The effects of cryogenically preserved sperm on the fertilization, embryonic development and hatching success of lumpfish *Cyclopterus lumpus*

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Abstract

Lumpfish (*Cyclopterus lumpus*) are used as cleaner fish in the Atlantic salmon (*Salmo salar*) farming industry to remove parasitic sea lice. At present, wild lumpfish broodstock are used which puts strain on wild populations. By successfully cryopreserving lumpfish sperm, the number of wild males required will be reduced and it enables the long-term storage of sperm for use in breeding programmes. The present study compared the use of fresh sperm and sperm which was cryogenically frozen for 24 hours to test whether it is a viable method of preservation. The fresh and frozen sperm from 5 males was used (in equal volumes) to fertilize eggs pooled from 5 females and the difference between fertilization success, percentage of eggs which reached the eyed stage, and the hatching success was measured. A group of 100 hatched larvae were on-grown for two weeks to test whether there was a difference weight between treatment groups. The results of the trial showed that fresh sperm produced a significantly higher percentage of fertilized eggs (fresh 92.6 ± 0.8 %, frozen 77.9 ±1.8 %, mean ± SEM), a higher percentage of eggs surviving to the eyed stage (fresh 93.9 ± 0.5 % and frozen 80.8 ± 1.4%) and had a more successful hatch rate (fresh 72.3 ± 6.6 % and frozen 63.6 ± 5.0%). There was no difference in mean weight (± SEM) of the two-week old larvae between treatments (fresh 0.63 g ± 0.024, frozen 0.59 g ± 0.028). In conclusion, this study showed that the same volume of cryogenically preserved lumpfish sperm produced fewer viable lumpfish larvae than fresh sperm. Despite this difference, the use of cryopreserved sperm did produce relatively high results at each stage of testing.

1. Introduction

Cleaner fish, like wrasses (Labridae) and lumpfish (*Cyclopterus lumpus* L.), may represent sustainable solutions for reducing the lice problem in the salmon industry (Treasurer, 2002; Imsland et al., 2014a-c, 2015a-b). Wrasse exhibit winter dormancy, proving ineffective delousers below 6˚C (Kelly et al., 2014), therefore there has been increasing interest in the use of lumpfish, which can target the parasite across all seasons (Imsland et al., 2014a-c, 2018; Eliasen et al., 2018; Powell et al., 2018). As lumpfish tolerate lower temperatures than wrasse species, their implementation was boosted principally in the northern parts of Norway (Imsland et al. 2014a), Scotland (Treasurer et al., 2018), the Faroe Islands (Eliasen et al., 2018; Johannesen et al., 2018) and Iceland (Steinarsson and Árnason, 2018). They are used as cleaner fish for all sizes of Atlantic salmon during their production cycle in sea (Imsland et al., 2014a, 2018). With the demand for lumpfish steadily increasing, this has resulted in gradually moving from wild caught broodfish towards intensive cultivation. Lumpfish production is currently utilizing technology and techniques used in halibut, wrasse and cod aquaculture, and a breeding program for lumpfish was established in 2017 (Imsland et al., 2021). Although wild-caught broodfish still is predominant, nearly all juvenile lumpfish used in salmon sea-pens are from intensive aquaculture production in Norway. In 2019, more than 38 million lumpfish were transferred to cages at commercial marine growth sites for salmonids, which constituted 70% of all cleaner fish used in Norway (Norwegian Directorate of Fisheries 2019). In numbers lumpfish is now the single most important cleaner fish species to date (Powell et al., 2018). Lumpfish are mainly used in the size range 20-300 g as they are most effective in sea lice grazing in this size range (Imsland et al., 2016, 2018).

In commercial production of lumpfish, the females are stripped for their eggs and the male's gonads are removed and mechanically ground to extract the milt (Jonassen et al., 2018). The difficulty during this process is that multiple females may become ready to spawn within a very short window of time and it is not advised to delay stripping them as it's possible that eggs will become overripe or the female may spawn in the tank. It is only possible to extract small volumes of milt from male lumpfish and even when attempted with multiple fully ripe males, less than 3 ml could be extracted from any one male (Norðberg et al., 2015). This volume is inadequate for fertilizing eggs on a commercial scale and alternatively, males are culled and through a time-consuming process, gonads are rinsed then ground to extract the milt. As a result, milt must be collected periodically and refrigerated until required. An extender solution is normally added which increases the lifespan of the sperm for around one week although the quality of sperm naturally degrades and as a result, fertilization rates may suffer. An effective solution to this issue is storing the milt using cryopreservation, a technique which is widely used for a number of other marine fish species e.g. Brazillian flounder *(Paralichthys brasiliensis*) (Lanes et al., 2008), Olive flounder (*Paralichthys olivaceus*) (Zhang et al., 2003), Atlantic haddock (*Melanogrammus aeglefinus*) (Rideout et al., 2004) and spotted wolffish (*Anarhichas minor*) (Gunnarsson et al., 2009). Cryopreservation is of interest not only for fish farming but also for the conservation and genetic improvement of resources. This technique has been well established in some freshwater fish species mainly, salmonid, sturgeons and carps, however, only in the last two decades research was focused on marine fish species (Cabrita et al., 2010; Beirao et al., 2019). The sperm of more than 40 marine fish species have now successfully been cryopreserved (Tsai and Lin, 2012; Magnotti et al., 2018). Moreover, with the expansion of global aquaculture production the capacity for long-term storage of cells, including sperm, can be pivotal for the creation of families in breeding

69 programmes and hatchery production (Butts et al., 2010; Martínez-Páramo et al., 2017;
70 Imsland et al., 2021).

