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

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1 **Abstract**

2 Lumpfish (*Cyclopterus lumpus*) are used as cleaner fish in the Atlantic salmon (*Salmo salar*)
3 farming industry to remove parasitic sea lice. At present, wild lumpfish broodstock are used
4 which puts strain on wild populations. By successfully cryopreserving lumpfish sperm, the
5 number of wild males required will be reduced and it enables the long-term storage of sperm
6 for use in breeding programmes. The present study compared the use of fresh sperm and sperm
7 which was cryogenically frozen for 24 hours to test whether it is a viable method of
8 preservation. The fresh and frozen sperm from 5 males was used (in equal volumes) to fertilize
9 eggs pooled from 5 females and the difference between fertilization success, percentage of
10 eggs which reached the eyed stage, and the hatching success was measured. A group of 100
11 hatched larvae were on-grown for two weeks to test whether there was a difference weight
12 between treatment groups. The results of the trial showed that fresh sperm produced a
13 significantly higher percentage of fertilized eggs (fresh 92.6 ± 0.8 %, frozen 77.9 ± 1.8 %,
14 mean \pm SEM), a higher percentage of eggs surviving to the eyed stage (fresh 93.9 ± 0.5 % and
15 frozen 80.8 ± 1.4 %) and had a more successful hatch rate (fresh 72.3 ± 6.6 % and frozen 63.6
16 ± 5.0 %). There was no difference in mean weight (\pm SEM) of the two-week old larvae between
17 treatments (fresh 0.63 g ± 0.024 , frozen 0.59 g ± 0.028). In conclusion, this study showed that
18 the same volume of cryogenically preserved lumpfish sperm produced fewer viable lumpfish
19 larvae than fresh sperm. Despite this difference, the use of cryopreserved sperm did produce
20 relatively high results at each stage of testing.

21

22 1. Introduction

23

24 Cleaner fish, like wrasses (Labridae) and lumpfish (*Cyclopterus lumpus* L.), may
25 represent sustainable solutions for reducing the lice problem in the salmon industry (Treasurer,
26 2002; Imsland et al., 2014a-c, 2015a-b). Wrasse exhibit winter dormancy, proving ineffective
27 delousers below 6°C (Kelly et al., 2014), therefore there has been increasing interest in the use
28 of lumpfish, which can target the parasite across all seasons (Imsland et al., 2014a-c, 2018;
29 Eliassen et al., 2018; Powell et al., 2018). As lumpfish tolerate lower temperatures than wrasse
30 species, their implementation was boosted principally in the northern parts of Norway
31 (Imsland et al. 2014a), Scotland (Treasurer et al., 2018), the Faroe Islands (Eliassen et al., 2018;
32 Johannesen et al., 2018) and Iceland (Steinarsson and Árnason, 2018). They are used as
33 cleaner fish for all sizes of Atlantic salmon during their production cycle in sea (Imsland et
34 al., 2014a, 2018). With the demand for lumpfish steadily increasing, this has resulted in
35 gradually moving from wild caught broodfish towards intensive cultivation. Lumpfish
36 production is currently utilizing technology and techniques used in halibut, wrasse and cod
37 aquaculture, and a breeding program for lumpfish was established in 2017 (Imsland et al.,
38 2021). Although wild-caught broodfish still is predominant, nearly all juvenile lumpfish used
39 in salmon sea-pens are from intensive aquaculture production in Norway. In 2019, more than
40 38 million lumpfish were transferred to cages at commercial marine growth sites for
41 salmonids, which constituted 70% of all cleaner fish used in Norway (Norwegian Directorate
42 of Fisheries 2019). In numbers lumpfish is now the single most important cleaner fish species
43 to date (Powell et al., 2018). Lumpfish are mainly used in the size range 20-300 g as they are
44 most effective in sea lice grazing in this size range (Imsland et al., 2016, 2018).

