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Improvements to the spotted wolffish (*Anarhichas minor*) sperm cryopreservation

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Abstract

Spotted wolffish (*Anarhichas minor*) is a promising candidate for cold water aquaculture. However, in captivity, the spotted wolffish lack gamete synchronization, and the males produce only a limited amount of sperm, which is a challenge for the aquaculture industry. In this context, it is crucial to develop protocols that allow sperm storage and maximization of its use, such as sperm cryopreservation. Current protocols for spotted wolffish sperm cryopreservation are sub-optimal and result in a significant decrease in fertilization success. Furthermore, the use of 0.5 ml straws is impractical because usually volumes of at least 25 ml cryopreserved sperm are needed for each liter of eggs.

In this study, the efficiency of antioxidants and membrane stabilizers added to the cryopreservation extender was tested. Both reduced glutathione (RG) at 1 mM and 5 mM and taurine at 10 mM and 50 mM were considered for antioxidants. BSA at 1 % and 5 % and egg yolk (EY) at 5 % and 10 % were considered for the membrane stabilizers. The sperm was cryopreserved 1:1 with an extender based on Kime and Tveiten (2002) + 10 % DMSO. Straws 0.5 ml were placed 4.5 cm over the liquid nitrogen (LN_2) for 10 min before being plunged directly into the LN_2. Thawing was done by holding the straws in a beaker filled with water (5 ºC) for 1 min.

The addition of 5 % BSA to the cryopreservation extender resulted in a significant increase in sperm motility compared to that of the cryo-control (sperm cryopreserved without antioxidants or membrane stabilizers), which made this the best out of the nine experimental treatments. A qualitative study of viability and lipid peroxidation was also conducted. The higher concentrations of antioxidants (5 mM taurine and 50 mM RG) had ~20 % increase in sperm viability compared to the cryo-control, but no improvements in the sperm motility were observed. The cryo-control presented higher lipid peroxidation values (measured as MDA concentration) than all antioxidant treatments.

In the next step, large volume cryopreservation was tested using 5 ml cryovials. The sperm samples were 1:1 diluted in the extender based on Kime and Tveiten (2002) with the addition of 10 % DMSO and 5 % BSA. Three different combinations of freezing heights (1.5 cm and 4.5 cm from LN_2) and thawing rates (6 min for 10 ºC and 4 min for 15 ºC) were tested. No significant difference was observed in sperm motility and VCL between the two treatments using freezing heights of 1.5 cm and cryo-control. While the treatment using a freezing height

of 4.5 cm had significantly lower motility and VCL, then the cryo-control. Also, the viability of the sperm using a freezing height of 4.5 cm was lower (~20 %) compared to the cryo-control (~65 %).

The results suggests that, spotted wolffish sperm should be cryopreserved by diluting 1:1 in the extender based on Kime and Tveiten (2002) with the addition of 10 % DMSO and 5 % BSA, regardless of whether the cryopreservation is done with the use of 0.5 ml straws or 5 ml cryotubes. When cryopreserving using 5 ml cryovials, the cryovials should be placed at 1.5 cm from the LN_2 for 10 min and thawed at 10 °C. The spotted wolffish lays minimum 5000 eggs and to fertilize this amount of eggs 25 ml of cryopreserved sperm is needed. These results, using 5 ml cryovials to cryopreserve sperm, could potentially decrease the time and workload during the reproductive period. For further studies it is suggested that a mix of 5 % BSA and either reduced glutathione or taurine be tested.

1.0 Introduction

With an annual production of 2.3 million tons (SSB 2019), Norwegian aquaculture is highly dominated by Atlantic salmon (*Salmo salar*). Several other species are currently cultured in Norway, for example, turbot (*Scophthalmus maximus*), rainbow trout (*Oncorhynchus mykiss*), Atlantic cod (*Gadus morhua*), Atlantic halibut (*Hippoglossus hippoglossus*), spotted wolffish (*Anarhichas minor)* and Arctic charr (*Salvelinus alpinus*). However, they represent only 5 % of the fish farmed in Norway (SSB 2019). In Norwegian aquaculture species diversification has been considered an essential mechanism to expand the aquaculture further.

1.1 The Wolffish

The family *Anarhichadidae* is classified into two genera (Figure 1): *Anarhichas* and *Anarrhichthys*. The *Anarrhichthys* genus includes a single species, the wolf eel (*A. ocellatus*), whereas the genus *Anarhichas* is composed of four species: Northern wolffish - *A. denticulatus*; Atlantic wolffish - *A. lupus*; spotted wolffish - *A. minor* and Bering wolffish - *A. orientalis* (Mecklenburg 2003; ADW 2019).

These are cold marine benthic species that live in various environments, from relatively shallow waters (from ~1 m) to slightly deep waters (to ~600 m). They are distributed from the North Pacific to the North Atlantic Ocean. A large head, blunt snout, and a long elongated body characterize the species. Its jaws, which are filled with strong canines anterior and large molariform teeth laterally, are excellent for feeding on echinoderms, crustaceans, molluscs and other fish (Nelson *et al.* 2016; FAO 2019a; FAO 2019b).

Kingdom	*Animalia*	
Phylum	*Chordata*	
Subphylum	*Vertebrata*	
Superclass	Gnathostomata	
Class	Actinopterygii	
Order	Perciformes	
Family	Anarhichadidae	
Genus	*Anarhichas*	*Anarrhichthys*
Species	*A. denticulatus*	*A. ocellatus*
	A. lupus	
	A. minor	
	A. orientalis	

Figure 1 Classification of generas Anarhichas and Anarrhichthys (Source Nelson et al. 2016).

Le François *et al.* (2002) evaluated a wide array of different marine fish species for their potential for diversifying the aquaculture industry in Atlantic Canada. The Atlantic wolffish and spotted wolffish came out with the highest scores of 91 % and 87 % respectively, the only difference being the R&D effort. Despite this, the spotted wolffish displayed superior traits with faster growth rate, higher egg volume per female, ability to sustain higher density, absence of aggressive behavior, better fillet yielding and later maturity (Moksness 1994; Le François *et al.* 2002; Foss *et al.* 2004; Foss & Sparboe 2009). According to Foss *et al*. (2004), the spotted wolffish has many desirable qualities which are beneficial in a species used for aquaculture purposes. At hatching, it is very well developed, with a small yolk reserve as the only real larval characteristic and can, therefore, be weaned directly on formulated feed (Figure 2). As a species, it is highly robust and can tolerate high stock densities, as well as significant variations to oxygen, carbon dioxide, ammonium, and salinity.

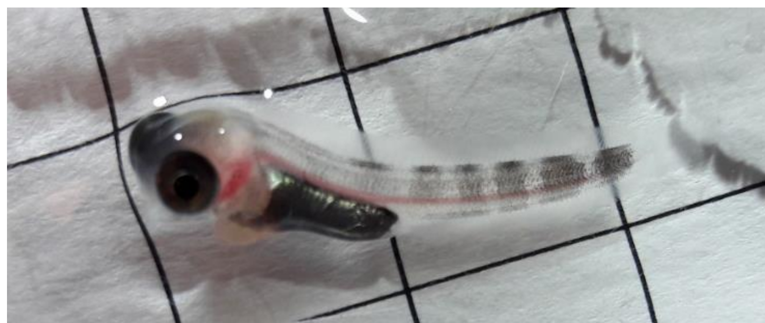

Figure 2 Spotted wolffish larvae 24 h post-hatch with fully developed digestive system (Source: José Beirão).

The spotted wolffish is found from the Barents Sea, down the coast of Norway, Faroe Islands, Iceland, and over to Greenland and eastern Canada (Figure 3). It lives over soft sandy mud and mud bottoms, often with boulders at depths between 25 – 590 m. It is usually found in waters ranging from -1.0 ºC to 7.5 ºC (Østvedt 1963; FAO 2019b). It can grow to a length of 120 cm. In nature, it grows slowly, with the length increasing by 10 cm each year until maturation.

Maturation occurs late, at 7 – 9 years (females mature 1 – 2 years earlier than males), and at a large size, 60 – 90 cm (Falk-Petersen *et al.* 1999).

Figure 3 Geographical distribution of Spotted wolffish (Source: FAO 2019a).

1.1.1 The spotted wolffish farming

In aquaculture, the spotted wolffish (Figure 4) has been of interest since 1994, when the first artificially fertilized spotted wolffish eggs were incubated. Despite limited investments being made, it had a very rapid progression as an aquaculture species. The first fish was slaughtered and sold in 2003 by Tomma Matifisk AS. The production peaked between 2003 – 2006, with an annual production of 100 – 130 tons. Unfortunately, in 2007 a large part of the broodstock was lost due to a pumping failure which caused the development of the spotted wolffish aquaculture to take a significant step backward. Tomma Steinbit took over the production from Tomma Matfisk AS and established a new broodstock. It was active but unused for several years due to asynchrony in the spawning of male and female wolffish (Foss & Sparboe 2009).

Figure 4 Spotted wolffish male (Source: José Beirão).

Today it is possible to rear spotted wolffish in either land-based facilities or flat-bottom circular sea cages with shelves (Foss *et al.* 2004). Results indicate that land-based facilities are the best option, even though it is much more expensive than sea cages due to high construction and operational costs. Spotted wolffish thrives at temperatures between 4 – 12 ^{o}C, which is easy to control with the use of recirculating aquaculture systems (RAS). RAS can also control other

water quality parameters like oxygen, carbon dioxide, salinity, and temperature (Moksness 1994; Lundamo, 1999; Hansen & Falk-Petersen, 2002; Foss *et al.* 2004).

Despite being an industry on the verge of commercial breakthrough, the production of spotted wolffish stalled in the early 2000s. Due to the cod fisheries collapsing in this period, interests for cod aquaculture was on the rise. Interests and capital shifted from the different new species being used for aquaculture purposes, to almost exclusively being concentrated on cod aquaculture. As the cod stock recovered, the interests for cod aquaculture declined, and interests for other species resurfaced (Foss & Sparboe 2009). In 2013, Aminor AS bought the broodstock of Tomma Steinbit, initiated commercial production of the spotted wolffish (Nystø *et al.* 2015) and delivered the first fish for slaughter in 2019. Research has been commenced to try and solve the remaining biological challenges. These include the lack of specific diets that fulfill its nutritional requirements, lack of knowledge of health and diseases, reduced control over the reproduction and maturation where male and female wolffish lack gamete synchronization during the reproductive season, as well as the lack of good quality sperm (Gunnarsson *et al.* 2009; Santana 2018).

1.1.2 Reproduction

The spawning season naturally occurs in late summer and early autumn at rather inaccessible grounds that have made an observation of its reproductive behaviour impossible to this date. However, behavioural studies suggest that mating pairs form months before the reproduction period and that the reproductive biology is like that of the Atlantic wolffish particularly that the fertilization is internal (Keats *et al.* 1985; Pavlov & Novikov. 1986; Falk-Petersen *et al.* 1999). The females are easy to recognize during the reproductive period due to the swollen belly, in which they store a large number of eggs (0.7 – 3.0 l). The eggs range in sizes from 5.4 – 6.5 mm and are enveloped in a viscous, gelatinous ovarian fluid. The males collect the eggs into a ball and guard them until hatching - 800 – 1000 d^{o} (Keats *et al.* 1985; Falk-Petersen *et al*. 1999; Hansen and Falk-Petersen 2001; Kime and Tveiten 2002; Foss *et al*. 2004).

In captivity, there is a change in the spawning behaviour of the spotted wolffish where they lack gamete synchronization during the reproductive season. Thus, the males do not show normal spawning behaviour, and the females release unfertilized eggs. Because of this, fertilization needs to be done artificially. The males produce a limited and irregular amount of sperm (0.5 – 6 ml) with variable quality and low concentrations (Foss *et al*. 2004; Gunnarsson *et al*. 2009; Beirao & Ottesen 2017; Santana 2018).

