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Large volume cryopreservation of lumpfish (*Cyclopterus lumpus L.*) sperm for commercial hatchery production

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Dedication

This research is dedicated to God and to the honor of my late Mum Mosunmola Comfort Opeifa and late Dad Jacob Oyewale Opeifa.

Acknowledgment

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Abstract

The growing Atlantic salmon (*Salmo salar*) aquaculture production is confronted with many challenges that limit the potential development of the industry. The major current problem threatening the salmon aquaculture production is the parasitic sea lice (*Lepeophtheirus salmonis*) that negatively affects both cultured salmon and wild stock populations. Several treatment options have failed to deliver universally accepted results without negative impacts on the fish or the environment. Therefore, the need for alternative solution made way for cleaner fish - lumpfish (*Cycloptherus lumpus*), a cold water species that can tolerate low temperatures without affecting its grazing capacity making it suitable for the biological control of sea lice infections in norther locations.

Despite the interest, some bottlenecks need to be solved to allow a reliable and commercially sustainable year-round production of lumpfish juveniles, including broodstock management and related gametes' availability issues. This study tries to establish a protocol for large volume cryo-preservation of lumpfish sperm for use under commercial production. The procedure involves cryopreservation of lumpfish sperm from crushed testes in a cryo-solution containing 10% DMSO diluted 50:50 in wolfish extender pre-frozen at two heights (2.5 cm and 4.8 cm) before storage in liquid nitrogen. Evaluation of sperm quality and viability was assessed based on single and combination of multiple criteria, including a) sperm motility parameters, b) functional integrity of sperms, and c) egg fertilization rates. Sperm concentration was measured through spermatocrit and the value varied widely between males, ranging 44-85%. The motility parameters (MOT, LIN, PROG and VCL) measured showed significantly reduced values between the control and treatment groups across all the parameters. The sperm quality measurement also followed a reduced pattern when comparison was made between percentages of motile sperm from CASA analysis with the percentage of live sperm cells evaluated through flow cytometry prior and after cryopreservation. Validation of methods via egg fertilization showed no significant differences between the control and the treated groups, most likely as a result of high sperm / egg ratio used in the experiment. A preliminary conclusion could only be reached because of lack of repetition of the experiments. A common trend about measurements indicated reduction in motility and quality of the sperm in the treated groups particularly in the 4.8 cm height, this then suggests that further work in this direction should be done focusing on heights around 2.5cm rather than 4.8cm.

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1. Introduction

1.1 Research background

The lumpfish (*Cyclopterus lumpus* L.) is an effective cleaner fish for controlling sea lice (*Lepeophtheirus salmonis*) infestations in farmed Atlantic salmon (*Salmo salar*). The rising demand by salmon producers, particularly for use in northern locations, has necessitated a steady increase of its intensive production. However, some bottlenecks need to be solved to allow a reliable and commercially sustainable year-round production of lumpfish juveniles. Among these are a better control of sexual maturation of wild-caught broodstock and other operations aiming to simplify broodstock management, including gametes' availability synchronization and short- or long-term storage of sperm samples for artificial fertilizations. Different sperm extenders (non-activating solutions) have been designed for short (1-2 weeks) and long-term sperm storage and are available on the market for use in different marine species but information on lumpfish is rather limited. However, a protocol has been established for cryopreservation of lumpfish sperm using small volume (250 µl) straws (Norðberg et al. 2015). Nevertheless, collection of sperm by abdominal stripping in this species is rather difficult and results in little volumes of milt representing a limiting factor when large egg batches need to be fertilized in commercial hatcheries. To meet the constant supply of large volume sperm requirement for commercial lumpfish production and to overcome the challenge of fresh sperm storage, there is need to establish alternative means of sperm conservation protocols through cryopreservation of lumpfish sperm in larger volumes while building on the existing protocols.

1.2 Atlantic salmon farming in Norway

Atlantic salmon (*Salmo salar*) aquaculture industry began in Norway in the 1970s (Heuch et al. 2005) and has grown to be commercially important in the 1980s followed by tremendous production (FAO, 2018). This growth has positioned Norway as the main Atlantic salmon producer in the world (1.2 mt) followed by Chile (0.55 mt) and United Kingdom (0.15 mt) (Directorate of Fisheries, 2019; Marine Harvest, 2018). The growth of Atlantic salmon aquaculture which promises better economic prospects has induced more interest and capital investment in the industry (Asche et al. 2011). This development has led to environmental and aquaculture-related problems of disease and pathogen spread (Jones et al. 2019), genetic impact on wild fish populations through farmed fish escapees (Jackson et al. 2015), pollution and other environmental issues (Bloodworth et al. 2019).

Large scale intensive Atlantic salmon production creates environment for both transmission and growth of parasites particularly salmon lice (*Lepeophtheirus salmonis)* which poses threats to both wild stock and farmed populations and the sustainability of the aquaculture industry as a whole (Torrisen et al. 2013, Abolofia et al. 2017). This parasite along with escaped farmed salmon are considered the main 'anthropogenic impact factors' identified as expanding threats to wild salmon populations in Norway in addition to other salmonids (Forseth et al. 2017).The effort to control lice infestation rose between 2013 and 2014 to around 9-23% of total cost of fish production per kg of fish in Norwegian salmon farms (Iversen et al. 2015, Jansen et al. 2016, Abolofia et al. 2017).

1.3 Sea lice

The major parasite that affects Atlantic salmon in most of the producing countries is salmon lice *L. salmonis* (Torrisen et al. 2013, Abolofia et al. 2017). This parasitic copepod naturally occurring in seawater is a major health threat for farmed finfish through epidemic infestations and has been blamed for sea trout *(Salmo trutta L.)* collapse in many countries while it remains a major threat to the young Atlantic salmon migrating to the sea through coastal and fjord waters (Forseth et al. 2017, Serra-Llinares et al. 2018). The female carries long (ca. 2 cm) paired egg strings estimated to carry about 700 eggs (Hayward et al. 2011). Salmon lice life cycle comprises the planktonic larvae (nauplii), the infective planktonic copepodite which is parasitic but not mobile, immature *chalimus* at which stage it attaches to the host's skin, the mobile pre-adult and free moving adult are also parasitic and can move around the surface of the fish skin (Hayward et al. 2011). Salmon lice infect its host externally by feeding on dermal tissues, body fluids and blood (Moen & Svensen, 2004, Bui et al. 2017).

The degree of infection of wild salmon population by salmon lice has been investigated to be low when compared to the wild stock within close proximity to intensive Atlantic salmon sea cages (Costello, 2009, Jones et al. 2019). This suggest that high density culture cages harbor dense populations of salmon lice (Torrissen et al. 2013).

The impact of salmon lice infestation leads to lesion and wounds which enhances fish stress and susceptibility to secondary infections with significant high economic consequences for the salmon industry in terms of production losses and high direct costs related to treatment or preventive actions (Abolofia et al. 2017). The cost of treatments against salmon lice was

estimated to be about 200 million NOK in 2012 (Abolofia et al. 2017) to which it should be added the damage to public opinion towards aquaculture industry (Van Geest et al. 2014).

There have been several actions taken towards controlling and reducing salmon lice impact on aquaculture production. The national action initiated in Norway in 1997 was through the joint collaboration between Animal Health Authority (AHA), fish farmers and fish health personnel described as The National Action Plan against salmon lice (Heuch et al. 2005). The objective was to reduce the impact of salmon lice on farmed fish through measures like established legal maximum number of lice per fish, monitoring of infections caused by salmon lice on wild stock populations and mandatory reporting to AHA (Heuch et al. 2005).

Efforts to fight against salmon lice threat on Atlantic salmon production include the use of non-chemical freshwater treatments which have proved effective to some degree when dealing with low fish biomass but ineffective at high fish densities in sea cages (Powell et al. 2015). Among chemical agents, immersion bath treatments involves the use of organophosphorus pesticides. For example, azamethiphos commonly used in Norway has been proved effective but its application through immersion is stressful to salmon (Kaur et al. 2015) and may affect non-target species in the marine environment (Urbina et al. 2018). Oxidative disinfectants such as hydrogen peroxide (H_2O_2) have also proved positive in the control of salmon lice, but depending on the organic load and dissolved organic carbon, water chemistry and interaction with the treatment chemicals, and have proved ineffective when large fish biomass treatment is required (Powell et al. 2015). Hydrogen peroxide has also been proved ineffective against the *chalimus* lice stage, coupled with high mortalities post treatment, and uncertainties regarding its dosage has necessitated the search for alternative methods (Overton et al. 2018). Chemotherapeutic in-feed treatment has been considered the most effective alternative solution for sea lice control (Davies & Rodger 2000, Bravo et al. 2015,). Incorporation of ivermectin into Atlantic salmon feeds proved effective but the high administered dose and poor fish absorption negatively impact the environment through accumulation of this chemical in the sediments. Urbina and co-workers (2019) reported that ivermectin contained in fish excretions and uneaten food results in toxic to benthic fauna species (Urbina et al. 2019).

Although, chemicals have proved effective in reducing the level of salmon lice infestation, they have negative impact on the surrounding environment (Van Geest et al. 2014, Bloodworth et al. 2019), they are expensive (Costello, 2009) and have been associated with development of resistant breed of salmon lice (Powell et al. 2015). These limitations associated with chemotherapeutic (bath and feed) treatments have created the need for seeking a more

sustainable treatment option which would have less or minimal negative impact on cultured species, the wild stock and the environment. The quest for a better treatment alternative has led to the adoption of less damaging biological methods to control sea lice infestations. The alternative treatment has been the use of cleaner fish as more sustainable option for controlling salmon lice in a co-culture relationship (McEwan et al. 2019).

1.4 Cleaner fish

An effective, long term, biological agents for controlling sea lice are cleaner fish that naturally graze on wild Atlantic salmon parasites in the ocean (Bolton-Warberg, 2018). Currently, the most widely used cleaner fish in salmonids' aquaculture in North Atlantic countries are lumpfish (*Cyclopterus lumpus*) and a number of species belonging to the wrasses group (Brooker et al. 2018).

1.4.1 Wrasses

Wrasses belongs to the Labridae family of marine fish that are extensively distributed in the South Atlantic Ocean, the western Baltic Sea up to mid-Norway, the North-Sea, the British Isle, Indian and Pacific coastal zones with more than 500 species (Moen & Svensen, 2004, Skiftesvik et al. 2014, Wainwright et al. 2018), Wrasses abundance in temperate coastal waters plays an essential role in the ecosystem food web as they prey on different kinds of invertebrates while some species have evolved with cleaning symbiosis relationship with other fish species (Skiftesvik et al. 2014).

The first experimental use of wrasses as a cleaner fish in sea lice control in Norway dates back to 1987 when two species, the rockcook (*Centrolabrus exoletus* L.) and the goldsinny (*Centrolabrus repestris* L.) proved successful in removing sea lice from infected farmed Atlantic salmon (Bjordal, 1988). Experimental use of wrasses as controlling agents for sea lice in commercial aquaculture farms extended to other producing countries including Ireland and Scotland (Bjordal, 1992; Deady et al. 1995). However, of all the wrasses group, the most commonly used specie in Norway for sea lice control is the ballan wrasse (*Labrus bergylta*) because of it fast growing potential, grazing capacity and activeness in winter (Steigen et al. 2015; Grant et al. 2016).

The species of wrasses have different features, depth preferences, different grazing capacities but a combination of different wrasses species was effective in sea lice control according to

Bjordal, (1988), and Moen & Svensen, (2004). In Norway, wrasses used in sea cages (e.g. ballan wrasse) are caught from the wild in certain periods of the year and transported to sea cages but are not sufficient to meet the growing industry demand. Concerns on overfishing the wild stocks have led to the establishment of tight regulations on wild catches by the Norwegian authorities (Directorate of Fisheries Norway, 2019). Efforts geared towards the development of intensive culture of wrasses (Skiftesvik et al. 2013) have not met expectations due to lack of development of appropriate hatchery protocols for a stable juvenile production and high production costs (Øie, et al. 2017). In addition, the geographical southern distribution of wrasses, their reduced feeding rate at lower temperatures (Deady et al. 1995, Imsland et al. 2019), smolt aggressive behavior towards wrasses when newly introduce in a co-culture system in sea cages (McEwan et al. 2019), sensitivity to temperature under 6 °C (Sayer & Reader, 1996; Grant et al. 2016) have set limits to their use by the industry. These characteristics behavior made wrasses unfit for use in the northern parts of Norway which is predominantly low in temperature. Moreover, an increase in salmon aquaculture development in northern Norway might face great challenges if there are no alternative biological control to wrasses.

These limitations in the use of wrasses species, including the ballan wrasse (*L. bergylta*) as a control agent for sea lice in the northern Norway made room for lumpfish (*Cyclopterus lumpus* L.) as cleaner fish based on its ability to tolerate lower range of temperatures and maintaining grazing activities during the winter for better use under commercial settings (Powell et al. 2018).

1.4.2 Lumpfish (C. lumpus)

Lumpfish (Fig. 1A) belongs to the order Scorpaeniformes and family Cyclopterinae with anteriorly and posteriorly compressed body having a polygonal transverse section in the middle part of its body (Davenport, 1985, Budney & Hall, 2010). Lumpfish has a wide natural distribution on the boreal regions of North Atlantic but also cover a wide range of distribution on the European coast of West Atlantic. *C. lumpus* is extensively distributed in the southwestern Greenland up to Hudson Bay, New Brunswick, Newfoundland, in Nova Scotia waters and along the Norwegian coastline (Fig. 1B) (Davenport, 1985, Rackovan & Howell, 2017, Jónsdóttir et al. 2018).
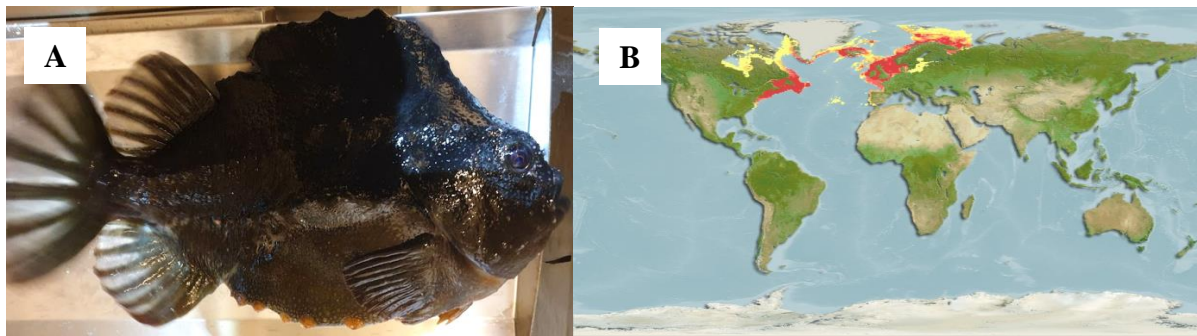
Figure 1. (A) Example of lumpfish (*Cyclopterus. lumpus*) broodstock used in the experiment, and (B) map of the geographic distribution of the species (Source: Aquamaps, www.fishbase.org).