71 Evaluation of sperm quality and viability can be assessed based on single or a
72 combination of multiple criteria, including sperm motility, ability of cryopreserved sperm to
73 fertilize eggs and or hatching rate (Hassan et al., 2015; Asturiano et al., 2017; Gallego and
74 Asturiano, 2018; Magnotti et al., 2018; Opeifa, 2019). Final evaluation of the viability of
75 cryopreserved sperm samples is normally performed by checking if the sperm functionality,
76 capacity to fertilize eggs and yield viable embryos or larvae is not compromised by the procedure
77 when compared to fresh samples (Hassan et al., 2015, Asturiano et al., 2017).

78 If lumpfish milt can be successfully preserved using cryopreservation without
79 damaging the sperm or their fertilization capacity, it would reduce the number of wild caught
80 males being used and create a more efficient, sustainable production process. Accordingly, the
81 aim of this study was to establish protocols for cryopreserving of lumpfish sperm without
82 negative effects on successful production of lumpfish larvae.

2. Materials and Methods

2.1 Experimental fish

The following experiment was carried out at Akvaplans-niva's FISK research station, Kraknes, Tromsø in April and June 2020. The lumpfish broodstock used in this facility are wild caught and used for commercial egg production. They are held in purpose-built tanks (8 m^3) at the station until the females are ready to spawn. The tanks run on a flow-through system with an average temperature of 6°C. Photoperiod manipulation (see Imsland et al., 2019a) was used to either suppress (reduced light exposure) or encourage (increase light exposure) spawning activity. The fish used in this trial were caught at Hekkingen (69°N), Malangen, Norway and brought to the research station by boat. They arrived the day prior to starting the experiment (23.04.2020) and were held in the dark overnight with males and females kept separately. The mean weight (±SD) and length of the males was 1.36 ± 0.66 kg, 30.5 ± 3.6 cm and mean weight and length of the females was 2.23 ± 0.78 kg, 35.8 ± 2.9 cm.

2.2 Milt collection

On day one of the trial (24.04.2020), 5 wild sexually mature male lumpfish were stripped to collect their milt. Males which were ready to spawn were identified by their appearance as they take on a red or orange hue when they are ready. The fish were killed with a blow to the head, dissected and their gonads removed. The gonads were washed using deionised water, patted dry to remove excess liquid and then passed through a sterile small kitchen grinder (Kitchen Basics 3 N 1 Manual Grinder) to create a paste. Wolffish extender

solution (Opeifa, 2019) was prepared one day before the main experiment by measuring 154 mM NaCl, 4.55 mM $CaCl_2$, 2.37 mM $MgSO_4$, and 4.8 cm^3 mM $KHCO_3$ salts and dissolving them in 1000 ml distilled water. The wolffish extender solution was added to the paste at a diluted 50:50 volume ratio and left for 10 minutes to soak. This mixture was then passed through a 50 µm sieve into a beaker to separate sperm from tissues. From the milt/extender liquid that was collected, 30 ml was removed and placed into sterile falcon tubes which were labelled according to each male. The remaining volume was stored in individual containers which were placed on stirring pads (50-75 rpm) in a refrigerator at 4°C.

2.3 Sperm analysis

2.3.1 Sperm counts

A small sample of milt from each male was kept aside (~1 mL) in individual cuvettes. A series of serial dilutions were set up in 1.5 ml cuvettes using the following volumes shown in Fig. 1. For the counts, 250 µL of the final dilution was added to a haemocytometer (Neubauer, Tek-Event Pty Ltd., NSW, Australia) until the counting chamber was flooded. The haemocytometer was viewed under a WPI inverted light microscope (40x0.6 objective). Cell counts were obtained by counting how many individual sperm were present in 5 small boxes (16 squares in each) and the total was used to calculate an average cell count using the following equation: Sperm/ml = (Number of sperm counted*Dilution factor)/(Volume*1000).

2.3.2 Sperm motility

To maintain a cool temperature for measuring sperm motility, all the equipment including pipette tips, cuvettes, counting chambers (Leja, Standard Count 4 Champer slide, 10 micron) seawater and sperm samples were kept refrigerated (4°C) and only removed for short periods of time. For each male, a fresh and frozen sample (24 h) were collected at the point of fertilizing the eggs. 900 µL of filtered (0.2 µm), chilled seawater was added to each cuvette. One cuvette was removed at a time and 100 µL of sperm was added and quickly mixed by inverting. A chilled pipette tip was used to load 6 µL of sample into the Leja chamber. The Leica acquire software (Leica Application Suite v3.8) captured 30 seconds of video at 40x0.65 magnification via a Leica Microsystems inverted light microscope. The process was repeated four times for each sample of sperm.

