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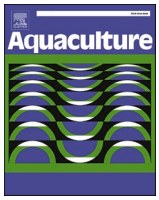

Effects of chilled storage and cryopreservation of sperm, and sperm to egg ratio on fertilization success in lumpfish (*Cyclopterus lumpus*)

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ABSTRACT

Lumpfish males usually release small amounts of milt and are therefore often sacrificed for surgical harvesting of gonads. To optimize the utilization of sperm obtained by dissection of the gonads, a series of small-scale fertilization experiments was carried out in petri dishes. Sperm extracted from homogenized gonads were diluted 1:1 with a commercial extender solution before use (AquaBoost®SpermCoat). Fertilization success was evaluated as percentage eyed eggs ~120 day-degrees post fertilization. Firstly, the effect of chilled storage (3 °C) of diluted milt were evaluated by fertilizing eggs (2.27×10^5 sperm/egg) from two freshly stripped females every 2nd or 3rd day over a 14-day period. Storage time did not affect the fertilization rates which were high throughout the 14-days trial period (89.1–99.8%). Secondly, the effect of sperm to egg ratio of cryopreserved and chilled stored sperm (3 °C for 3 days) on fertilization rate was investigated and compared by fertilizing eggs from three separate females with six different sperm to egg ratios (1.25×10^5 to 2×10^9). For both storage methods, only the lowest sperm to egg ratio (1.25×10^5) resulted in a significant lower but still high fertilization rate (80.7%). Using freshly extracted sperm at lower sperm to egg ratios (1.25×10^5 to 3.1×10^4) to fertilize eggs from three females revealed, however, no relationship between sperm to egg ratio and fertilization rates as they were high for all ratios (91.2–93.4%). Two additional experiments were undertaken at a real scale to validate the petri dish results at higher egg numbers and volumes. A fixed volume of 40 ml eggs (~4800 eggs) was fertilized with four sperm to egg ratios ranging from 3.1×10^4 to 5×10^5. All sperm to egg ratios resulted in high fertilization rates ranging from 93.7 to 99%. In the second real scale experiment increasing volumes of eggs (1 up to 160 ml) were fertilized with a fixed sperm to egg ratio (2.5×10^5). The fertilization success (95.9–97.5%) was not affected by egg volume. In conclusion, the current study showed that lumpfish sperm can be chilled stored at 3–4 °C for 14 days and can be cryopreserved and thawed without any significant reduction in fertilization success under the conditions used in this study. Compared to other marine teleosts, lumpfish require a low sperm to egg ratio for successful fertilization, but the critical low ratio to obtain high fertilization was not revealed in this study.

1. Introduction

Lumpfish (*Cyclopterus lumpus* L.) and wrasses are frequently used as a biological means to reduce the sea lice infestation problem in the Atlantic salmon farming. As lumpfish tolerate lower temperature than wrasses their implementation was initially in the northern parts of Norway (Imsland et al., 2014), Scotland (Treasurer, 2018), the Faroe Islands (Eliasen et al., 2018) and Iceland (Steinarson and Árnarson, 2018).

Initially, lumpfish eggs and sperm were collected from wild caught

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brood fish only. As the demand for lumpfish increased, there has been a gradual shift towards an intensive production using farmed brood stock, and the first breeding program for lumpfish started in 2017 (Imsland et al., 2021). In 2021 approximately 27.5 million lumpfish, all farmed juveniles, were deployed in salmon cages in Norway alone (Fiskeridirektoratet, 2022).

In commercial production of lumpfish multiple females are stripped for eggs at the same time. Lumpfish males release none or very small volumes of milt when hand stripped in captivity and these small volumes are inadequate for fertilizing eggs on a commercial scale. Our own observations are consistent with Norðberg et al. (2015) who reported that no >3 ml of milt could be extracted from any one male. Because of this, milt is usually collected post-mortem after euthanasia as the gonads are ground to extract larger milt volumes (Jonassen et al., 2018). This method has also been used for zebrafish and other small sized fish species (Sun et al., 2010; Diogo et al., 2019), but is a time-consuming process. Thus, efficient milt storage methods are essential for a better utilization of the limited volumes of lumpfish milt.

Both chilled storage (short-term) and cryopreservation (long-term) can be used for sperm preservation in aquaculture (Migaud et al., 2013). Short-term storage requires storage of milt in extenders (Ladoktha et al., 2017; Vuthiphandchai et al., 2009) at low temperatures to reduce sperm metabolism (Babiak et al., 2006; Mansour et al., 2004). Temperatures ranging from close to 0 °C to 4 °C are recommended (Gallego and Asturiano, 2019; Babiak et al., 2006; Mansour et al., 2004) and the most common approach is constant stirring or rocking of the milt samples and use of open shallow containers for air exchange (Santos et al., 2018; Dorsey et al., 2011). Pountney et al. (2020a) tested five different milt extender solutions for short-term storage of lumpfish milt; Modified Turbot Extender (Babiak et al., 2006), Herring Ringers Solution (Pillai et al., 1994), AquaBoost®SpermCoat, Mounibs solution (Mounib, 1978) and Mounib's with 1% BSA inclusion. Sperm stored in the two Mounib's solutions lost their motility after 7 days storage while the motility of sperm stored in the other three extenders was significantly reduced after 7 days with a further significant reduction after 14 days (79.8%–98.9%).

Cryopreservation is an effective long-term storage of sperm which is useful for several purposes such as synchronization of gamete availability of both sexes, more efficient use of the total volume of available milt, more simple off-season spawning (Contreras et al., 2019), as well as safe transport of sperm of high genetic quality for e.g. genetic purposes. This method is widely used for a number of other marine fish species e.g. Atlantic haddock (*Melanogrammus aeglefinus*) (Rideout et al., 2004), Spotted wolffish (*A.minor*) (Gunnarsson et al., 2009), Brazilian flounder (*Paralichthys brasiliensis*) (Lanes et al., 2008) and turbot (*Scophthalmus maximus* L.) (Suquet et al., 1995; Dreanno et al., 1997; Suquet et al., 1998; Chereguini et al., 1999).

Norðberg et al. (2015) made the first attempt at cryopreservation of lumpfish sperm using three different variants of Mounib's solutions as milt extenders. The modified Mounib's no GHS solution resulted in the best milt motility recovery after thawing. The fertilization rates were quite low with no significant difference in fertilization rates between eggs fertilized with fresh (30.8%) and cryopreserved sperm (27.3%).

Imsland et al. (2022) tested cryopreservation of lumpfish sperm using the protocol described by Opeifa (2019). This includes a different milt extender (wolffish extender) than the ones used by Pountney et al. (2020a) and Norðberg et al. (2015). The percentages of fertilized eggs were high for both fresh (92.6%) and cryopreserved sperm (77.9%) and thus considerably higher than what was reported by Norðberg et al. (2015). The percentage of eyed eggs was significantly higher with fresh sperm (94%) than cryopreserved sperm (81%) while differences in hatching rate and larval growth for 14 days post hatch were non-significant Imsland et al. (2022).