Spotted wolffish sperm is motile at stripping and remains motile for about 48 h. This is one of the main differences between the sperm from fish with internal fertilization, and the sperm from fish with external fertilization, for example, the Atlantic salmon, where the sperm is immotile at stripping. It is currently not possible to immobilize and reactivate spotted wolffish sperm, and thus refrigeration as a storage method is not the best option. Cryopreservation is the preferred method for sperm storage, where sperm can be kept for a prolonged period without the quality decreasing over time. Cryopreservation of sperm is an option that has been used for different fish species, including wolffish, with success (Le François *et al.* 2008; Gunnarsson *et al.* 2009). However, the protocols were not optimized, and the results are sub-optimal.

1.2 Sperm quality assessment

Sperm quality is defined as a measurement of the ability of sperm to successfully fertilize an egg (Rurangwa *et al*. 2004; Fauvel *et al*. 2010). Any quantifiable physical parameter that directly correlates with the fertilization capacity of sperm could be used as a measure of sperm quality (Rurangwa *et al*. 2004). Over the years many biomarkers have been used, including sperm motility, density, membrane viability seminal plasma osmolarity, pH and chemical composition, enzymatic activity, ATP concentration, lipid peroxidation, morphology, and several others (Billard & Cosson 1992; Ciereszko & Dabrowski 1993, 1994; Billard *et al*. 1995a,b; Lahnsteiner et al. 1996, 1998; Fauvel *et al*. 1998; Geffen & Evans 2000; Chowdhury & Joy 2001; Migaud *et al*. 2013; Valdebenito *et al*. 2013; Cabrita *et al*. 2014)

Motility is regarded as the most practical and useful indicator of sperm quality. It refers to the spermatozoa ability to move and is essential regarding the sperm's ability to achieve and fertilize an egg (Rurangwa *et al*. 2004). Several studies have reported a high correlation between sperm motility and fertilization ability (Bozkurt & Secer 2006; Liu *et al*. 2007; Beirão *et al*. 2011; Gallego *et al*. 2013; Gallego *et al*. 2017). It has been most commonly analyzed subjectively by simple evaluation under the microscope, which has given variable results depending on the person doing the analysis (Gallego *et al*. 2018). However, computer-assisted sperm analysis (CASA) that gives an objective assessment of sperm quality has become widely used.

Other studies have considered sperm velocity as a better biomarker for sperm quality (Figure 5), which includes VCL - curvilinear velocity, VSL - straight-line velocity and VAP - average path velocity (Rurangwa *et al*. 2001; Viveiros *et al*. 2010; Gallego *et al*. 2017).

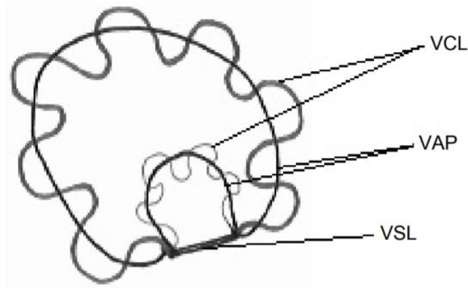

Figure 5 Example of two tracks with different VCL - curvilinear velocity and VAP - average path velocity but the same VSL - straight-line velocity – (Source: Nagy et al. 2015)

In many fish such as salmonids, silurids, and cyprinids, the sperm has been reported to have a smooth gently curved trajectory which has made VAP and VCL almost identically (Kime *et al*. 1996, 2001; Kime & Tveiten 2002; Rurangwa *et al*. 1998). However, in a species such as wolffish, where the fertilization takes place within a gelatinous egg mass, the sperm has been shown to follow a zigzag path, which is like that of mammalian sperm. This type of movement, where the VAP values are much lower than the VCL, suggests that the sperm needs some wobbling movement to traverse through the ovarian fluid (Kime and Tveiten, 2002; Elofsson *et al*. 2003).

Viability is the sperms capability to move and fertilize an egg. Nonetheless, when doing sperm analysis, viability refers to the percentage of live sperm, where the integrity of the sperm cell membrane is intact. This parameter is particularly important if sperm motility is low, to differentiate between non-motile sperm and dead sperm (Rurangwa et al. 2004). Viability can be assessed by using non-permeable and permeable dyes that stain the cells differently depending on whether the sperm cell membrane is intact or not. The percentage of viable spermatozoa is then counted either manually under a microscope, by flow cytometry or by an automatic counter (Cabrita et al. 2010; Fauvel et al. 2010).

Sperm produces reactive oxygen species (ROS), typically hydrogen peroxide (H_2O_2), superoxide anions (O_2^-), and hydroxyl radicals (OH^-), spontaneously which are required in small amounts to drive important functions. However oxidative stress is induced if the production of ROS exceeds the sperm`s antioxidant defenses (Ball 2008; Aitken et al. 2009; Taylor et al. 2009; Li et al. 2010).

Polyunsaturated lipids, which are abundant in sperm cells, are highly susceptible to oxidative attack and are one of the main manifestations of ROS induced damages to sperm (Ayala *et al*. 2014). The oxidative attack results in a chain reaction which produces end-products such as

MDA, malondialdehyde (Ayala *et al*. 2014). Both lipid peroxidation and the increasing levels of ROS have been associated with the reduction of sperm motility, the fertility potential of sperm and its ability to create normal, healthy embryo (Lenzi *et al*. 1993; Agarwal *et al*. 1994; Armstrong *et al.* 1999; Conquer *et al*. 1999; Gagnon *et al*. 1999; Zarghami & Khosrowbeygi 2005; Ball 2008; Aitken *et al*. 2009; Li *et al*. 2010; Badade *et al.* 2011; Cabrita *et al*. 2014).

Sperm ROS formation can be a possible explanation of poor sperm quality, either in freshly collected semen or after processing for use in various reproductive technologies (Guthrie & Welch 2006).

1.3 Cryopreservation

Cryopreservation can be defined as the storage of biological materials using, most commonly, liquid nitrogen as a coolant that sustains a temperature of -196 ºC. That suspends the chemical, biological, and physical processes (Chao & Liao 2001). It is a technique which can theoretically preserve cells in a suspended viable form for up to 32 000 years (Ashwood-Smith 1980)

It is an essential tool in the aquaculture industry allowing a simplification of the broodstock management by 1) reducing the number of used males; 2) manipulating the spawning season; 3) avoiding vertical transmission of diseases; 4) conserving and maintaining genetic variability and diversity; 5) enabling easier transportation of gametes and; 5) helping with species which have reproductive problems, such as lack of synchronization in the gamete production or low sperm production (Rana *et al*. 1995; Suquet *et al*. 2002; Asturiano *et al*. 2004, 2017; DeGraaf & Berlinsky 2004; Cabrita *et al*. 2010; Chao & Liao 2011; Martínez-Páramo *et al*. 2017; Judycka *et al*. 2019).

During cryopreservation, the temperature decreases below the medium`s freezing point, and extracellular ice crystals start forming. At this point the cells are protected by a cell membrane that hinders the formation of intracellular ice formation; however, the water within the cells is now in a “super-cooled” state and will have a higher chemical potential than the extracellular fluids. This results in cellular dehydration due to water being transported out of the cell to maintain equilibrium between the intra and extracellular fluids, where the worst-case scenario is an eventual collapse of the cell membrane (Piironen 1992; Rana 1995). This can be avoided by controlling the freezing rate: to low freezing rates cause the concentration of extracellular solutions to increase, to high freezing rates increase the chance for the formation of intracellular ice which can damage the cell (Muldrew & McGann 1990; Piironen 1992; Rana 1995; Benson *et al*. 2012).

As a result, during the process of cryopreserving, the cells undergo different types of stress, such as changes in the osmotic balance and temperature changes during cooling, freezing, and thawing. (Holt 2000). These changes can potentially be lethal; thus, the cells need to be protected. This protection is attained by using a buffer (extender) which is added to maintain the correct osmolality after adding a cryoprotectant. Cryoprotectants act by lowering the freezing point of the solution and protecting the cells against ice crystal formation (Rana 1995; Elliot *et al*. 2017).

Different cryoprotectants, that permeate the cell membrane, are added to the extender and are used to increase fluidity and partly dehydrate the cell to lower the freezing point. This, in turn, decreases the number of intracellular ice crystals which are formed. The cryoprotectants are, however toxic to the sperm cells, and this toxic effect is related to the concentration and exposure time (Swain & Smith 2010). Thus, a balance between cryoprotectant effectiveness and toxicity needs to be found in each case.

Substances, such as antioxidants and membrane stabilizers, can also be added to the extender, which are less toxic and can help to mitigate the damage caused by cryoprotectants. They inhibit ice growth and help stabilize internal solute concentrations under osmotic stress, which reduces the amount of cryoprotectants needed (Quinn *et al.* 1980; Anchordoguy *et al.* 1988; Crowe *et al*. 1988; Swain & Smith 2010).

1.3.1 The use of antioxidants and membrane stabilizers

To overcome the adverse effects of sperm oxidative stress during the cryopreservation process, antioxidants can be supplemented as an addition to the cryopreserving extender. Antioxidants are considered the primary defense factor against oxidative stress. (Wang *et al*. 1997; Agarwal *et al*. 2004; Ebisch *et al*. 2007; Ball 2008; Li *et al*. 2010; Silva *et al*. 2011). There are two types of antioxidants: enzymatic and non-enzymatic. The enzymatic antioxidants participate in the sperm`s natural antioxidant defense system and include superoxide dismutase, catalase, glutathione peroxidase or reduced glutathione. (Alvarez et al. 1987; Alvarez and Storey 1989; Kefer *et al*. 2009; Silva *et al*. 2011; Partyka *et al*. 2012). The-non enzymatic antioxidants are supplemented through diet and include reduced glutathione, taurine, hypotaurine, selenium, zinc, urate, ascorbic acid, vitamin E, carotenoids or ubiquinones (Alvarez and Storey 1989; Therond *et al*. 1996).

Several studies have been conducted on the addition of different antioxidants. For example, taurine has been frequently used as an antioxidant and demonstrated to have an improvement

in fish sperm quality. Martínez-Páramo *et al.* (2013) found slight improvements (not significant) when using 1 mM taurine to cryopreserve European sea bass (*Dicentrarchus labrax*) sperm. Kutluyer *et al.* (2016) used 1, 2 and 4 mM taurine when cryopreserving goldfish (*Carassius auratus*) sperm with significant improvements. Red seabream (*Pagrus major*) sperm was cryopreserved using 50 mM taurine resulting in significant improvements (Liu et al. 2014). However, Riesco *et al.* (2017) got no improvements with 50 mM taurine when used with dusky grouper (*Epinephelus marginatus)* sperm cryopreservation. Reduced glutathione has also frequently been used as an antioxidant, and Gadea *et al*. (2011) found significant improvements after using 1 mM in human sperm cryopreservation. Lahnsteiner *et al.* (2011) used 1 mM reduced glutathione with both brook trout (*Salvelinus fontinalis*) and the rainbow trout sperm cryopreservation with no improvements. Paula *et al.* (2012) got no improvements with the use of 0.5, 1.0, and 1.5 mM on streaked prochilod (*Prochilodus lineatus*) sperm cryopreservation. Neither did Sariözkan *et al*. (2009) find any improvements to sperm quality after using 2 mM on cattle bull (*Bos taurus*) sperm cryopreservation.

Some of the lethal damages that cryopreservation can cause to the spermatozoa include an increase in membrane fragility of live sperm cells, loss of membrane permeability and changes in membrane stability, which results in increased permeability of membranes to water and cations (Cabrita *et al*. 1999; Cabrita *et al*. 2001a). Membrane stabilizers are added to the extender to protect the cells against such damages, and several membrane stabilizers have been widely used when cryopreserving, such as egg yolk, bovine serum albumin (BSA), trehalose, sucrose, and proline.

