping (manually collecting milt from) fish. While this experiment illustrates the changes in sperm motility with refrigeration, it may not be the most reliable when considering raw values of sperm quality. Furthermore, fish were anaesthetized each time sampled. This repeated anaesthetic use could also have impacted sperm quality. Wagner et al. (2002) found that duration of motility may be affected by tricaine (MS222) use. Billard et al. (1988) found the same thing but contested that reductions in sperm quality had to do with the shock in reduced pH when in anaesthetic.

While the upmost care was taken when activating sperm, there is a potential bias in results due to mechanical error of the activation. A 1% BSA solution was used as an activation medium to reduce sperm sticking to microscope slides. Some spermatozoa despite the addition of BSA in the activating medium still got stuck to the microscope slide and were interpreted as static by CASA. Additionally, static sperm were also often categorized as motile due to Brownian movement, which is the flow of water under a cover slip. Quality control measures were taken by removing said sperm to not impact my analyses, but the proportion of deleted motile sperm to static sperm may have impacted the accuracy of my measurements. On average, 3.41+/- 0.411 motile sperm were deleted from each sample while 1.42 +/- 0.510 static sperm were deleted from each sample. Biases in removed data may impact the accuracy of my measurements.

Despite having samples last for 2 weeks and retain motility and velocity, only 2 of the 6 fish samples lasted to the 2-week (384 hour) mark. In the case of the first two fish sampled, samples had become dehydrated. The third and fourth fish samples were viable, while the 5th and 6th fish sampled were contaminated (likely by ciliates, though this was not confirmed). This experiment was not designed with the intent to store milt for this long, and samples were analysed because the opportunity presented itself as some samples were still viable. In future studies and practices, improved methods to limit contamination should be explored. In future studies where samples are held for longer periods of time, more diluent or extender may need to be added to prevent dehydration. Samples were kept in microcentrifuge tubes and were not aerated. Improved vessels for sperm storage that would minimize contamination while also allowing for improved aeration would be beneficial.

Although motility and velocity can be used as a proxy for ATP production, future studies should explicitly examine the ATP production of activated sperm without storage and following storage of activated sperm. ATP assays have been done on sperm cells (Lhanister et al., 1996). Further research tying the link between motility, different kinematic parameters and ATP production should be explored. Flagellar beat frequency typically increases with increases in temperature due increased ATP utilization (Billard and Cosson, 1986). Increases in velocity are typically a result of the axoneme activity which is linked to this beat cross frequency. Furthermore, if samples are not aerated well and oxidative phosphorylation is the main mode of ATP production, ROS and its harmful products may damage the cell resulting in reduced quality once reoxygenated (Dietritch et al., 2021). Examining the oxidative damage of sperm following storage could further uncover the physiological changes sperm undergo when in short-term storage.

Given that HBSS has shown efficacy as an extender and diluent, it would be interesting to note whether addition of other extenders or added components could extend the duration of short-term storage. In many instances, custom extender and diluents are used for different fish species. These extenders can be made by hand or are a combination of a variety of extenders. DMSO is an extender used in cryopreservation of tilapia spermatozoa (Bozkurt et al., 2019). Egg yolks have also been used in Tilapia cryopreservation (Harvey and Kelley). Other future directions could include the use of altogether different extenders as well. Evaluating the efficacy of extenders meant for cryopreservation for short-term uses may be interesting to see how long sperm can be stored short-term before needing to be cryopreserved. By using different extenders, combining extenders, or creating new extenders, we may discover which methods are best in the long- and short-term storage of tilapia milt.

Through this study, I was able to successfully store Blue Tilapia milt in HBSS over the span of 18 hours, and as luck would have it 2 weeks with no significant reduction in velocity. However, there was almost a statistically significant effect of sperm storage on percent motility, and there was a significant reduction in percent motile after storage of 2 weeks at 9 minutes post activation. There may be some effects of time stored on percent motility. This study shows that storage time has little to no consequences on sperm quality between 1 and 18 hours. The goal of this study was to provide a practical time frame for individuals working with sperm to inform them how different quality parameters may change with shot-term refrigeration. This study allows for further study into the reproductive biology of Blue Tilapia. By being able to control sperm quality and by extension fertilization, production of Tilapia may be improved in aquaculture and conservation settings.

Appendix

Table 3. Means of kinematic velocity parameters of different treatment groups. Velocity parameters were measured using CASA hardware and software.

Time stored in fridge (hrs)	Time post activation (seconds)	Number of subjects	Mean VCL (μm/s)	Mean VAP (μm/s)	Mean VSL (μm/s)
0	60	6	65.3224	45.6567	40.5333
1	60	6	58.2893	41.3736	38.1736
9	60	4	65.1151	42.903	37.294
18	60	4	60.7162	42.7862	38.7598
384	60	2	58.258	38.4325	34.7189
0	180	6	61.0678	40.9168	37.0446
1	180	6	49.4358	33.7388	30.99
9	180	4	59.533	40.4153	36.4937
18	180	4	45.5893	30.9267	27.8463
384	180	2	56.6821	37.9551	34.7215
0	360	6	47.8317	32.5127	27.1339
1	360	6	40.3704	28.4077	26.0414
9	360	4	40.6469	26.8308	23.9912
18	360	4	34.7833	23.875	21.013
384	360	2	43.0325	26.1714	23.4945
0	900	6	43.0274	27.9522	24.0496
1	900	6	43.9811	28.7111	25.1265
9	900	4	41.0868	24.0199	20.3991
18	900	4	46.6082	27.5162	21.4255
384	900	2	36.2124	23.1256	20.0967

Table 4. Means and variances of percent motility of different treatment groups. Velocity parameters were measured using CASA hardware and software.

