Cryopreservation of lumpfish *Cyclopterus lumpus* (Linnaeus, 1758) milt

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This study has established a successful protocol to cryopreserve lumpfish *Cyclopterus lumpus* (Linnaeus, 1758) milt. Three cryosolutions were tested based on Mounib's medium; the original medium including reduced l-glutathione (GSH), the basic sucrose and potassium bicarbonate medium without GSH, or with hen's egg yolk (EY). Dimethyl sulphoxide (DMSO) was used as the cryoprotectant along with all three diluents in a 1 to 2 dilution. Cryopreservation was performed with the mentioned cryosolutions at two freezing rates. Motility percentages of spermatozoa were evaluated using ImageJ with a computer assisted sperm analyzer (CASA) plug-in. Findings revealed that spermatozoa cryopreserved in Mounib's medium without GSH had a post-thaw motility score of 6.4 percentage points (pp) higher than those in the original Mounib's medium, and an addition of EY to the modified Mounib's medium lowered the post-thaw motility score by 19.3 pp. The difference in motility between both freezing rates was 13.0 pp, and samples cryopreserved on a 4.8 cm high tray resulted in a better post-thaw motility score. On average, cryopreserved milt had a 24.1 pp lower post-thaw motility score than fresh milt. There was no significant difference in fertilisation success between cryopreserved and fresh milt. Cryopreservation of lumpfish milt has, to our knowledge, never been successfully carried out before. The established protocol will be a main contributing factor in a stable production of lumpfish juveniles in future.

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11   1. Introduction

13   Cryopreservation is an effective method for long-term storage of viable spermatozoa in fish

14   (Blaxter, 1953). This technique offers several benefits including artificial fertilisation, which

15   allows for efficient use of milt. This is especially useful in species from which milt samples are

16   difficult to obtain (Ohta & Izawa, 1996), or when limited volumes can be stripped (Clearwater &

17   Crim, 1995). Other advantages of the technique include the option to preserve declining stocks

18   (Tian et al., 2008) and to retain genetic variability in broodstocks (Suquet et al., 2000).

20   Milt cryopreservation has been well established for some freshwater fish species belonging to

21   families of salmonids (Billard, 1992), cyprinids (Billard et al., 1995) and siluroids (Legendre,

22   Linhart & Billard, 1996). Cryopreservation efforts for marine fish species were ongoing already

23   in the 80's (Bolla, Holmefjord & Refstie, 1987), and (several) successful cryopreservation

24   protocols have been defined (Suquet et al., 2000), for e.g. Atlantic haddock (*Melanogrammus*

25   *aeglefinus*) (Rideout, Trippel & Litvak, 2004), Olive flounder (*Paralichthys olivaceus*) (Zhang et

26   al., 2003), Brazillian flounder (*Paralichthys brasiliensis)* (Lanes et al., 2008), and Atlantic

27   halibut (*Hippoglossus hippoglossus*) (Ding et al., 2011). However, it is a challenging task to

28   determine an optimal protocol for cryopreservation of milt for any particular fish species as

29   diluent and cryoprotectant selection, dilution ratio, as well as freezing and thawing rates are

30   parameters that interact with one another, and have all been found to vary greatly between

31   species (Suquet et al., 2000; Rideout, Trippel & Litvak, 2004).

32

33   Recent research has shown that lumpfish (*Cyclopterus lumpus*), an epibenthic-pelagic fish

34   species naturally found in the North Atlantic Ocean, is an effective cleaner fish in combating

35   infestation with sea lice (*Lepeophtheirus salmonis* and *Caligus elongats*) among farmed salmon

36   (Imsland et al., 2014), which is a growing problem in that industry (Costello, 2009a).