Identifying an optimal sperm to egg ratio (n° of sperm/egg) is important to avoid wasting sperm, especially in species releasing small volumes of milt as is the case for lumpfish, and to reduce the number of male brood fish needed. Excess sperm on the other hand can inhibit fertilization (Levanduski and Cloud, 1988, Rurangwa et al., 2005). The optimal sperm to egg ratio varies between fish species and individuals and may also be influenced by the sperm quality (Rurangwa et al., 2004). The minimum sperm to egg ratio required for successful fertilization rates varies from 10^2 to 10^3 in several fish species e.g., turbot *Scopthalmus maximus* (Chereguini et al., 1999), pufferfish *Takifugu niphobles* (Gallego et al., 2013), African catfish *Calrias geriepinus* (Rurangwa et al., 1998) to $>10^5$ sperm per egg in some marine species like Atlantic halibut *Hippoglossus hippoglossus* (Tvedt et al., 2001) and Atlantic cod *Gadus morhua* (Butts et al., 2009; Babiak et al., 2012). So far there is no information in the literature about the optimal sperm to egg ratio for lumpfish.

The purpose of the present study was to a) develop a reliable method for chilled storage of lumpfish milt, b) refine the protocol for use of cryopreserved milt with the aim of obtaining similar fertilization rates as when using fresh or chilled milt and c) optimize the sperm to egg ratio regarding fertilization success.

2. Material and methods

This study includes three small-scale and two real-scale fertilization experiments with lumpfish eggs. In the small-scale experiments a low number of eggs were incubated in petri dishes while flow-through incubators were used for incubation of a higher number of eggs in the real-scale experiments. The last two experiments were done to verify that the petri dish results are valid also with larger egg volumes.

2.1. Broodstock

Wild lumpfish brood fish were caught by gill nets at the coast of Norway during the peak spawning season in March and transported for two hours in a 1 m^3 tank with oxygen supplementation to the Nofima research facility in Sunndalsøra, distributed to 4 m^3 tanks, fed to satiation using a commercial feed (Silk 4.5 mm, Skretting, Nutreco N·V, Netherlands) at ambient temperature (6.5–8.0 °C) under low light intensity. Sex was determined using ultrasound imaging (6.5 mHz, Log.Q book XP vet, GE medical systems, USA) of the body cavity, and the ratio females:males was approximately 3:1. The age of the brood fish was unknown as they were all wild caught. Mean weights of females was approximately 1200 g and of males 500 g.

Before stripping of eggs, maturation status was assessed both visually and using ultrasound imaging of the body cavity using a five-point scale as described by Pountney et al. (2020b). Females were considered ready for spawning when the gonads were significantly enlarged, single ovarian lobes filled the image and free hydrated oocytes were apparent on the dorsal region of the ovarian lobe.

2.2. Gamete collection

Flumequin (75 ml/100 l of seawater) was used to anaesthetize the female brood fish before the females were hand stripped and the eggs stored at 4 °C until fertilization within 3–4 h after stripping. The males were killed by an overdose of anesthetics before dissection of the gonads to collect the milt. Blood vasculature and connective tissue were removed using a scalpel before grinding the gonads twice. Then the sperm/gonad mix was passed through a tea strainer to get rid of any remaining debris. The milt was finally diluted 1:1 with a commercial extender (AquaBoost®SpermCoat) and stored in cell flasks at 3 °C. The flasks were tilted gently by hand three times a day or placed on a shaking machine to allow the sperm cells sufficient access to oxygen during storage.

2.3. Assessment of sperm concentration and motility

A SDMG Photometer, calibrated for lumpfish milt, was used to determine the sperm cell concentrations (n° of sperm/ml). This is a

quick and simple method, frequently used for salmon milt. Prior to the measurement, 20 μl of milt was diluted in 4 ml physiological saltwater (9 ppt NaCl). The motility of the sperm was measured using a microscope with 10 × magnification. A milt droplet was placed on a microscope slide with a cover glass placed on the top of the droplet. A small volume of 3 °C seawater was added to the edge of the cover glass while looking into the microscope. Seawater activates the sperm, and an immediate reaction can be seen through the microscope when the motility is high. The motility of the sperm cells was evaluated subjectively using a score from 1 to 3 (1 ≤ 50%, 2 = 50–75%, and 3 = 75–100% motile sperm). Only sperm with motility score 3 was used in the experiments.

2.4. Calculation of fertilization rates

Lumpfish eggs are highly pigmented, which makes it hard to observe early cell division. For this reason, the fertilization success (n° *eyed eggs/ total n° fertilized eggs) * 100)* was evaluated at the eyed egg stage (Fig. 1) approximately 120 day-degrees after fertilization at 8 °C, i.e.,15 days post fertilization. Imsland et al. (2022) showed a strong correlation between percent fertilized and eyed lumpfish eggs.

Photos were taken of each replicate petri dish (approximately 100 eggs/dish) at the eyed-egg stage for Experiment 1, 2, 3 and 4. For Experiment 5, photos were made of a random sample of about 300 eggs from each of the six different volumes of eggs (147 from the lowest volume).

2.5. Water treatment

All incoming seawater was filtered through a 10 μm drum filter and UV-treated. The seawater used for incubation in petri dishes was additionally filtered through 1 μm cartridge filters followed by additional UV treatment and finally filtered through a 0.2 μm filter by suction.

2.6. Overview experiments

Table 1 gives an overview of the experimental conditions for each of the five experiments included in this study.

2.7. Experiment 1: Chilled conservation of sperm

The aim of this experiment was to investigate how long lumpfish sperm can be stored before the quality of the sperm deteriorates.

Gonads were dissected from five males, ground and strained through a tea strainer. The milt from all five males had high sperm concentrations and motility score 3. After assessment of sperm concentration and motility, an equal amount of milt from each of the five males was mixed and diluted 1:1 in AquaBoost®SpermCoat and stored in cell flasks at 3 °C the following 14 days. The sperm concentration of the pooled and diluted milt solution was then measured (2.3×10^5/ml). An equal portion of the pooled and diluted milt was used to fertilize eggs from two different new females separately every 2nd or 3rd day over a period of 14 days, i.e., 14 different females in total.

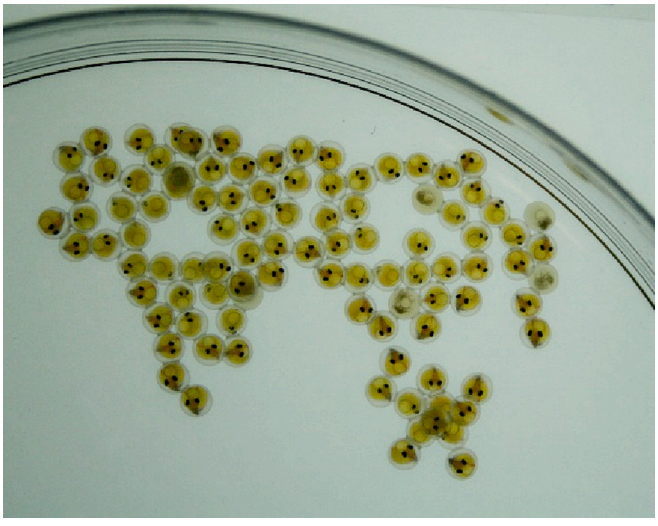

Fig. 1. Photos of eyed eggs were used to calculate the percentage of eyed eggs in lumpfish.