2.4 Cryopreservation of sperm

Sperm was cryogenically frozen according to the protocol described by Opeifa (2019), but with minor modifications. For each fresh sperm sample (1:1 volume of sperm: extender), dimethyl sulfoxide (DMSO) cryoprotectant was added at 10% volume (30 ml solution = 3 mL DMSO) and mixed thoroughly. DMSO has a freezing point of 19°C therefore it was stored in a water bath at 21°C to prevent it solidifying. Pre-labelled cryo straws (2 mL, Minitübe GmbH, Tiefenbach, Germany) were loaded with sample (1 mL) and sealed with a stainless-steel ball at each end. 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 above the liquid nitrogen. No attempt was made to measure the motility after the equilibration time. The straws were loaded onto a metal rack embedded in Styrofoam (5 x 5 cm, 1 cm thick) which was floating on a bath of liquid nitrogen (5 cm, Fig. 2). The floating rack held the straws 2.5

cm above the surface of the liquid nitrogen where the vapor cools them gradually to the lower temperature. The cooling was done uncovered. The straws (4 straws at a time) sat on the raft for 10 minutes, after which the rack was tilted upward, and the straws were plunged into the liquid nitrogen. This process was repeated for each male. Protective thermal insulating gloves and a face shield were worn at all times when handling liquid nitrogen.

2.5 Egg collection

On day 2 of the trial (25.04.2020), five sexually mature female lumpfish were selected for stripping. The females were assessed for swelling of the abdomen and a protruding, swollen urogenital area as these indicate readiness to spawn. The fish were removed from the tank individually using a net, euthanised, and their eggs stripped and held in individual, sterile jugs. The eggs of each female were poured from one sterile jug to another 5 times to mix and equally distribute the ovarian fluid. From this egg/ovarian fluid mix, 200 mL was taken per female and added to a separate, sterile jug and the mixture was then stirred thoroughly. The jug was left to stand for three minutes to allow the ovarian fluid to separate from the eggs. The ovarian fluid was then siphoned from the top using a 10 mL pipette and added to a separate sterile measuring jug. The eggs were stirred thoroughly a second time and 15 mL of the mixed eggs (approx. 1000~1050 eggs) were added to 33 individual (280 mL) plastic containers (N=33). On top of each batch of eggs, 7 mL of ovarian fluid was added using the 10 mL pipette to cover the surface.

2.6 Fertilization

The eggs were fertilized on day 2 of the trial with 100 µL of either fresh or frozen sperm from each male. The cryo straws of frozen sperm were held in a flask of liquid nitrogen on the bench. The containers of fresh sperm were removed from the fridge as and when required. This research group has previously done preliminary trials on several aspects in relation to storage and use of fresh milt (Opeifa, 2019; Lauri Kapari, Akvaplan-niva, pers. comm.) and fresh sperm can be refrigated for short period (1-2 days) without negative effects. Using a 200 µL pipette (Accumax A series, Accumax Lab Devices Pvt. Ltd, India), 100 µL of fresh sperm was added to each one of the three pots for each male. The sperm was mixed using a sterile pipette to distribute around the eggs. Following this, 2 mL of seawater was added on top of the eggs to activate the sperm and the mix was thoroughly stirred. The next stage was to thaw the frozen sperm. For each male, 4 individual 2 mL straws were thawed and pooled. The straws were removed from the liquid nitrogen using tweezers and plunged into a freshwater bath which was maintained at an average temperature of 5°C. The straws thawed in the water bath for 2 minutes. Individually, the straws were removed from the water bath, dried to remove any water and one end cut off with sterile scissors. All four straws were emptied into one sterile container which was then mixed and from which, 100 µL was taken per pot of eggs. The same procedure was carried out for fresh and frozen for all 5 males and a set of three controls was set up whereby eggs were left unfertilized. The egg / sperm ratio used in the fertilization validation was 3 ml eggs = ca. 100 eggs fertilized with 100 microliters sperm at a concentration of 109 – 183 cells/µl 10^6 approximately 11-18 x10^7 sperm cells /egg.

The eggs were left undisturbed for 24 hours in a cool (4°C) temperature room. They were then treated with Buffodine ® disinfectant (solution of 100 ml Buffodine® per 10 litres water) on top of each cake) for 10 minutes. The treatment was rinsed off and each container

was then re-filled with seawater. The seawater used throughout this fertilization process had a temperature of 5.5°C and a salinity of 33.5 ppt.

2.7 Incubator set-up

The day after fertilization, the embryos were transferred to individual 1 L incubators which were set up as part of a flow-through system on a bench in the commercial egg production room (Fig. 3). The room held 30 individual incubators each with their own inflow and outflow pipes. Inflow was through a re-directed pipe from the main water supply and outflow ran via a small section of tubing from each incubator into a central pipe which fed directly into the drain system. The eggs sat in metal baskets which allowed for water circulation around the eggs but prevented larvae from escaping as they hatched. Temperature and oxygen were measured daily using a handheld OxyGuard. The average water temperature was 7.9°C and oxygen saturation 108%.

2.8 Egg sampling

24 hours post fertilization, approximately 100 eggs were sampled from each incubator. Eggs were carefully removed (by hand) from two adjacent sides of the cake to better represent the overall population. They were placed into welled chambers and carefully pulled apart using tweezers and put in individual rows. Approximately 1 mL of a 10% acetic acid and seawater solution was added to each row of embryos to increase contrast and make the cells easier to distinguish. The eggs were checked for fertilization (formation of an embryo) by the presence of cell division (Fig. 4) using a compound microscope (Leica Microsystems, Wetzlar,