Several unique features of lumpfish make it distinct from other cleaner fish species. The body is lined with seven longitudinal rows of compressed small rounded projections three on each side while the middle one that divide the turbercles into three on each side run across the dorsal fin backward. The head is thick and short, with blunted snout which featured a terminal mouth arranged with rows of small, conical simple teeth. The eye is moderate and lateral while the ventral part between the pectoral fins feature a large suction disc in the belly which it uses to attach to objects for support (Moen & Svensen, 2004, Saraiva et al. 2019).

Male lumpfish can be 25–30 cm in body length with a maximum of ca. 50 cm, while the females generally measure 30-40 cm reaching occasionally 60 cm in length. C. *lumpus* has been found to exhibit different color ranges between bluish-grey and blackish-grey with males featuring purple, red or orange colors during spawning periods and they can weigh up to about 5.5kg (Danielsen, 2016,) or 9.6 kg according to Johannesson (2006). Sexually mature lumpfish migrate for spawning from around February upwards to late spring and early summer into the coastal shallow waters where the females undergo several spawning. Overall, the lumpfish breeding season is considered to range from March to August although, although, mature fish can be found around the year. After spawning, females return to deep waters while males remain in shallow waters to guard the eggs (Rackovan & Howell, 2017). However, lumpfish has been observed to live both as pelagic and demersal species at depth ranging between 5m to 1700m (Zhukova et al. 2018,). The newly hatched fry remain in shallow waters while they attach to sea weeds with the help of their sucker until dispersed by currents into the open sea where juveniles inhabit the semi-pelagic waters like the adult (Rackovan & Howell, 2017).

In the last decade, wild catches of lumpfish have intensified to meet industry demand which may not be sustainable (Powell et al., 2018). Sexually mature male and female broodstock are collected from the wild for producing juveniles which are supplied to salmon farms where they

are introduced in co-culture with farmed fish (Imsland et al., 2018). This has significantly increased the pressure on the wild stocks leading to fluctuations and declines which called for precautionary management approaches like in the case of other cleaner fish species (Eriksen et al., 2014). The rising demand for lumpfish juveniles has necessitated a steady increase of its intensive production from few thousands fish in 2010 to approximately 30 million juveniles in 2016 (Powell et al., 2018). The same authors highlight that lumpfish production needs to increase further to reach ca. 50 million fish annually in order to meet future industry demands. Figure 2 shows the number of farmed lumpfish, ballan wrasse and other cleaner fish sold to Norwegian salmon and rainbow trout (*Oncorhynchus mykiss*) producers in the period 2012-2017 (Directorate of Fisheries, 2019).
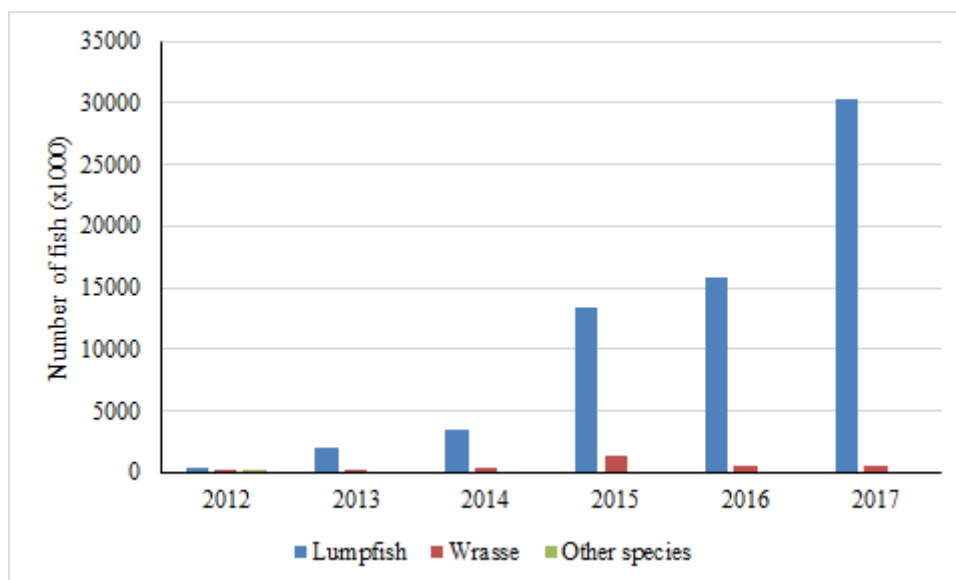

Figure 2. Sale of farmed cleaner fish to salmon and trout producers in 2012-2017 (Directorate of Fisheries, 2019).

Despite its advantages as cultured species for ensuring a stable juvenile production and as effective cleaner fish for use in northern locations, lumpfish aquaculture presents some bottlenecks and challenges that need to be solved (Powell et al., 2018). Among these are a better control of sexual maturation for year-round production (Powell et al., 2018) and other operations aiming to simplify broodstock management. For example, the morphological features exhibited by lumpfish make collection of sperm by abdominal stripping rather difficult and yield little volumes of milt which represents a limiting factor when large egg batches need to be fertilized under commercial settings (Norðberg et al. 2015; Akvaplan-niva, pers. comm.).

Cryopreservation of milt in this species is therefore seen as a possible solution to solve the above issues by ensuring a sufficient and constant supply of male gametes for a stable production of fry (Norðberg et al., 2015) and experimental work related to this species.

1.5. Fish sperm cryopreservation

Long-term storage of sperm via cryopreservation helps simplifying broodstock management allowing gamete availability synchronization, banking of milt from individuals with desirable genetic traits, and easy transportation of samples from different locations (Cabrita, et al., 2010) for use in commercial operations and breeding programs (Muchlisin, 2005). Cryopreservation protocols has been investigated in over 200 cultured fish, mostly freshwater or anadromous fish species like African catfish, (*Clarias gariepinus*), European catfish (*Silurus glanis*), salmonids such as brown trout (*Salmo trutta*), Atlantic salmon (*S. salar*) and rainbow trout (*O. mykiss*), sturgeons like beluga (*Huso huso*) and starlet, *Acipenser ruthenus* (Cabrita et al. 2010), and various cyprinids (Asturiano et al. 2017). However, there is a gap in knowledge between sperm cryopreservation of freshwater species and marine species with the latter being far less investigated as shown by the number of articles published in the period 2012-2017 alone (Fig.3). This gap in the amount of research in protocols for marine species sperm cryopreservation has been attributed to the fact that most of these spawns naturally in tanks which makes artificial fertilization not always required, thus reducing the need for such gamete management techniques (Martínez-Páramo et al. 2017). Among the cold-water marine species, sperm cryopreservation protocols have been developed for Atlantic cod (*Gadus morhua*), turbot (*Scophthalmus maximus*), spotted wolffish (*Anarhichas minor*), haddock (*Melanogrammus aeglefinus*) and Atlantic halibut *(Hippoglossus hippoglossus)* as reviewed by Suquet et al. (2000) and Martínez-Páramo et al., (2017).
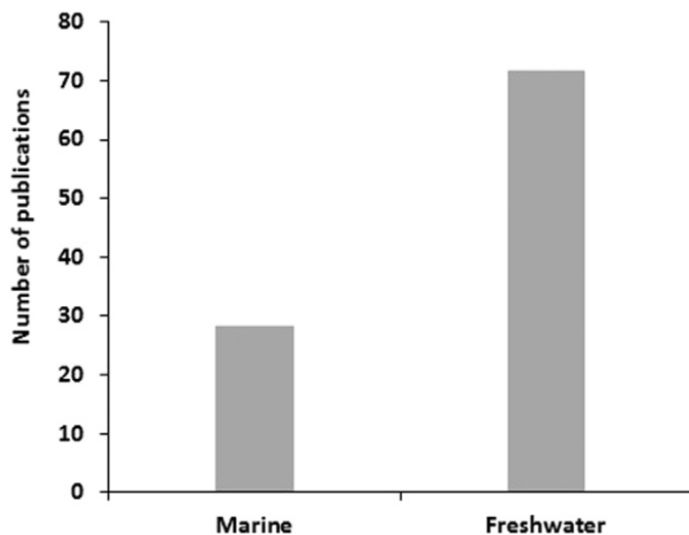
Figure 3: Number of published articles on sperm cryopreservation in marine species *vs.* freshwater species in the period 2012-2017 (Figure from: Martínez-Páramo et al, 2017).

However, while several successful efforts in sperm cryopreservation have been achieved on other marine fish species, lack of standardization of specific protocols has limited their implementation in commercial production (Asturiano et al. 2017). Overall, there is limited research on new cultured marine species (Martínez-Páramo et al. 2017) including the lumpfish. To date, the only published sperm cryopreservation protocol for this species being the one reported by Norðberg et al. (2015). In this work, the authors established a successful protocol to cryopreserve lumpfish milt obtained by abdominal stripping diluted with a general milt extender (modified Mounib's medium) and using small volume (250 μl) cryo-straws, which may not be fully applicable under commercial conditions.

1.6 Sperm quality assessment

Evaluation of sperm quality and viability can be assessed based on single or a combination of multiple criteria, including a) sperm motility, b) morphological and functional integrity of sperm's membranes and organelles, and c) ability of cryopreserved sperm to fertilize eggs and or hatching rate (Tiersch et al. 2007; Hassan et al. 2015; Asturiano et al. 2017).

Computer assisted sperm analysis (CASA) has been employed as a rapid and relatively simple quantitative tool for analysis of various environmental or physiological parameters that could affect fish sperm quality for differences in applications, including the effect of cryopreservation

procedures in cultured species (Asturiano et al. 2017). It serves as a quick and effective way of measuring the post-thaw viability of sperm relative to the one of fresh samples to enhance the efficiency of cryopreservation protocols (Kollár et al. 2018, Gheller, 2019).

However, further sperm quality assessment which involves cellular properties has been performed using flow cytometry (Martínez-Páramo et al. 2017). Flow-cytometry is one of the alternative techniques used in evaluating the quality and viability of spermatozoa. In particular, the method allows estimating the proportion of LIVE and DEAD spermatozoa via employment of two DNA fluorescent stains, namely SYBR14 – staining live cells - and Propidium iodide (PI) for dead cells. This improves the rate and speed of quantifiable measurement of the effects of cryopreservation on sperm viability via assessment of the level of cell membranes' integrity (Gallo et al. 2018).

Final evaluation of the viability of cryopreserved sperm samples is normally performed by checking if the sperm functionality, capacity to fertilize eggs and yield viable embryos or larvae is not compromised by the procedure when compared to fresh samples (Tiersch et al. 2007; Hassan et al. 2015, Asturiano et al. 2017).

1.7 Aim of the study

The goal of this research seek to establish protocols for large volume cryopreservation of lumpfish as a means to contribute to commercial application of cryopreservation protocols in this species. The aim in the current thesis will build upon the successful protocol established by Norðberg and co-workers (2015) for small volume cryopreservation of lumpfish sperm extending the analyses to the use of larger volume straws employing a sperm extender developed for cold-water marine species (wolfish extender) for use under commercial conditions. The results will be analyzed in terms of sperm motility, viability and fertilizing potential using CASA, flow cytometry and egg fertilization rates.

2.0 Materials and methods

2.1 Locations and experimental period

There were two locations used for the pilot and the main cryopreservation experiments. The initial pilot trials were carried out at Akvaplan-niva Research and Innovation Station (FISK; Kraknes, Tromsø), between July and October 2018. Subsequent trials and the main experiments were conducted at the Department of Arctic and Marine Biology, UiT Arctic University of Norway, between December 2018 and April 2019.

2.2 Origin of fish and handling

The lumpfish broodstock used for the experiment were collected from the wild through gill net at Hekkingen (Malangen, Norway) and were stored in flow through circular tanks at 6°C for a few days at FISK to acclimatize and enhance egg maturation through stimulations by male fish before the experiment (Beirão et al. 2019). Males with reddish coloration were selected and paired with the female having bulging abdomen. Several observations were carried out on the male fish relative to its behavior towards the female fish in the same tank. The males that were observed sitting in the artificial spawning troughs place in the circular tanks were selected for dissection. Females with reddish and swollen urogenital openings were considered ready to spawn and selected for the experiment.

2.3 Sperm collection

Mature males were removed from the tanks and their body wet weight were measure before the testicles were dissected. All the fish used for the cryopreservation experiments were first stripped but the volume of sperm collected through stripping was too small for use in the experiment, therefore, the fish were killed by a blow to the head before testicles were dissected out (Fig. 4). The testicles were dissected out from each male and processed through crushing. After dissection, the testicles were rinsed with distilled water to remove any blood spill from the milt sac, thereafter gently blotted with paper towels before being crushed through a manual small kitchen grinder. The crushed milt was then sieved through two layers of fine (0.2mm) mesh into a clean beaker (Beirão et al. 2019). The crushed milt volume collected from each male ranged between 20 to 40 ml (Fig.4).
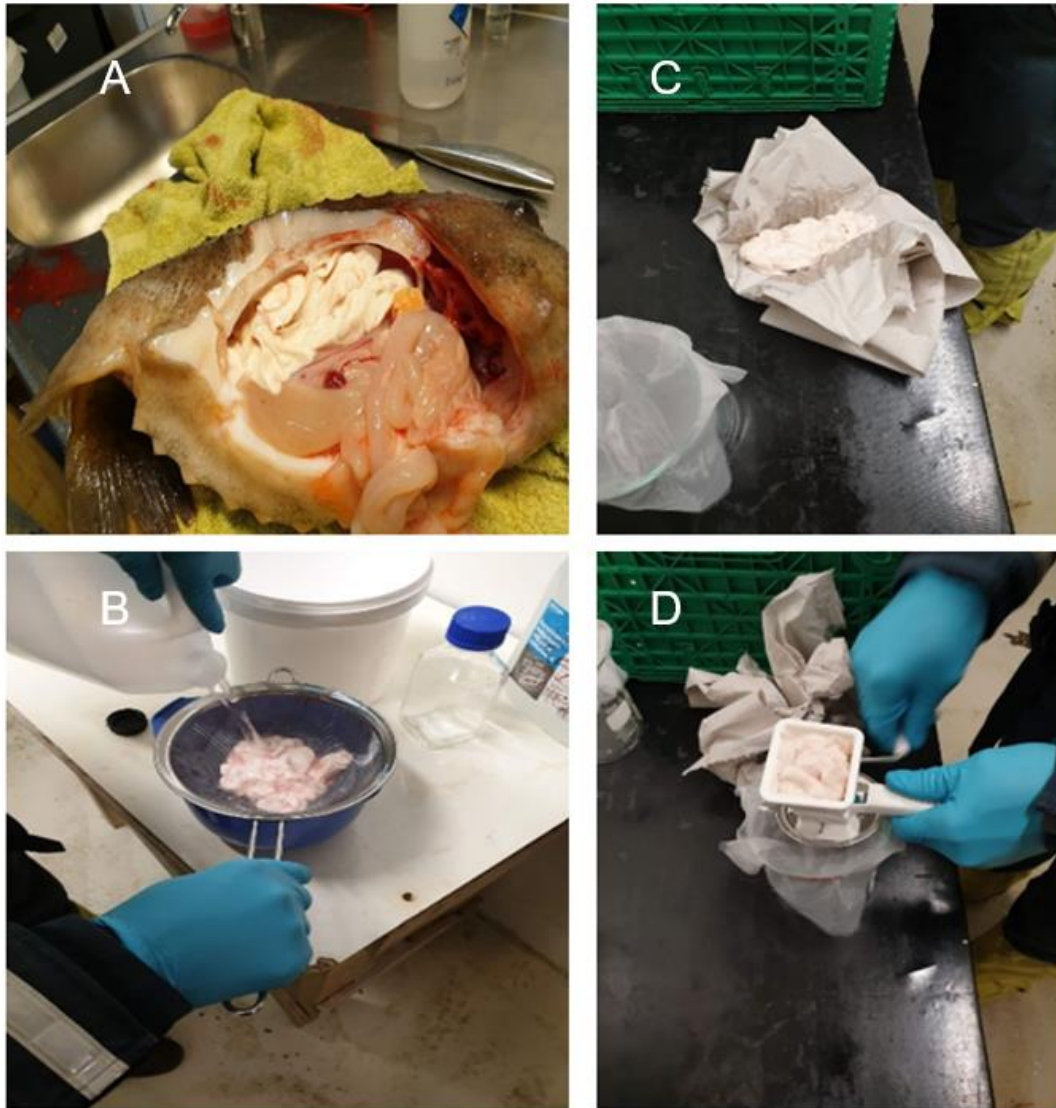
Figure 4. Procedure used for fish dissection (A), collection of male testes (B) rinsing of testes (C), blotting with paper and (D) crushing of testes for sperm extraction.