Egg yolk is used extensively, and with excellent results in several cases. Butts *et al.* (2010) and Babiak *et al*. (2012) demonstrated significant improvements after using 10 % egg yolk in Atlantic cod sperm cryopreservation, as did Cabrita *et al*. (2001a) on rainbow trout sperm cryopreservation with the same concentration. However, a study done on gilthead seabream (*Sparus aurata*) sperm cryopreservation by Beirão *et al*. (2010), found a significant decrease in sperm quality when using 10 % egg yolk. Also, BSA has been used extensively as a membrane stabilizer. Beirão *et al*. (2010) used 0.1 % BSA on gilthead seabream sperm cryopreservation resulting in significantly higher motility. Cabrita *et al*. (2001a) also found significantly higher motility after using 0.4 % BSA to cryopreserve rainbow trout sperm. While Uysal & Bucak (2007) showed significant improvement after using 0.5 %, 1 % and 2 % BSA on ram (*Ovis aries*) sperm cryopreservation. However, Sariözkan *et al.* (2009) found no improvement after

using 0.5 % BSA on cattle bull sperm cryopreservation. Neither did Riesco *et al.* (2017) after cryopreserving sperm from dusky grouper using 1 % BSA.

1.3.2 Large volume cryopreservation

Cryopreservation of sperm in low volumes, typical straws with a volume of 0.5 ml, is useful for small scale production, or for species that only require small volumes of sperm. At a commercial scale, many straws are needed to thaw due to the fertilization of large volumes of egg simultaneously. According to Santana (2018) spotted wolffish needs cryopreserved sperm concentration of 5×10^5 spz / egg to obtain the same fertilization rates as when using fresh sperm. The spotted wolffish lay 5000 - 15000 eggs and has an average sperm cell concentration between 10^6 and 10^8/ml. Fertilizing 5000 eggs would, therefore, need 25 ml sperm (10^8) or 100 x 0.5 ml straws. Cryopreserving using larger volumes could therefore potentially decrease the time and workload required for the process of both freezing and thawing.

There have been several studies on large volume cryopreservation. Some report similar sperm quality between large and low volume cryopreservation, while others report lower sperm quality comparing large volume cryopreservation to low volume cryopreservation. Yao *et al.* (2000), Cabrita *et al.* (2001b), Gunnarsson *et al*. (2002) and Nomura *et al.* (2018) cryopreserved sperm (ocean pout - *Macrozoarces americanus L.,* rainbow trout, spotted wolffish, Japanese eel - *Anguilla japonica* respectively) using different volumes (0.25 ml vs. 1.8 ml, 0.5 ml vs. 5 ml, 0.5 ml vs. 1 ml and 0.25 ml v. 5 ml) resulting in no significant difference to sperm motility between the volumes. However, a study done by Várkonyi *et al.* (2018) found significant difference to sperm motility after cryopreserving common carp (*Cyprinus carpio*) sperm using 5 ml and 10 ml cryotubes, resulting in significantly reduced sperm motility compared to the frozen control.

1.3.3 Earlier studies on cryopreservation of spotted wolffish sperm

There have been three previous attempts to establish a protocol for spotted wolffish sperm cryopreservation with some success. The first study was conducted by Le François *et al*. (2008), who tried to identify a suitable extender for use in cryopreservation of both Atlantic wolffish and spotted wolffish sperm. In addition, this study identified the presence of antifreeze proteins (AFP) in the wolffish seminal fluid. Evaluation of the toxicity of two cryoprotectants (10 % DMSO and methanol) was also conducted. The evaluation was done subjectively at 15 ºC, and the commercial extender Cryo-Fish was chosen as the best medium for sperm cryopreservation, giving the sperm 60 % motility compared to the sperm cryopreserved with no addition giving

25 % sperm motility. In the study by Gunnarson *et al*. (2009) the effect of different concentrations of DMSO, different freezing rates and different sizes of straws (0.5 and 1.0 ml) on post-thaw motility of spotted wolffish sperm were analyzed. The evaluation was done subjectively at room temperature (temperature not detailed in the study but perhaps above 20 ^{o}C), and hardly any difference in post-thaw motility was recorded in the trials for DMSO concentration and freezing rates. The different straw sizes did not affect sperm survival. Finally, Santana (2018) studied the toxicity of three different cryoprotectants (DMSO, 1, 2-propanediol and methanol) at three different concentrations (5, 10 and 20 %) on post-thaw motility of spotted wolffish. He further tested different freezing rates (1.5, 2.5, 4.5 and 7.5 cm from the liquid nitrogen) and thawing rates (1 min at 5 ^{o}C and 25 s at 10°C) using 0.5 ml straws. The evaluation was done objectively using a CASA system at 6 ^{o}C. Motility decreased for all the cryoprotectants after freezing; however, DMSO gave the highest motility at all freezing heights and thawing rates. Santana (2018) suggests using an extender based on Kime & Tveiten (2002), with the addition of 10 % DMSO, when using 0.5 ml straws. A freezing height of either 4.5 or 7.5 cm from the surface of liquid nitrogen for 10 min and thawing rate of 1 min at 5 ^{o}C were also recommended.

1.4 Objectives

As documented in the introduction presented above, the efficient cryopreservation of spotted wolffish sperm is one of the necessities needed so that the spotted wolffish aquaculture can be more profitable and successful. There is currently a protocol in place for cryopreservation of spotted wolffish sperm using 0.5 ml straws. Nonetheless, this protocol is suboptimal, and the use of 0.5 ml straws is impractical since volumes of minimum 25 ml cryopreserved sperm are needed to fertilize 1 litre of eggs. The main objective of this thesis was to improve the usability of sperm cryopreservation in commercial spotted wolffish farming. The specific objectives were:

- Testing the usefulness of membrane stabilizers and antioxidants in an extender.
- Testing the cryopreservation of wolffish sperm in large volumes using 5 ml cryotubes.

2.0 Material and method

2.1 The broodstock

For the present thesis, we used spotted wolffish broodstock of farmed origin from the commercial producer AMINOR AS. The experiments were mainly carried out at Mørkvedbukta research station (Nord University, Bodø). Later the experiments were carried out at AMINOR`s facilities in Halsa, due to the fish moving from Mørkvedbukta to Halsa.

At the Mørkvedbukta research station, the fish were held at all times in a semi-closed system with water pumped from 50-meter depth through a 200 µm filter. Out of the spawning season, the fish were kept in covered tanks located outside. At the beginning of the spawning season, the fish were moved indoors to three 10-meter circular tanks five square tanks of 2 x 2 m, but with the same water source.Both had a water height of 0.4 m. The fish were fed 3 times a week based on appetite, with the use pellets from Skretting (Vitalis CAL/Vitalis REPRO). The broodstock was kept under natural photoperiod, at a 34 ‰ salinity and temperature of the water changing from 5 ºC in the winter/spring up to 11 ºC in the summer/autumn. Oxygen measurements of the outgoing water were to be held at over 80 % but rarely dropped below 90 %. The average temperature outside during the experiment was 2.62 ºC. The conditions the broodstock were kept at did not change when it was moved from the covered tanks outside to the circular tanks indoor.

The first fish chosen to be used in the experiment were males producing good quality semen during the previous reproductive season. The males rested at least four weeks between samplings. The sperm samplings took place from October 2017 to February 2018 at Mørkvedbukta, corresponding to the natural spawning season, and on June 2018 at Halsa from individuals kept on reverse photoperiod.

2.2 *Sperm sampling of spotted wolffish*

Using a hand net, the fish were moved into a 25 liter container for sedation in seawater containing 500 ppm of MS-222. When the fish were thoroughly anesthetized (checked by squeezing the tail area of the fish and turning it upside down), they were picked up and placed on a bench which was covered by a wet towel. Fresh seawater was poured over the gills before covering the head and gills with a towel. To decrease the chance of the samples being contaminated with urine or feces, the abdominal area was massaged to release the sperm. The urogenital area was cleaned by using a dry paper towel, taking away urine, feces, and seawater. Pre-cut Pasteur pipettes (as shown in Figure 7) were kept cold on ice in a cooling box until use. The pipette was placed with a slight suction on the urogenital papillae, and the fish was stripped.

The stripping was done by pressing down on the lateral area of the abdomen (Figure 6) where the gonads are located, by applying a downward dragging motion, from where the pectoral fins are located, and down towards the urogenital papillae. This was done on one side of the wolffish first, and then on the other side. It was essential to change pipettes often to decrease the chance of the samples being contaminated by urine or blood. If there was any indication of urine, the urogenital papillae were cleaned with a paper towel before placing a new pipette. If blood came out of the urogenital papillae, sampling was stopped. When a pipette was filled, it was immediately put back into the cooling box in a rack over ice until analysis

The acceptable threshold for motility (%) was set to 50 %, and the sperm from at least three different males was pooled for each sample. However, for the preliminary trial, this threshold was not retained.

Figure 7 Pasteur pipette has been cut for milt sampling (Source: Stian Flengstad)

Figure 6 Sperm collection from a spotted wolfish male (Source: João Santana)

2.3 Sperm analysis

2.3.1 Analysis of motility (%) and curvilinear velocity (VCL) using the CASA system

Using a CASA system (Computer Assisted Sperm Analysis) SCA 6.2 – Motility module (Microptic, Barcelona, Spain), both the motility and the curvilinear velocity (VCL) were measured. VCL was selected instead of VAP since, as explained in the introduction section, spotted wolffish sperm presents a zigzag path, and thus, VAP does not represent the actual velocity.

The system was attached to a microscope (Nikon Eclipse Ci, Tokyo, Japan) which had a camera (Basler acA1300-200uc, Ahrensburg, Germany) on top for image recording, as well as a stage temperature controller (Linkam T95-PE, Tadworth, United Kingdom) set to 6 ºC. The CASA system was adjusted to 20 frames/s, 1 s acquisition time, 10 – 50 μm^2 for head area. Cells that were moving slower than 9 µm/s were considered drift.

Sperm analysis (Figure 8) was conducted within 1 - 3 h after the first sperm was sampled. The samples were kept in the fridge (4 ºC) before being taken out in batches. They were held on a rack in ice in a cooling box while analyzing. The motility was measured by diluting the sperm with the extender based on Kime and Tveiten (2002), selected based on the preliminary trial (see 2.5.1), with the addition of 1 % BSA to decrease cell stickiness to the microscope slide. The dilution ratio was 3 µl sperm and 17 µl extender. This was then mixed by pipetting the solution a few times. Four µl of the diluted sperm was added to a microscope slide and covered with a coverslip for analysis in the microscope. When placing the slide under the microscope, there was almost always a slight drift movement which usually stopped after a few minutes. The CASA video was set to record after the drift had stopped.

Figure 8 Sample being prepared for motility analysis, CASA system in the background (Source: José Beirão).

2.3.2 Analysis of sperm viability

Viability was measured using a commercial kit (LIVE/DEAD^{TM} Sperm Viability Kit) by Invitrogen. The fluorescence dyes, SYBR-14, and propidium iodide (PI) were used. SYBR-14 is a permeable dye which stains living cells, with functioning cell membrane, in green. Propidium iodide (PI) is a non-permeable dye which stains cells with damaged cell membrane in red.

A SYBR-14 working solution was prepared by diluting 2 µl of SYBR-14 1 mM stock solution (already in aliquots) with 18 µl of the extender solution based on Kime and Tveiten (2002) giving a concentration of 100 nM. Sperm was also diluted 1:10 in an eppendorf to a volume of 200 µl in the extender solution based on Kime and Tveiten (2002). 0.5 µl of the 100 nM SYBR-14 working solution was added to the diluted sperm and incubated in the dark for 10 min at 4˚C. After the 10 min incubation period 1.5 µl of PI 2.4 mM stock solution was added to the Eppendorf, which gave a final concentration of 12 µM, and incubated for five more minutes. After the incubation time was over 2 µl of the cell suspension was pipetted onto a microscope slide and read in a fluorescent microscope (Figure 9). At least 100 cells were counted per slide, and three slides were read per sample. The number of green and red-stained cells were counted, and the percentage of viable cells calculated.