226 Germany). Percentage fertilization rate was calculated by dividing the number of fertilized
227 embryos by the total egg count and multiplying this by 100. Ten days later (04.05.2020) a
228 further sample of 100 eggs was taken from each cake to check for the presence of an eyed
229 embryo (Fig. 5). From this, a percentage-eyed value for each cake was calculated.
230
231 *2.9 Sampling of larvae*
232
233 Due to space limitations, larvae from each replicate were pooled with 30 individuals
234 from each (A, B, C) being added to a separate flow-through set-up of 10 small tanks to be
235 grown on. The other fish were euthanised with an overdose of anaesthetic (^{TM18}Finquel, 150
236 mg L^{-1}) and kept in separate containers for counting.
237 The larvae which were kept on, were hand fed at 2-h intervals throughout the working
238 day with 0.4 g at each feeding of 250-360 µm compound fish feed (Otohime B, Marubeni
239 Nisshin) between 8am-4pm. At the end of each day, 30 minutes after the final feed, the tanks
240 were cleaned to remove excess feed and waste. Due to space limitations the larvae were
241 euthanized with an overdose of anaesthetic (^{TM18}Finquel, 150 mg L^{-1}) after two week of rearing
242 and were individually weighed (mg) using a fine balance scale (Mettler Toledo, Columbus,
243 Ohio, USA) and measured (mm) using pictures processed with ImageJ software. From this
244 data, an average condition factor was calculated for each treatment to test whether there was
245 a significant difference in growth between the groups over 14 days. Condition factor (K) was
246 calculated using Fultons formula: $K = 100*\text{weight}/\text{length}^3$.
247
248 *2.10 Statistical analysis*
249

Statistical analysis was performed using Prism8 statistical software. A Pearsons-r test was performed to test for a correlation between sperm count and sperm motility. A Kolmogorov-Smirnov test (Zar, 1984) was used to assess for normality of distributions. The homogeneity of variances was tested using the Levene's F test (Zar, 1984). A two-way ANOVA (Zar, 1984) was used to compare the fertilization, embryonic development and hatching success between groups treated with cryopreserved compared to fresh sperm.

3. Results

3.1 Sperm characteristics

The results of the sperm count analysis indicated that males 2 and 5 had a lower sperm count than males 1, 3 and 4. Sperm motility was high across all males (> 73%, Table 1). There was not a significant correlation between sperm count and motility ($P > 0.40$).

3.2 Egg fertilization and development

3.2.1 Fertilization

The egg batches which were fertilized using fresh sperm, had a higher fertilization success than those fertilized with cryogenically preserved sperm (fresh 92.6 ± 0.8 %, frozen 77.9 ±1.8 %, mean ± SEM, two-way ANOVA, $P < 0.05$, Fig. 6). Overall, both treatments resulted in high fertilization percentages across all groups (>68%). There was no significant difference between males and fertilization percentage (two-way ANOVA, $P > 0.70$, Fig. 6).

3.2.2 Embryonic development

There was a significantly higher percentage of eyed embryo in batches treated with fresh sperm, than those treated with cryopreserved sperm (two-way ANOVA, $P < 0.01$, Fig. 7). The average percentage of embryos with developed eyes from egg batches fertilized with fresh sperm, was 94 ± 0.5% (mean ± SEM), compared to cryopreserved sperm 81 ± 2.3%.

There was no significant difference between males and the percentage of embryos which has developed eyes (two-way ANOVA, $P > 0.35$, Fig. 7).

3.3 Hatching

3.2.1 Hatching success

Hatching began at 31 days post fertilization. All incubators began to hatch within 1 hour of each other. There was a relatively high hatching success across groups (> 52%) and a large amount of variation between groups (Fig. 8). For males 3 and 4, there was a significant difference in hatching success between treatments (fresh 72.3 ± 6.6 % and frozen 63.6 ± 5.0%, two-way ANOVA, $P < 0.05$, Fig. 8), whereas male 1 had a higher hatching success for frozen sperm (fresh 65.4 ± 9.2 % and frozen 78.2 ± 4.4 %). Overall, the method of sperm storage had no significant effect on the percentage of larvae which successfully hatched (two-way ANOVA, $P > 0.25$, Fig. 8).

3.2.2 Larval growth

There was no significant difference in size between treatment groups at day 14 post hatch (fresh 0.63 g ± 0.02, frozen 0.59 g ± 0.03). Condition factors were not significantly different between groups treated with fresh sperm compared to those with cryopreserved either at day 0 (fresh 3.87 ± 0.14, frozen 3.89 ± 0.17) nor at day 14 (fresh 2.95 ± 0.11, frozen 2.87 ±0.13).

4. Discussion

4.1 Characterisation of males

The success of fertilization depends at least partly on the quality of the gametes. In terms of the spermatozoa, 'quality' may be defined as their ability to swim, reach and then fertilize the oocyte. Motility is an important biomarker as sperm must be able to swim to reach the oocyte and so it's commonly used as a test for sperm quality (Gallego and Asturiano, 2018; Nusbaumer et al., 2019). A ranking system used for assessing sperm motility has 5 rankings with the top being 75-100% motility (Agarwal, 2011). The sperm samples taken from the males in this experiment showed an average motility of above 73% for all males and although there was lower sperm count found for males 2 and 5 no correlation between sperm count and motility was found. The lack of individual male effect is reflected in the follow-up studies as the results showed no significant correlation between the male fish used, and fertilization percentage, embryonic development or early larval growth.