At each of the seven fertilization events (See Table 1 for details), eggs from two females were fertilized and incubated separately to elucidate the effect of possible differences in egg quality between females. 1 ml of eggs (approximately 120 eggs) from each of the two females were distributed into each of four petri dishes, i.e., 8 petri dishes in total per fertilization event. Because the lumpfish eggs get sticky and attach to each other on contact with seawater and form dense lumps within a few minutes (Davenport, 1985), a sterile spatula was used to gently position the eggs into one single layer. 20 μl of the pooled milt solution was quickly mixed with 1 ml of seawater and poured over the eggs. Three minutes later, the eggs were rinsed three times with seawater to remove the excess milt. Finally, 20 ml of seawater with 10% of Penicillin-Streptomycin antibiotics (VWR) was added to each of the 112 (= 14 × 8) petri dishes. The petri dishes were stored at 8–9 °C until the eyed egg stage at 120 day-degrees, i.e., approximately 15 days post fertilization. During this period, the water in the petri dishes was changed twice a week by sucking off water with a large syringe (50 ml) before refilling with 20 ml seawater with 10% of Penicillin-Streptomycin.

2.8. Experiment 2: Cryopreserved vs. chilled sperm and sperm to egg ratio

The aim of this experiment was to compare the fertilization success using chilled stored or cryopreserved sperm, each tested with six different sperm to egg ratios using pooled milt from three males.

Cryopreserved or chilled stored sperm was used to fertilize a random sample of 68 ml eggs (approx. 8100 eggs) from three separate females at six different sperm to egg ratios, i.e., 3 replicates/female per sperm to egg ratio/ and sperm storage method. Eggs from three females were fertilized separately to elucidate the effect of varying egg quality of females on the fertilization success.

The milt was extracted from gonads as described in Experiment 1. Half of the milt volume from each of the four males was stored at 3 °C in the fridge for three days, while the other half was sent overnight on ice to Cryogenetics for cryopreservation. Milt from one male was discarded due to low sperm concentration (6.8 x 10^9sperm/ml) and poor motility. The sperm concentration of the three males used for cryopreservation was 17.3×10^9, 13.5×10^9 and 12.4×10^9 sperm/ml. The milt from each of these three males was frozen in 0.5 ml straws with a standardized concentration of 2×10^9 sperm per ml, i.e., 1×10^9 sperm per straw.

Chilled or thawed cryopreserved sperm from the three males was mixed before fertilizing the eggs. Both the chilled and the cryopreserved sperm was diluted in 50 ml of AquaBoost®SpermCoat to obtain double volumes of the sperm solution (120, 60, 45, 30, 15 and 7.5 μl), equivalent to 2×10^6, 1×10^6, 7.5×10^5, 5×10^5, 2.5×10^5 and 1.25×10^5 sperm/egg, respectively. Three females were stripped for eggs on the day the cryopreserved sperm returned to Nofima, i.e., three days after dissection of the male gonads.

Firstly, the six predetermined sperm densities of chilled sperm were used to fertilize (as described in chapter 2.7) two replicates each of 1 ml eggs from each of the three females, i.e., in total 6 replicates per sperm density. Thereafter, the cryopreserved sperm was used to fertilize 6 replicates from the same three egg batches as used for the chilled sperm. The cryopreserved sperm was thawed in a water bath at 25 °C for 30 s. Apart from the thawing procedure, the procedures for egg fertilization and evaluation of fertilization success were the same as for fertilization with chilled sperm. Due to the high number of treatments and replicates,

Table 1
Experimental design of the five experiments.

		Incubation		Numbers			Temperature		
Exp. no.	Title	Units	Volume	Males	Females	Replicates	Sperm	Eggs	Sperm to egg ratio
1	Chilled conservation of sperm	Stagnant	20 ml	5 single	2/ fertilization	8	3	8–9 °C	Fixed: 2.27×10^5
2	Experiment 2 Cryopreserved vs. chilled sperm and sperm to egg ratio	Stagnant	20 ml	3 mixed	3 separate	6	3 & cryo	8–9 °C	2×10^6, 1×10^6, 7.5×10^5, 5×10^5, 2.5×10^5, 1.25×10^5
3	Sperm to egg ratio – fresh sperm	Stagnant	20 ml	2 mixed	3 separate	6	3	8–9 °C	1.25×10^5, 6.2×10^4, 3.2×10^4
4	Different sperm to egg ratios tested with a larger egg volume	Flow through	3 L	3 mixed	3 separate	2	3	8–9 °C	5.0×10^5, 2.5×10^5, 1.25×10^5, 3.1×10^3
5	Effect of increasing egg volume on fertilization success	Flow through	3 L	3 mixed	3 mixed	1	3	8–9 °C	Fixed: 2.5×10^5

the fertilization process was done with no >20 petri dishes at a time.

2.9. Experiment 3: Sperm to egg ratio – Fresh sperm

Because none of the males used for Experiment 2 released running sperm, the effect of sperm to egg ratio when using fresh sperm was tested separately. Because also the lowest sperm to egg ratio tested for Experiment 2 (1.3×10^5 sperm/egg) resulted in high fertilization rates (87–95%), 1.25×10^5 sperm/egg was chosen as the highest ratio followed by 6.25×10^4 and 3.1×10^4 sperm/egg in this experiment. To elucidate a possible effect of differences in egg quality between females, each of the three sperm to egg ratios were used to fertilize two random samples of eggs from each of three females, i.e., 6 replicates per each sperm to egg ratio.

Gonads were extracted from two males and prepared as described for Experiment 1. After counting of sperm density (sperm/ml) and check of sperm motility, an equal amount of sperm from each of the two males was mixed and diluted in 50 ml of AquaBoost®SpermCoat. The sperm concentration for each male was standardized to 2×10^9/ml, and the dilution volume was determined using the formula ((sperm concentration/2×10^9) x 2).

1 ml of sperm was then added to this volume of AquaBoost®SpermCoat. The three desired sperm to egg ratios (3.1×10^4, 6.25×10^4 and 1.25×10^5 sperm/egg) were obtained by using 3.5, 7.5 and 15 μl of the diluted sperm solution per approximately 120 eggs (1 ml). A sample of 1 ml eggs from each of the three females was transferred to each of 6 petri dishes, i.e., 6 dishes per sperm to egg ratio, 18 in total. The eggs were fertilized as explained in paragraph 2.4.