2.4 Egg collection

Two female fish ready to spawn were sampled by checking for bulging abdomen and reddish coloration around urogenital opening (Fig. 5). Prior to stripping, the two female lumpfish fish were killed by a blow to the head. Two fingers were gently placed through the urogenital opening with gentle pressure which breaks the egg sac and allowed free flow of egg when gentle pressure was applied to the abdomen (Fig. 5). A total volume of egg stripped from the two female lumpfish was 400 and 600 ml. Stripped eggs were stored on ice and transported from FISK Akvaplan-niva research station (Kraknes, Tromsø) to the physiology laboratory, UiT

within 1.5 hours before fertilization procedure was initiated. Equal volume (200 ml) of eggs were collected from the two females and pooled to avoid female effect in the fertilization experiment.
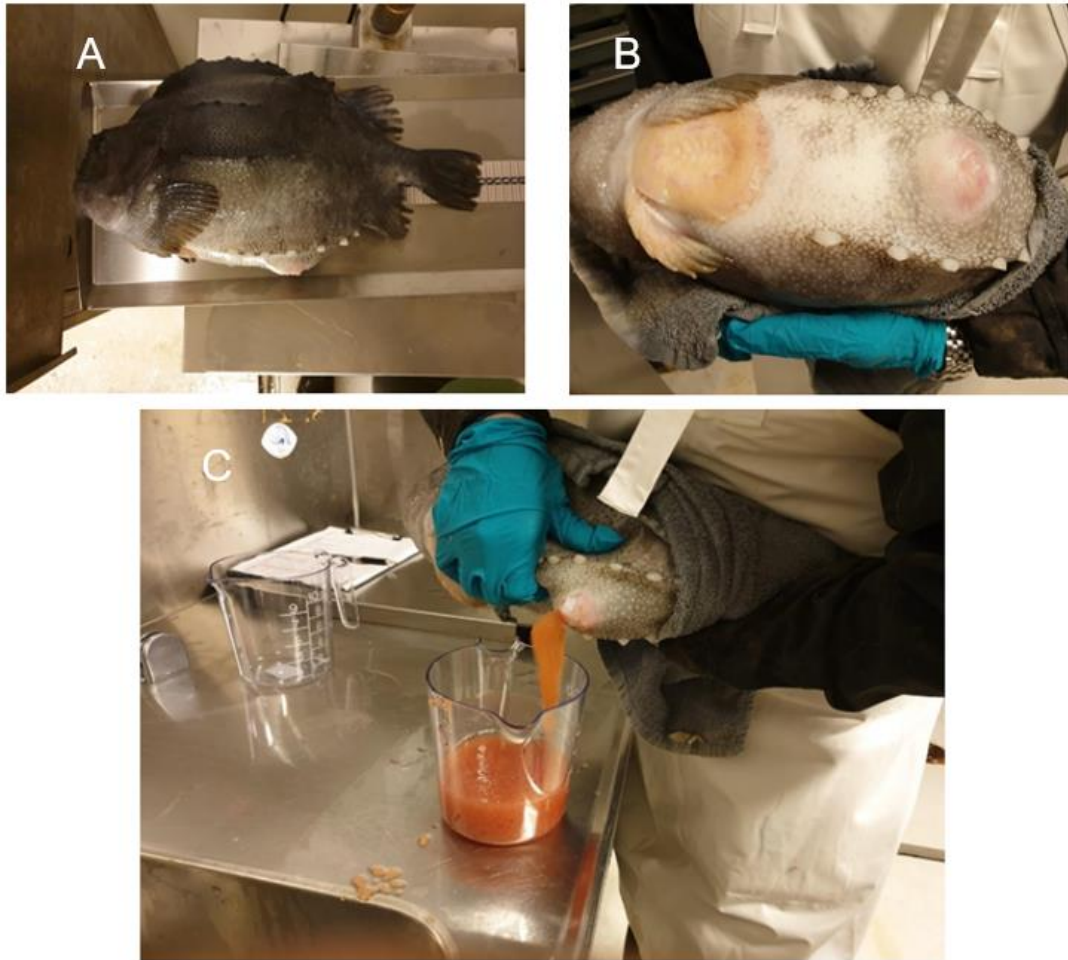

Figure 5. Procedure for egg stripping (A) Female lumpfish weight and length measurement (B) swollen and red urogenital opening showing that the fish was ready to spawn and (C) eggs stripping.

2.5 Preliminary analyses

The following section gives a brief summary of pilot trials conducted prior to one main experiment.

Trial 1: This pilot experiment was designed to find a good but cheaper alternative to the commercially available fish extender Aquaboost® (Cryogenetics AS, Hamar, Norway) for short- and long-term storage of milt. The high cost and limited storage time of this extender forms part of the basis for looking for a suitable substitute, therefore, comparison was made between Aquaboost® and an extender optimized for spotted wolffish, *Anarhichas minor* (Kime and Tveiten, 2002) if the outcome could yield similar or better result than the commercially available extender. Preliminary work in this direction was carried out at FISK by: 1) dissection, grinding and sieving of whole male testes; 2) dilution of fresh milt collected from crushed testes in AquaBoost® or the wolfish extender before short-term storage (few days); 3) comparison of quality of sperm stored in the above extenders using egg fertilization trials. In this pilot trial, the quality of the sperm stored in AquaBoost® was observed to drop rapidly after only three days while the wolffish extender preserved at best sperm quality and fertilization capacity (data not shown). A decision was taken to concentrate only on the use of wolfish extender in subsequent trials and main experiment.

Trials 2-4: Other variables tested at FISK involved the comparison of 0.5ml cryo-straws with 2ml cryo-bags (Nasco Wirl-Pak, P/N: 99100007, USA) and 2ml cryo-tubes (ThermoFisher Scientific AS, Norway). The outcome from these trials shows poor post-cryopreservation result when validated through fertilization of eggs. The poor results was attributed to some of the observations during pre and post-cryopreservation procedure of freezing and thawing revealed damages to cryo-bags while the cryo-tubes appears to be too thick which was suspected to have affected the freezing curve even though it could not be measured for lack of micro thermometer. Sperm viability was judged through fertilization results which was low for both bags and cryo-tubes used. The poor outcome from the trials narrowed the scope down to the choice of commercially available 2.5ml cryo-straws (Minitübe GmbH, Tiefenbach, Germany) (data not shown).

Trial 5: The last trial involved using 2.5ml cyo-straws while multiple variables considered include; comparison between stripped and crushed sperm viability, testing three different freezing heights (2.5 cm, 3.5cm and 4.8 cm) and three different freezing times (5 minutes, 7.5minutes and 10 minutes). The outcome from this trial pointed towards 10 min freezing time

and freezing heights below 4.8 cm would likely give better results than the prior variables tested (data not shown). The direction from the last trial informed the design of the final experiment around five male lumpfish each with three triplicate samples tested at two freezing heights (2.5 cm and 4.8 cm) for 10min freezing time. Cryo-preservation results were validated through fertilization tests involving eggs pooled from two female lumpfish (data not shown).

2.6 Main experiment

The main experiment included grained testis from five males and pooled eggs from two females. On the 4th of April, five lumpfish males were collected from tanks at FISK, length and weight of each fish was measured (mean weight 2.13 kg, SD: ± 1.03 and mean length: 37.40 cm, SD: ± 3.73) before they were dissected and testicles collected and grained through a small kitchen grinder. The grained sperm was sieved through a fine mesh size 0.2 mm into a clean beaker. The content of each beaker was then emptied into a falcon tubes, total volume measured in ml and diluted 50:50 in wolffish extender before placing each falcon tube containing different males' sperm on paper towel laid on ice and transporting to physiology laboratory, UiT where the experiment took place. The first part of the experiment required motility check through CASA, sperm concentration analysis through both CASA and spermatocrit, followed by live and dead sperm cells count through flow-cytometry analysis. The experimental set up is as shown in Fig. 6.
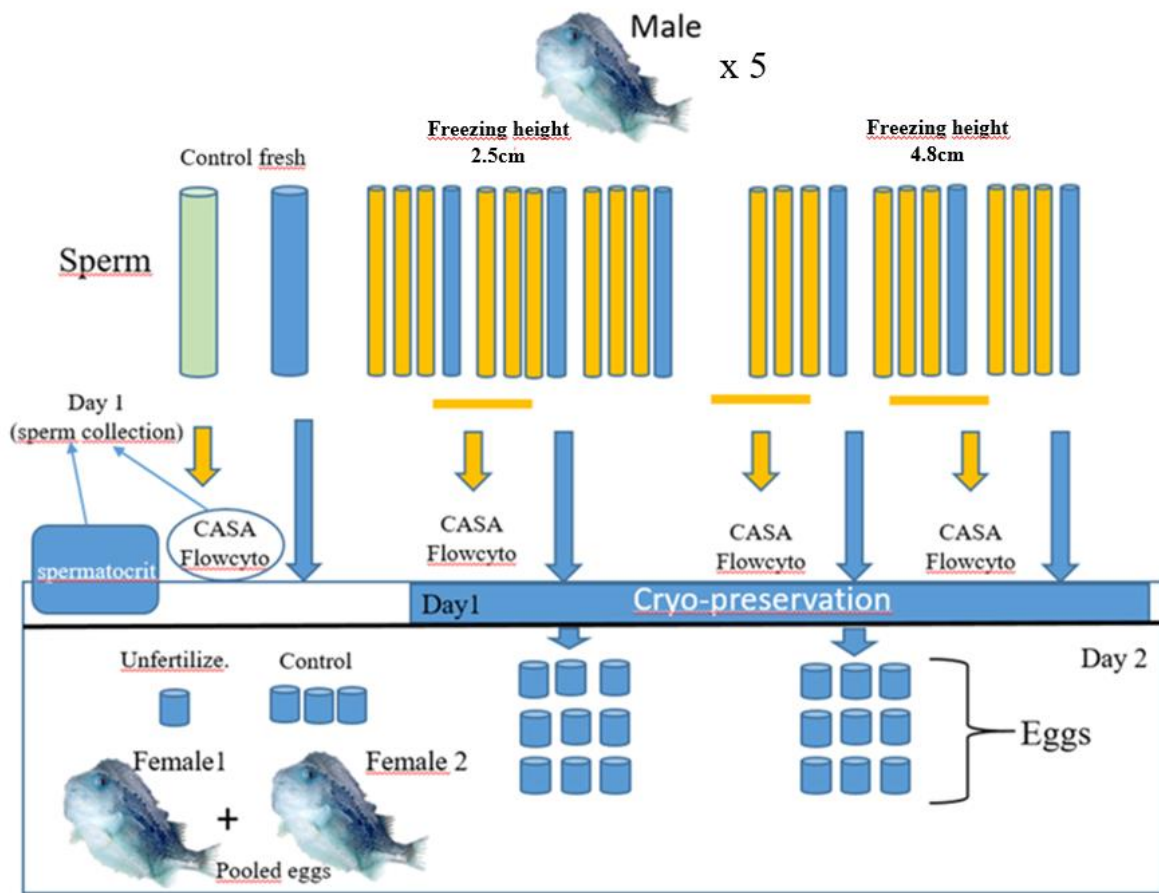
Figure 6: Schematic presentation of experimental set-up of the main experiment. Grained sperm from five individual males were cryopreserved in 2.5 ml cryo straws (n=24 straws per male) at two different freezing curves (2.5 and 4.8 cm freezing trays: n=12 straws per freezing height). Eggs pooled from two female lumpfish were used for all fertilization tests of fresh (control) sperm and cryopreserved sperm. (Day1) sperm quality check (CASA and flow-cytometry), sperm concentration check (spermatocrit) and cryopreservation of sperm, (Day2) sperm quality check for cryopreserved sperm and egg fertilization.

2.7 Preparation of wolffish extender

Wolffish extender was prepared one day before the main experiment by measuring 154mM NaCl, 4.55mM $CaCl_2$, 2.37mM $MgSO_4$, and4.8 cm3mM $KHCO_3$ (Table 1) salts and dissolving them in 1000 ml distilled water.

Table1: Salt concentrations and weight used to prepare 1000ml wolffish extender

Ingredient	Weight (g)
NaCl	9.005
$CaCl_2$	0.670
$MgSO_4$	0.581
$KHCO_3$	0.486

The process of mixture was carried out on a magnetic mixer which enabled homogeneous solution before autoclaving and storage in refrigerator. Prior to use, 600 ml wolfish extender was measured and 6g of BSA and 1.11g glucose added shortly before the start of the experiment to produce cryo-solution.