45 In commercial production of lumpfish, the females are stripped for their eggs and the
46 male's gonads are removed and mechanically ground to extract the milt (Jonassen et al., 2018).
47 The difficulty during this process is that multiple females may become ready to spawn within
48 a very short window of time and it is not advised to delay stripping them as it's possible that
49 eggs will become overripe or the female may spawn in the tank. It is only possible to extract
50 small volumes of milt from male lumpfish and even when attempted with multiple fully ripe
51 males, less than 3 ml could be extracted from any one male (Norðberg et al., 2015). This
52 volume is inadequate for fertilizing eggs on a commercial scale and alternatively, males are
53 culled and through a time-consuming process, gonads are rinsed then ground to extract the
54 milt. As a result, milt must be collected periodically and refrigerated until required. An
55 extender solution is normally added which increases the lifespan of the sperm for around one
56 week although the quality of sperm naturally degrades and as a result, fertilization rates may
57 suffer. An effective solution to this issue is storing the milt using cryopreservation, a technique
58 which is widely used for a number of other marine fish species e.g. Brazillian flounder
59 (*Paralichthys brasiliensis*) (Lanes et al., 2008), Olive flounder (*Paralichthys olivaceus*)
60 (Zhang et al., 2003), Atlantic haddock (*Melanogrammus aeglefinus*) (Rideout et al., 2004) and
61 spotted wolffish (*Anarhichas minor*) (Gunnarsson et al., 2009). Cryopreservation is of interest
62 not only for fish farming but also for the conservation and genetic improvement of resources.
63 This technique has been well established in some freshwater fish species mainly, salmonid,
64 sturgeons and carps, however, only in the last two decades research was focused on marine
65 fish species (Cabrita et al., 2010; Beirao et al., 2019). The sperm of more than 40 marine fish
66 species have now successfully been cryopreserved (Tsai and Lin, 2012; Magnotti et al., 2018).
67 Moreover, with the expansion of global aquaculture production the capacity for long-term
68 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.

83 **2. Materials and Methods**

84

85 *2.1 Experimental fish*

86

87 The following experiment was carried out at Akvaplans-niva's FISK research station,
88 Kraknes, Tromsø in April and June 2020. The lumpfish broodstock used in this facility are
89 wild caught and used for commercial egg production. They are held in purpose-built tanks (8
90 m³) at the station until the females are ready to spawn. The tanks run on a flow-through system
91 with an average temperature of 6°C. Photoperiod manipulation (see Imsland et al., 2019a) was
92 used to either suppress (reduced light exposure) or encourage (increase light exposure)
93 spawning activity. The fish used in this trial were caught at Hekkingen (69°N), Malangen,
94 Norway and brought to the research station by boat. They arrived the day prior to starting the
95 experiment (23.04.2020) and were held in the dark overnight with males and females kept
96 separately. The mean weight (\pm SD) and length of the males was 1.36 ± 0.66 kg, 30.5 ± 3.6 cm
97 and mean weight and length of the females was 2.23 ± 0.78 kg, 35.8 ± 2.9 cm.

98

99 *2.2 Milt collection*

100

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

107 solution (Opeifa, 2019) was prepared one day before the main experiment by measuring 154
108 mM NaCl, 4.55 mM CaCl₂, 2.37 mM MgSO₄, and 4.8 cm³ mM KHCO₃ salts and dissolving
109 them in 1000 ml distilled water. The wolffish extender solution was added to the paste at a
110 diluted 50:50 volume ratio and left for 10 minutes to soak. This mixture was then passed
111 through a 50 µm sieve into a beaker to separate sperm from tissues. From the milt/extender
112 liquid that was collected, 30 ml was removed and placed into sterile falcon tubes which were
113 labelled according to each male. The remaining volume was stored in individual containers
114 which were placed on stirring pads (50-75 rpm) in a refrigerator at 4°C.