37   Commercial production of lumpfish juveniles is ongoing in Norway (Willumsen, 2001; Schaer &

38 Vestvik, 2012), and on the Faroe Islands our aim is to establish a year-round production of
39 lumpfish juveniles.
40
41 The lumpfish breeding season is considered to range from March to August (Johannesson 2006,
42 Kennedy et al., 2014), but in Faroese waters, mature lumpfish have been caught as late as in
43 November (own observation). During the breeding season, brood fish are obtained along the
44 coast lines, and are primarily captured by local fishing vessels using gillnets as the fish travel
45 from deep waters towards the shore for spawning (Holst, 1993; Muus, 1999). Males specifically,
46 are found around the shores during the breeding season as they prepare their territory and wait
47 for females to arrive, so spawning can take place (Goulet, Green & Shears, 1986; Kennedy et al.,
48 2014). Females lay their eggs in a nest site and then leave the spawning grounds for the males to
49 stay and protect their offspring from predators (Muus, 1999; Kennedy et al., 2014). It has been
50 reported that lumpfish males have a breeding period that lasts for 6 – 10 weeks (Davenport,
51 1985).
52
53 Reddish colouration is an indication that males have reached sexual maturity (Kells & Carpenter,
54 2011). Our observation is however, that this is not always a guarantee that stripping is successful.
55 Additionally, stripping most often results in small volumes of milt: in our trials volume ranged
56 from 0.08 to 3.2 ml. This is a limiting factor when a large number of eggs from females are to be
57 fertilized at the same time. Delaying stripping of females is unadvisable as eggs may become
58 overripe or the female may release roe before artificial fertilisation can be carried out (e.g. at
59 night), resulting in unfertilised roe (own observation). Cryopreservation of lumpfish milt could
60 solve the issues mentioned above and ensure a year-round supply of male gametes for a
61 sustainable juvenile production. Additionally, storing of milt from individuals with desirable
62 genetic qualities may be important if breeding programmes are to be initiated for this species.
63
64 In a pilot study (supplementary data), we tested three cryosolutions (see supplementary table 1)
65 previously found successful in the cryopreservation of milt from other marine fish species,
66 including Atlantic haddock (*Melanogrammus aeglefinus*) and Atlantic cod (*Gadus morhua*)
67 (Rideout, Trippel & Litvak, 2004), Olive flounder (*Paralichthys olivaceus*) (Zhang et al., 2003)
68 and Atlantic halibut (*Hippoglossus hippoglossus*) (Ding et al., 2011). We found that out of the

69   three solutions, only spermatozoa cryopreserved in potassium bicarbonate and sucrose; the

70   modified Mounib's medium with 10% DMSO (Dimethyl sulfoxide), as used by Ding et al.

71   (2011), resulted in motile spermatozoa post cryopreservation (see supplementary table 2). The

72   original Mounib's medium also includes 6.5mM reduced L-Glutathione (GSH) (Mounib, 1978),

73   which is known to prevent free radicals from oxidising the lipid bilayer in the cell membrane,

74   and thus prevents cell degradation. The role of sucrose in the medium is to stabilise the

75   liposomal membrane of spermatozoa during cryopreservation (Quinn, 1985; Gwo, 2000).

76

77   Freezing rate has an effect on motility post-cryopreservation (Suquet et al., 2000). The height of

78   the tray used in the cryopreservation process has a great effect on the rate. Rideout et al. (2004)

79   found that a 3 cm high tray resulted in samples reaching -90º C in 90 seconds whereas it took 12

80   minutes on a tray 5.5 cm high. They also found better survival in the spermatozoa when the

81   freezing rate was slow. In accordance with this, our pilot study indicated better motility post

82   cryopreservation when samples were cryopreserved on a 4.8 cm tray compared to a 2.5 cm tray

83   (supplementary table 2).