2.8. Sperm quality assessment

2.8.1. Sperm concentration

Sperm concentration for the five males employed in the study was evaluated by Packed Cell Volume (PCV) or spermatocrit and automatically by CASA (see section 2.8.2). For spermatocrit, an aliquot of each sperm sample was first collected into a 1.5ml Eppendorf tube and then siphoned into 10µl micro-hematocrit capillary tubes in triplicates for each male sample (Fig.7a) before ultra-centrifuging (Hettich EBA 12, Hettich GmbH, Germany) at 5400g for 10min following Peruzzi et al., (2009) (Fig.7b). Sperm concentration was then calculated by use of a calibrated scale dividing the measured pack cell volume over the total volume (seminal fluid + pack cell volume) multiplied by 100. Because the sperm samples used for this analysis has been diluted 50:50 with wolffish extender, the spermatocrit results were multiplied by 2 to compensate for the initial dilution.
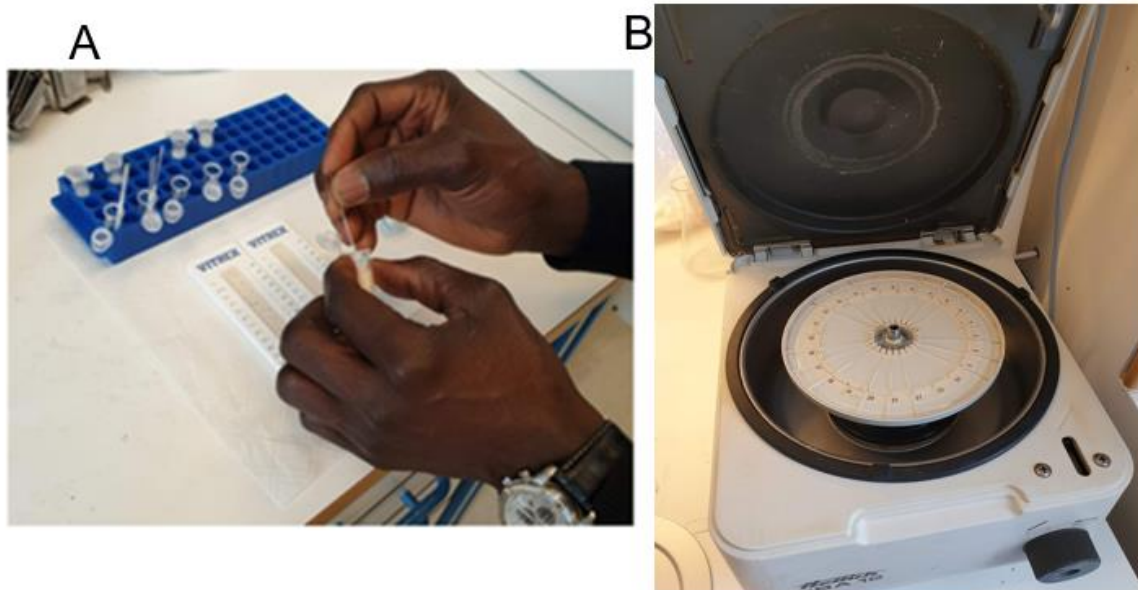
Figure 7. Procedure for sperm concentration analysis using spermatocrit. (A): Collection of aliquot of sperm samples into 1.5ml Eppendorf tube and siphoning into 10µl micro-hematocrit capillary tubes. (B): ultracentrifugation at 5400g for 10min.

2.8.2 Sperm motility and viability

Sperm motility and viability of the five males used in the experiment were analyzed by computer assisted software analyser (CASA) at 0, 24 and 48 hours after cryopreservation. The 0 hour refers to analysis of the fresh sperm sample prior to cryopreservation, while 24 and 48 hours refers to post cryo-preservation analysis. Wolffish extender (Kime and Tveiten, 2002) with pH 6.5 was used to dilute the fresh sperm at 50:50 dilution ratio before the analysis. The extender used at this stage contained glucose and BSA a non-penetrating cryo-protectant which provided energy source to the sperm cells.

The step that followed in the sperm analysis involved sperm dilution and activation in this proportions; 5µl of fresh sperm (50:50 dilution in wolffish extender) added to 750µl wolffish extender and activated with 750µl cool seawater (final sperm dilution of 1:1700). 6µl of activated sperm was injected unto the specialized microscope slide (Leica® Standard Count 2 Chamber Slide 20 micron) placed on a hollow cold plate cooled by a continuous water flow regulated at 4°C before and during sperm motility analysis. The parameters; motility (MOT), linearity (LIN), progressiveness (PROG) and curve linear velocity (VCL) were measured

within five different fields for 2 minutes. The first 30 seconds of the measurement was not considered which ensured there were no drifting of the sample and to maintain a steady image capture. The images captured during CASA motility analysis as shown in Figs.13-15, shows the differences in motility parameters measured between control groups and the treated groups. The color, shape, length and abundance suggest the quality difference between the control and treated groups. The red coloration indicate rapid progression which in this case was not found in any of the groups. Green colors shows sperm that have medium progressiveness while the blue coloration shows sperm cells that are alive but not progressive and the yellow dots represents dead or immotile sperm cells.

The sperm motility parameters were measured on computer assisted analysis system (CASA) coupled to a phase contrast microscope (Nikon E-2OO, Nikon, Tokyo, Japan) with x10 negative phase contrast objective connected to a temperature regulated cooling system for the slide (Fig.8). Motility parameters were evaluated with the included Leica Application SUIT (LAS) software for both fresh and cryopreserved sperm following Figueroa et al., (2016) and Horokhovatskyi et al., (2018).

The procedure for analyzing the cryo-preserved sperm samples involved thawing of cryo-preserved samples in water bath regulated to $37^{o}C$ for 35 seconds immediately after removing it from liquid nitrogen. The thawed samples followed the same dilution used for fresh (control) sperm sample; 5µl of cryopreserved sperm (50:50 dilution in wolffish extender before cryopreservation) added to 750µl wolffish extender and activated with 750µl cool seawater (final sperm dilution of 1:1700) were immediately analyzed for motility through CASA and sperm quality flow cytometry which followed the same procedure used for the control samples.

Figure 8. Experimental setup for sperm quality check using CASA system. (A) Laptop equipped with software analyzer. (B) A phase contrast microscope (Nikon E-2OO, Nikon, Tokyo, Japan) with x 10 negative phase contrast objective. (C) CASA system. (D)Temperature regulator cooling device. (E) Hose supplying chilled water to the slide.

2.8.3 Live and dead sperm cells analysis

Live and dead sperm viability was measured through flow cytometer (Sysmex-Partec Cube8, Görlitz, Germany) equipped with three lasers 488 nm, 638 nm and 561 nm and a UV LED (Fig. 9) followed the methods adopted by Maria et al., (2006). Two control samples were established as live control (fresh sperm sample) and dead control (killed sperm sample). The protocol for killing the sperm sample was established by collecting 1ml fresh sperm sample into an Eppendorf tube that was incubated in a water bath at 40° for one hour. Thereafter, the Eppendorf tube containing the killed sperm was removed and placed on ice until flow-cytometry analysis was carried out. These controls were used in calibrating the regions for image captured for both live and dead sperm samples and as standards for the treatments.

LIVE/DEAD sperm kit (Molecular Probes, USA) SYBR 14 green and PI red were the double stains used for staining the sperm cells before evaluating samples through flow-cytometer. The staining procedure involved; staining for live sperm cells with SYBR 14 for 10 minute and thereafter staining for dead sperm cells with Propidium Iodide (PI) for another 15 minutes. The estimation of live/ dead sperm cells count was possible through the help of laser 488 nm in combination with FL1 536/40 nm filter and 561 nm laser combined with FL5 610/40 nm filter incorporated to the flow-cytometer. There are two fluorescence measurements involved in the analysis; forward scatter and side scatter fluorescent. The value of each fluorescent differ from each other which account for position difference. The measurement through forward scatter (FCS) gives an idea of the size particles being analyzed and also estimate values of debris available in the count. Side scatter fluorescent on the other hand shows the position of the particles being analyzed in the regions.

Although, two hundred thousand (200,000) sperm cells events count was set as pre-defined count, the values measured for each analysis were slightly above the set limit (machine errors). The analysis show the proportion of dead, live and immotile sperm cells. With the help of combine fluorescents (FSC and SSC), images captured by these two lasers individually or together were distributed in three regions (2, 3 and 4) (Fig. 19 and 20). Region 2 represent the total events measured for both live and dead sperm cells counted from FSC and SSC. Regions 3 and 4 was gated for images estimation for dead and live sperm cells as captured by side scatter fluorescent SSC.

For the control groups the unstained sperm cells was located outside the gated regions which could not be justified as either dead or alive because the lasers could not detect any stain on the sperm cells, therefore they were position outside the regions. However, the visual observation of the captured image shows that the unstained sperm cells were only concentrated within a region outside regions 3 and 4. For the dead sperm cells located within region 3, any sperm cells outside region 3 could be classified as either live or moribund sperm cells. On the other hand, sperm cells within region 4 are classified as live sperm cells and any sperm cells outside this region could be classified as either moribund or dead cells (Fig. 19 or 20).
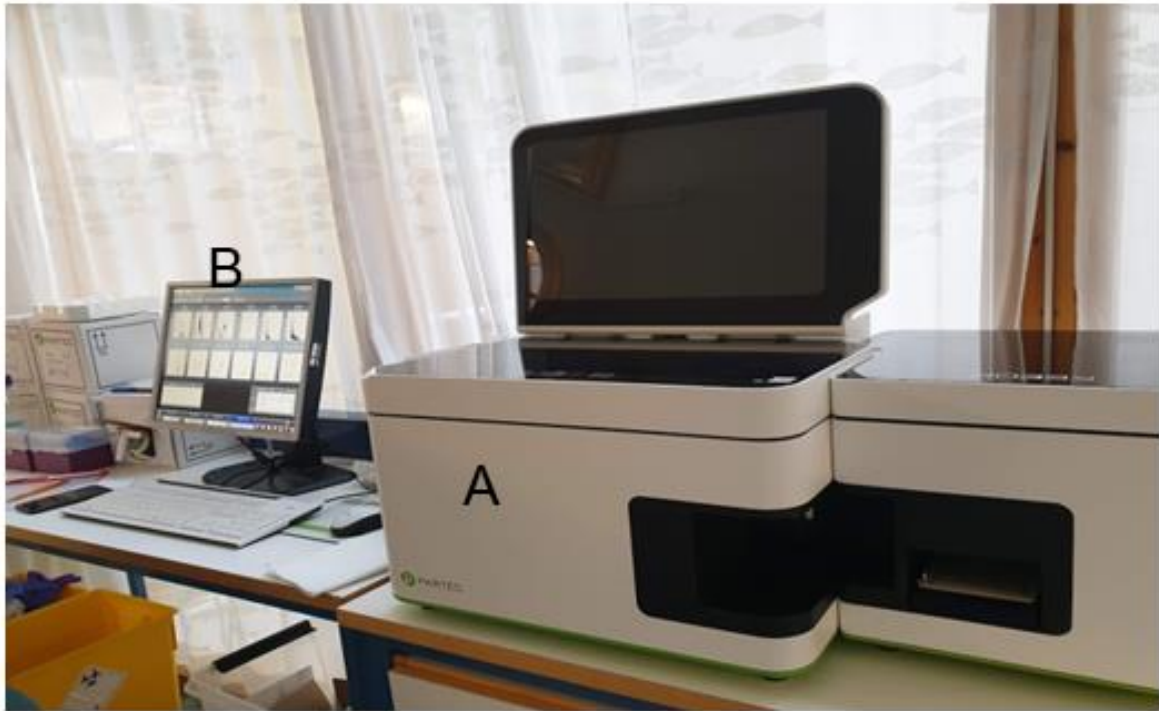
Figure 9. Setup for live / dead sperm analysis. (A): Flow cytometer (Sysmex-Partec Cube8, Görlitz, Germany). (B): Computer screen showing image capture and parameter estimation.

2.9 Sperm cryopreservation

A volume of 30ml diluted (1:1 volume) sperm with wolfish extender from five males was measured into five falcon tubes and 10% (3ml) DMSO added as penetrating cryo-protectant to the 2.5 cm and 4.8 cm treatment groups. The mixture was gently mixed prior to filling twelve pieces of 2.5 ml minitube cryo-straws. The same procedure was repeated for the five males and for each treatment height. The resulting twelve minitube cryo-straws for each treatment comprised three triplicate samples for CASA and flow-cytometry analysis plus an extra straw added for egg fertilization making four straws x 3 for each male (see section 2.6 and Fig. 6). The straws were then kept on ice for 10 min (equilibration time) from adding 10 % DMSO to the falcon tube until freezing the straws on one of the freezing trays (2.5 and 4.8 cm freezing heights, respectively) above the liquid nitrogen (Fig.10 A). After ten minutes freezing on one of the freezing trays, frozen sperm were plunged directly into liquid nitrogen below the trays and later stored overnight in liquid nitrogen (Fig.10B).
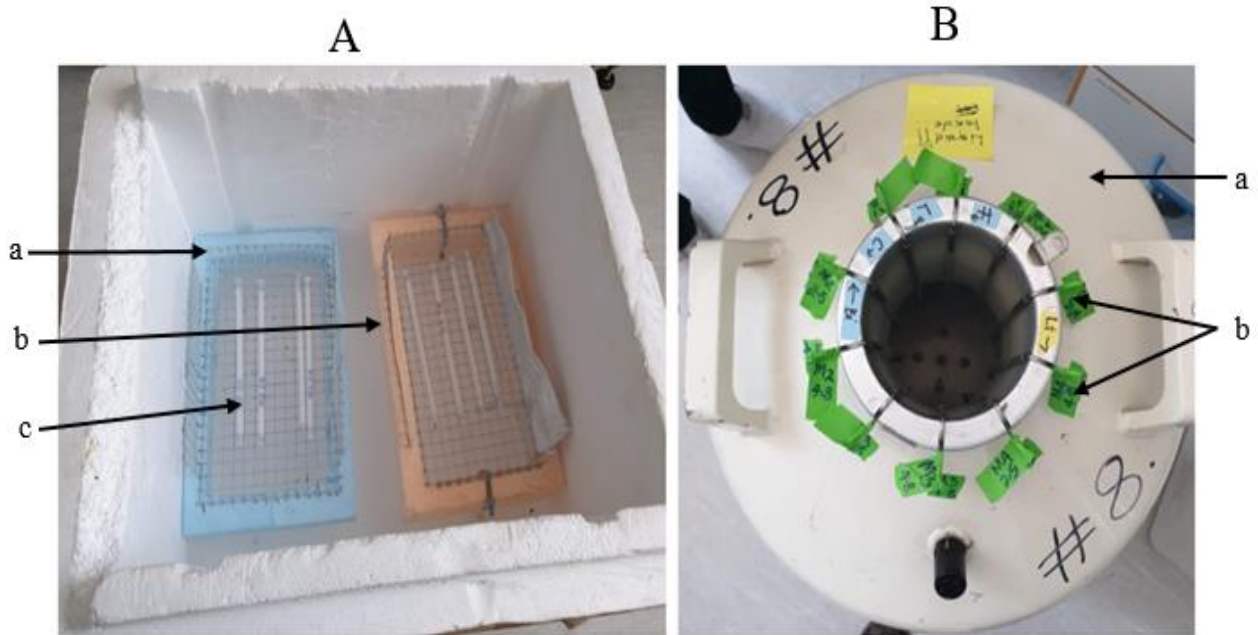
Figure 10. Procedure for cryopreservation of lumpfish sperm. (A): Freezing of sperm over liquid nitrogen. (Aa):2.5 cm freezing tray. (Ab): 4.8 cm freezing tray. (Ac) Straws filled with 50:50 diluted lumpfish sperm freezing over liquid nitrogen vapor. (Ba): Liquid nitrogen tank used for overnight storage of frozen sperm. (Bb): Treatment samples stored in labelled different canisters.