115

116 *2.3 Sperm analysis*

117

118 *2.3.1 Sperm counts*

119

120 A small sample of milt from each male was kept aside (~1 mL) in individual cuvettes.
121 A series of serial dilutions were set up in 1.5 ml cuvettes using the following volumes shown
122 in Fig. 1. For the counts, 250 µL of the final dilution was added to a haemocytometer
123 (Neubauer, Tek-Event Pty Ltd., NSW, Australia) until the counting chamber was flooded. The
124 haemocytometer was viewed under a WPI inverted light microscope (40x0.6 objective). Cell
125 counts were obtained by counting how many individual sperm were present in 5 small boxes
126 (16 squares in each) and the total was used to calculate an average cell count using the
127 following equation: Sperm/ml = (Number of sperm counted*Dilution factor)/(Volume*1000).

128

129 *2.3.2 Sperm motility*

130

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

141

142 *2.4 Cryopreservation of sperm*

143

144 Sperm was cryogenically frozen according to the protocol described by Opeifa (2019),
145 but with minor modifications. For each fresh sperm sample (1:1 volume of sperm: extender),
146 dimethyl sulfoxide (DMSO) cryoprotectant was added at 10% volume (30 ml solution = 3 mL
147 DMSO) and mixed thoroughly. DMSO has a freezing point of 19°C therefore it was stored in
148 a water bath at 21°C to prevent it solidifying. Pre-labelled cryo straws (2 mL, Minitübe GmbH,
149 Tiefenbach, Germany) were loaded with sample (1 mL) and sealed with a stainless-steel ball
150 at each end. The straws were then kept on ice for 10 min (equilibration time) from adding 10
151 % DMSO to the falcon tube until freezing the straws on one of the freezing trays above the
152 liquid nitrogen. No attempt was made to measure the motility after the equilibration time. The
153 straws were loaded onto a metal rack embedded in Styrofoam (5 x 5 cm, 1 cm thick) which
154 was floating on a bath of liquid nitrogen (5 cm, Fig. 2). The floating rack held the straws 2.5

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

160

161 *2.5 Egg collection*

162

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

176

177 *2.6 Fertilization*

178

179 The eggs were fertilized on day 2 of the trial with 100 μ L of either fresh or frozen
180 sperm from each male. The cryo straws of frozen sperm were held in a flask of liquid nitrogen
181 on the bench. The containers of fresh sperm were removed from the fridge as and when
182 required. This research group has previously done preliminary trials on several aspects in
183 relation to storage and use of fresh milt (Opeifa, 2019; Lauri Kapari, Akvaplan-niva, pers.
184 comm.) and fresh sperm can be refrigerated for short period (1-2 days) without negative effects.
185 Using a 200 μ L pipette (Accumax A series, Accumax Lab Devices Pvt. Ltd, India), 100 μ L of
186 fresh sperm was added to each one of the three pots for each male. The sperm was mixed using
187 a sterile pipette to distribute around the eggs. Following this, 2 mL of seawater was added on
188 top of the eggs to activate the sperm and the mix was thoroughly stirred. The next stage was
189 to thaw the frozen sperm. For each male, 4 individual 2 mL straws were thawed and pooled.
190 The straws were removed from the liquid nitrogen using tweezers and plunged into a
191 freshwater bath which was maintained at an average temperature of 5°C. The straws thawed
192 in the water bath for 2 minutes. Individually, the straws were removed from the water bath,
193 dried to remove any water and one end cut off with sterile scissors. All four straws were
194 emptied into one sterile container which was then mixed and from which, 100 μ L was taken
195 per pot of eggs. The same procedure was carried out for fresh and frozen for all 5 males and a
196 set of three controls was set up whereby eggs were left unfertilized. The egg / sperm ratio used
197 in the fertilization validation was 3 ml eggs = ca. 100 eggs fertilized with 100 microliters
198 sperm at a concentration of $10^9 - 10^{10}$ cells/ μ l approximately $10^{11} - 10^{18}$ sperm cells /egg.