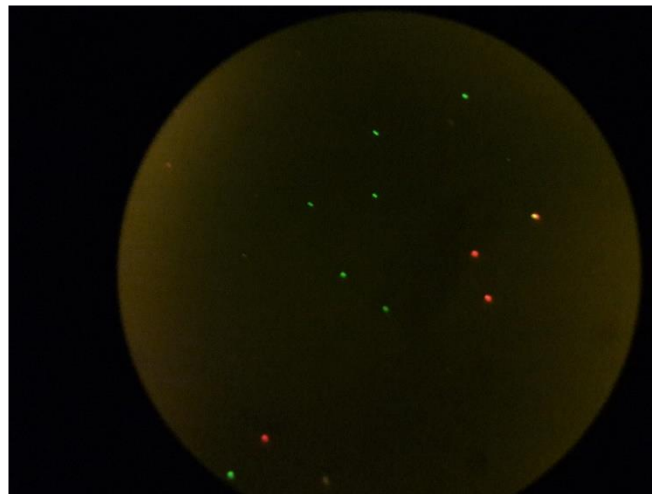

Figure 9 Assesment of sperm cell viability. Propidium iodide stains damaged cells red, while SYBR-14 stains living cells green (Source: José Beirão).

2.3.3 Analysis of sperm lipid peroxidation

Sperm lipid peroxidation was measured using a commercial kit (Lipid Peroxidation (MDA) Assay kit) by Sigma-Aldrich®. The lipid peroxidation is determined by the reaction between malondialdehyde (MDA) and thiobarbituric acid (TBA) which forms a colorimetric/fluorometric product. All the samples and standards were read in duplicates.

For the standard curve, 10 µl aliquots of 0.1 M MDA were prepared in advance by diluting 10 µl of the 4.17 M MDA standard with 407 µl distilled water. Then 20 µl of the 0.1 M MDA solution was diluted with 980 µl distilled water to make a 0.002 M MDA standard solution. Lastly, 20 µl of the 0.002 M MDA standard solution was diluted with 980 µl distilled water to make a 0.04 mM MDA standard solution.

Five Eppendorf (curve point) were made ready by adding 0, 0.5, 1, 2, 4, and 6 µl of the 0.04 mM MDA standard solution. Distilled water was added into each of the eppendorfs which brought the total volume of each Eppendorf to 200 µl and a final concentration of 0, 0.1, 0.2, 0.4, 0.8 and 1.2 µM.

The sperm sample was prepared on ice by mixing 100 µl of sperm with 300 µl of MDA lysis buffer containing 3 µl BHT (100x). The sample was then vortexed before being centrifuged at 13 000 × g for 10 min at 4 °C to remove insoluble material. Two hundred µl of the supernatant was placed in an Eppendorf.

TBA solution was prepared by diluting the kit TBA powder with 7.5 ml glacial acetic acid and adjusting the final volume to 25 ml using distilled water. Six hundred µl of the TBA solution was added into each Eppendorf tube, containing standard curve point and sample, to form the MDA-TBA adduct. To enhance sensitivity, 300 µl of 1-butanol was added to the reaction mix. Each Eppendorf tube was then incubated at 95 °C for 60 min in the dark. It was important to keep an eye on the tubes when incubated since lids kept opening due to pressure, which resulted in evaporation. After the incubation time, the samples and standards were placed on ice to cool for 10 min, after which samples and standards were centrifuged at 16 000 × g for 3 min at 4 °C.

The sample readings were done by transferring 200 µl of each sample and standard curve point into a 96 well plate for reading. The well plate was covered with aluminum foil to keep it from being contaminated by light. The well plate was read with a Fluostar Optima plate reader (BMG LABTECH) and measured the colorimetric assay with an absorbance of 516 nm. Due to the low number of pools for the lipid peroxidation, no statistics were conducted.

2.3.4 Sperm concentration

The sperm cells concentration was done using an optical microscope with a 40x magnification and a Bürker counting chamber, MARIENFELD, Paul Marienfeld GmbH, Germany (Figure 10).

The samples were read in triplicates, and each sample was made ready by diluting 20 µl sperm with 180 µl extender, making it a dilution rate of 10. Next, placing a coverslip on a Bürker counting chamber with a drop (c. 50 µl) of sperm on the sample induction point of the chamber. Four squares in the Bürker counting chamber was counted diagonally for each triplicate (Figure 10).

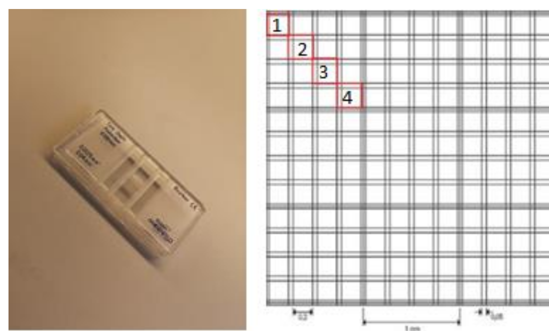

Figure 10 Assessment of sperm concentration with Bürker`s counting chamber. Counting started at the first square in the upper left corner, moving diagonally downward against the lower right corner (Source: Stian Flengstad).

2.5 Experiment 1 – The use of membrane stabilizers and antioxidants in cryopreservation of spotted wolffish sperm using 0.5 ml straws.

2.5.1 Preliminary trial - Selection of extenders solution

A preliminary trial was conducted to decide which extender to be used. The two extenders were tested in triplicate (3 pools) and an average of these sperm pools taken. The first extender tested was based on SR - Smith and Ryan (2010) who analyzed *Xiphophorus nigrensis* sperm, which, like wolffish, has internal fertilization.

It contained:

207 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.49 mM $MgCl_2$, 0.41 mM $MgSO_4$ and 10 mM Tris (pH: 7.5 and osmolality: 357 mOsm/kg).

The second extender tested was based on KT - Kime and Tveiten`s (KT) preliminary spotted wolffish seminal fluid analysis.

It was composed of:

154 mM NaCL. 3.55 mM $CaCl_2$, 4.83 mM $KHCO_3$, 2.37 mM $MgSO_4$, 1.00 mM glucose (pH: 7.5 and osmolality: 310 mOsm/kg).

Semen was then incubated with the extenders at a dilution of 1:1 in a fridge at 4 ºC. The sperm was tested at 0, 3, 24, 48, 72, and 96 h for motility (%) and VCL (µm/s) using a CASA system. The viability (%) was checked at 0, 3, 24, and 48 h using a fluorescent microscope.

2.5.2 Selection of antioxidants and membrane stabilizers

Two antioxidants (taurine at 5 mM and 10 mM, and reduced glutathione (RG) at 10 mM and 50 mM) and two membrane stabilizers (BSA at 1 % and 5 %, and egg yolk at 5 % and 10 %) were selected to be added to the extender. The antioxidants, membrane stabilizers, and the concentrations were selected based on the bibliography that exists for other fish species.

2.5.3 Cryopreservation of sperm using 0.5 ml straws.

The sperm was diluted 1:1 with the extender based on Kime and Tveiten (2002) +10 % DMSO + treatment (membrane stabilizer or antioxidant) and incubated for 2 min (the extender used was based on chapter 2.5.1) as equilibration time. In total, nine different treatments were tested (2 membrane stabilizers × 2 concentrations + 2 antioxidants x 2 concentrations + control) as seen in Table 1. The control was based on the standard protocol, using the extender and 10 %

DMSO without membrane stabilizers or antioxidants. During the equilibration time, the 0.5 ml straws were filled with the diluted sperm. The straws were kept on ice packs before and after being filled (Figure 11).

After the equilibration, the straws were placed on a 4.5 cm thick floating rack which had been placed inside a Styrofoam box filled with nitrogen (Figure 11). After 10 min on the rack, the straws were placed directly into the nitrogen. The straws were then stored in a LN_2 storage tank until thawing for analysis.

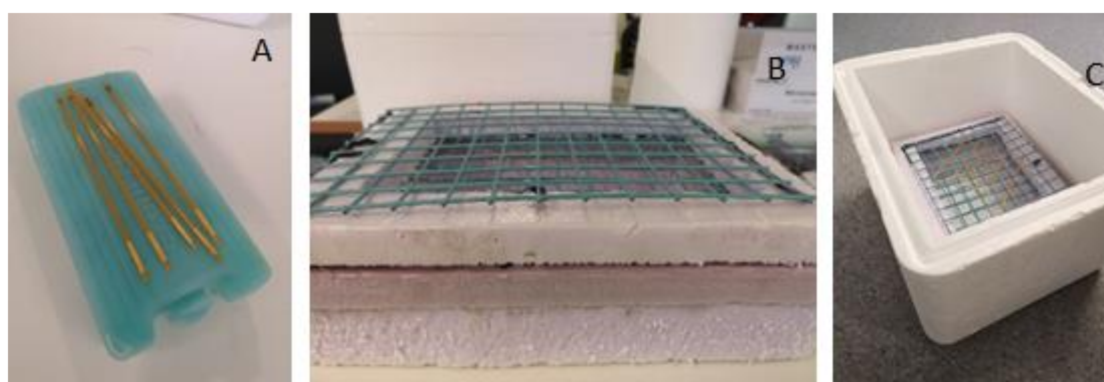

Figure 11 (A) Straws filled with sperm during the equilibration on ice packs. (B) Floating rack which is placed into a container (C) filled with nitrogen. Straws are placed on the floater for 10 min before being placed directly into the nitrogen (Source: Stian Flengstad).

Table 1 Two different membrane stabilizers and two different antioxidants were tested at different concentrations with the extender.

Membrane stabilizers		Antioxidants	
BSA	Egg yolk	Taurine	RG
1 %	5 %	10 mM	1 mM
5 %	10 %	50 mM	5 mM

2.5.4 Thawing of sperm cryopreserved using 0.5 ml straws

When thawing the straws, the same setup, which was found adequate in a previous study done by Santana (2018) was used.

A beaker filled with water was placed inside a cooling box filled with ice (Figure 12). The temperature was 5 ^{o}C. One straw at a time was picked up from the nitrogen and then thawed in the waterfilled beaker using forceps. The straws were thawed in water for 1 min. The straws were continuously being stirred to keep ice from forming outside the straws. After thawing, the

straws were immediately emptied into Eppendorf tubes. They were analyzed for motility (%), VCL (µm/s), viability (%), and lipid peroxidation as MDA concentration. Lipid peroxidation was only analyzed for the control treatment, and samples cryopreserved with antioxidants, not for fresh sperm.

Figure 12 Thawing the cryopreserved sperm in a water bath (Source: Stian Flengstad).

2.6 Experiment 2: Large volume cryopreservation with the use of 5.0 ml cryotubes.

2.6.1 Preliminary trials – Selection of freezing height and thawing rate for large volume cryopreservation

A preliminary trial was conducted to decide which freezing heights and thawing rates to test with 5 ml cryotubes (Figure 13). Due to the high volume of sperm being used, we only did one pool for each treatment. However, each treatment was analyzed in triplicate and an average of the triplicates taken. Using the same method as the one used in experiment 1 (2.5), the cryotubes were filled with 1:1 of sperm and extender based on Kime and Tveiten (2002) with the addition of 5 % BSA. Floating racks of three different heights were used: 1.5, 4.5, and 7.5 cm. The freezing height and the thawing rate used is as seen in Table 2. The control used the same extender with the addition of 5 % BSA and used 0.5 ml straw cryopreserved at 4.5 cm height and thawed at 5 ºC for 1 min.