4.2 Fertilization and embryonic development

Fertilization was high across all of the groups, although there was a significantly lower percentage of fertilized embryos in groups treated with cryopreserved sperm. As explained above, the starting quality of the sperm samples were high, and this can account for the successful fertilization of embryos across all the groups. In vitro fertilization trials are a necessary step for validating the effectiveness of sperm cryopreservation protocols due to close correlations between sperm motility and fertility (Dziewulska et al., 2011; Zadmajid et

al., 2019). The decreased fertilization rate in the eggs treated with cryo sperm, could be as a result of sperm being damaged by the freezing process (Fernandez-Díez and Herráez, 2018; Zadmajid et al., 2019), or potentially an error in the freezing or thawing technique. Sperm cryopreservation generates different levels of DNA damage, mostly fragmentation and base oxidation, as has been reported in different species (Herráez et al., 2015; Fernandez-Díez and Herráez, 2018). Sperm is highly temperature sensitive (Majhi et al., 2013; de Costa et al., 2019) and if temperature fluctuated during the cryopreservation method it is almost inevitable that this fluctuation caused sperm damage.

Other studies have noted the potential differences in egg quality as a factor which influences fertilization where multiple females are used (e.g. Kanyilmaz et al., 2016; Reading et al., 2018). For this trial the eggs were pooled from multiple females and so the effect of one female having higher quality eggs would not likely be a large influencing factor.

Assessing embryos for the presence of eyes is a commonly used marker of development in aquaculture (Kanyilmaz et al., 2016; Imsland et al., 2019b). The results showed that the number of embryos which reached eyed stage, was similar to the fertilization results. It is likely that the method of storage of sperm did not have any additional effect during 4-cell to eyed embryo stage and that the difference in the number of eyed embryos is a reflection of the difference in percentage fertilization.

4.3 Hatching success

The results showed that hatching success was not significantly affected by the method of sperm preservation. There was a large degree of variation in the results of the hatching success which could have resulted from a number of factors. On the day of hatching, a

mechanical fault resulted in the embryos in all incubators received water that was two degrees cooler than they were accustomed to. Another factor could be the density of the egg cakes. They were packed into rings which possibly created a cake which was too thick. The sticky nature of lumpfish eggs means that they stick together in lumps (hence the name lumpfish or lumpsucker) (Davenport, 1985; Norðberg et al. 2015; Jonassen et al., 2018) but if they were too densely packed, water circulation and therefore oxygenation of the eggs may have been reduced, particularly around eggs close to the centre.

The lack of consistent pattern suggests that the method of sperm storage had little influence on the hatching success of lumpfish larvae and that instead it was more a result of environmental factors. Changes in temperature, salinity and light are known alter the hatching rhythm (Imsland et al., 2019b), where far more larvae hatch at one time in the response to a change in their surrounding environment. This being said, the fact that they all hatched in such a short period of time, meant that it is possible that a pattern was missed. It would have been useful to see whether there was a significant difference in time it took for the larvae to begin hatching to see if treatment had an effect. To capture the very first larvae hatching, a rigged camera may have been useful to be able to look back and observe any patterns.

4.4 Growth

There was no significant difference in size between the two treatment groups. Cryogenically preserved sperm has been known to caused deformities and reduced fertilization rate as freezing and thawing lead to structural damage of sperm that cause lower fertilization and hatching of eggs (Billard, 1983; Lahnsteiner et al., 1992). Lower hatching rates were observed during eggs fertilized with frozen-thawed sperm compared with fresh

sperm (Zilli et al., 2003; Nahiduzzaman et al., 2011). Cryopreserved sperm affected embryonic development and reduced survival of the eyed-eggs and larvae of trout (Pérez-Cerezales et al., 2011). In some studies use of cryopreserved sperm have led to lower larval growth (Nusbaumer et al., 2019), whereas other studies have not found this (Chereguini et al., 2002; Sarder et al., 2013; Akter et al., 2016). In the present study growth of the two groups of lumpfish larvae was similar suggesting that once the larvae are hatched out, there is no detrimental effect of cryopreservation on the early growth of lumpfish larvae.

5. Conclusion

In conclusion, cryopreservation has shown promising results as a method of storage for lumpfish sperm. The results of this trial indicate that the main effect of using cryogenically preserved sperm, rather than fresh, is a small but significant reduction in percentage of eggs fertilized. It appeared to have no influence on the success of the larvae in terms of hatching and does not influence their early larvae stage.

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Author statement

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Conflict of interest

There is no conflict of interest in relation to this study.

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Figure legends

Fig. 1. Serial dilutions used for sperm count analysis in the present study.

Fig. 2. Cropreservation of sperm samples. (a) Straws are acclimatised on a raft which floats 2.5 cm above the suface of liquid nitrogen. (b) Staws are plunged into liquid nitrogen,

Fig. 3. An illustration of a single incubator used in the study. The blue arrows indicate direction of waterflow through the system.