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

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

204

205 *2.7 Incubator set-up*

206

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

216

217 *2.8 Egg sampling*

218

219 24 hours post fertilization, approximately 100 eggs were sampled from each incubator.
220 Eggs were carefully removed (by hand) from two adjacent sides of the cake to better represent
221 the overall population. They were placed into welled chambers and carefully pulled apart using
222 tweezers and put in individual rows. Approximately 1 mL of a 10% acetic acid and seawater
223 solution was added to each row of embryos to increase contrast and make the cells easier to
224 distinguish. The eggs were checked for fertilization (formation of an embryo) by the presence
225 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

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

256

257 **3. Results**

258

259 *3.1 Sperm characteristics*

260

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

264

265 *3.2 Egg fertilization and development*

266

267 *3.2.1 Fertilization*

268

269 The egg batches which were fertilized using fresh sperm, had a higher fertilization
270 success than those fertilized with cryogenically preserved sperm (fresh $92.6 \pm 0.8\%$, frozen
271 $77.9 \pm 1.8\%$, mean \pm SEM, two-way ANOVA, $P < 0.05$, Fig. 6). Overall, both treatments
272 resulted in high fertilization percentages across all groups ($>68\%$). There was no significant
273 difference between males and fertilization percentage (two-way ANOVA, $P > 0.70$, Fig. 6).

274

275 *3.2.2 Embryonic development*

276

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

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

283

284 *3.3 Hatching*

285

286 *3.2.1 Hatching success*

287

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

296

297 *3.2.2 Larval growth*

298

299 There was no significant difference in size between treatment groups at day 14 post
300 hatch (fresh $0.63 \text{ g} \pm 0.02$, frozen $0.59 \text{ g} \pm 0.03$). Condition factors were not significantly
301 different between groups treated with fresh sperm compared to those with cryopreserved either
302 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
303 ± 0.13).

304 **4. Discussion**

305

306 *4.1 Characterisation of males*

307

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

319

320 *4.2 Fertilization and embryonic development*

321

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

328 al., 2019). The decreased fertilization rate in the eggs treated with cryo sperm, could be as a
329 result of sperm being damaged by the freezing process (Fernandez-Díez and Herráez, 2018;
330 Zadmajid et al., 2019), or potentially an error in the freezing or thawing technique. Sperm
331 cryopreservation generates different levels of DNA damage, mostly fragmentation and base
332 oxidation, as has been reported in different species (Herráez et al., 2015; Fernandez-Díez and
333 Herráez, 2018). Sperm is highly temperature sensitive (Majhi et al., 2013; de Costa et al.,
334 2019) and if temperature fluctuated during the cryopreservation method it is almost inevitable
335 that this fluctuation caused sperm damage.

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

340 Assessing embryos for the presence of eyes is a commonly used marker of
341 development in aquaculture (Kanyilmaz et al., 2016; Imsland et al., 2019b). The results
342 showed that the number of embryos which reached eyed stage, was similar to the fertilization
343 results. It is likely that the method of storage of sperm did not have any additional effect during
344 4-cell to eyed embryo stage and that the difference in the number of eyed embryos is a
345 reflection of the difference in percentage fertilization.

346

347 *4.3 Hatching success*

348

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

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

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

368

369 *4.4 Growth*

370

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

376 sperm (Zilli et al., 2003; Nahiduzzaman et al., 2011). Cryopreserved sperm affected
377 embryonic development and reduced survival of the eyed-eggs and larvae of trout (Pérez-
378 Cerezales et al., 2011). In some studies use of cryopreserved sperm have led to lower larval
379 growth (Nusbaumer et al., 2019), whereas other studies have not found this (Chereguini et al.,
380 2002; Sarder et al., 2013; Akter et al., 2016). In the present study growth of the two groups of
381 lumpfish larvae was similar suggesting that once the larvae are hatched out, there is no
382 detrimental effect of cryopreservation on the early growth of lumpfish larvae.

383

384 **5. Conclusion**

385

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

391

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400 **Author statement**

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402 Supervision; Writing – original draft; Writing – review & editing; Emily Purvis, Investigation;
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406

407 **Conflict of interest**

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

409

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412

413

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580

581 **Figure legends**

582

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

584

585 Fig. 2. Cryopreservation of sperm samples. (a) Straws are acclimatised on a raft which floats
586 2.5 cm above the surface of liquid nitrogen. (b) Straws are plunged into liquid nitrogen,

587

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

590

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

593

594 Fig. 5. An eyed lumpfish embryo. The arrow indicates the presence of a developed eye at c.
595 100 day-degrees post-fertilization. The diameter of the egg is 2.2 mm.