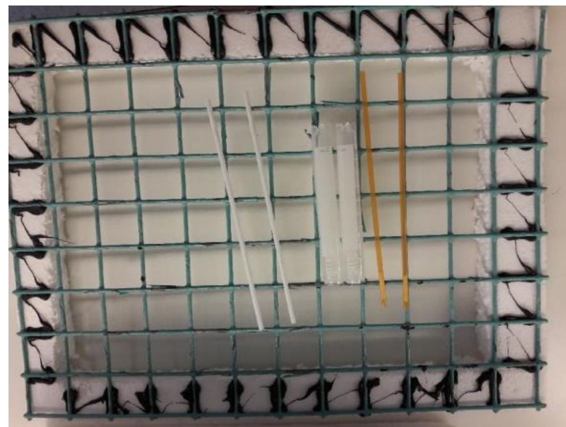

Figure 13 Visual difference between 0.5 ml straws and 5 ml cryovial (Source: José Beirão).

Table 2 Different freezing heights and thawing rates tested. The first row shows the control used.

Freezing height (cm)	Thawing rate (ºC)	Thawing rate (min)
4.5 cm (control)	5 ºC	1 min
1.5 cm	5 ºC	10 min
1.5 cm	10 ºC	6 min
1.5 cm	15 ºC	4 min

1.5 cm	20 ºC	3.5 min
4.5 cm	15 ºC	4 min
7.5 cm	15 ºC	4 min

2.6.2 Cryopreservation with the use of 5 ml cryotubes

The cryotubes were tested using two different freezing heights (Table 3) found from chapter 2.6.1. Motility (%) and VCL (µm/s) were assessed in this trial using a CASA system, and a fluorescent microscope for viability (%).

The sperm was diluted 1:1 with the extender based on Kime and Tveiten (2002), 10 % DMSO, and 5 % BSA. A 2 min equilibration time on ice was given. After this period, the cryotubes were placed on a floating rack (different height options) which had been placed inside a Styrofoam box filled with nitrogen. After 10 min on the rack, the cryotubes were placed directly into the nitrogen. The cryotubes were stored in a LN_2 storage tank until thawing for analysis.

Table 3 Freezing and thawing rates used for cryopreservation of large volumes.

Freezing height (cm)	Thawing rate (ºC)	Thawing rate (min)
1.5 cm	10 ºC	6 min
1.5 cm	15 ºC	4 min
4.5 cm	15 ºC	4 min

2.6.3 Thawing of sperm using 5 ml cryotubes

The thawing rate was based on chapter 2.6.2 (Table 3).

A beaker filled with water was placed inside a cooling box filled with ice. The chosen temperature was adjusted using a thermometer and lukewarm water and ice. Using forceps, one cryotube at a time was picked up and held under the water for the thawing duration. While being kept under water with the help of forceps, the cryotube was continuously stirred. After thawing, cryotubes were immediately taken to be analyzed for motility (%), VCL (µm/s), and viability (%).

2.7 *Statistical analysis*

All the data were tested for normality using a one-sample Kolmogorov-Smirnov and homogeneity of variance by Bartlett´s test before being analysed with a one-way ANOVA using R statistical software 3.5.3 (R Core Team, 2019) with the RStudio addition (RStudio Inc). When the ANOVA showed that there was a significant difference, a *post-hoc* Tukey test was used to specify the differences between treatments. If the data did not meet the ANOVA assumptions, three solutions were tried to fix the data. A transformation using a log or square root, a Kruskal-Wallis test, or removing an outlier. In all tests, a significant level (α) 0.05 was considered. The statistical analysis was conducted between each of the antioxidant treatments and between each of the membrane stabilizer treatments. Only the motility (%) and VCL (µm/s) in experiment 1 and 2 were analysed. No statistical analysis was conducted for the viability and lipid peroxidation due to insufficient data (pools ≤ 2). The reason for the low number of pools for viability and lipid peroxidation was a LN_2 tank which ran dry. Viability and lipid peroxidation were therefore analysed qualitatively.

3.0 Results

3.1 Experiment 1 – The use of membrane stabilizers and antioxidants in cryopreservation of spotted wolffish sperm using 0.5 ml straws.

3.1.1 Preliminary trial - Selection of extender solution

At 0 h, the average values of motility, VCL (Figure 14) and viability (Figure 15) were 48.47 %, 21.68 µm/s, and 93 %, respectively. After three hours, the sperm using an extender based on SR had lower motility, VCL, and viability then KT (14.76 % vs. 36.61 %, 14.90 µm/s vs. 18. 94 µm/s and 61.15 % vs. 77.03 % respectively). At 24 h, motility (36.61 %) and VCL (16.4 µm/s) increased, while viability decreased (52.25 %) compared to 3 h for SR. KT had at 24 h lower motility (21.99 %), VCL (14.60 µm/s) and viability (44 %) compared to SR. Both SR and KT had approximately the same values for motility (31.23 % vs. 34.42 %), VCL (19.29 µm/s vs. 17.67 µm/s) and viability (49.99 % vs. 47.89 %) at 48 h. Three days in and the motility SR was lower than KT (20.50 % vs. 30.13 %), yet the VCL (16.95 µm/s vs. 17.94 µm/s) was approximately the same. Four days into the experiment there was no difference between SR and KT., motility (18.63 % vs. 20.46 %) and VCL (16.65 µm/s vs. 15.54 µm/s) remained the approximately the same.

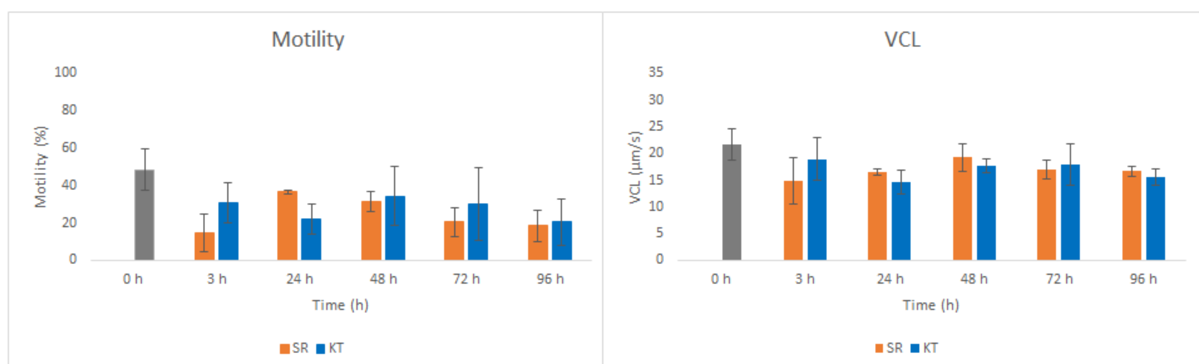

Figure 14 Preliminary trials comparing changes to motility (%) - to the left and VCL (µm/s) - to the right, of sperm using two extenders. Orange bars:extender of Smith and Ryan (2010); blue bars; extenders of Kime and Tveiten (2002); grey bar: fresh control at 0 h. Data are mean ± standard deviation for three technical replicates (n=1)

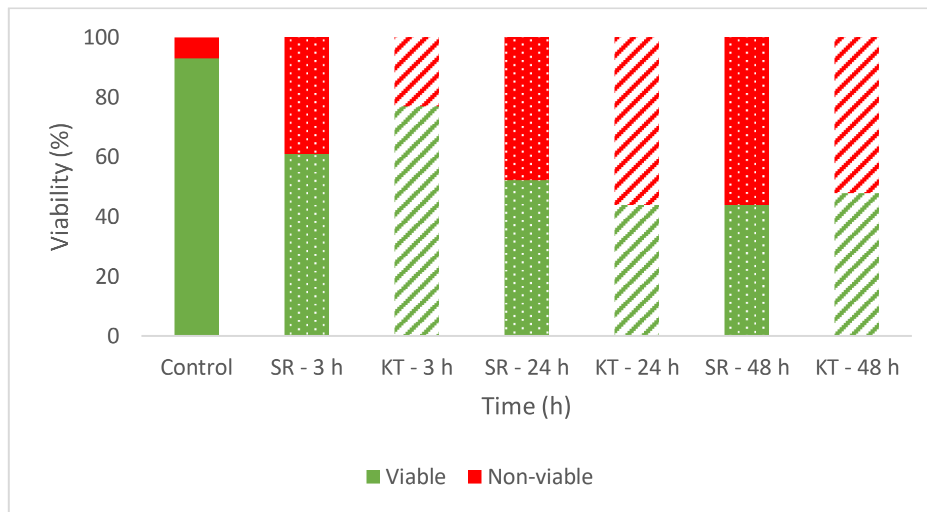

Figure 15 Preliminary trial comparing the change to the viability of sperm diluted in two extenders, Smith and Ryan (2010) - small dots pattern - and Kime and Tveiten (2002) – striped - over 48 h (n=1).

3.1.2 The addition of taurine and reduced glutathione to the extender solution during cryopreservation.

The average initial values of motility (%) and VCL (µm/s) were 60.38 ± 7.25 % and 27.89 ± 3.95, µm/s respectively (Figure 16). The ANOVAs revealed that there were differences for the motility ($F_{5,36}$=10.810, P<0.001) and the VCL ($F_{5,36}$=2.999, p = 0.023) between the treatments for the antioxidant trial. After the freeze-thawing, the 1 mM and 5 mM RG (38.60 ± 7.42 % and 40.07 ± 5.81 %), 10 mM and 50 mM taurine (43.73 ± 8.11 % and 37.78 ± 4.89 %) and cryo control (42.95 ± 4.89 %) treatments all had lower motility (%) than the control as detected by the post-hoc Tukey test. Furthermore, the test revealed no significant difference between the treatments and the cryo control. For the VCL (µm/s) the post-hoc Tukey test revealed that the fresh sperm control was significantly higher than the 10 mM, and 50 mM taurine treatments (21.87 ± 2.41 µm/s and 22.78 ± 3.01 µm/s), but not compared with the cryo control (23.26 ± 2.69 %) and the 1 mM and 5 mM RG treatment (22.42 ± 2.41 µm/s and 22.59 ± 3.67 µm/s). For the viability (Figure 17), no statistics were conducted due to the n=1, however, the viability value was lower in the cryo control (36.8 %) compared to that of the treatments using 1 mM and 5 mM RG (39.56 % and 59.80 %), and 10 mM and 50 mM taurine (44.3 % and 56.79 %) treatments. The results from the lipid peroxidation (Figure 18) indicated, that cryo control (22.36 ± 7.14 µM MDA10^8) had higher MDA levels then the treatments using 1 mM and 5 mM RG (17.51 ± 0.37 and 18.15 ± 1.34 µM MDA/10^8) and 10 mM and 50 mM taurine (19.14 ± 5.59 and 15.63 ± 5.41 µM MDA/10^8).