2.10. Experiment 4: Different sperm to egg ratios tested with larger egg volumes

The eggs in Experiments 1, 2 and 3 were all fertilized in petri dishes, each containing approximately 120 eggs (1 ml). The present experiment was done to verify that the fertilization rates obtained in the three small-scale experiments are valid also with a much higher number of eggs per batch.

Four sperm to egg ratios (5×10^5, 2.5×10^5, 1.3×10^5, 3.1×10^4 sperm/egg) were used to fertilize 40 ml of eggs, i.e., approximately 4800 eggs. Eggs from two females were used to elucidate possible effects of female variability in egg quality. The eggs were incubated in small flow-through incubators with two replicate incubators per female and sperm to egg ratio, i.e., 4 replicates per sperm to egg ratio.

Gonads were dissected from three males, ground and strained. After measuring sperm concentration and assessment of sperm motility, the milt was diluted in 50 ml AquaBoost®SpermCoat to enable fertilization with low sperm to egg ratios. The sperm density after dilution was 3.7×10^9/ml. The four desired sperm to egg ratios were obtained using 4.1, 16.1, 32.2 and 64.5 μl of the diluted sperm solution per ~4800 eggs.

40 ml of eggs from each female were distributed to each of eight perforated aluminum boxes, i.e., 16 aluminum boxes in total. The egg mass was flattened with a spatula to form a thin layer of eggs. Each perforated box was placed in a plastic box to ensure good contact among eggs, sperm, and water during the fertilization process.

Each of the four predetermined volumes of diluted milt was added to approximately 50 ml of water and shaken quickly before the milt solution was poured over the eggs. 2–3 min later, more seawater was added until the water covered the eggs. Five minutes later, the eggs were rinsed three times with seawater. After rinsing, the perforated boxes with eggs were transferred to incubator cells and incubated at 8–9 °C until the eyed stage (120 day-degrees).

2.11. Experiment 5: Effect of egg volume on fertilization success

The objective of this experiment was to investigate if an increasing number of eggs affects the fertilization success when being fertilized with a fixed number of sperm (2.5×10^5 sperm per egg). Six different volumes of eggs 1, 10, 20, 40, 80 and 160 ml, were tested corresponding to approximately 120, 1200, 2400, 4800, 9600, 19,200 eggs, respectively.

Gonads were dissected from three males, ground and strained, after which an equal number of sperm from each male was mixed following a sperm motility chec and diluted 1:1 in AquaBoost®SpermCoat.

An equal number of eggs from two females were mixed before fertilization. The eggs were then distributed to six perforated aluminum boxes placed in a plastic box, one box per egg volume. A ratio of 2.5×10^5 sperm per egg was used for fertilization of the eggs. A predetermined volume of milt was mixed quickly with an equal volume of seawater and poured over the eggs. After 2 min, additional water was added until all eggs were covered. The eggs were left for another 5 min before rinsing three times with seawater. Fertilization of the lowest egg volume (1 ml) was done in a petri dish. Five minutes after fertilization, these eggs were moved from the petri dish into a perforated box. The perforated boxes with eggs were then transferred to flow-through incubators and incubated at 8–9 °C until the eyed egg stage. At the eyed egg stage (120 day-degrees), photos were taken of a random sample of about 300 eggs from each of the six volumes of eggs (147 eggs from the lowest volume) from which the fertilization rate of each egg volume was calculated.

2.12. Statistical analyses

Experiment 1: Chilled storage of sperm.

The effect of day of fertilization and female on percent eyed eggs was analyzed by the following model:

$$y_{ijk} = D_i + F_{ij} + e_{ijk} \quad (1)$$

where

y_{ijk} is the fertilization rate; D_i is the fixed effect of day i ($i = 1, 2,.., 7$);

F_{ij} is the fixed effect of female j ($j = 1, 2$) on day i ($i = 1, 2,.., 6$); and e_{ijk} is the random error effect of the petri dish k within F_{ij} and D_i.

Experiment 2: Cryopreserved vs. chilled stored sperm and number of sperm to egg ratio.

The effects of storing the sperm (cold vs. cryopreserved) and of the number of sperm to egg on fertilization rate were analyzed by the following model:

$$y_{ijkl} = TS_i + NS_j + TSxNS_{ij} + F_k + e_{ijkl} \quad (2)$$

where

y_{ijkl} is the fertilization rate; TS_i is the fixed effect of type of sperm storage i ($i = 1, 2$); NS_j is the fixed effect of the number of spermatozoa/egg j ($j = 1, 2,.., 6$); $TSxNS_{ij}$ is the fixed interaction effect between TS_i and NS_j; F_k is the fixed effect of female k ($k = 1, 2, 3$); and e_{ijkl} is the random error effect of the replicated petri dish l within $TSxNS_{ij}$ and F_k.

Experiment:3 Sperm to egg ratio – fresh sperm.

The effect of a lower sperm to egg ratio (than in Experiment 2) on fertilization rate was analyzed by the following model:

$$y_{ijk} = NS_i + F_j + NSxF_{ij} + e_{ijk} \quad (3)$$

where

y_{ijk} is the fertilization rate; NS_i is the fixed effect of number of sperm to egg ratio i ($i = 1, 2, 3$); F_{ij} is the fixed effect of female j ($j = 1, 2$); $NSxF_{ij}$ is the fixed effect of the interaction between NS_{ij} and F_{ij}, and e_{ijk} is the random error effect of petri dish k (k = 1, 2, 9) within NS_i and F_j.

Experiment 4: Different sperm to egg ratios with larger egg volumes.

The effect of fertilizing a much higher number of eggs per batch (than in Experiment 1, 2 and 3) with four different of sperm to egg ratios (5.0×10^5, 2.5×10^5, 1.25×10^5, 3.1×10^4) was analyzed by the same model as in Experiment 3 (Model 3).

Experiment 5: Effect of egg volume on fertilization success.

The effect of fertilizing an increasing number of eggs with a fixed number of sperm to egg on the fertilization rate was analyzed using a simple linear regression model with number of eggs as a covariate.

The five statistical models were run using the Proc GLM procedure in SAS/STAT software, Version 9.4 for Windows. Copyright© SAS Institute Inc.

3. Results

3.1. Experiment 1: Chilled storage of sperm

The effect of storage time on percent eyed eggs was not significant different from zero ($p = 0.526$), and the percentage of eyed eggs was high (89.1–99.8%) for all groups except for eggs from one of the two females used at day 3, 10 and 14 with 32.7, 67.2 and 65.1% eyed eggs, respectively (Fig. 2).

3.2. Experiment 2: Cryopreservation vs. chilled stored sperm and sperm to egg ratio

The interaction effect between sperm storage method (chilled or cryopreserved) on percent eyed eggs was not significantly different from zero ($P = 0.91$) and therefore omitted from the model. Neither the effect of storage method nor female affected the percent eyed eggs significantly ($P = 0.55$ and $P = 0.15$, respectively).