596

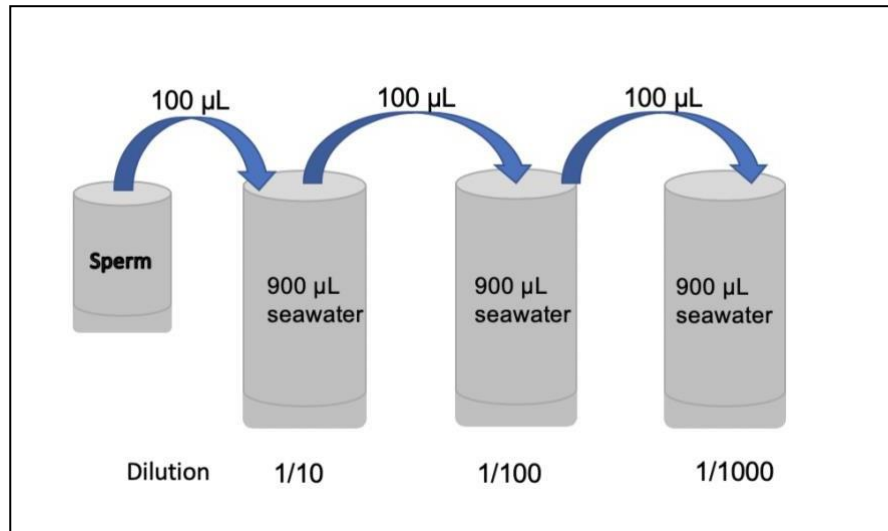
597 Fig. 6. The average percentage off eggs fertilized from a sample of 100 taken from each
598 replicate (N=3). Eggs were fertilized in triplicate batches with samples of fresh and
599 cryogenically preserved sperm from 5 male lumpfish.

600

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

604

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).



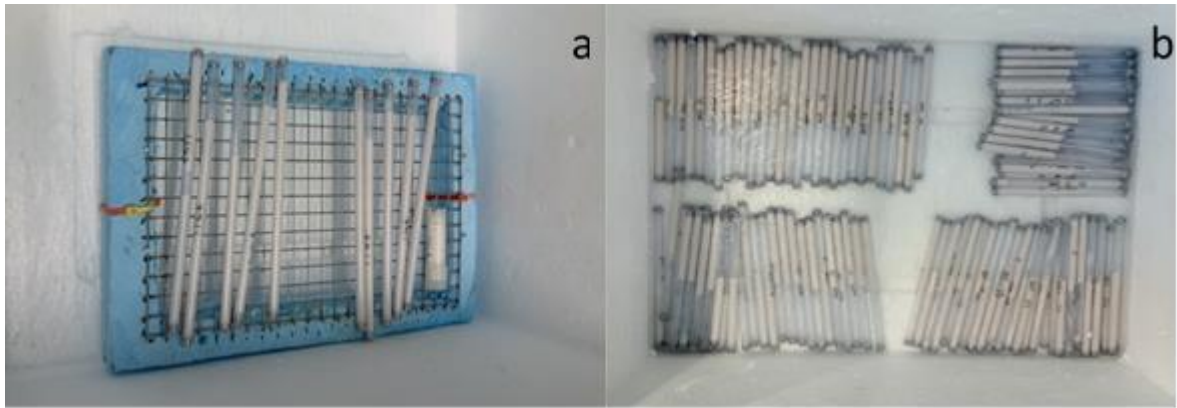
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610 Fig. 1. Imsland et al.