2.10 Egg fertilization

3ml of pooled eggs were measured into falcon tubes used for incubation of the eggs. The egg distribution follows this order; two control sets. (a) Unfertilized egg sample and (b) triplicate of fresh sperm fertilized eggs. Three triplicate egg samples were prepared for each treatment's groups (n=45 tubes in total). Ovarian fluid even distribution on the eggs was managed by separating it from the eggs before egg distribution and was equally distributed across all the egg samples in the treatment groups. This was to ensure uniformity of condition for all the treatments (Fig. 11a). The egg / ratio used in the fertilization validation was 3ml eggs = ca. 100 eggs fertilized with 50 microliters sperm at a concentration of 109 – 183 cells/µl 10^6 approximately 5-9 x107 sperm cells /egg. All the groups were fertilized with 100µl of 50:50 diluted sperm (fresh or cryopreserved sperm). Control groups were fertilized with 50:50 diluted fresh sperm stored overnight in a cool room regulated at 4ºC over an agitator. Fertilized eggs were incubated in a cool room regulated at 4ºC (Fig. 11b).
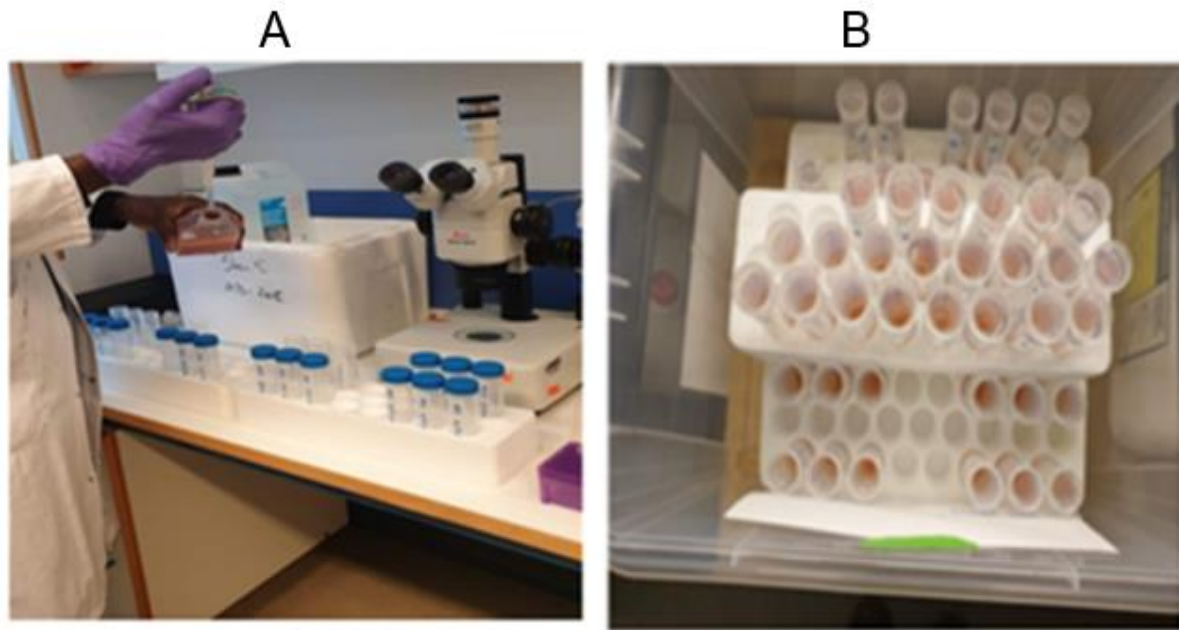
Figure 11. Procedure for egg fertilization. (A): Egg and ovarian fluid distribution into Falcon tubes before fertilization with fresh and cryopreserved sperm. (B): Egg incubation in Falcon tubes (still water) in a cool room regulated at 4°C.

Fertilization success was assessed by observing the different egg treatment batches under a light microscope. Ninety-six pieces of eggs were counted for each of the egg samples (control and two treatments) into a counting chamber before observation was carried out under the microscope. The percentage of fertilized eggs were measured 18 hours to 24 hours post fertilization. Two microscopes were used for this assessment. Initial counting started with the small light microscope (Leica WILD M10, 0.63x) but poor analysis from the small microscope for the first batch of eggs (control male 1 eggs) required the need to switch to a bigger dissecting stereomicroscope (Nikon Profile projector V – 12B). The success of fertilization was measured through blastomere division which observation shows 2-cells and 4-cells division (Fig.12). The observation was similar in all cases between the control groups and the two treatments groups. The egg batches that were counted late (i.e. fertilized after 48 h cryopreservation of sperm) had their water changed with fresh cool sea water to prevent the egg quality drop and death.
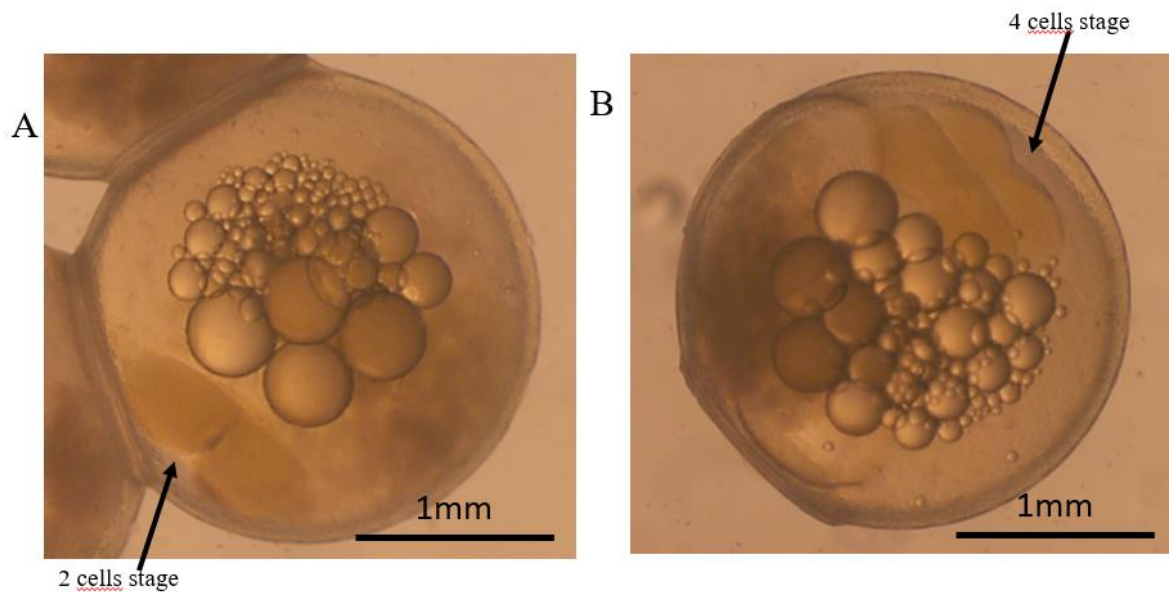
Figure 12. Procedure for verifying egg fertilization. (A): Arrows indicate the blastomere (2-cells stage). (B): Arrows indicate the blastomere (4-cells stage) 18 - 24 hours post fertilization. Scale bar indicates egg size measured in mm.

2.11 Statistical analyses

Linear regression analysis was carried out to determine the correlation between sperm concentrations estimated by spermatocrit and sperm cells counts. Sperm motility and viability results from lumpfish sperm cryopreservation experiment were analyzed through a non-parametric statistical analysis. Motility parameters (MOT), (LIN), (PROG) that were in percentages were log transformed except for (VCL) measurement that was not in percentage. Similarly, the data for live stained sperms and fertilization rates that were in proportions were ARCSIN transformed in other to improve normality. Motility (CASA), sperm quality (flow-cytometry data) and fertilization rates were checked for normality (Shapiro-Wilk) and homogeneity of variance (Levene's test) to satisfy the assumption of ANOVA. Normality and variance assumptions were violated in all the cases tested, therefore motility parameters, sperm quality and fertilization rates were analyzed with a non-parametric Welch ANOVA test. Robust tests of equality of means was performed for motility parameters except for Log LIN, sperm quality data and fertilization rate where it could not be performed because at least one group has zero variance. However, Gabriel's post Hoc test was applied for pairwise comparisons between the control and treated groups to see where the difference lied since equality of means test showed no significance. All analyses were performed using the IBM SPSS Statistics v.25

software. The critical P value was set at 0.05. Data are presented as means ± SD of means, unless otherwise stated.

3.0 Results

Overall, there were significant differences in the quality of the cryopreserved sperm relative to their controls groups as measured for motility parameters, sperm quality parameters and fertilization rates. The results obtained for pre and post-thaw sperm quality analysis are presented under sperm concentration, CASA, flow-cytometry analysis and egg fertilization.

3.1 Sperm concentration analysis

The sperm concentration for the 5 males as measured by spermatocrit and automatically by CASA is shown in Tab. 4. The concentrations varied from 44 to 85 percent indicating a large variation among males. Male 4 is particularly high in sperm concentration value in comparison to the other males. Evaluation of sperm concentrations by CASA also varied greatly among the 5 males, ranging 115-183 cells/ml 10^9 and with male 4 showing the highest value.

There was no significant positive correlation between the sperm concentrations obtained by spermatocrit (PCV, %) and CASA (cells/ml) in the five males (Fig.X).

Table 4. Sperm concentration as measured by spermatocrit and sperm count (CASA) for the five males. Data are presented as mean ±SD of n=3 (spermatocrit) and n=5 (CASA) replicates, respectively.

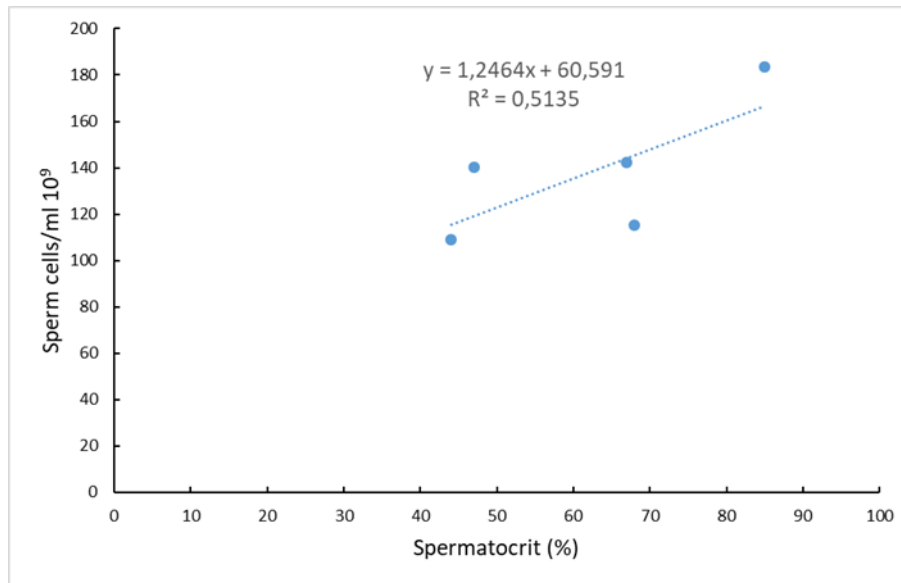| Male	Spermatocrit value(%)	Sperm count (CASA) (cells/ml 10^9)
Male 1	44 ± 2.75	109,14 ± 3,84
Male 2	67 ± 0.70	142,33 ± 10,66
Male 3	68 ± 1.20	115,33 ± 10,80
Male 4	85 ± 1.53	183,55 ± 30,55
Male 5	47 ± 1.04	140,22 ± 23,96

Fig. 13 Correlation between values of sperm concentration as measured by spermatocrit and sperm cell counts by CASA.

3.2 CASA – Motility parameters

The CASA image for motility parameters shows high variance between the control groups and the treated groups. Visual observation of the captured images appears to show some level of variance between the control groups and their treatment groups. There appears to be more medium progressive sperm cells (green) in the in the control group than observed in the two treated groups. The proportions of non-progressive sperm cells (blue) appears to be more in treatment 4.8 cm than medium progressive sperm (green) for the 2.5 cm treatment. However, treatment 4.8 cm appears to have the lowest proportions of medium progressive sperm (green), but with more proportions of immotile or dead sperm cells (yellow) than observed in the 2.5 cm treatment and the control group (Fig.14 - 16).

Fig.14: CASA image representing motility parameters measured for the control groups. (a): Green coloration represent medium progressive sperm cells.(b): Blue denote non-progressive sperm. (C): Yellow dots represent immotile or dead sperm cells.