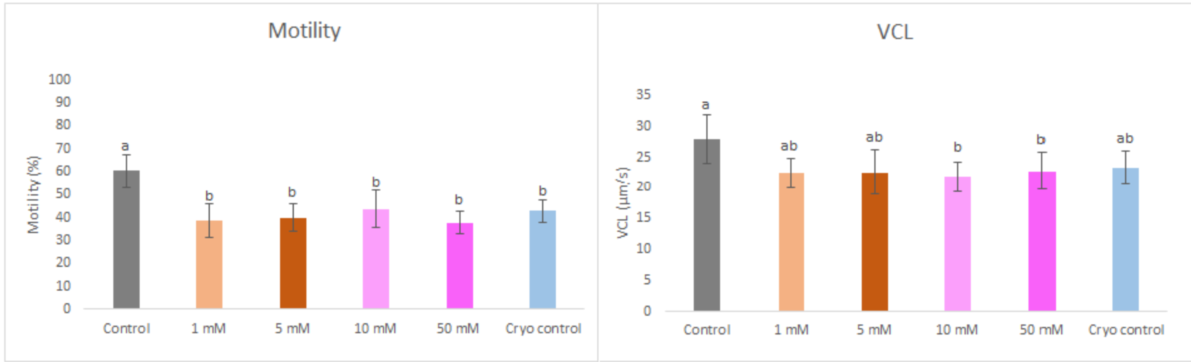

Figure 16 Comparing sperm motility (%) - to the left and VCL (µm/s) - to the right, when adding two different antioxidants at two different concentrations. Fresh sperm / control (grey bar), 1mM and 5 mM reduced glutathione (brown bars), 10 mM and 50 mM taurine (pink/purple bars) and cryopreserved control (blue bar). Data are mean ± standard deviation (n=7). Different letters show differences between treatments (p < 0.05).

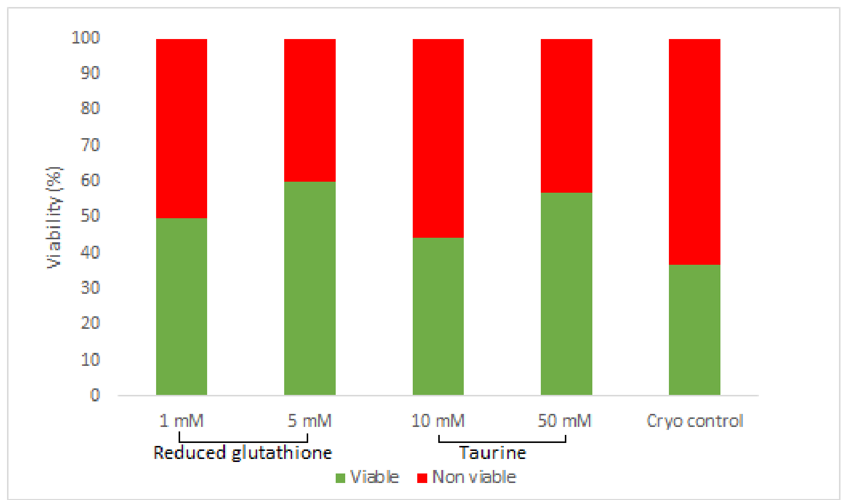

Figure 17 Sperm viability (%) when adding two different antioxidants, reduced glutathione and taurine, at two different concentrations. Cryo control using only sperm with the addition of extender (n=1).

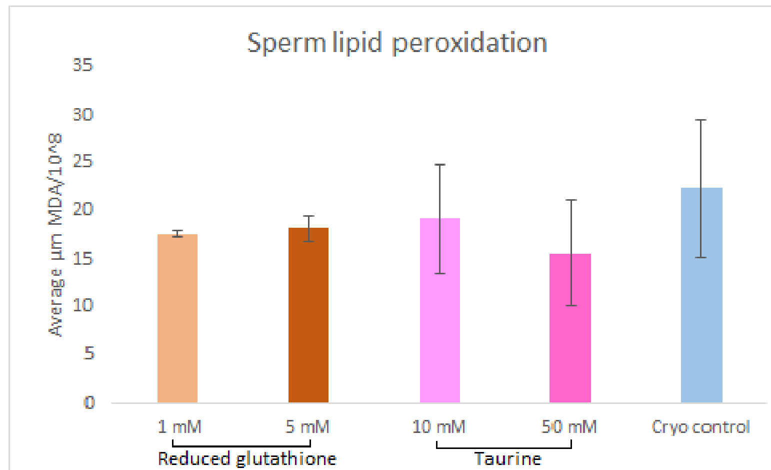

Figure 18 Sperm lipid peroxidation when adding two different antioxidants at two different concentrations. 1 mM and 5 mM reduced glutathione (brown bars), 10 mM and 50 mM taurine (pink/purple bars), and cryopreserved control (blue bar). Data are mean ± standard deviation (n=2).

3.1.3 The addition of BSA and egg yolk to the extender solution during cryopreservation.

The average initial values of motility (%) and VCL (µm/s) were 57.32 ± 3.83 % and 25.46 ± 3.08 µm/s respectively (Figure 19). The ANOVAs revealed that there were significant differences for the motility ($F_{5,24}$=10.840, P<0.001) and the VCL ($F_{5,24}$=2.988, P = 0.030) between the treatments for the membrane stabilizers. The samples cryopreserved with 5 % and 10 % egg yolk (45.42 ± 5.03 % and 41.89 ± 6.70 %) and the cryo control (50.11 ± 4.88 %) had lower motility (%) than the fresh control as detected by the post-hoc Tukey test. However, the 5% BSA (61.30 ± 5.46 %) had significantly higher motility (%) then the cryo control (50.11 ± 4.88 %) and the egg yolk treatments (less than 46 % motile cells). For the VCL (µm/s), the post-hoc Tukey test revealed that there were significant differences between the treatments. The fresh control (25.46 ± 3.08 µm/s) had significantly higher VCL than the cryo control (20.17 ± 2.05 µm/s) and 5 % egg yolk (20.23 ± 1.09 µm/s). No significant differences were found between the cryo control and the different treatments. Regarding the viability (Figure 20), there were similar values among the groups (80 – 90 %), except for 1 % BSA, which had 15 – 20 % lower viability compared to the rest.

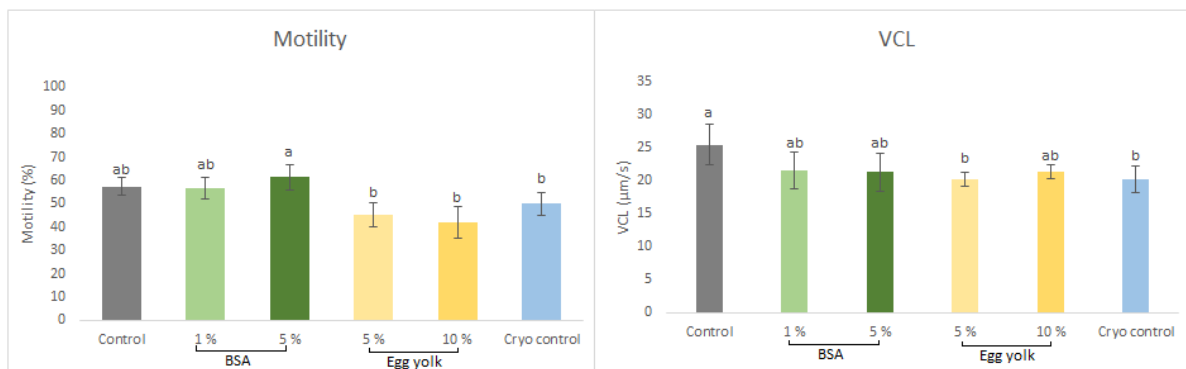

Figure 19 Sperm motility (%) - to the left and VCL - to the left, when adding two different membrane stabilizers at two different concentrations. Fresh sperm / control (grey bar), 1 and 5 % BSA (green bars), 5 and 10 % egg yolk (yellow bars) and cryopreserved control (blue bar). Data are mean ± standard deviation (n=5). Different letters show differences between treatments ($p < 0.05$).

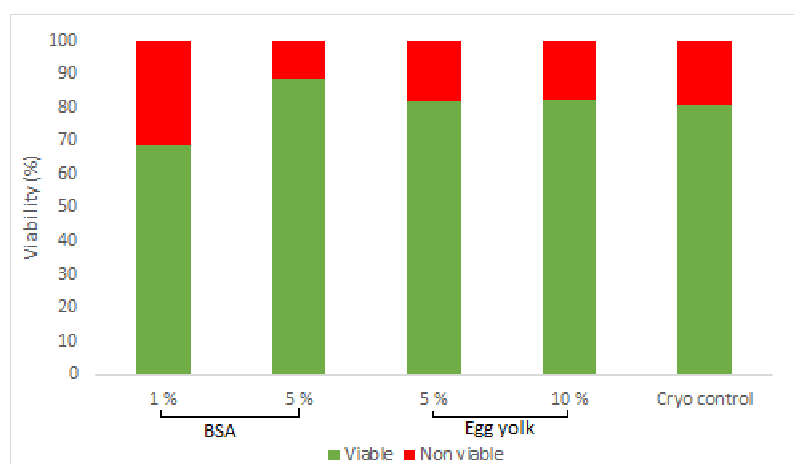

Figure 20 Sperm viability (%) when adding two different membrane stabilizers, BSA, and egg yolk at two different concentrations. Cryo control using only sperm with the addition of the extender (n=1).

3.5 Experiment 2: Large volume cryopreservation of spotted wolffish sperm with the use of 5.0 ml cryotubes.

3.5.1 Preliminary trials – Selection of freezing height and thawing rate for large volume cryopreservation

The trial conducted to decide which freezing heights and thawing rates to test with 5 ml cryotubes. The average control values of motility (%) and VCL (µm/s) were 30.47 % and 20.74 µm/s respectively (Figure 21). These values were relatively low as compared with the other fresh sperm control values since, due to the difficulties in obtaining high quality sperm, a threshold value of 50 % sperm motility was not applied in the preliminary trial. Six different cryotubes were prepared with sperm and extender and after that, cryopreserved using different freezing height and thawing rate. Four prepared cryotubes were cryopreserved using FR of 1.5 cm and thawed at 20 °C, 15 °C, 10 °C, and 5 °C for 3.5, 4, 6 and 10 min respectively. The sperm cryopreserved with these four cryotubes had 15.47 %, 22.3 %, 16.25 % and 12.8 % motility respectively, and 18.64 µm/s, 16.64 µm/s, 20 µm/s and 17.08 µm/s VCL respectively. Two prepared cryotubes were cryopreserved using a freezing height of 4.5 cm and 7.5 cm and both thawed at 15 °C for 4 min. The sperm from these two cryotubes had 8.92 % and 0 % motility, and 14.01 µm/s and 0 µm/s VCL respectively.

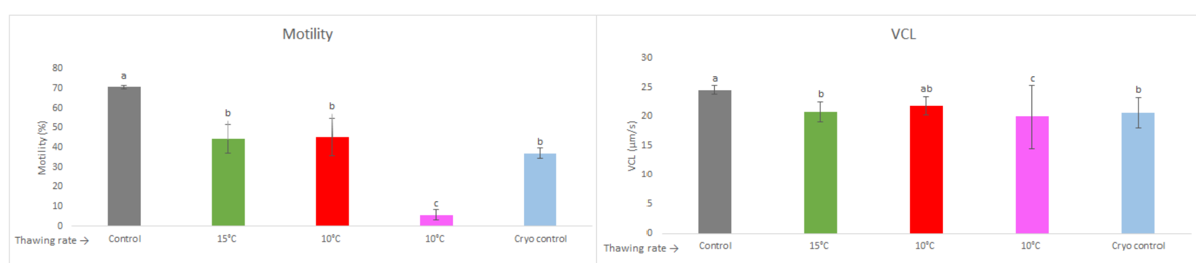

Figure 21 Sperm motility (%) - to the right and VCL (µm/s) - to the left, using different freezing heights and thawing rates. Fresh sperm / control (grey bar), sperm cryopreserved using freezing height of 1.5 cm and thawing rate of 3.5 min at 20 °C (blue bar), using freezing height of 1.5 cm and thawing rate of 4 min at 15 °C (green bar), using a freezing height of 7.5 cm with a thawing rate of 4 min at 15 °C (no bar = 0 motility and VCL), using freezing height 4.5 cm with a thawing rate of 4 min at 15 °C (yellow bar). Using a freezing height of 1.5 cm and a thawing rate of 6 min at 10 °C (red bar) and using a freezing height of 1.5 cm at a thawing rate of 10 min at 5 °C (orange bar). Data are mean ± standard deviation for three technical replicates (n=1).