Number of sperm to egg had a significant ($P = 0.004$) effect on percent eyed eggs because of the lowest sperm to egg ratio (1.25×10^5 sperm/egg). For this ratio, percent eyed eggs were 78.9% for chilled sperm and 82.6% for cryopreserved sperm. For all other sperm to egg ratios the estimated percentage of fertilized eggs ranged from 93.2 ± 1.1 to 94.9 ± 1.1% (Fig. 4).

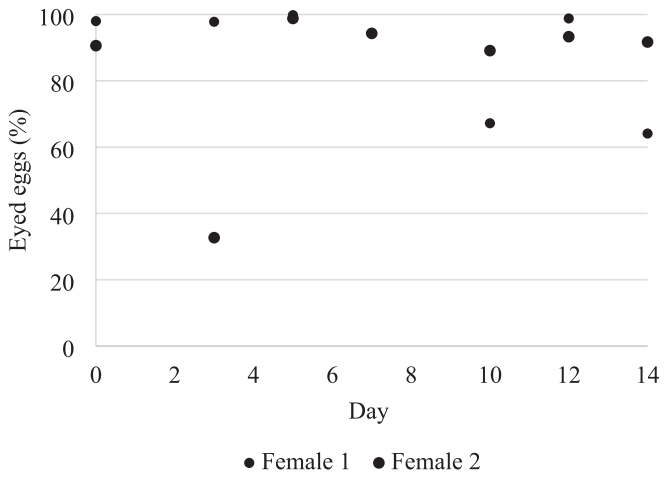

Fig. 2. Percentage eyed eggs for 14 batches of lumpfish eggs fertilized with pooled milt from four lumpfish males at seven different days post dissection of gonads. The eggs were hand stripped from two new females on day of dissecting the gonads (day zero) and every 2nd or 3rd day thereafter until day 14.

3.3. Experiment 3: Sperm to egg ratio – Fresh sperm

The interaction effect between the sperm to egg ratio and female on percent eyed eggs was not significantly different from zero ($P = 0.538$) and was therefore omitted from the model. The mean percentages of eyed eggs were all high ranging from 91.2 to 93.4% (Fig. 3). Neither number of sperm/egg nor female affected the percentage of eyed eggs were not significantly ($P = 0.43$ and $P = 0.19$, respectively).

3.4. Experiment 4: Different sperm to egg ratios with larger egg volumes

The mean percentages of eyed eggs were all very high, ranging from 93.7 to 99.0% for the lowest to the highest number of sperm per egg (Fig. 4), and was 95.9 and 97.5% for each of the two females. The interaction effect between the sperm to egg ratio and female on percentages of eyed eggs was not significantly different from zero ($P = 0.53$) and was therefore omitted from the model. Neither the effect of number of sperm/egg nor female affected the percentage of eyed eggs significantly ($P = 0.12$ and $P = 0.32$, respectively).

3.5. Experiment 5: Effect of egg volume on fertilization success

Fertilizing an increasing number of eggs (a 160-fold increase) with a fixed number of sperm to egg did not affect the percentage of eyed eggs

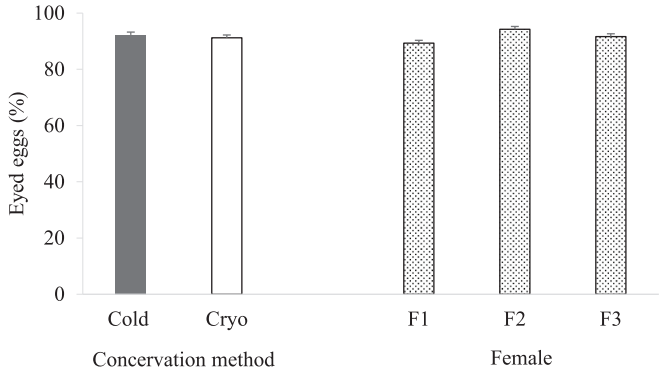

Fig. 3. Percentage of eyed eggs for lumpfish eggs originating from three females and fertilized either with chilled (white) or cryopreserved sperm (black). The three dotted columns show the mean results (chilled and cryopreserved sperm) for each female. Bars indicate standard errors of the estimates.

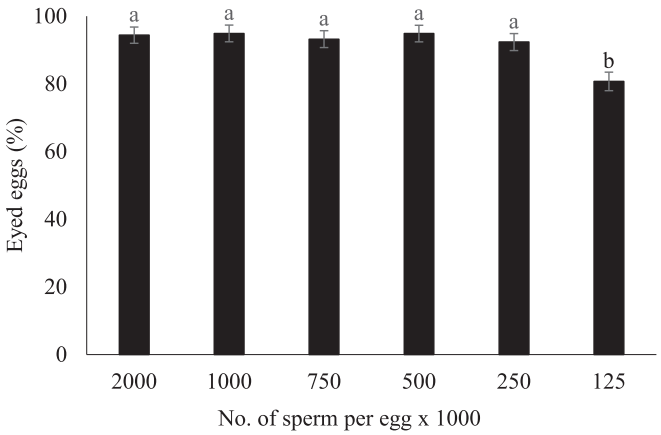

Fig. 4. Percentage of eyed eggs for lumpfish eggs fertilized with different sperm to egg ratios. Bars indicate standard errors of the estimates. Different letters indicate significant differences ($P = 0.004$).

(>97%) significantly ($P = 0.70$).

4. Discussion

Generally, the fertilization rates measured as percentage of eyed eggs were high in all experiments, showing that both chilled and cryopreserved lumpfish sperm has a high fertilization capacity when stored properly.

4.1. Chilled storage

Chilled storage of milt can be used for short to medium-term periods while preserving the sperm fertilizing ability (Bobe and Labbe, 2008). The survival of chilled stored sperm is species specific and varies from a few hours in seabass (Stoss and Holtz, 1983) to 95 days in Atlantic halibut (*Hippoglossus hippoglossus*) (Babiak et al., 2006).

Dilution of sperm in a physiological solution (extender) improves the sperm storage capacity due to improved oxygen supply to each sperm cell, and a good extender keeps the sperm cells inactive, thus reducing their need for oxygen (Beirão et al., 2019). This has been documented in several species like e.g., seabass *Disentrarchus labrax*, turbot *Scopthalmus maximus and* cod *Gadhus morhua* L (Bobe and Labbe, 2008). In the present study, a commercial extender (AquaBoost®SpermCoat, dilution ratio 1:1) was successfully used resulting in fertilization rates >90% up to 14 days of chilled storage (3 °C). The current experiment was done at the end of the spawning season and ended after 14 days because no more females released eggs after that. However, the results indicate that lumpfish sperm can be stored for two weeks without any loss in their ability to fertilize eggs under the conditions used in the present study. Pountney et al. (2020a) reported a 93.3% reduction in sperm motility after 14 days chilled storage in AquaBoost®SpermCoat. One explanation for the significant reduction in sperm motility could be that they used a sperm to extender dilution of 1:5 instead of the recommended 1:1 dilution. In the present study the motility scores were high (3) for all sperm used to fertilize eggs during the 14-days trial period. Saad et al. (1988) showed that sperm in carp (*Cyprinus carpio*) underwent morphological changes during chilled storage causing a reduction in the fertilization rate. Apparently, lumpfish sperm used in the present study did not start to deteriorate during the 14 days of chilled storage. However, it must be considered that the high sperm to egg ratio used in the present study may have contributed to the high fertilization success throughout the 14-days experimental period. The significant variation in fertilization success between individual females at day 3, 10 and 14 confirms that both egg, and sperm quality are of importance for the fertilization success.