Fig.15: CASA image representing motility parameters measured for 2.5 cm treated groups. (a): Green coloration represent medium progressive sperm cells.(b): Blue denote non-progressive sperm. (C): Yellow dots represent immotile or dead sperm cells.
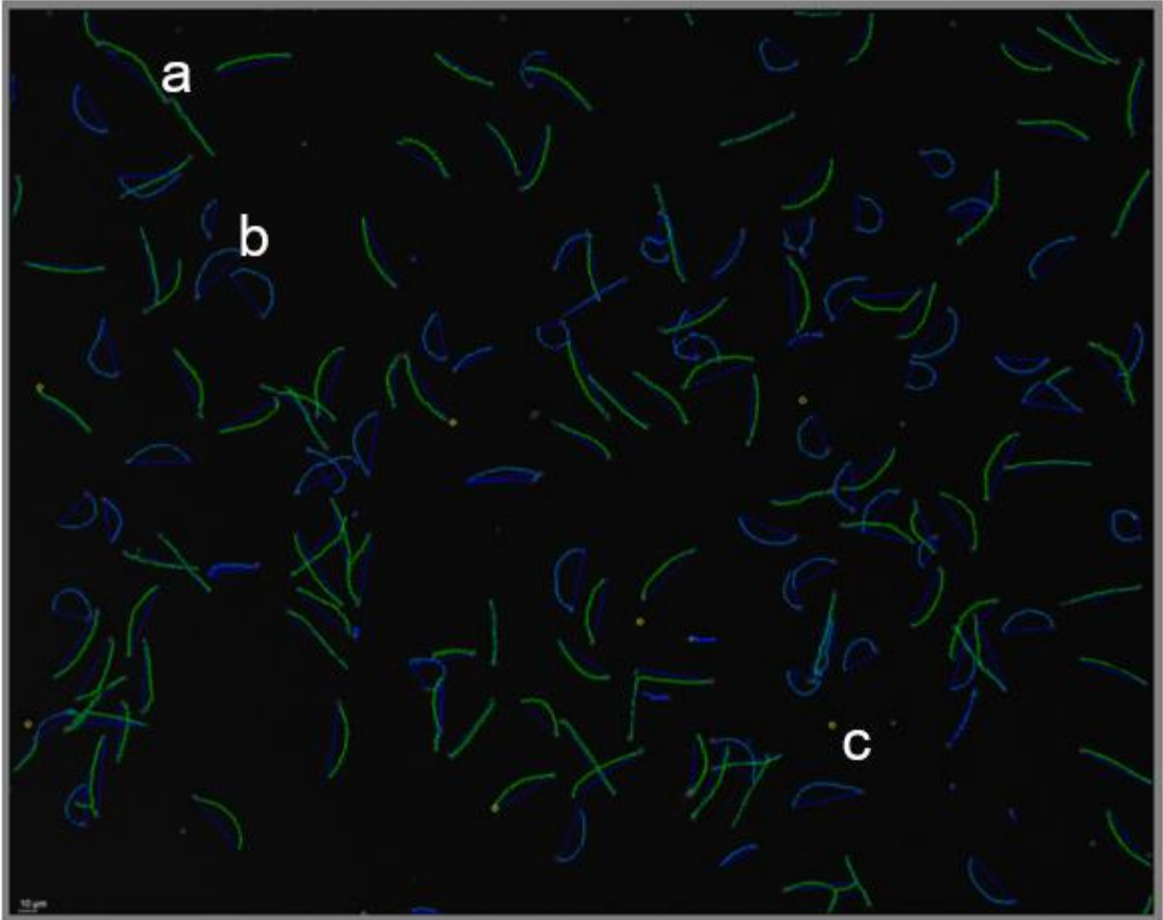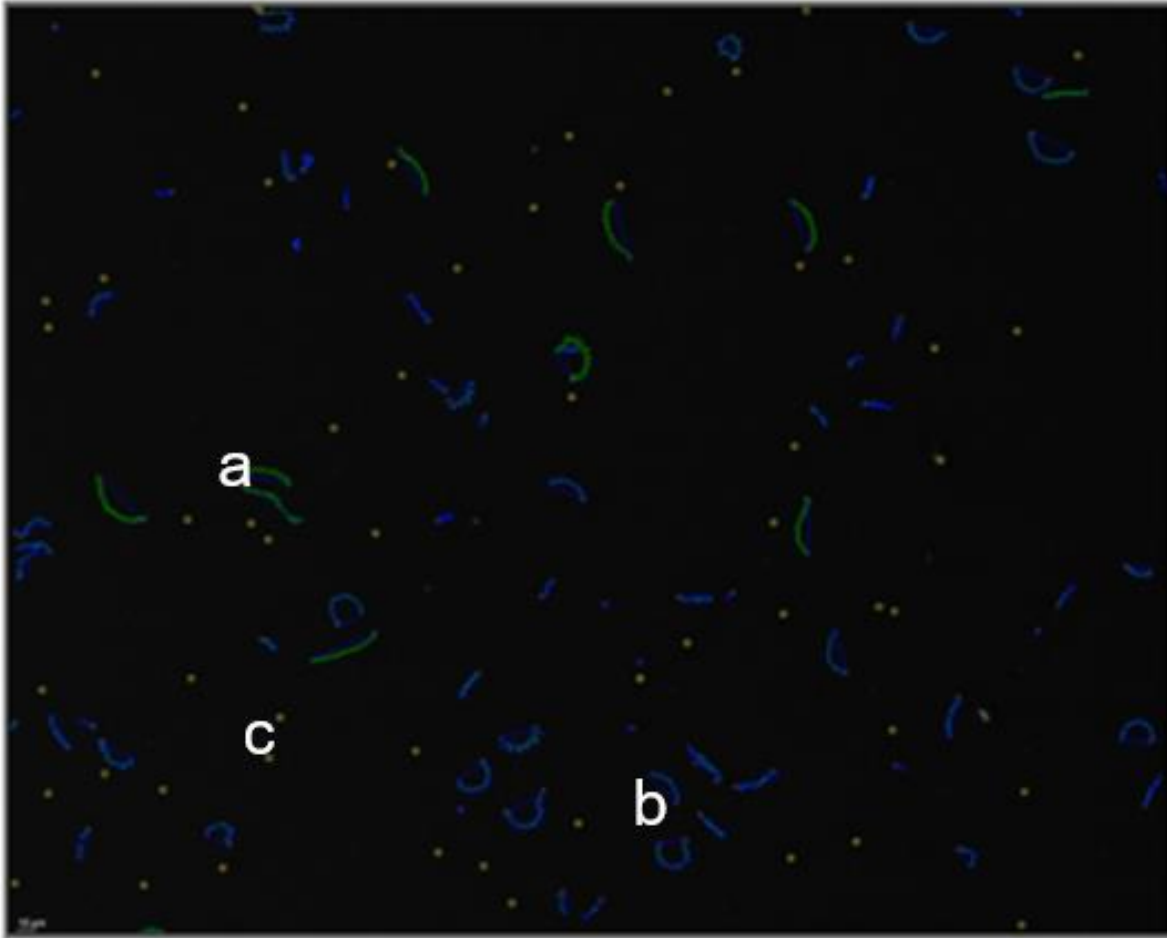
Fig.16: CASA image representing motility parameters measured for 4.8 cm treated groups. (a): Green coloration represent medium progressive sperm cells.(b): Blue denote non-progressive sperm. (C): Yellow dots represent immotile or dead sperm cells.
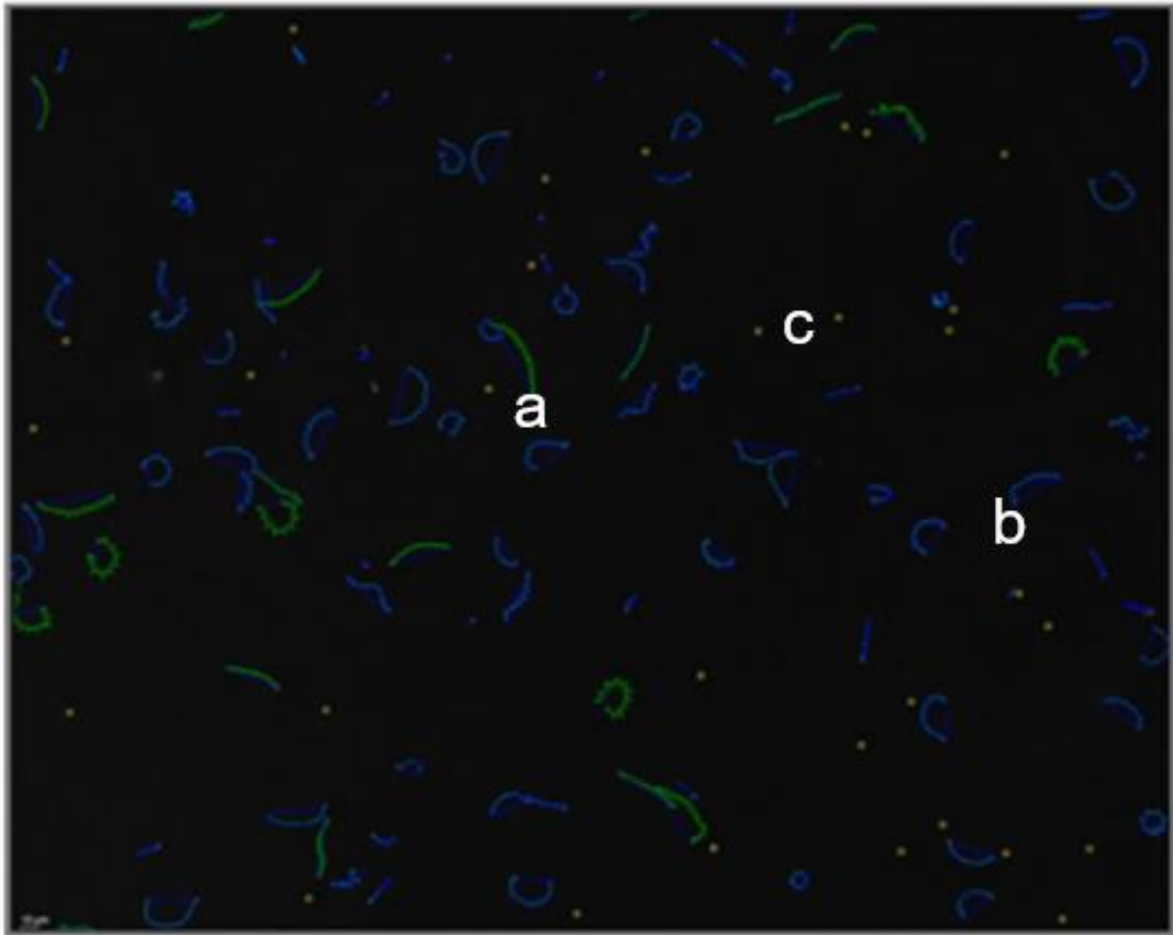
3.2.1. Motility

For motility, differences between the control and the treatment groups could only be observed in male 1, male 3 and male 5 (Fig.17). Significant difference was observed in male 1 for 4.8 cm treatment ($p < 0.007$) and for male 3 for both of its treatments 2.5 cm treatment ($p < 0.01$), and 4.8 cm treatment ($p < 0.01$) and male 5 significantly different from its 4.8 cm treatment ($p < 0.03$) whereas, the other treated groups shows no significant difference to their control (Fig. 17).

Figure 17. Difference in the mean values for MOT between the control groups and their treatments groups. The error bars indicate standard deviation. The difference in letters represents statistically significant difference.

3.2.2 Linearity

For linearity, four out of five treated groups differ from their control groups except for male 2 that did not show any difference between the control and its treatment groups (Fig. 18). For male 1, significance difference was observed between control and the two treatments 2.5 cm at ($p<0.001$), 4.8 cm at ($p<0.001$). There exists significant difference between male 3 and its 4.8 cm treatment ($p<0.001$). For male 4, the control is different from its 2.5 cm treatment ($pp<0.001$) and 4.8 cm treatment ($p<0.001$) but the two treatments are not different from each other. However, difference was observed for male 5, at significant level of ($p<0.001$) for 2.5 cm treatment and 4.8 cm treatment at ($p<0.001$) (Fig. 16).
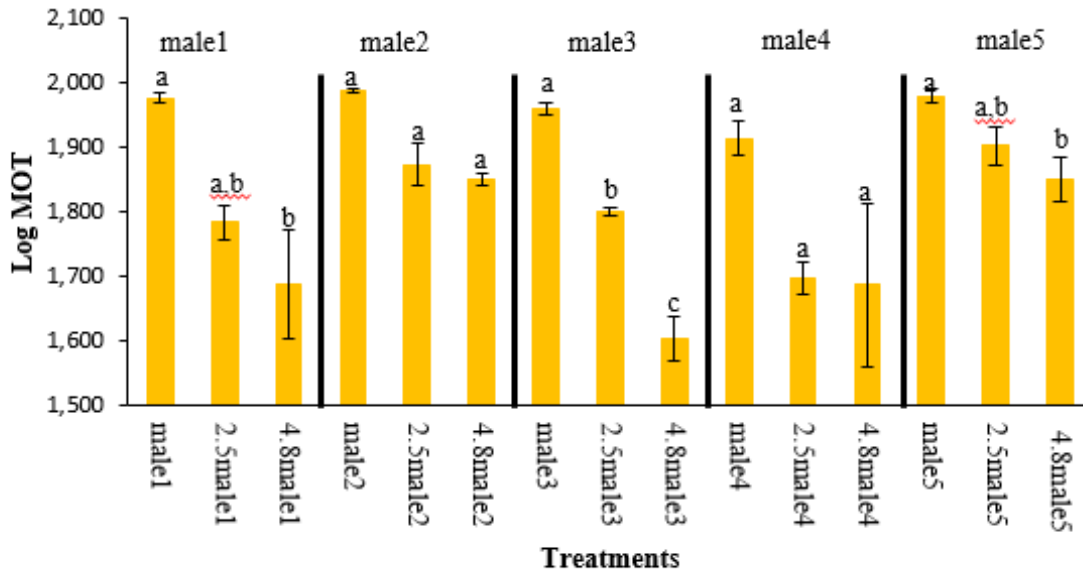
Figure 18. Difference in the mean values for LIN between the control groups and their treatment groups. The error bars indicate standard deviation while difference in letters represents statistical significant difference.

3.2.3. Progressiveness

There were no differences observed in progressiveness between male 2 control group and its 2.5 cm and 4.8 cm treatments but other four treatment group shows differences to their controls groups (Fig. 19). Significant difference was observed between male 1 in its 4.8 cm treatment group (p=0.030). Similarly, a significant interaction exist between male 3 and its 4.8 cm treatment (p<0.001). For male 4, significant difference were observed in 2.5 cm treatment (p=0.037) and 4.8 cm treatment p<0.001. Further still, there exist significant interaction between male 5 control and the treatment groups, 2.5 cm treatment (p<0.001) and 4.8 cm treatment at (p<0.051) respectively. (Fig.17).
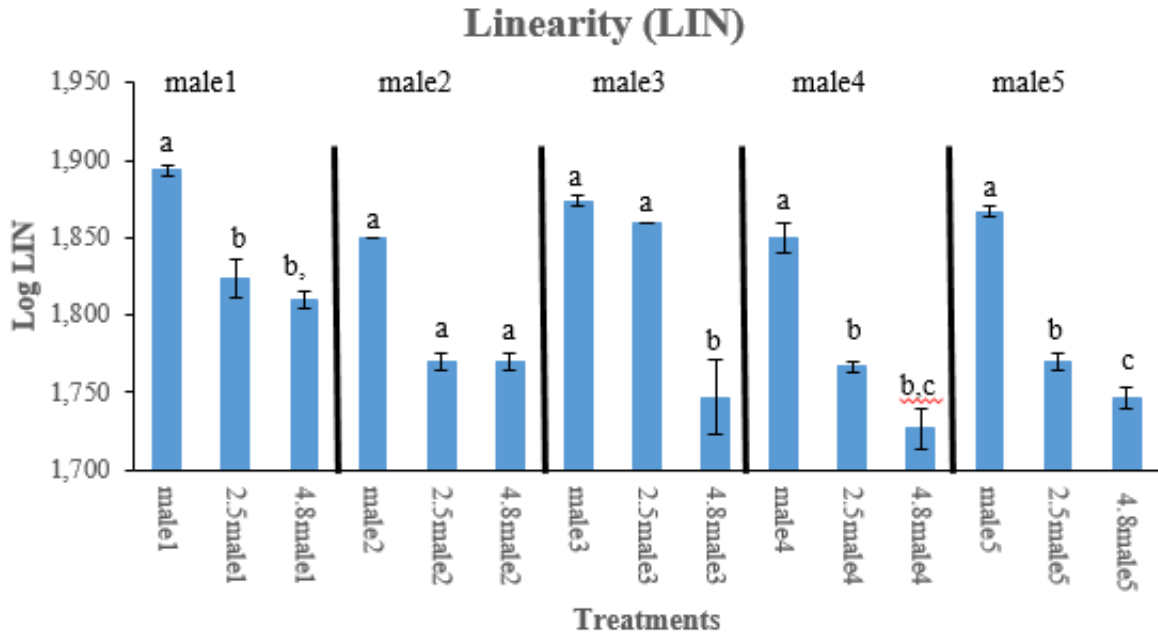
Figure 19. Difference in the mean values for PROG between the control groups and their treatment groups. The error bars indicate standard deviation while difference in letters represents statistical significant difference.