3.5.2 *Experiment 2: Spotted wolffish sperm cryopreservation with the use of 5 ml cryotubes at different freezing height and thawing rates.*

The average initial values of motility (%) and VCL (µm/s) were 70.62 ± 5.44 % and 24.65 ± 0.84 µm/s respectively (Figure 22). Sperm cryopreserved using 0.5 ml straws were in this experiment used as cryo control. The sperm from the cryo control had an average value of 37.09 ± 8.13 % motility and 20.64 ± 0.89 µm/s VCL. The ANOVAs revealed that there were significant differences in the motility between the sperm samples cryopreserved using cryotubes. ($F_{4,15}$=39.400, P<0.001). For the VCL (µm/s), the Bartlett test indicated that there was no homogeneity of variances (p = 0.0003672), most likely because of the variability observed for the sperm cryopreserved in cryotubes using a freezing height of 4.5 cm. A Kruskal-Wallis test was decided to be used instead on the dataset for VCL. The Kruskal-Wallis test found significant difference between the sperm cryopreserved in cryotubes using a freezing height of 4.5 and the fresh control, cryo control and both sperm using a freezing height of 1.5 cm (p = 0.02092 - same p for all of them.). After the freeze-thawing, the samples frozen at 1.5 cm and thawed at 15 ºC and 10 ºC (44.35 ± 9.30 % - 20.81 ± 0.92 µm/s and 45.41 ± 9.23 % - 21.91 ± 0.59 µm/s respectively) showed no significant difference to that of the cryo control as detected by Kruskal-Wallis test. However, the sperm sample using a freezing height of 4.5 cm (4.39 ± 4.38 % and 13.58 ± 5.54 µm/s) had significant lower motility (%) and VCL (µm/s) compared to the rest. The viability (%), evaluated qualitatively (Figure 23), indicated the same trend as for the motility (%) and VCL (µm/s). Both the sperm samples cryopreserved using cryotubes at freezing height of 1.5 cm and thawed at 15 ºC and 10 ºC (80.81 % and 75.47 %), respectively, had viability like that of the cryo control (66.60 %). While the sperm samples cryopreserved using a freezing height of 4.5 cm had much lower viability than the rest (18.60 %).

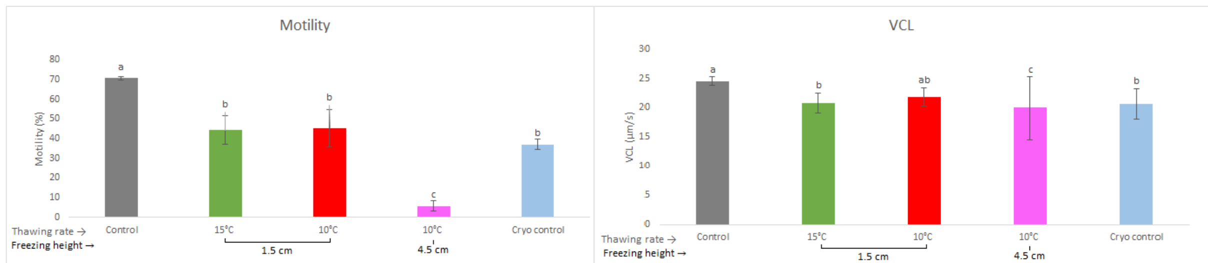

Figure 22 Sperm motility (%) - to the right, and VCL (μm/s) - to the left, using different freezing heights and thawing rates. Fresh sperm/control (grey bar), cryopreserved control using straws (blue bar), sperm cryopreserved using a freezing height of 1.5 cm and a thawing rate of 6 min at 10 °C (red bar) , sperm cryopreserved using a freezing height of 1.5 cm and a thawing rate of 4 min at 15 °C (green bar) and sperm cryopreserved using freezing height of 4.5 cm at a thawing rate of 6 min at 10 °C (purple bar). Data are mean ± standard deviation (n=4). Different letters show differences between treatments ($p < 0.05$).

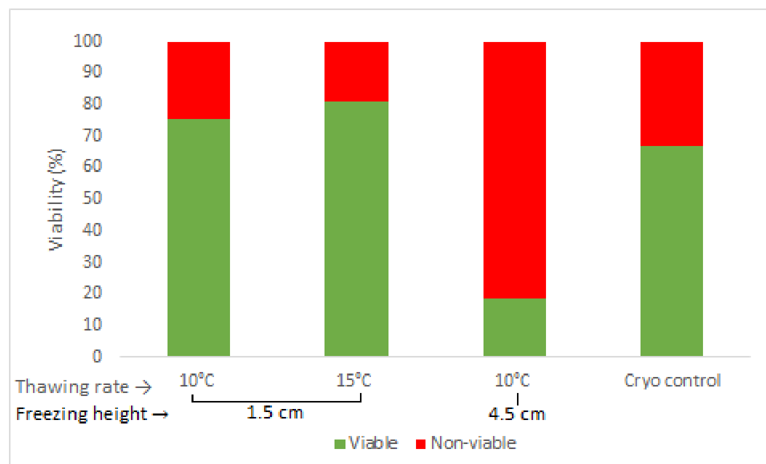

Figure 23 Sperm viability (%) when using different freezing heights and thawing rates (n=2).

4.0 Discussion

In this study, I improved the protocol for sperm cryopreservation of spotted wolffish by testing the addition of antioxidants and membrane stabilizers to the extender solutions; Also, I tested the use of cryotube, which be used for large-scale cryopreservation of the spotted wolfish sperm.

4.1 Preliminary trial - Selection of extender solution

In this study, the spotted wolffish sperm remained motile for at least 96 h (SR – 18 ± 8.37 % and KT – 20 ± 12.30 %) and had almost the same motility as that at 3 h (SR – 14.76 ± 9.88 % and KT – 30.85 ± 10.54 %). The results are unique for this species as there have been no reports of sperm remaining motile for more than 48 h, as stated by Kime and Tveiten (2002). The sperm was stored in a fridge at 4 °C and was only out of the fridge (kept in ice) for a few minutes for analysis. It was decided to end the experiment after 96 h, maybe prematurely as it would have been interesting to see how long the motility would last. Ocean pout, a species with internal fertilization, has sperm that can remain motile for 5 days at 4 °C without losing much activity when either in semen or in an extender. However, the motility diminishes rapidly after this period (Yao *et al.* 1999). Further studies, preferably with more pools, are needed to figure out how long spotted wolffish sperm remain motile.

4.2 Experiment 1 – The use of membrane stabilizers and antioxidants in cryopreservation of spotted wolffish sperm

In the first experiment, I tested the addition to the extender of the antioxidants (10 mM and 50 mM taurine, and 1 mM and 5 mM reduced glutathione) and membrane stabilizers (1 % and 5 % BSA, and 5 % and 10 % egg yolk). For the antioxidants, there was no significant difference between the treatments and cryo control when comparing motility (%) and VCL (µm/s). The results regarding the use of taurine are similar to that of Martínez-Páramo *et al.* (2013) and Riesco *et al.* (2017) who used 1 mM on European sea bass sperm and 50 mM on dusky grouper sperm respectively. None of these authors found significant improvements in motility (%) and VCL (µm/s) with the use of taurine. However, a study done by Kutluyer *et al.* (2016) on goldfish sperm found significant improvements in motility (%) when using 1, 2 and 4 mM taurine (~30 – 35 % motile cells) compared to the post-thaw control (~15 % motile cells). Furthermore, Liu *et al.* (2014) obtained significant improvements on motility using 25-, 50- and 100 mM taurine (~75 – 77 % motile cells) compared to the post-thaw control (66 % motile cells) in red seabream

sperm cryopreservation. Regarding VCL, there was no significant difference after using 50 mM taurine on red seabream.

Regarding the use of reduced glutathione, my results are similar to that of Paule *et al.* (2012), Lahnsteiner *et al.* (2011) and Sariözkan *et al.* (2009) who used 0.5, 1 and 1.5 mM in streaked prochilod sperm, 1 mM on brook (*Salvelinus fontinalis*) and rainbow trout sperm and 2 mM on cattle bull sperm cryopreservation respectively. None of these authors found significant differences after comparing the sperm cryopreserved with and without reduced glutathione. However, Gadea *et al*. (2011) reported significant improvements of motility after using 1 mM reduced glutathione (40.07 % motile sperm) on human sperm compared to the post-thaw control (38.53 %) but not to VCL.

Viability (%) was evaluated qualitatively, and our results seem to indicate that the higher concentrations of antioxidants used (5 mM reduced glutathione and 50 mM taurine) might have had a positive effect (59.80 % and 56.79 % viable cells respectively) compared with the cryo control (36.87 % viable cells). The lower concentrations of antioxidants used (49.56 % viable cells in 1 mM reduced glutathione and 44.30 % viable cells in 10 mM taurine) seemed to have a positive effect compared to the cryo control, but not as much as the higher concentrations. Other studies reported higher viability when using higher concentrations of antioxidants. Perumal *et al*. (2013) cryopreserved gayal (*Bos frontalis*) sperm using no addition of taurine (~70 %), 25 (~80 %), 50 (~85 %) and 100 (~70 %) mM taurine, having an optimum at around 50 mM taurine. the higher concentration (100 mM taurine) had similar results as that of the treatment using no addition of taurine. Ansari *et al.* (2010) found significant increased sperm viability after cryopreserving Nili-Ravi buffalo (*Bubalus bubalis*) sperm with the addition of reduced glutathione at no addition (~70 %), 0.5 (~75 %), 1.0 (~80 %), 2.0 (89 %) and 3.0 (~65%) mM reduced glutathione. For both Perumal *et al*. (2013) and Ansari *et al.* (2010), after using higher concentrations of antioxidants (100 mM taurine and 3 mM reduced glutathione) in sperm cryopreservation, there was a significant drop in sperm quality when compared to lower concentrations used. While other authors have had no change to sperm viability when comparing the addition of different concentration of antioxidants in sperm cryopreservation (Gadea et al. 2011; Gadea et al. 2013; Slanina *et al*. 2018).

The addition of antioxidants to the sperm in our study gave a slight improvement to the lipid peroxidation. Both concentrations of reduced glutathione (1 mM - 17.51 μM MDA/10^8 and 5 mM - 18.15 μM MDA/10^8), and taurine (10 mM - 19.14 μM MDA/10^8 and 50 mM - 22.36 μM

MDA/10^8) had lower concentrations than the cryo control (22.36 µM MDA/10^8). Unfortunately, it was not possible to conduct a test on the fertilizing capacity, but a decrease in the MDA could be indicative of an improvement that reflects in the fertilizing capacity. Sarıözkan *et al.* (2010) cryopreserved Angora buck (*Capra aegagrus hircus*) sperm using different extenders finding that the fertilization capacity (76.5 %, 52.9 %, 47.1 %, and 27.8 %) has a negative trend just like the MDA concentrations (1.64 ± 0.26 nmol/l, 2.64 ± 0.18 nmol/l, 2.82 ± 0.28 nmol/l and 4.39 ± 0.06 nmol/l respectively). Unfortunately, due to logistic limitations, we could not evaluate the MDA in freshly collected samples, only in cryopreserved samples that we transported to our lab, 3 h driving away from the fish farm. Thus, we should be cautious to comment on how the cryopreservation process affects the sperm quality in the spotted wolffish. However, in studies done in cattle bull, human, Atlantic salmon and Indian red jungle fowl (*Gallus gallus murgha*), MDA values increased at least 80 % after the freeze-thaw process (Cheema *et al*. 2009; Karimfar *et al*. 2015; Figueroa *et al.* 2018; Ansari *et al.* 2019).