4.2. Cryopreservation

The freezing/thawing process can damage sperm at several levels such as plasma membrane, mitochondria, and cell morphology (Cabrita et al., 2001, 2005; Gallego et al., 2012) and ultimately affect both cell viability and fertilization capacity. The motility rate of frozen-thawed sperm is generally higher in marine fish species (Suquet et al., 2000) than in freshwater species such as rainbow trout (Lahnstainer et al., 1996), carp (Babiak and Glogowski, 1997) and tilapia (Chao et al., 1987). In seabass *Dicentrarchus labrax*, the fertilization capacity of frozen-thawed sperm was approximately 65% of that of fresh sperm (Fauvel et al., 1998), and in turbot *Scophthalmus maximus* approximately 83% (Dreanno et al., 1997). In the current study there was no significant difference between the two storing methods, and the fertilization rates were high for both chilled (92.2%) and cryopreserved (91.2%) sperm. This minor difference corresponds well with the findings of Norðberg et al. (2015), who showed only a 3.5% reduction in fertilization success for cryopreserved as compared to fresh lumpfish sperm, although the fertilization rates were generally low (27.3 and 30.1%, respectively). Nordberg et al. (2020 tested three different variants of Mounib's as sperm extenders and found a similar, but smaller reduction (24.1%) in sperm motility recovery after thawing like the one Pountney et al. (2020a, 2020b) found. The minor difference in fertilization success found by Norðberg et al. (2015) could be due to surplus sperm per egg as they used a 3.0×10^4 sperm to egg ratio, corresponding to the lowest ratio used in the present study. Imsland et al. (2022) achieved high, but significantly different percentages of eyed eggs for fresh and cryopreserved sperm (94% and 81%, respectively). They used a very high sperm to egg ratio ($11–18 \times 10^7$) compared to Norðberg et al. (2015) and the ones used in the present study (3.1×10^4 to 2×10^9). The high fertilization success and minor differences between the two storage methods in the present study and the studies by Norðberg et al. (2015) and Imsland et al. (2022) demonstrate that cryopreservation can be used for conservation of lumpfish sperm. It should, however, be considered that possible negative effects of cryopreservation may be uncovered at even lower sperm to egg ratios.

4.3. Sperm to egg ratio

The minimum required sperm to egg ratio for high fertilization varies among fish species and seem to be higher in marine fish species compared to freshwater species (Beirão et al., 2019). In the present study there was a significant reduction in percentage eyed eggs at the lowest sperm to egg ratio (1.25×10^4) in Exp. 2 using cryopreserved and chilled stored sperm. In the follow-up experiment (Exp. 3) sperm from newly harvested gonads was used for fertilization of the eggs at even lower sperm to egg ratios. However, sperm to egg ratio did not affect the fertilization rates, and even the lowest ratio (3.1×10^4) resulted in 91.1% eyed eggs. This indicate that both cryopreservation and chilled storage may have a small negative effect on the fertilization ability compared to fresh milt. The results also indicate that we did not reach a minimum sperm to egg ratio required to obtain high fertilization in lumpfish.

5. Conclusion

In conclusion, the current study showed that lumpfish sperm can be chilled stored at 3–4 °C for 14 days without any significant reduction in fertilization success when using an appropriate extender, and that cryopreservation can be used for long-term storage of lumpfish sperm. The results also indicate that a low sperm to egg ratio is required to obtain high fertilization in lumpfish. It must, however, be considered that surplus sperm may have masked negative effects of both chilled storage and cryopreservation as we did not reach a critical low sperm to egg ratio in the present study. Differences in egg quality influenced the fertilization rates more than the maximum number of days of chilled

storage tested in this study. The last two experiments showed that decreasing the sperm to egg ratio and increasing the egg volumes did not alter the results obtained in the first three small-scale fertilization experiments, i.e., the small-scale results are valid even in a real-scale situation.

Author contribution statement

Ingrid Lein: conceptualization, investigation, writing and editing. Adriana Neeltje de Vries: conceptualizations, investigation, review, and editing. Helge Tveiten: conceptualization, review, and editing. Terje Refstie: conceptualizations, review, and editing. Bjarne Gjerde: conceptualization, statistical analysis, writing and editing.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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References

Babiak, I., Glogowski, J., 1997. Cryopreservation of sperm of common carp, *Cyprinus carpio* L. Aquac. Res. 28, 567–571. https://doi.org/10.1046/j.1365-2109.1997.00897.x.

Babiak, I., Ottesen, O., Rudolfsen, G., Johnsen, S., 2006. Chilled storage of semen from Atlantic halibut *Hippoglossus hippoglossus* L. In: Optimizing the protocol. Theriogenology, 66, pp. 2025–2035. https://doi.org/10.1016/j.theriogenology.2006.06.002.

Babiak, I., Marshhäuser, V., Ottesen, O., Rudolfsen, G., Eggen, B., Babiak, J., 2012. Effects of extender, storage and sperm-to-egg ratio on cryopreservation success of Atlantic cod (*Gadus morhua* L.) sperm. J. Appl. Ichthyol. 28, 941–947. https://doi.org/10.1111/jai.12063.

Beirão, J., Boulais, M., Gallego, V., O'Brien, J.K., Peixoto, S., Robeck, T.R., Cabrita, E., 2019. Sperm handling in aquatic animals for artificial reproduction. Theriogenology 133, 161–178. https://doi.org/10.1016/j.theriogenology.2019.05.004.

Bobe, J., Labbe, C., 2008. Chilled storage of sperm and eggs. In: Cabrita, E., Robles, V., Herráez, P. (Eds.), Methods in Reproductive Aquaculture, Marine and Freshwater Species. Setion IV. Sperm and Egg Cryopreservation. CRC Press, Boca Raton, 18p.

Butts, I.A.E., Trippel, E.A., Litvak, M.K., 2009. The effect of sperm to egg ratio and gamete contact time on fertilization success in Atlantic cod *Gadus Morhua* L. Aquaculture 286, 89–94. https://doi.org/10.1016/j.aquaculture.2008.09.005.

Cabrita, E., Anel, L., Herraéz, M.P., 2001. Effect of external cryoprotectants as membrane stabilizers on cryopreserved rainbow trout sperm. Theriogenology 56, 623–635. https://doi.org/10.1016/S0093-691X(01)00594-5.

Cabrita, E., Robles, V., Rebordinos, L., Sarasquete, C., Herráez, M.P., 2005. Evaluation of DNA damage in rainbow trout (*Oncorhynchus mykiss*) and gilthead sea bream (*Sparus aurata*) cryopreserved sperm. Cryobiology 50, 144–153. https://doi.org/10.1016/j.cryobiol.2004.12.003.