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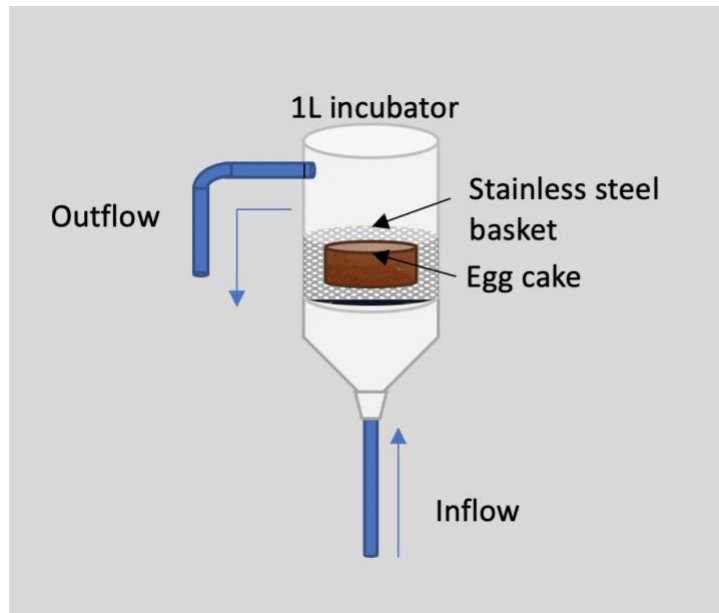


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615 Fig. 2. Imsland et al.

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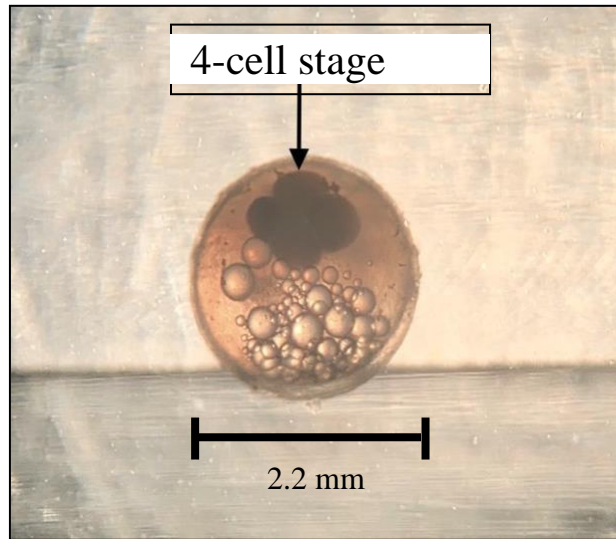
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619 Fig. 3. Imsland et al.

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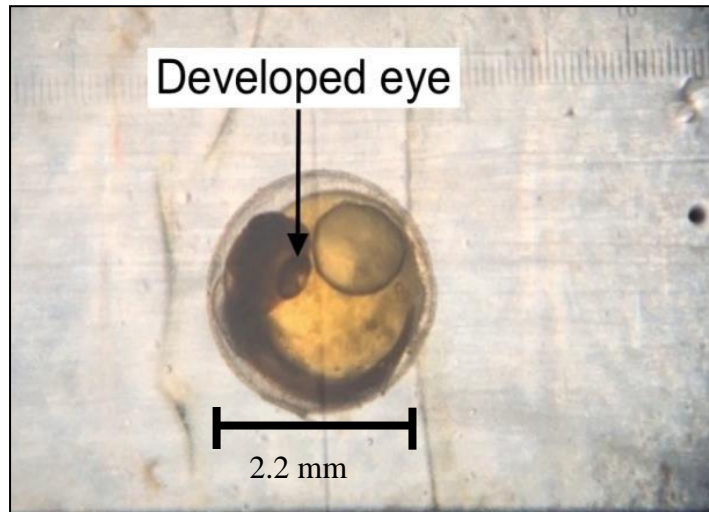
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624 Fig. 4. Imsland et al.