Both the tested concentrations of taurine (10 mM and 50 mM) and reduced glutathione (1 mM and 5 mM) seem to result in a higher sperm cell viability and lower peroxidation then that of the cryo control. The higher sperm cell viability and lower peroxidation could impact the post-thaw sperm fertilization ability. However, the impossibility to analyse a higher number of samples and conduct fertilization trials does not let to make a definitive conclusion.

For the membrane stabilizers, only 5 % BSA (61.30 ± 5.46 %) gave significant improvements to the motility (%) compared to the cryo control (50.11 ± 4.88 %) and had even similar motility values to the fresh control using fresh sperm (57.32 ± 3-83 %). No significant difference was found when comparing VCL of sperm cryopreserved with 5 % BSA (22.59 ± 3.67 µm/s) and the cryo control (23.26 ± 2.69 µm/s) and fresh control (27.89 ± 3.95 µm/s). Evaluating the viability qualitatively, it seems like 5 % BSA (88.61 %) was approximately the same as the cryo control (80.78 %). Our results regarding 5 % BSA are similar to those found by Carbita *et al*. (2001a), Uysal & Bucak (2007) who both found increased sperm quality when adding BSA in different concentrations. Carbita *et al*. (2001a) cryopreserved rainbow trout sperm with the addition of 0.4 % BSA getting an increase of ~20 % sperm motility compared to the cryo control. However, the rainbow trout sperm viability was significantly lower compared to the cryo control (~17 % sperm viability). Uysal & Bucak (2007) cryopreserved ram semen with the addition of 0.5, 1 and 2 % BSA (45.0 %, 48.5 % and 51.2 % respectively) resulting in a significant increase in sperm motility compared to the cryo control (39.5 %). The viability for

the ram sperm also increased significantly (73.2 %, 69.3 %, and 78.2 % respectively) compared to the cryo control (50.7 %). Beirão *et al*. (2010) also got significantly higher motility and VCL when using 0.1 % BSA (66.20 % and 122.54 µm/s) compared to the post-thaw control (40.5 % and 82.87 µm/s) after cryopreserving gilthead seabream sperm.

There was no significant difference between 1 % BSA and the cryo control and fresh control when comparing motility (56.76 ± 4.44 %) and VCL (21.55 ± 2.72 µm/s). The viability for 1 % BSA (68.84 %), which was evaluated qualitatively, was slightly lower than the cryo control (80.78 %). The results from our study using 1 % BSA is similar to that of Sariözkan *et al.* (2009) and Riesco *et al.* (2017). They evaluated cryopreservation of sperm with the addition of BSA and finding no significant difference between the treatment using BSA and the post-thaw control. Sariözkan *et al.* (2009) found no significant different to sperm motility when comparing cryopreserved cattle bull sperm using 0.5 % BSA (44.07) with post-thaw control (49.81 %).Riesco *et al.* (2017) cryopreserved dusky grouper sperm using 1 % BSA finding no significant differences to either sperm motility (48.16 %) or viability (70.29 %) when compared to the post-thaw control (51.94 % motility and 67.04 % viability).

BSA is known to eliminate free radicals generated by oxidative stress and protect the membrane integrity of sperm cells from heat shock during freezing/thawing (Uysal *et al.* 2005). Spotted wolffish is known to have antifreeze proteins (AFP) which helps to stabilize the sperm by reducing the changes in the protein pattern induced when freezing (Beirão *et al*. 2012; Desjardins *et al.* 2007; Desjardins *et al.* 2012; Zilli *et al.* 2014). The properties from both BSA and AFP might have had a synergic effect which contributed to improving sperm quality after thawing.

In this study, no significant improvements were found to the sperm motility when cryopreserving using 5 % and 10 % egg yolk in addition to the extender (45.42 ± 5.03 % and 41.89 ± 6.70 % respectively) nor sperm VCL (20.23 ± 1.09 µm/s and 21.31 ± 1.09 µm/s) when compared to the cryo control (50.11 ± 4.88 % and 20.17 ± 2.05 µm/s). Other studies have shown that the egg yolk gave significant improvements when used. Babiak *et al*. (2012) found significant improvements in the motility after cryopreserving Atlantic cod sperm with Hanks' solution (HBSS) + 10 % egg yolk (~65 % motile cells) compared to the post-thaw control using only HBSS (~8 % motile cells). Butts *et al.* (2010) used 10 % egg yolk together with glycol and both Mounib's diluent (Mounib) or Hanks' Solution (HBSS) and found significantly increased sperm motility (~50 % and ~55 %) compared with Mounib and HBSS without egg yolk (~20

% and ~15 % respectively). Cabrita *et al.* (2001a) cryopreserved sperm from rainbow trout and found it to have a significant increase in motility after using 10 % egg yolk (42.2 %) compared to the post-thaw control (17.7 %). However, Beirão *et al*. (2010) got a significant decrease in motility and VCL when using 10 % egg yolk (1.08 % and 39.45 μm/s) compared to the post-thaw control (40.5 % and 82.87 μm/s) after cryopreserving gilthead seabream sperm.

Egg yolk seemed to be particularly challenging to work with in our study. The CASA system struggled with differentiation between spermatozoa and small particles that came from the egg. This might have affected the evaluation negatively. In a study by Pérez-Cerezales *et al.* (2010), it was used low-density lipoprotein (LDL) fraction, instead of pure egg yolk when cryopreserving *Oncorhynchus mykiss* sperm. The use of 12 % LDL (37.6 % motility and ~60 % eyed embryos) had a positive effect post-thaw with increased sperm quality compared to the use of 10 % egg yolk (13.7 % motility and 27 % eyed embryos). It might be speculated if the results would be different if a LDL fraction was used or even pre-prepared egg yolk (from a commercial laboratory chemical producer).

4.3 Experiment 2 – Spotted wolffish sperm cryopreservation with the use of 5 ml cryotubes

4.3.1 Preliminary – Selection of freezing height and thawing rate for large volume cryopreservation

At a commercial scale, many straws are needed to thaw due to the fertilization of large volumes of egg simultaneously. According to Santana (2018) spotted wolffish needs cryopreserved sperm concentration of 5 x 10^5 spz / egg to obtain the same fertilization rates as when using the fresh sperm. The spotted wolffish lay 5000 - 15000 eggs and has an average sperm cell concentration between 10^6 and 10^8/ml. Fertilizing 5000 eggs would, therefore, need 25 ml sperm (10^8) or 100 x 0.5 ml straws. Cryopreserving using larger volumes could therefore potentially decrease the time and workload required for the process of both freezing and thawing.

The preliminary trial was conducted for experiment 2 in order to decide which freezing height (1.5 cm, 4.5 cm, and 7.5 cm) and thawing rate (5 ºC for 10 min, 10 ºC for 6 min, 15 ºC for 4 min and 20 ºC for 3.5 min) would be used further in the experiment.

The freezing heights that was used in the preliminary trial are based on studies done by Santana (2018) and Gunnarsson *et al*. (2002) who found no significant differences when cryopreserving

spotted wolffish sperm (1.5, 2.5, 4.5, 4,7, 5.5, 7.1 and 7.5 cm). It was in our study seen that freezing height played an important role for sperm motility when cryopreserving. The best motility (%) was achieved using FR of 1.5 cm using a thawing rate of 20 ºC (15.44 %), 15 ºC (22.30 %) and 10 ºC (16.25 %) compared to the samples which used FR of 4.5 cm (8.92 %) and 7.5 cm (0 %).

4.3.2 Spotted wolffish sperm cryopreservation with the use of 5 ml cryotubes at different freezing height and thawing rates

In the second experiment, we tested the use of 5 ml cryovials for cryopreserving spotted wolffish sperm. The two best combinations of freezing height / thawing rate (1.5 cm from the LN_2 / 10 ºC and 1.5 cm from the LN_2 / 15 ºC) resulted in similar values of motility (45.41 ± 9.45 % and 44.35 ± 7.20 %), VCL (21.91 ± 1.60 µm/s and 20.81 ± 1.76 µm/s) and viability (75.47 % and 80.81 %) to the ones obtained with the cryo control using 0.5 ml straws (37.09 ± 2.66 % motility, 20.65 ± 2.66 µm/s VCL and 66.60 % viability). The third combination of freeing height / thawing rate (4.5 cm / 10 ºC) had significantly lower motility (4.39 ± 2.12 %) and VCL (13.58 ± 7.45 µm/s) and lower viability (18.60 %) compared to the cryo control. These results are comparable with other authors results, also using large scale cryopreservation. There was no significant difference in motility after Cabrita *et al*. (2001b) cryopreserved rainbow trout sperm using 0.5 ml straws (57.7 %), 1.8 ml straws (43.3 %) and 5 ml cryotubes (~43 %). However, the evaluation was subjectively, and the viability was significantly lower for the 0.5 ml straw (35 %) compared to the 1.8 ml straw (39 %) and 5.0 ml cryotube (61.9 %). Gunnarsson *et al*. (2002), evaluated cryopreservation of spotted wolffish sperm using 0.5 ml and 1.0 ml straws subjectively resulting in no significant difference in motility between the two (33 % and 60 % respectively). Four different volumes (0.5 ml, 1.8ml, 2.5 ml, and 5 ml) was used for cryopreserving yamú (*Brycon amazonicus*) sperm, Velasco-Santamaría *et al.* (2006), found no significant difference in the sperm motility (35 % 39 % 33 % and 39 % respectively) using these volumes. Nomura *et al*. (2018) did not find any significant difference in Japanese eel sperm motility after cryopreserving using 0.25 ml straws (49.9 %), 2.5 ml straws (33.3 %) and 5 ml cryotubes (54.8 %). Regarding the freezing rates, obtained with different freezing heights from the LN_2, Santana (2018) found no differences in sperm motility after cryopreserving 0.5 ml straws using freezing heights of 1.5, 2.5, 4.5 or 7.5 cm from the LN_2 (~30 %, ~35 %, ~35 % and ~40 % motile cells respectively). Furthermore, Gunnarsson *et al.* (2002) found no significant differences after cryopreserving using different freezing heights of 4.7 cm (29 %), 5.5 cm (20 %) and 7.1 cm (31 %) over LN_2. However, in our study, using cryovials, it was

observed that the freezing height had a critical impact on motility (%), VCL (%), and viability (%) then thawing rate. It can be speculated that due to the higher volume used, 5 ml cryotubes, in this study compared to that of Gunnarsson *et al.* (2002) and Santana (2018) who used lower sperm volumes (0.5 ml and 1.0 ml) might have affected the freezing rate (°C/min) and consequently the motility. Most authors report a decrease in sperm quality after cryopreservation using large volumes, but this decrease is compensated by the possibility of storing sperm in large volumes. Twenty-five ml of cryopreserved sperm is needed to fertilize 5000 spotted wolffish eggs, according to Santana (2018). The use of large cryopreservation volumes is therefore highly advantageous for an industry that commercially has been using 0.5 ml straws. It makes the process of fertilizing a large number of eggs more cost-efficient and less time-consuming. It is important to mention that each species respond differently to the protocols, however, these results, using BSA in combination with freezing height of 1.5 cm and thawing rate of 10 °C (or 15 °C) with 5 ml cryotubes have had a good effect on both motility, VCL and viability post-thaw.

5.0 Conclusion

This study suggests that for cryopreservation of spotted wolffish sperm, the extender should at least incorporate 5 % BSA, regardless of whether the cryopreservation is done with the use of 0.5 ml straws or 5 ml cryotubes. Regarding the use of antioxidants, both the higher tested concentrations of reduced glutathione and taurine seemed promising, but further studies are needed, especially the use of these antioxidants together with BSA. When cryopreserving using 5 ml cryovials, the cryovials should be placed at 1.5 cm from the LN_2 for 10 min and thawed at 10 ^oC. For each liter of spotted wolffish eggs (around 5 000 eggs) 5 of these cryovials should be used to secure the highest fertilization rates.

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