3.2.4 Curvilinear velocity

For curve linearity, differences between the control groups and the treatment groups could only be observed in male 1, male 3, male 4 and male 5 except for male 2 (Fig. 20). Significant difference was observed in male 1 for both 2.5 cm treatment ($p < 0.001$) and 4.8 cm treatment ($p < 0.001$). For male 3, the significance was observed in 4.8 cm treatment ($p < 0.001$) but male 4 significant interaction was observed in its two treatment at significant levels ($p < 0.004$) for 2.5 cm treatment and 4.8 cm treatment at ($p < 0.001$) while, the significant difference could only be observed between control 5 in 4.8 cm treatment ($p < 0.001$) (Fig. 18).
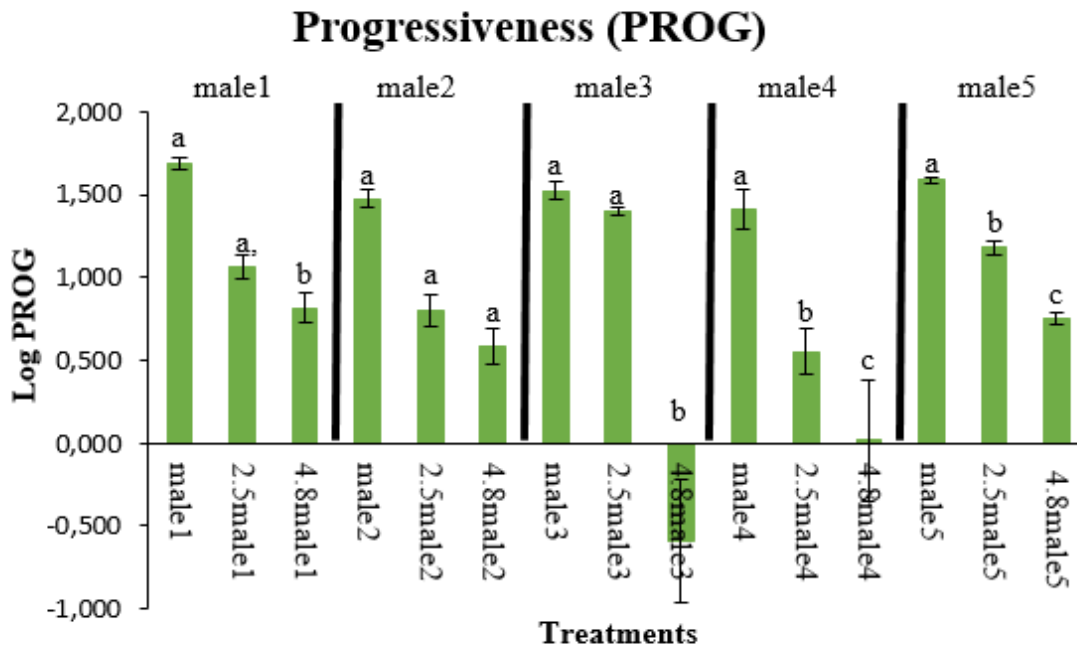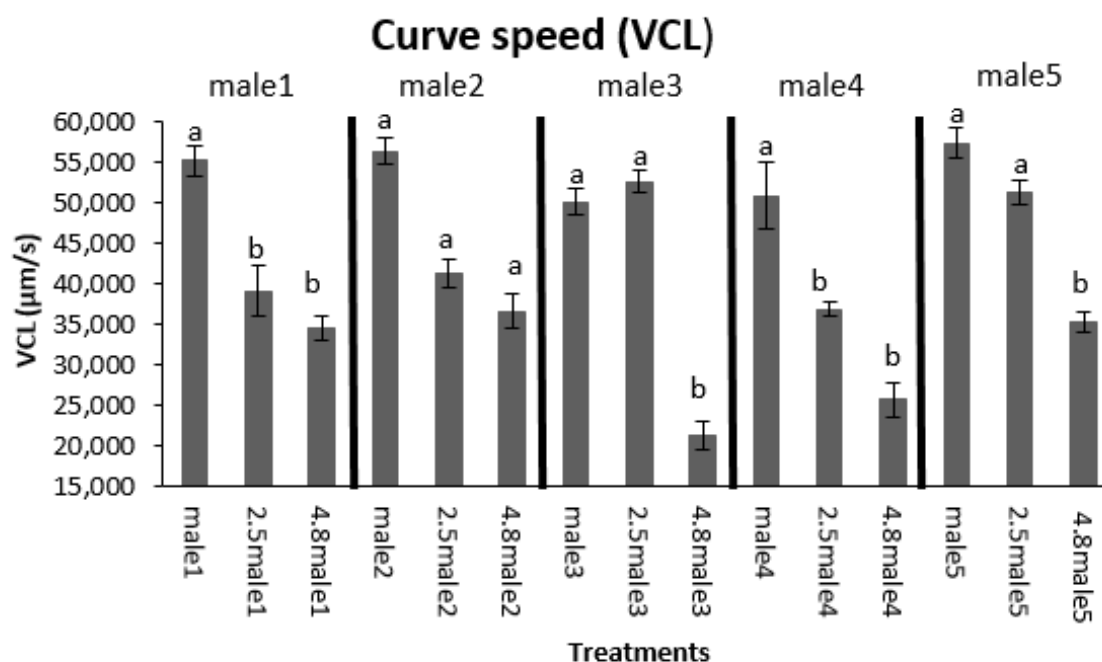
Figure 20. Difference in the mean values for VCL between the control groups and their treatment groups. The error bars indicate standard deviation while difference in letters represents statistical significant difference.

3.3. Flow cytometry Analysis

The images captured from flow cytometer analysis for both fresh and cryopreserved sperm cells are shown in different regions with differences between the control and the treatment groups are represented as live and dead sperm cells distribution across different regions. Visual observation of the images captured by flow-cytomer (Fig. 21) shows a great reduction in the percentage of live sperm cells (region 4) concentrations post cryo-preservation between the control and the treated groups. The reduction was more visible in 4.8 cm treatment groups than observed in 2.5 cm treatment groups post cryopreservation relative to the proportions in the control groups (Fig.22a and Fig. 22b).

Figure 21. Images for control groups from Flow cytometer (Sysmex-Partec Cube8, Görlitz, Germany) equipped with three lasers 488 nm, 638 nm and 561 nm and a UV LED. (A): All event captured by combined images from both forward and side scatter lasers. (B): Control unstained sperm cells located outside the regions. (C): Control dead sperm cells captured by side scatter laser located in region 3. (D): Control live sperm cells captured by side scatter laser located in region 4.

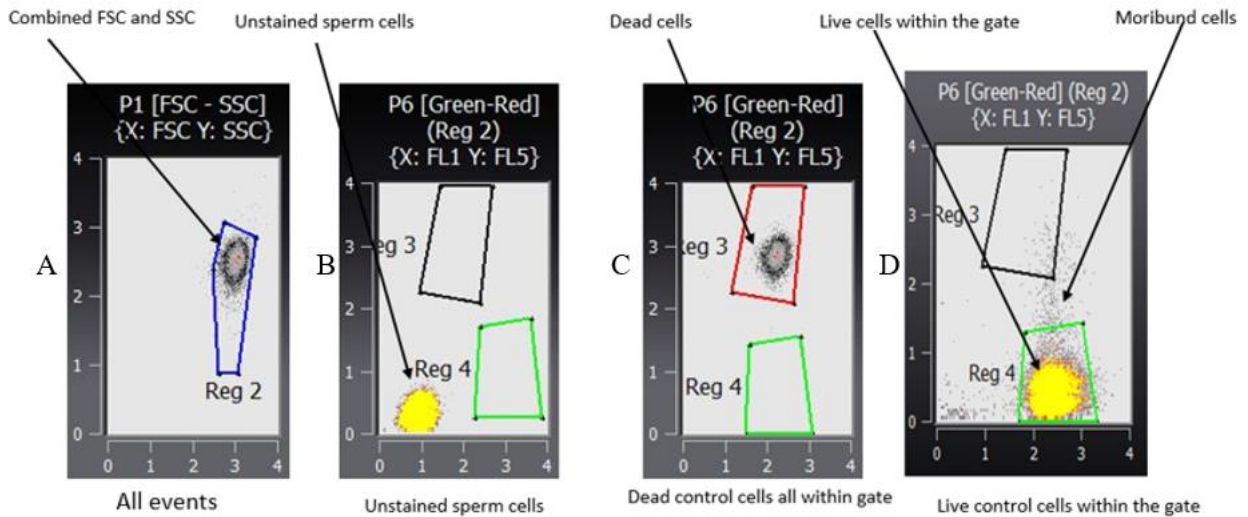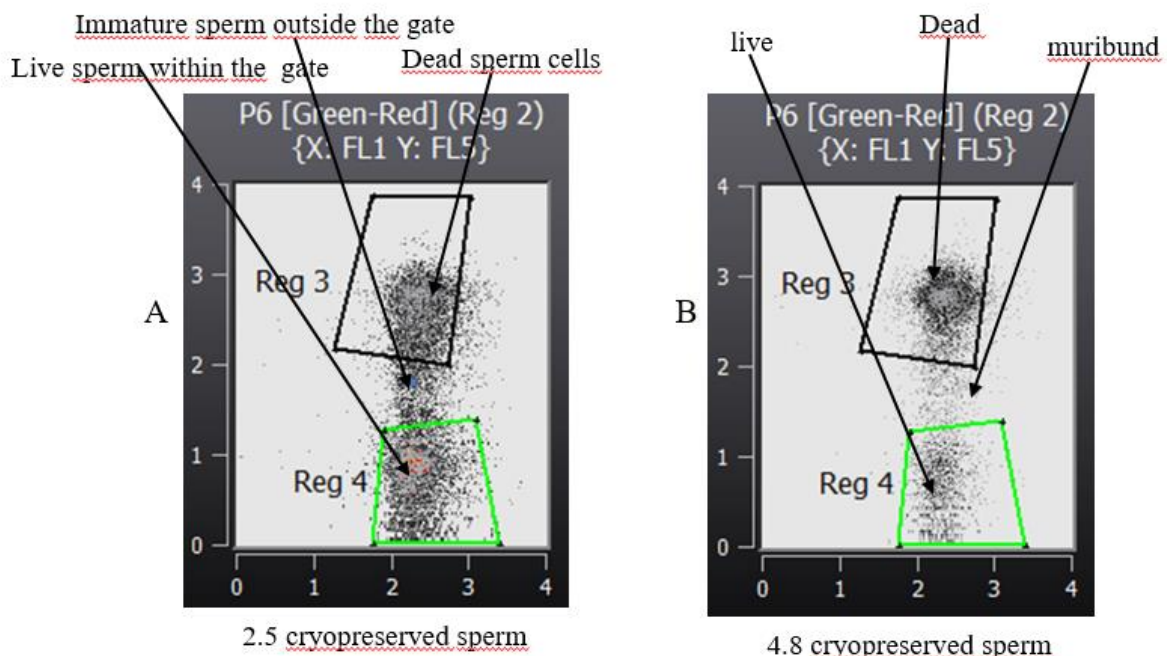**Figure 22**. Images representation for treatment groups post cryopreservation from Flow cytometer (Sysmex-Partec Cube8, Görlitz, Germany) equipped with three lasers 488 nm, 638

nm and 561 nm and a UV LED. (A): Treatment 2.5 cm with sperm cells distribution post-cryopreservation across live and dead regions. (B): Treatment 4.8 cm with less sperm cells within live region and most of the sperm cells represented in dead region post cryopreservation.

3.3.1 Live stained sperm cells

For live stained sperm cells, differences could be observed between the control groups and across all the treatment groups (Fig. 23). Significant difference was observed for male 1 in both 2.5 cm (p<0.001) and 4.8 cm (p<0.001) treatments, in male 3 for both treatments 2.5 cm (p<0.001) and 4.8 cm (p<0.001), in male 4 for treatment 2.5 cm (p<0.001) and 4.8 cm (p<0.001) treatment, and also in male 5 for both of its treatments (p<0.001) and 4.8 cm (p<0.001). However, male 2 only show significant difference in 4.8 cm (p<0.001). For all the groups there were no difference observed between the treatment groups for each male except for male 2 that its treatment groups differ from each other (Fig. 21).
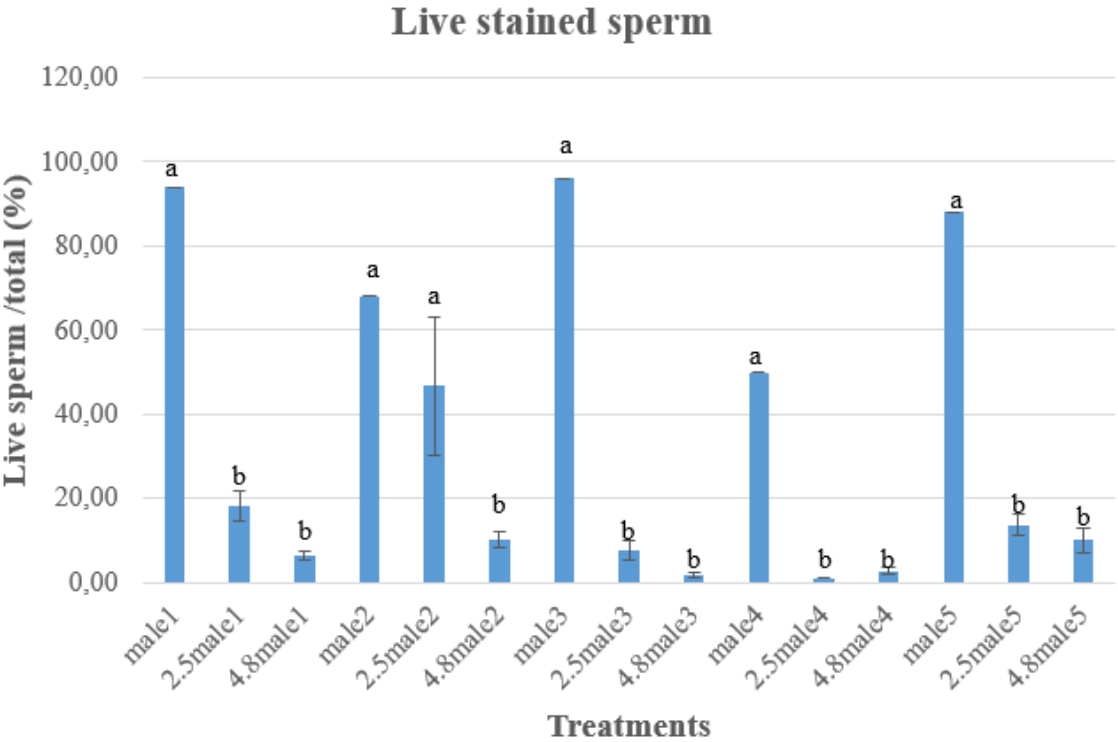
Figure 23. Difference in mean values for live stained sperm cells between the control groups and treatment groups. Error bars indicate standard deviation while differences in letters represents statistical significant difference.