Time stored in fridge (hr)	Time post activation (seconds)	Number of subjects	Mean % motility	Variance of the mean
0	60	6	24.7709	85.3491
1	60	6	30.1641	168.457
9	60	4	22.9015	260.068
18	60	4	20.4889	35.5142
384	60	2	10.4684	11.9813
0	180	6	18.9118	87.1273
1	180	6	16.5468	163.777
9	180	4	15.2329	116.117
18	180	4	11.3151	15.89
384	180	2	9.10194	0.5547
0	360	6	12.8412	58.8517
1	360	6	17.3808	75.8181
9	360	4	10.6948	16.7227
18	360	4	8.61583	16.435
384	360	2	5.78377	12.426
0	900	6	6.56264	5.46478
1	900	6	9.8129	63.6646
9	900	4	7.54656	10.6002
18	900	4	4.97225	2.41575
384	900	2	2.29601	4.03701

REFERENCES

Alavi, S. M., Rodina, M., Viveiros, A. T. M., Cosson, J., Gela, D., Boryshpolets, S., & Linhart, O. (2009). Effects of osmolality on sperm morphology, motility and flagellar wave parameters in Northern pike (*Esox lucius L.*). *Theriogenology*, *72*(1), 32–43.

Alavi, S., & Cosson, J. (2005). Sperm motility in fishes. I. Effects of temperature and pH: A review. *Cell Biology International*, *29*(2), 101–110.

Alavi, S., & Cosson, J. (2006). Sperm motility in fishes. (II) Effects of ions and osmolality: A review. *Cell Biology International*, *30*(1), 1–14.

Beirão, J., Boulais, M., Gallego, V., O'Brien, J. K., Peixoto, S., Robeck, T. R., & Cabrita, E. (2019). Sperm handling in aquatic animals for artificial reproduction. *Theriogenology*, *133*, 161–178.

Billard, R. (1986). Spermatogenesis and spermatology of some teleost fish species. Reproduction Nutrition Développement, 26(4), 877–920.

Billard, R., & Cosson, M. P. (1992). Some problems related to the assessment of sperm motility in freshwater fish. *Journal of Experimental Zoology*, *261*(2), 122–131.

Blake Grant, F., Pang, P. K. T., & Griffith, R. W. (1969). The twenty-four-hour seminal hydration response in goldfish (*Carassius auratus*)—I. sodium, potassium, calcium, magnesium, chloride and osmolality of serum and seminal fluid. *Comparative Biochemistry and Physiology*, *30*(2), 273–280.

Blake Grant, F., Pang, P. K. T., & Griffith, R. W. (1969). The twenty-four-hour seminal hydration response in goldfish (Carassius auratus)—I. sodium, potassium, calcium, magnesium, chloride and osmolality of serum and seminal fluid. Comparative Biochemistry and Physiology, 30(2), 273–280.

Bozkurt, Y., Yavaş, I., Bucak, M. N., & Yeni, D. (2019). Effect of Different Cryoprotectants (Glycerol, Methanol and Dimethyl Sulfoxide) on Post-thaw Quality, Viability, Fertilization Ability and DNA Damage of Cryopreserved Nile Tilapia (*Oreochromis niloticus*) Spermatozoa. *Cryo Letters*, *40*(1), 11–17.

Butts, I., Roustaian, P., & Litvak, M. (2012). Fertilization strategies for winter flounder: Effects of spermatozoa density and the duration of gamete receptivity. Aquatic Biology, 16(2), 115–124.

Cosson, J. (2004). The Ionic and Osmotic Factors Controlling Motility of Fish Spermatozoa. *Aquaculture International*, *12*(1), 69–85.

Cosson, M. P., Billard, R., & Letellier, L. (1989). Rise of internal Ca^{2+} accompanies the initiation of trout sperm motility. *Cell Motility and the Cytoskeleton*, *14*(3), 424–434.

Dietrich, M. A., Judycka, S., Słowińska, M., Kodzik, N., & Ciereszko, A. (2021). Short-term storage-induced changes in the proteome of carp (*Cyprinus carpio L.)* spermatozoa. *Aquaculture*, *530*, 735784.

Dzyuba, B., Legendre, M., Baroiller, J. F., & Cosson, J. (2019). Sperm motility of the Nile tilapia (*Oreochromis niloticus*): Effects of temperature on the swimming characteristics. *Animal Reproduction Science*, *202*, 65–72.

El-Sayed, A. F. M. (2019). *Tilapia Culture: Second Edition*. Elsevier Science.