Chao, N.H., Chao, W.C., Liu, K.C., Liao, I.C., 1987. The properties of tilapia sperm and its cryopreservation. J. Fish Biol. 30, 107–118. https://doi.org/10.1111/j.1095-8649.1987.tb05737.x.

Chereguini, O., García de la Banda, I., Inmaculada, R., Fernández, A., 1999. Growth and survival of young turbot (*Scophthalmus maximus* L.) produced with cryopreserved sperm. Aquac. Res. 33, 637–641. https://doi.org/10.1046/j.1365-2109.2002.00703.x.

Contreras, P., Dumorné, K., Ulloa-Rodígez, P., Merino, O., Elías, M., Figueroa, E., Farías, Jorge G., Valdebenito, I., Rosopatrón, J., 2019. Effect of short-term storage on sperm function in fish semen: a review. Rev. Aquac. 12, 1373–1389. https://doi.org/10.1111/raq.12387.

Davenport, J., 1985. Synopsis of biological data on the lumpsucker, *Cyclopterus lumpus* (Linnaeus, 1758). FAO Fish. Synop. 147, 6–10. Available at. http://www.fao.org/docrep/017/ap950e/ap950e.pdf. Available at.

Diogo, P., Martins, G., Eufrásio, A., Silva, T.S., Cabrita, E., Gavaia, P., 2019. Selection criteria of zebrafish male donors for sperm cryopreservation. Zebrafish 16, 189–196. https://doi.org/10.1089/zeb.2018.1660.

Dorsey, K.M., Guthrie, H.D., Welch, G.R., Mohler, J., Theisen, D.D., Siewerdt, F., Vinyard, B.T., Woods, L.T., 2011. Quality assessment of wild Atlantic sturgeon semen under conditions of short-term storage. N. Am. J. Aquac. 73, 418–425. https://doi.org/10.1080/15222055.2011.629945.

Dreanno, C., Suquet, M., Quemener, L., Cosson, J., Fierville, F., Normant, Y., Billard, R., 1997. Cryopreservation of turbot (*Scophtalmus maximus*) spermatozoa. Theriogenology 48, 589–603. https://doi.org/10.1016/S0093-691X(97)00276-8.

Eliasen, K., Danielsen, E., Johannesen, Á., Joensen, L., Patursson, E.J., 2018. The cleaning efficacy of lumpfish (*Cyclopterus lumpus* L.) in Faroese salmon (*Salmo salar* L.) farming pens in relation to lumpfish i<e and seasonality. Aquaculture 488, 61–65. https://doi.org/10.1016/j.aquaculture.2018.01.026.

Fauvel, C., Suquet, M., Dreanno, C., Zonno, V., Menu, B., 1998. Cryopreservation of sea bass (*Disentrarchus labrax*) spermatozoa in experimental and production conditions. Aquat. Living Resour. 11, 387–394.

Fiskeridirektoratet, 2022. Statistics by the Norwegian Fisheries Directory. https://www.fiskeridir.no/Akvakultur/Tall-og-analyse/Akvakulturstatistikk-tidsserier/Rensefisk.

Gallego, V., Asturiano, J.F., 2019. Fish sperm motility assessment as tool for aquaculture research: a historical approach. Rev. Aquac. 11, 697–724. https://doi.org/10.1111/raq.12253.

Gallego, V., Peñaranda, D.S., Marco-Jiménez, F., Mazzeo, I., Pérez, L., Asturiano, J.F., 2012. Comparison of two techniques for the morphometry study on gilthead seabream (*Sparus aurata*) spermatozoa and evaluation of changes induced by cryopreservation. Theriogenology 77, 1078–1087. https://doi.org/10.1016/j.theriogenology.2011.10.010.

Gallego, V., Pérez, L., Asturiano, J.F., Yoshida, M., 2013. Relationship between spermatozoa motility parameters, sperm/egg ratio, and fertilization and hatching rates in pufferfish (*Takifugu niphobles*). Aquaculture 416-417, 238–243. https://doi.org/10.1016/j.aquaculture.2013.08.035.

Gunnarsson, S., Sigurdsson, S., Thorarensen, H., Imsland, A.K., 2009. Cryopreservation from spotted wolffish. Aquac. Int. 17, 385–389. https://doi.org/10.1007/s10499-008-9210-x.

Imsland, A.K., Reynokds, P., Eliassen, G., Hangstad, T.A., Foss, A., Vikingstad, E., Elvegård, T.A., 2014. The use of lumpfish (*Cyclopterus lumpus* L.) to control sea lice (*Lepeophtherus salmonis* Krøyer) infestations in intensively farmed Atlantic salmon (*Salmo salar* L.). Aquaculture 424-425, 18–23. https://doi.org/10.1016/j.aquaculture.2013.12.033.

Imsland, A.K.D., Reynolds, P., Hangstad, T.A., Kapari, L., Maduna, S.N., Hagen, S.B., Jónsdóttir, Ó.D.B., Spetland, F., Lindberg, K.S., 2021. Quantification of grazing efficacy, growth and health score of different lumpfish (Cyclopterus lumpus L.) families: possible size and gender effects. Aquaculture 530, 735925. https://doi.org/10.1016/j.aquaculture.2020.735925.

Imsland, A.K.D., Purvis, E., Reinardy, H.C., Kapari, L., Watts, E.J., Hangstad, T.A., 2022. The effects of cryogenically preserved sperm on the fertilization, embryonic development and hatching success of lumpfish *C.lumpus*. Aquaculture 547 (2022). https://doi.org/10.1016/j.aquaculture.2021.737466, 737466.

Jonassen, T.M., Lein, I., Nytrø, A.V., 2018. Hatchery management of lumpfish. In: Treasurer, J.W. (Ed.), Cleaner Fish Biology and Aquaculture Applications. 5M publishing ltd, Sheffield, pp. 114–136. https://doi.org/10.1016/j.aquaculture.2013.12.03.

Ladoktha, P., Ponchunchoovong, S., Udomkarn, C., 2017. Preservation of black sharkminnow, *Labeo chrysophekadion* (Bleeker, 1849) spermatozoa. Aquac. Res. 48, 3837–3847. https://doi.org/10.1111/are.13211.

Lahnstainer, F., Berger, B., Wismann, T., Patzner, R.A., 1996. The influence of various cryoprotectants on semen quality of the rainbow trout (*Oncorhynchus mykiss*) before and after cryopreservation. J. Appl. Ichthyol. 12, 99–106. https://doi.org/10.1111/j.1439-0426.1996.tb00070.x.

Lanes, C.F.C., Okamoto, M., Cavalcanti, P.V., Collares, T., Campos, V.F., Deschamps, J.C., Robaldo, R.B., Marins, L.F., Sampaio, L.A., 2008. Cryopreservation of Brazilian flounder (*Paralichthys orbignyanus*) sperm. Aquaculture 275 (1–4), 361–365. https://doi.org/10.1016/j.aquaculture.2007.12.025.