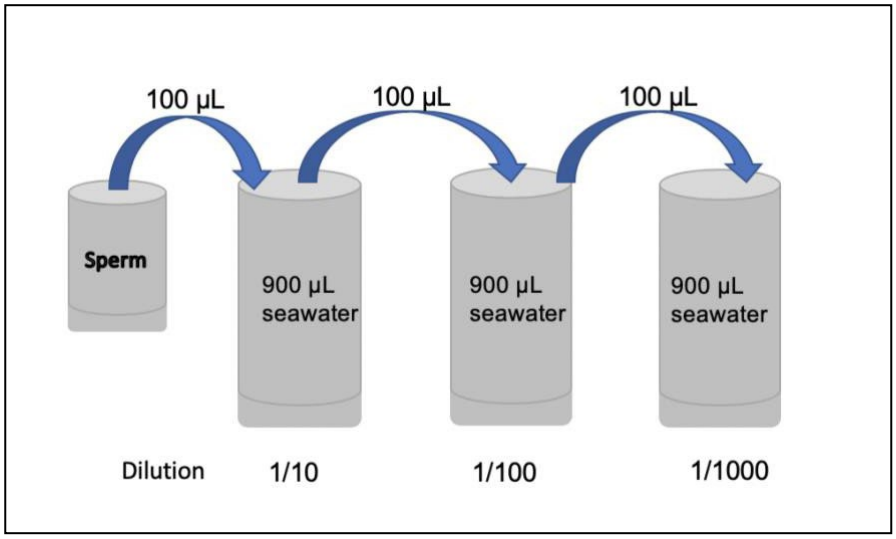
Fig. 4. The 4-cell stage (c. 4 day-degrees (dd) post fertilization (PF)) viewed through a compound microscope. The diameter of the egg is 2.2 mm.

Fig. 5. An eyed lumpfish embryo. The arrow indicates the presence of a developed eye at c. 100 day-degrees post-fertilzation. The diameter of the egg is 2.2 mm.

Fig. 6. The average percentage off eggs fertilized from a sample of 100 taken from each replicate (N=3). Eggs were fertilized in triplicate batches with samples of fresh and cryogenically preserved sperm from 5 male lumpfish.

Fig. 7. Percentage of eggs which survived to eyed stage measured from a sample of 100 developing eggs taken from each replicate. Percentages are given as an average of the three triplicates per treatment per male (N=3).

605 Fig. 8. Comparison between the average percentage of larvae which successfully hatched from
606 eggs fertilized with fresh sperm, compared to cryopreserved sperm. Percentages are given as
607 an average of the three triplicates per treatment per male (N=3).

608 

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610 Fig. 1. Imsland et al.

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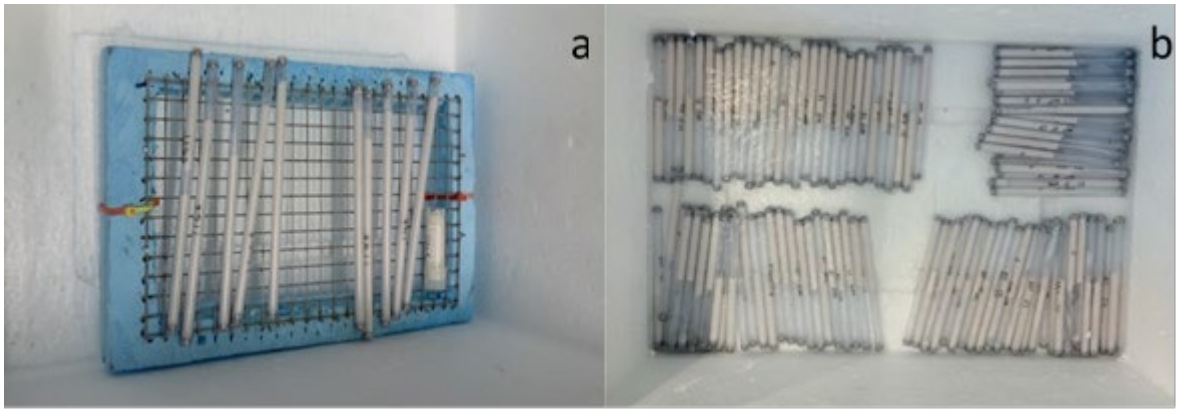

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615 Fig. 2. Imsland et al.

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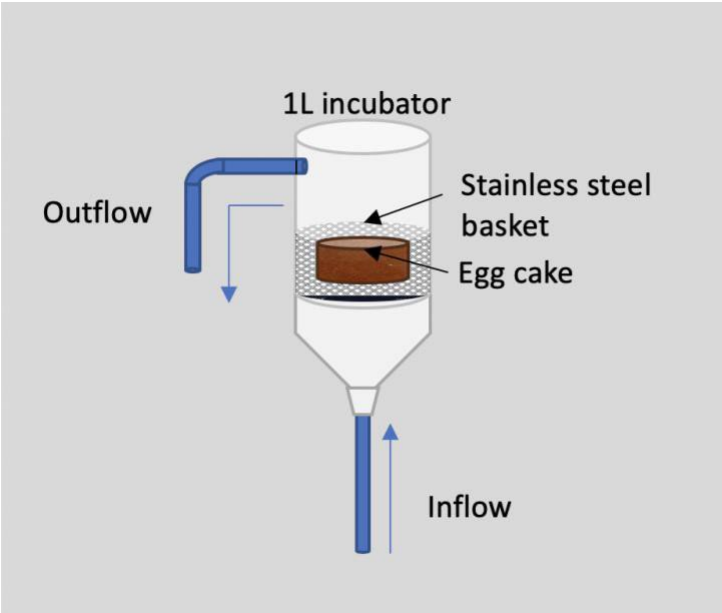

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619 Fig. 3. Imsland et al.