3.4. Egg Fertilization
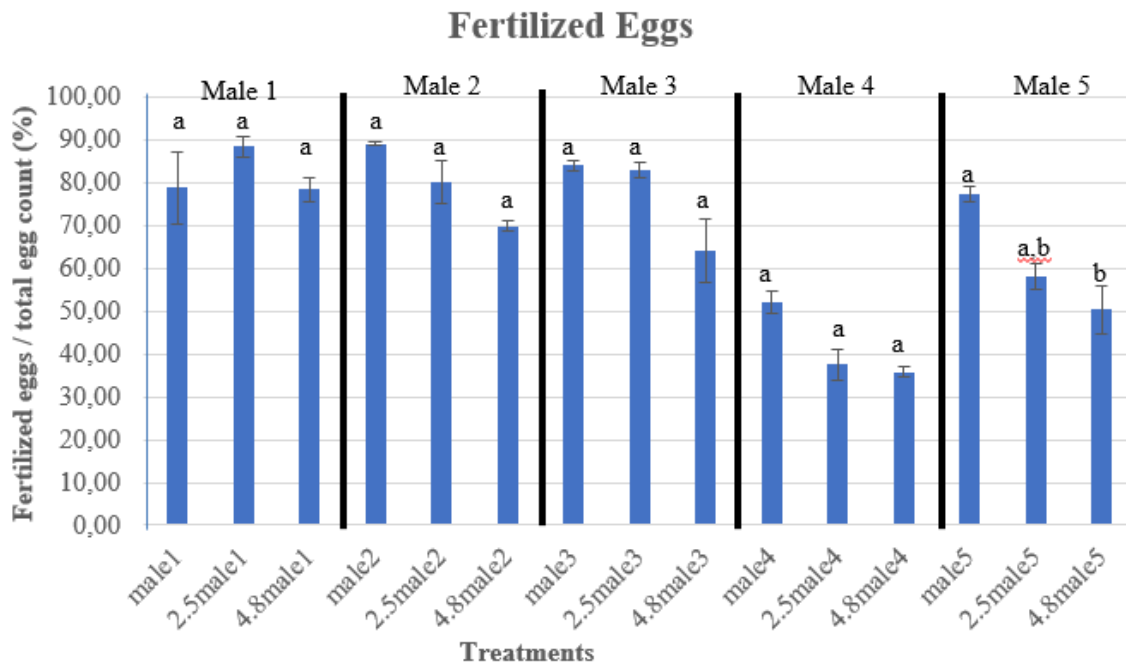
Figure 24. Mean difference values for egg fertilization between the control groups and treatment groups. Error bars indicate standard deviation while statistical difference is shown with differences in letters.

In terms of egg fertilization rates, apart from male 5 where some differences could be observed between control fresh and treated sperm, in all other males the percentage of eggs fertilized with cryopreserved sperm did not differ from their respective controls. As for male 5, only the treatment at 4.8 cm resulted in egg fertilization rates higher ($p < 0.03$) than the control (Fig 22).

4.0 Discussion

The main objective of this thesis was to work towards establishing a protocol for large volume lumpfish sperm cryopreservation in view of improving brood stock management in commercial farming. This was achieved by use of established methods like CASA analysis, flow-cytometry and egg fertilization rates commonly employed for the evaluation of sperm quality following cryopreservation in a wide range of cultured species.

In terms of sperm concentration, the problem of stripping cannot be dissociated from the morphological body structure of lumpfish (Saraiva et al., 2019) which suggests that the only available option of retrieving large volume of sperm from this species would be to sacrifice the fish to meet the requirements of commercial producers. Stripped sperm contains mature sperm cells while sperm obtained from crushed testes is composed of a mixture of mature and immature sperm cells (Beirão et al., 2019). In this experiment the spermatocrit values of milt from crushed testes ranged between 44% and 85%, varying greatly among the 5 males and with one male (M4) being considerably higher than the remaining ones. However, spermatocrit is not as effective in marine fish as in freshwater species because spermatozoa may not sediment properly due to their density being similar to that of seminal plasma inducing variability in results (Beirão et al., 2018). Nevertheless, estimation of sperm concentrations obtained by CASA also varied considerably among the samples, being higher in M4 too. When comparing my results on sperm cell concentration from crushed testes (range: 109-183 x 10^9 cells ml^{-1}) these appear to be considerably higher than the values obtained on stripped lumpfish sperm measured by manual counting by Norðberg et al., (2015) averaging 31.44 x 10^9 cells ml^{-1}. The difference in sperm concentrations between these two works most likely arise from the fact that sperm from stripped males contains only mature sperm while sperm from crushed testes contains also a large proportion of immature sperm cells resulting in higher cells' counts.

Regarding motility parameters, the results shows that the treatment groups generally display a significant drop in motility across all four parameters measured in this experiment compared to their respective controls but with a drop trend observed slightly towards the 4.8 cm treatment more than the 2.5 cm treatment. This is like the result from Norðberg et al., 2015 which shows lower motility for cryopreserved sperm than for fresh milt. This is also comparable to

cryopreservation of halibut, Atlantic cod, ocean pout (*Zoarces americanus*), turbot and other cold-water marine species as reviewed in Suquet et al. (2000), and from Horokhovatskyi, et al. 2018 who observe post-cryopreservation motility recovery dropped relative to the control.

This unique difference observed in male 2 across the four motility parameters and sperm quality suggest that, there is the possibility to cryopreserve lumpfish sperm in large volume around the lower 2.5 cm height. This trend was similar to the Cabrita et al., (2001) who demonstrated faster freezing rate of 5 ml straws of rainbow trout sperm exposed to liquid nitrogen vapor at 2 cm. Similarly, Nomura et al., (2018) has demonstrated freezing height can be significant factor that affect cooling, freezing rate and quality of cryopreserve sperm as they achieved better result for Japanese eel (*Anguilla japonica*) for 2.5 ml and 5 ml at a lower freezing height of 1 cm than the observed drop in sperm quality and freezing rate when freezing height was increased. However, Norðberg et al. (2015) findings contradicts the lower height suggested outcome from this experiment which could be explained because of small volume straws used in their experiment but they were able to get better result at a higher height. The difference between Norðberg et al. (2015) finding and my finding around different height suggest that there is a relationship between volume and freezing height.

In terms of sperm quality, the result from flow cytometry in this experiment shows great drop in the milt quality (% live sperm) basically observe in all the treated groups while there was an increase in the percentage of the DEAD or moribund cells (Appendix 1). For instance, it was only male 2 in 2.5 cm treatment that there was no significant difference for flow-cytometry live-dead sperm viability analysis while significant difference was observed in all other treatments relative to their respective control groups. The comparison between CASA motile and non-motile sperm analysis with flow cytometry live and dead sperm percentage shows corresponding drop in the measured parameters which followed the same trend observed for measured motility parameters. However, it was observed that CASA motile sperm measurement was higher than the live percentages measured by flow cytometer particularly for the treatment groups (Appendix 1). This difference could be attributed to time frame before the analysis of the treatment samples. CASA analysis was made immediately post thaw while flow cytometry samples had to wait for a minimum of twenty-five minutes before analysis could be carried out because of the time required for double staining. The difference in the evaluation

could be the impact of the penetrating cryo-protectant DMSO on the sperm cells resulting from a prolonged exposure (Li et al., 2013). This difference in the values of estimated live sperm cells by flow cytometry may not be enough facts to substantiate the ineffectiveness of the cryopreservation procedure followed in this experiment if judged from indifferent fertilization rate result from both the controls and the treatment groups.

This reduction in the percentage of live cell in the cryopreserved and increase in the dead or moribund cells indicate a negative effect of cryopreservation on the sperm cells and suggest damage to cells morphology. The viability drop in percentage correlates to the reduction obtained from CASA motility analysis which shows significant drop in the percentage of motile cryopreserved sperm. This is comparable to the cryopreservation of red seabream sperm which shows about 30% sperm cells damage post cryopreservation (Liu et al 2007). It is similar to the findings from other species, like the Brazilian flounder (*Paralichthys orbignyanus*), haddock (*Melanogranmus aeglefinus*) and Atlantic cod that showed positive correlation between sperm motility and sperm quality which suggest that the two values follows the same trend whereas, in European catfish (*Silurus glanis*) there was no correlation between sperm motility and sperm viability (Lanes et al. 2008).

In terms of egg fertilization, apart from male 1 where I found some different result for the control which seems lower though not significantly lower (Fig. 24), it could be because the first batch of eggs were observed under a different microscope (Leica WILD M10, 0.63x) where some eggs were possibly not seen as fertilized than the rest of the eggs which were observed under stereomicroscope (Nikon Profile projector V – 12B) which was much better. There were no significant difference between the fertilization rates of the control groups and their treatments groups except in male 4 where there was differences between the control and the two treatment groups. The difference observed in male 4 could be attributed to the low quality of sperm which was suspected to contain less matured sperm (matured and immature sperm cells) judging by its highest sperm concentration than obtained from the other male samples and coupled with the cryopreservation effects.

The volume of sperm to eggs ratio (5-9 x 10^7 sperm cells /egg) used in this experiment could be responsible for the no difference observed between the fertilization rate of the control and the treated groups despite very low CASA motility estimation and very poor sperm quality evaluation of the cryopreserved. The ratio was way too high and has been described as 2-fold

to 10 – fold excess of sperm that would ignore low motility factor of sperm that can prevent egg from fertilization according to Kime et al. (2001). This is comparable to the validation method used about a decade ago when sperm validation test was based on fertilization or hatching rate and not on egg / ratio (Kime et al., 2001). Comparing the ratio of sperm to egg used in this experiment (1: 5-90.000) which was way too high with what Norðberg et al used (1:30.000) although the results were similar in that, there was no significant difference between fertilization rate for the control and the treated groups for the two experiments. This factor could be explained on the basis of high sperm to egg ratio used in this experiment which enhances the meeting of viable sperm with ovum despite reduced cell viability (Lanes et al. 2008).

In comparing the egg / sperm ratio used in this experiment to that of the study conducted on optimal egg / sperm ratio for spotted wolfish (*A. minor*) where they suggest sperm /egg ratio of 5×10^5, the concentration used in my study was about 100 times higher than the optimal recommended for spotted wolfish for artificial inseminations with fresh sperm and about 170-3000 times higher than the one used in lumpfish for cryopreserved sperm (Beirão & Ottesen, 2018). According to Butts et al. (2012) the sperm / egg ratio differ from species to species for instance, for turbot (*Scophthalmus maximus*) the ratio ranges from few thousands of sperm to an egg while millions sperm to an egg in herring (*Clupea harengus*), and for turbot, optimal ratio of sperm to egg is 6000 (Lanes et al. 2008). However, it would have been ideal to use a discriminating sperm ratio to test the viability of the sperm in this experiment between the control and the treated groups which would have been appropriate to justified the findings from Kim et al. (2001) that if 50% of the sperm is immotile, then sperm fertilizing ability would be affected.

5.0 Conclusion

The trend in this study shows that cryopreservation affects the sperm cells and therefore the percentages of live one was significantly reduced in both treatments tested but most in 4.8 cm treatment. All the measured parameters across the groups follow this pattern. If time had accommodated multiple practical, I could have attempt to draw conclusions based on multiple comparison between experiments but instead, a preliminary conclusions could only show a common trend about measurements tend to indicate reduction in motility and quality of the sperm in the treated groups particularly in the 4.8 cm height, This would indicate that further work in this direction should be done focusing on heights around 2.5 rather than 4.8. It would be necessary to work towards establishing the freezing curves which would require incorporation of thermocouple temperature sensor (0.1 mm) into the cryo-straws while freezing. Further still, it would be necessary to include possible correlation between % live sperm and motility in future work. Finally, future work should focus on the optimization of the fertilization protocols in lumpfish by estimating the correct egg/sperm ratio to be used in cryopreservation or other type of fertilization.

The findings in this study has indicated that using individual parameters measured in this study may not be sufficient evidence to reach a conclusion, but overall interactions between motility and sperm quality parameters could be very important criteria to judge spermatozoa viability in determining a successful result (Horokhovatskyi et al, 2018).

This study can therefore preliminarily conclude that cryopreservation protocol in large volume for commercial production is possible.

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APPENDIX 1: CASA analysis of Percentages motile, immotile and progressiveness and non-progressiveness. Flow cytometry analysis of percentage live and dead.

	CASA ANALYSIS						
	Prog+P6:P46ression		Motile	FLWOCYTO		CASA	FLWOCYTO
	Progressive (PR)			% live	Non-progressive (N	Immotile (IM)	%Dead
M1 LIVE	58,51		97,81	94,00	39,30	2,19	0,00
M1 DEAD	44,83		92,89	0,00	48,06	7,11	98,00
M2 LIVE	8,26		53,93	68,00	45,66	46,07	5,00
M2 DEAD	13,94		65,80	0,00	51,86	34,20	99,00
M3 LIVE	13,47		63,73	96,00	50,26	36,27	0,00
M3DEAD	8,61		69,87	0,00	61,26	30,13	84,00
M4 LIVE	4,35		35,87	50,00	31,52	64,13	3,00
M4 DEAD	7,24		47,08	0,00	39,84	52,92	99,00
M5 LIVE	38,31		94,58	88,00	56,27	5,42	1,00
M5 DEAD	26,43		98,13	0,00	71,70	1,87	99,00
2.5male1	6,93		70,19	16,00	63,26	29,81	50,00
2.5male1a	4,09		67,85	25,00	63,77	32,15	48,00
2.5male1b	9,00		86,13	13,00	77,13	13,87	62,00
4.8male1	6,49		69,80	4,00	63,31	30,20	78,00
4.8male1a	3,02		68,41	8,00	65,39	31,59	69,00
4.8male1b	2,96		73,92	7,00	70,97	26,08	76,00
2.5male2	29,76		90,12	14,00	60,37	9,88	62,00
2.5male2a	42,84		95,57	63,00	52,73	4,43	15,00
2.5male2b	26,50		63,44	63,00	36,94	36,56	15,00
4.8male2	22,17		61,01	14,00	38,84	38,99	59,00
4.8male2a	27,18		65,04	8,00	37,86	34,96	66,00
4.8male2b	0,00		46,64	9,00	46,64	53,36	65,00
2.5male3	1,45		38,81	11,00	37,35	61,19	64,00
2.5male3a	0,12		35,42	3,00	35,30	64,58	66,00
2.5male3b	15,29		72,67	9,00	57,39	27,33	67,00
4.8male3	33.64		87,44	1,00	53,80	12,56	81,00
4.8male3a	3,53		54,29	1,00	50,77	45,71	74,00
4.8male3b	2,02		44,48	3,00	42,47	55,52	76,00
2.5male4	6,34		51,87	1,00	45,53	48,13	91,00
2.5male4a	1,20		58,73	1,00	57,53	41,27	91,00
2.5male4b	4,12		71,60	1,00	67,49	28,40	90,00
4.8male4	0,23		27,23	1,00	27,01	72,77	88,00
4.8male4a	35,92		91,21	3,00	55,29	8,79	83,00
4.8male4b	39,53		98,34	4,00	58,80	1,66	82,00
2.5male5	13,67		76,94	18,00	63,27	23,06	63,00
2.5male5a	13,79		73,05	14,00	59,26	26,95	70,00
2.5male5b	18,01		91,57	9,00	73,56	8,43	78,00
4.8male5	4,91		60,00	14,00	55,09	40,00	69,00
4.8male5a	5,98		75,21	4,00	69,23	24,79	87,00
4.8male5b	6,35		77,78	12,00	71,43	22,22	73,00