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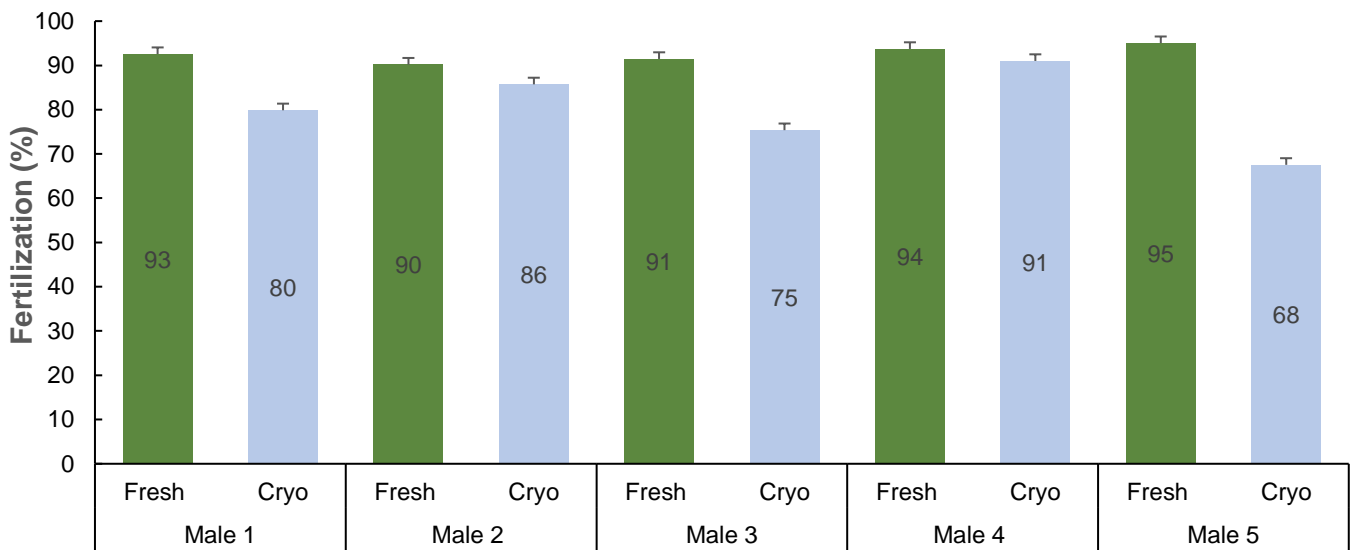


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627 Fig. 5. Imsland et al.

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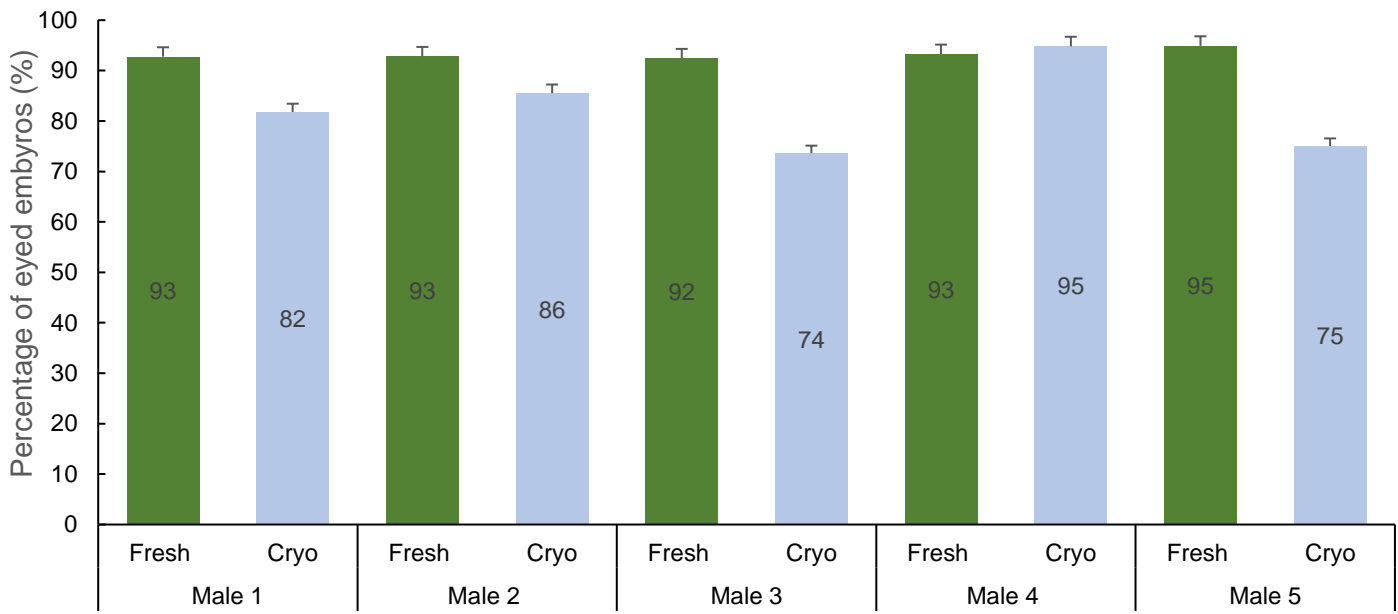