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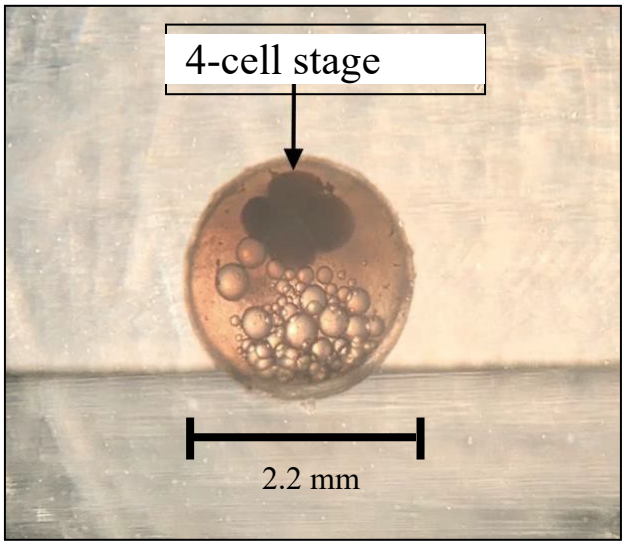

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624 Fig. 4. Imsland et al.

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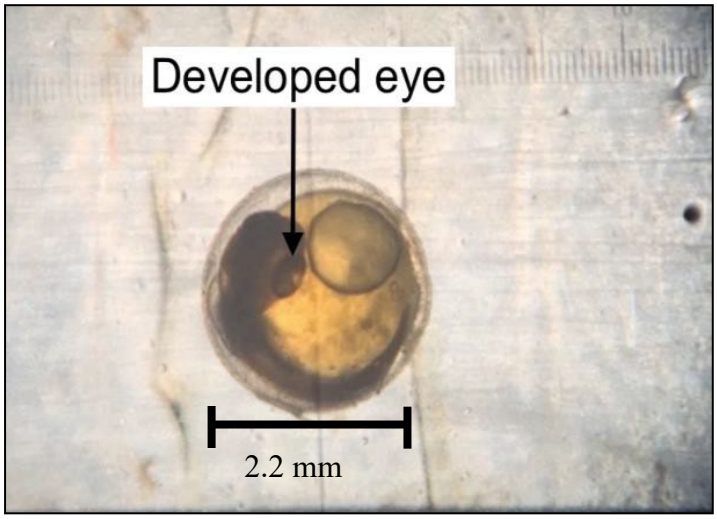

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627 Fig. 5. Imsland et al.

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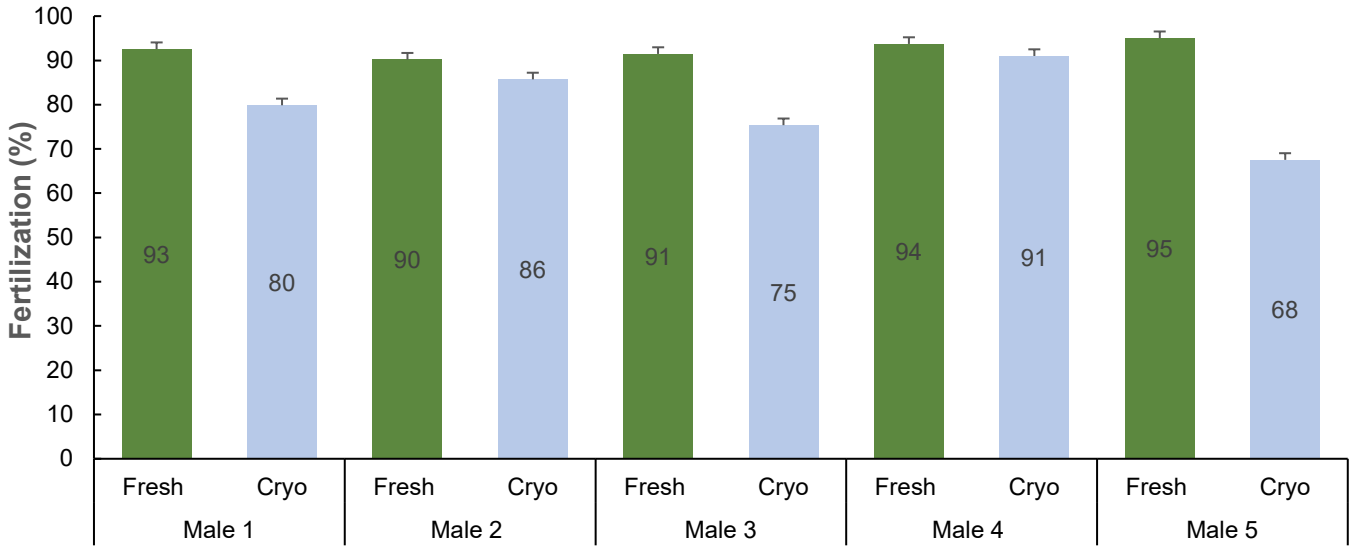

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631 Fig. 6. Imsland et al.