Levanduski, M.J., Cloud, J.G., 1988. Rainbow trout (*Salmo gairdneri*) semen: effect of non-motile sperm on sperm fertility. Aquaculture 75, 171–179. https://doi.org/10.1016/0044-8486(88)90030-0.

Mansour, N., Lahnsteiner, F., Berger, B., 2004. Characterization of the testicular semen of the African catfish, *Clarias gariepinus* (Burchell, 1822), and its short-term storage. Aquac. Res. 35, 232–244. https://doi.org/10.1111/j.1365-2109.2004.00993.x.

Migaud, H., Bell, G., Cabrita, E., McAndrew, B., Davie, A., Bobe, J., Herraez, M.P., Carrillo, M., 2013. Gamete quality and broodstock management in temperature fish. Rev. Aquac. 5, 1–30. https://doi.org/10.1111/raq.12025.

Mounib, M.S., 1978. Cryogenic preservation of fish and mammalian spermatozoa. J. Reprod. Fertil. 53, 13–18.

Norðberg, G., Johannesen, A., Arge, R., 2015. Cryopreservation of lumpfish *Cyclopterus lumpus* (Linnaeus, 1758) milt. PeerJ 3, e1003. https://doi.org/10.7717/peerj.1003.

Opeifa, B.T., 2019. Large Volume Cryopreservation of Lumpfish (*Cyclopterus lumpus* L.) Sperm for Commercial Hatchry Production. MSc thesis. UiT the Arctic University of Norway, 53 pp. https://munin.uit.no/handle/10037/16143.

Pillai, M.C., Yanagimachi, R., Cherr, G.N., 1994. In vivo and in vitro initiation of sperm motility using fresh and cryopreserved gametes from the Pacific herring, *Clupea pallasi*. J. Exp. Xool. 269, 62–68. https://doi.org/10.1002/jez.1402690108.

Pountney, S.M., Migaud, H., Davie, A., 2020a. Short term cold storage and sperm concentration assessment of lumpfish (*Cyclopterus lumpus*. L) milt. Aquaculture 529, 1–6. https://doi.org/10.1016/j.aquaculture.2020.735646Get.

Pountney, S.M., Lein, I., Migaud, H., Davie, A., 2020b. High temperature is detrimental to captive lumpfish (Cyclopterus lumpus, L) reproductive performance. Aquaculture 522, 735121. https://doi.org/10.1016/j.aquaculture.2020.735121.

Rideout, R.M., Trippel, E.A., Litvak, M.K., 2004. The development of haddock and Atlantic cod sperm cryopreservation techniques and the effect of sperm age on cryopreservation success. J. Fish Biol. 65, 299–311. https://doi.org/10.1111/j.0022-1112.2004.00449.x.

Rurangwa, E., Roelants, I., Huyskens, G., Ebrahimi, M., Kime, D.E., Ollevier, F., 1998. The minimum effective spermatozoa:egg ratio for artificial insemination and the effects of mercury on sperm motility and fertilization ability in *Clarias gariepinus*. J. Fish Biol. 53, 402–413. https://doi.org/10.1111/j.1095-8649.1998.tb00989.x.

Rurangwa, E., Kime, D.E., Ollevier, F., Nash, J.P., 2004. The measurement of sperm motility and factors affecting sperm quality in cultured fish. Aquaculture 234, 1–28. https://doi.org/10.1016/j.aquaculture.2003.12.006.

Rurangwa, E., Roelants, I., Huyskens, G., Ebrahimi, M., Kime, D.E., Ollevier, F., 2005. The minimum effective spermatozoa: egg ratio for artificial insemination and the effects of mercury on sperm motility and fertilization ability in *Clarias grienpinus*. J. Fish Biol. 53 (2) https://doi.org/10.1111/j.1095-8649.1998.tb00989.x, 402–313.

Saad, A., Billard, R., Theron, M.C., Hollebecq, M.G., 1988. Short-term preservation of carp (*Cyprinus carpio*) semen. Aquaculture 71, 133–150. https://doi.org/10.1016/0044-8486(88)90280-3.

Santos, M., Soares, F., Moreira, M., Beirao, J., 2018. Evaluation of different extenders for the cold storage of meagre (*Argyrosomus r*egius) semen. Aquac. Res. 49, 2723–2731. https://doi.org/10.1111/are.13733.

Steinarson, A., Árnarson, T., 2018. Rearing of cleaner fish in Iceland. In: Treasurer, J.W. (Ed.), Cleaner Fish Biology and Aquaculture Applications. 5M Publishing Ltd, Sheffield, pp. 420–435. https://doi.org/10.1016/j.aquaculture.2018.01.026.

Stoss, J., Holtz, W., 1983. Successful storage of chilled rainbow trout (*Salmo gairdneri*) spermatozoa for up to 34 days. Aquaculture 31, 269–274. https://doi.org/10.1016/0044-8486(83)90318-6.

Sun, C., Huang, C., Su, X., Zhao, X., Dong, Q., 2010. Optimization of handling and refrigerated storage of guppy *Poecilia reticulata* sperm. J. Fish Biol. 77, 54–66.

Suquet, M., Billard, R., Csson, J., Normant, Y., Fauvel, C., 1995. Artificial insemination in turbot (*Scophthalmus maximus*): determination of the optimal sperm to egg ratio and time of gamete contact. Aquaculture 133, 83–90. https://doi.org/10.1016/0044-8486(94)00395-5.

Suquet, M., Dreanno, D., Dorange, G., Normant, Y., Quemener, L., Gaignon, J.L., Billard, R., 1998. The ageing phenomenon of turbot spermatozoa: effects on morphology and concentration, intraccellular ATP content, fertilization and storage capacities. J. Fish Biol. 52, 31–41. https://doi.org/10.1111/j.1095-8649.1998.tb01550.x.

Suquet, M., Dreanno, C., Fauvel, C., Cosson, J., Billard, R., 2000. Cryopreservation of sperm in marine fish. Aquac. Res. 31, 231–243. https://doi.org/10.1046/j.1365-2109.2000.00445.x.

Treasurer, J., 2018. Cleaner Fish Biology and Aquaculture Applications. 5M Publishing, Sheffield, pp. 122–145. https://doi.org/10.1111/jfb.13830.

Tvedt, H.B., Benfey, T.J., Martin-Robichaud, D.J., Power, J., 2001. The relationship between sperm density, spermatocrit, sperm motility and fertilization success in Atlantic halibut, *Hippoglossus hippoglossus*. Aquaculture 194, 191–200. https://doi.org/10.1016/S0044-8486(00)00516-0.

Vuthiphandchai, V., Thadsri, I., Nimrat, S., 2009. Chilled storage of walking catfish (*Clarias macrocephalus*) semen. Aquaculture 296, 58–64. https://doi.org/10.1016/j.aquaculture.2009.07.018.