Garcia, R. R. F., Vasconcelos, A. C. N., Povh, J. A., Oberst, E. R., Eloy, L. R., & Streit Junior, D. P. (2016). Different extenders solutions for tambaqui semen cooling. *Pesquisa Agropecuária Brasileira*, *51*(6), 780–784.

Harvey, B., & Kelley, R. N. (1984). Chilled storage of *Sarotherodon mossambicus* milt. *Aquaculture*, *36*(1–2), 85–95.

Jenkins-Keeran, K., & Woods, L. C. (2002). An Evaluation of Extenders for the Short-Term Storage of Striped Bass Milt. *North American Journal of Aquaculture*, *64*(4), 248–256.

Kholodnyy, V., Gadêlha, H., Cosson, J., & Boryshpolets, S. (2020). How do freshwater fish sperm find the egg? The physicochemical factors guiding the gamete encounters of externally fertilizing freshwater fish. *Reviews in Aquaculture*, *12*(2), 1165–1192.

Krasznai, Z., Márián, T., Izumi, H., Damjanovich, S., Balkay, L., Trón, L., & Morisawa, M. (2000). Membrane hyperpolarization removes inactivation of Ca^{2+} channels, leading to Ca^{2+} influx and subsequent initiation of sperm motility in the common carp. *Proceedings of the National Academy of Sciences*, *97*(5), 2052–2057.

Kristan, J., Dadras, H., & Policar, T. (2021). Optimization of short-term storage of pikeperch semen: An applicable approach. *Animal*, *15*(1), 100001.

Lahnsteiner, F., Berger, B., Weismann, T., & Patzner, R. A. (1996). Motility of spermatozoa of *Alburnus* (Cyprinidae) and its relationship to seminal plasma composition and sperm metabolism. *Fish Physiology and Biochemistry*, *15*(2), 167–179.

Little, D. C., & Hulata, G. (2000). Strategies for tilapia seed production. In M. C. M. Beveridge & B. J. McAndrew (Eds.), *Tilapias: Biology and Exploitation* (pp. 267–326). Springer Netherlands.

Locatello, L., Rigoni, G., Soriano, M. E., Rasotto, M. B., & Poli, F. (2020). Going beyond conventional parameters to unveil sperm quality in fish: The use of fibre optic technology to assess mitochondrial respiratory performance. *Biology Open*, bio.053306.

Merino, O., Figueroa, E., Cheuquemán, C., Valdebenito, I., Isachenko, V., Isachenko, E., Sánchez, R., Farías, J., & Risopatrón, J. (2017). Short-term storage of salmonids semen in a sodium alginate-based extender. *Andrologia*, *49*(5), e12661.

Morita, M., Takemura, A., & Okuno, M. (2003). Requirement of Ca2+ on activation of sperm motility in euryhaline tilapia *Oreochromis mossambicus*. *Journal of Experimental Biology*, *206*(5), 913–921.

Polge, C., Smith, A. U., & Parkes, A. S. (1949). Revival of Spermatozoa after Vitrification and Dehydration at Low Temperatures. *Nature*, *164*(4172), 664–666.

Prakash, S., Prithiviraj, E., Suresh, S., Lakshmi, N. V., Ganesh, M. K., Anuradha, M., Ganesh, L., & Dinesh, P. (2014). Morphological diversity of sperm: A mini review. *Iranian Journal of Reproductive Medicine*, *12*(4), 239–242.

R Core Team (2021). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.URL https://www.R-project.org/.

Rana, K. (1988). Reproductive Biology and the Hatchery Rearing of Tilapia Eggs and Fry. In J. F. Muir & R. J. Roberts (Eds.), Recent Advances in Aquaculture (pp. 343–406). Springer Netherlands.

Rothbard, S., & Pruginin, Y. (1975). Induced spawning and artificial incubation of Tilapia. *Aquaculture*, *5*(4), 315–321.

Rurangwa, E., Kime, D. E., Ollevier, F., & Nash, J. P. (2004). The measurement of sperm motility and factors affecting sperm quality in cultured fish. *Aquaculture*, *234*(1–4), 1–28.

Stoss, J. (1983). 6 Fish Gamete Preservation and Spermatozoan Physiology. In Fish Physiology (Vol. 9, pp. 305–350). Elsevier.

Wagner, E., Arndt, R., & Hilton, B. (2002). Physiological stress responses, egg survival and sperm motility for rainbow trout broodstock anesthetized with clove oil, tricaine methanesulfonate or carbon dioxide. *Aquaculture*, *211*(1–4), 353–366.

Yang, H., Hazlewood, L., Walter, R. B., & Tiersch, T. R. (2006). Effect of osmotic immobilization on refrigerated storage and cryopreservation of sperm from a viviparous fish, the green swordtail *Xiphophorus helleri*, *52*(2), 209-218.

Yang, H., Hazlewood, L., Walter, R. B., & Tiersch, T. R. (2006). Effect of osmotic immobilization on refrigerated storage and cryopreservation of sperm from a viviparous fish, the green swordtail Xiphophorus helleri. Cryobiology, 52(2), 209–218.