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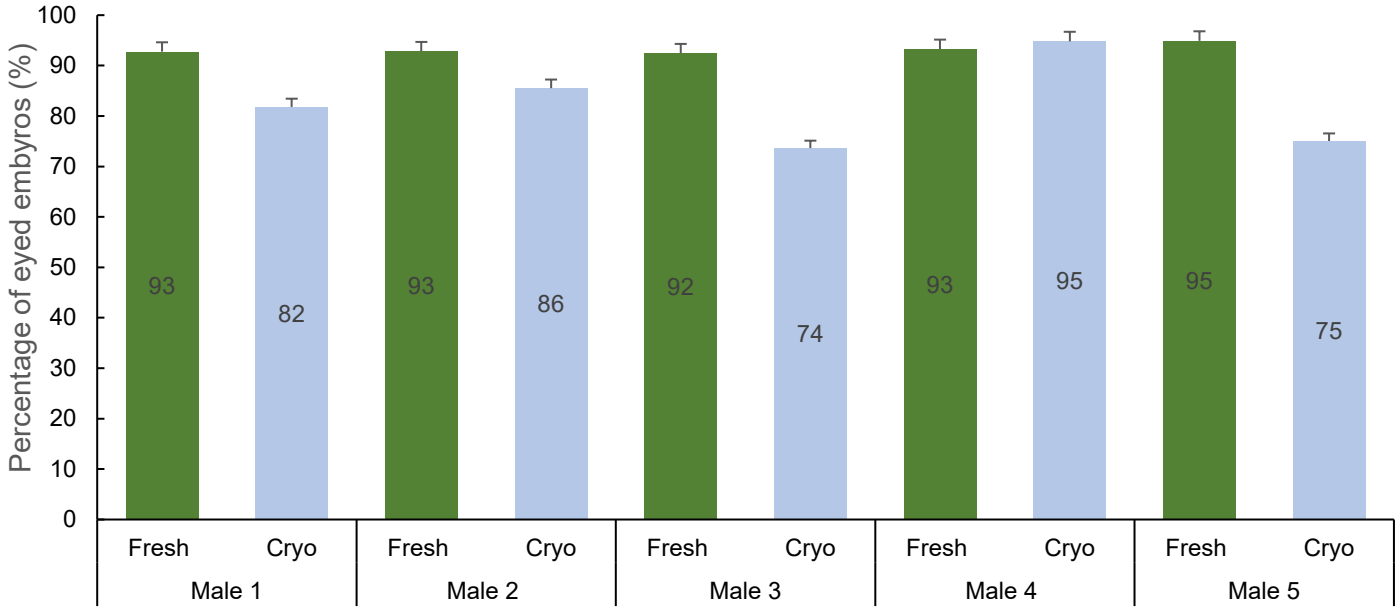

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635 Fig. 7. Imsland et al.

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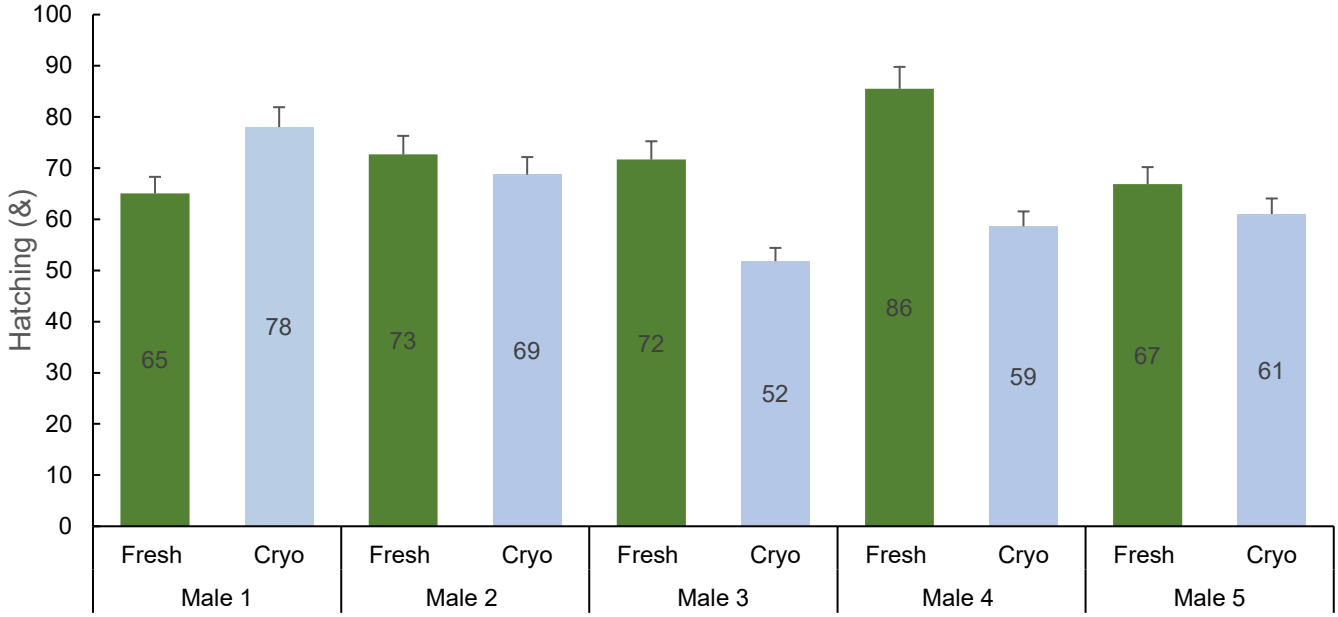

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639 Fig. 8. Imsland et al.

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641 Table 1. Sperm characteristics measured following collection on day 1 of the trial. Sperm
642 count was measured using a haemocytometer as cells per ml for each male. Sperm motility as
643 assessed using Leica microscope video software.
644

	Sperm count (cells ml^{-1})	Sperm motility (% sperm motile, mean ± SE, N= 4 per male)
Male 1	$1.1x(10^9)$	81.8 ±1.5 %
Male 2	$6.5x(10^8)$	85.8 ±1.1 %
Male 3	$1.2x(10^9)$	84.8 ±0.5 %
Male 4	$1.1x(10^9)$	79.4 ±1.3 %
Male 5	$9.5x(10^8)$	73.6 ± 0.6 %

645