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631 Fig. 6. Imsland et al.

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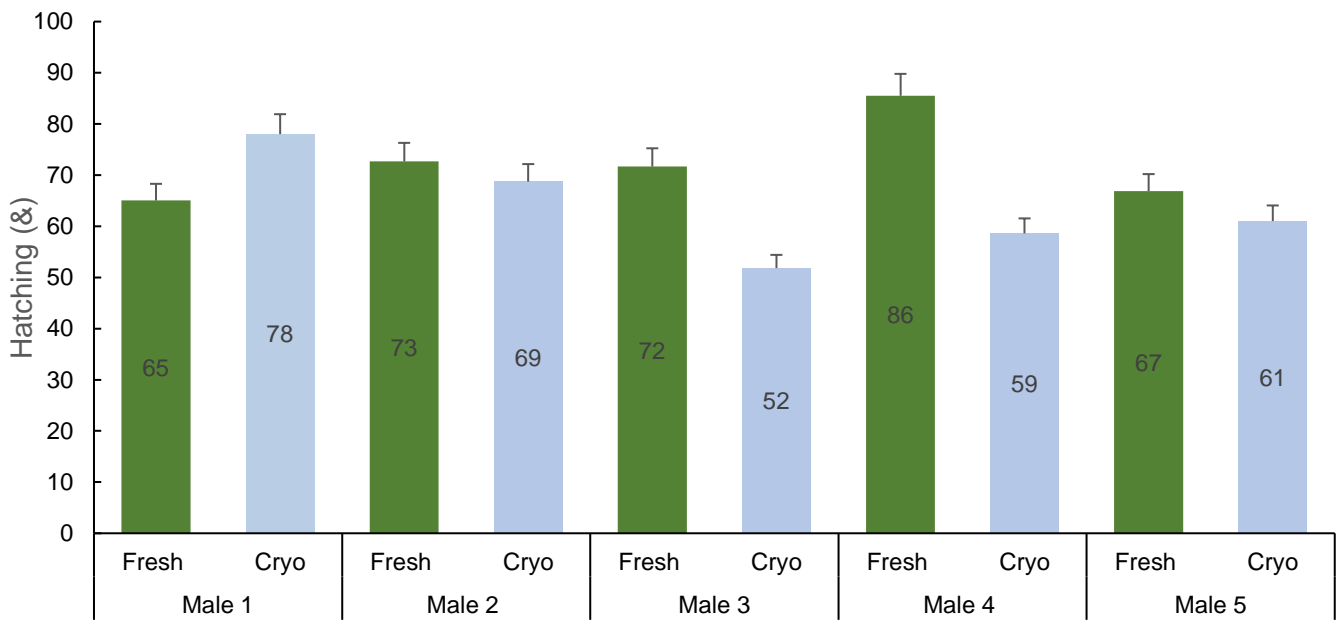


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635 Fig. 7. Imstrand et al.

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638

639 Fig. 8. Imsland et al.

640

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