84

85   Thawing temperature may also affect post-cryopreservation motility. For marine fish species,

86   applied thawing temperatures typically vary between 10 and 40º C (Suquet et al., 2000), these

87   are lower than temperatures used for milt samples from freshwater fish species, which vary

88   between 30 and 80º C (Suquet et al., 2000). These findings correspond well to our pilot study

89   where two thawing rates were tested; 37º C and 50º C and results showed clearly that lumpfish

90   milt thawed in 37º C resulted in higher motility scores (supplementary table 2).

91

92   Our pilot study was unfortunately only based on milt samples from one lumpfish (stripped in

93   2012), so there were no replicates to base any firm conclusions on. Additionally, we did not have

94   optimal equipment (cryo vials instead of straws) and were unable to carry out computer assisted

95   sperm analysis (CASA).

96

97   Based on pilot study findings detailed above, we tested the efficacy of cryopreserving lumpfish

98   milt (stripped in 2014) in three different diluents, all being variations based on Mounib's

99   medium (100 millimolar (mM) potassium bicarbonate ($KHCO_3$), and 125 mm sucrose) as earlier

100   findings indicated its success. We also tested the effect of two different freezing tray heights (4.8

101   and 6.4 cm). Motility performances of fresh and cryopreserved spermatozoa were analysed using

102   a modern CASA system. Fertility post-cryopreservation was compared to the fertility of fresh

103   milt.

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106   2. Materials & Methods

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108   *2.1. Experimental design*

109   Stripped milt from seven lumpfish males (weight: mean ± SD; 459.53 ± 348.10 g, and length:

110   22.44 ± 5.21 cm) was used in this experiment (some males were stripped more than once, so

111   n=16 samples). Stripping volume ranged from 0.3 to 2.7 ml per sample (0.97 ± 0.73)

112   (supplementary table 3). Motility percentage, pH (6.55 ± 0.21), osmolality (0.463 ± 0.06

113   mOsmol kg^{-1}), and milt concentration (31.44 x 10^9 ± 8.35 x 10^9 cells ml^{-1}) were measured after

114   stripping each male. In the cryopreservation experiment, milt samples (n=16) from seven fish

115   were cryopreserved in triplicates in the following diluents: 1) Mounib's medium without GSH

116   "Mounib no GSH", as in Ding et al. (2011), 2) Mounib's original medium (Mounib, 1978)

117   "Mounib", and 3) Mounib's medium as in "Mounib no GSH" but with added hen's egg yolk

118   (EY), which is a non-penetrating cryoprotectant that is commonly used in cryopreservation

119   (Jamieson, 1991) "Mounib plus EY". The cryoprotectant DMSO (10%) was used with all three

120   diluents (table 1). Additionally, we test two tray heights: 1) the previously successful 4.8 cm tray

121   and, 2) a higher tray of 6.4 cm to ascertain whether an even slower freezing rate may further

122   increase motility. All milt samples were tested at two freezing rates except "Mounib", which was

123   only tested at one of these (tray height: 4.8 cm) due to insufficient volumes of milt. For every

124   milt sample used in the cryopreservation experiment, a matching sample of fresh milt (obtained

125   from the same stripping) served as a control.

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135 **Table 1** The chemical composition of the three cryosolutions tested in this experiment. All are based on Mounib's
136 basic medium with and without reduced l-glutathione and hen's egg yolk. Mounib no GSH and Mounib plus EY
137 were tested on two freezing trays (height 4.8 and 6.4 cm), and Mounib was only tested on one tray (height 4.8 cm).
138 The concentration in every sample was: milt to diluent (1:2) and 10% of the diluent volume was DMSO.
139

Cryosolution:	Diluent:	Dilution:	Cryoprotectant
Mounib	100 mM KHCO3, 125 mM Sucrose and 6.5 mM L-Gluthatione (reduced)	1:2	10% DMSO
Mounib no GSH	100 mM KHCO3 and 125 mM Sucrose	1:2	10% DMSO
Mounib plus EY	100 mM KHCO3, 125 mM Sucrose and 10% hen egg yolk	1:2	10% DMSO

140 All reagents in this table are purchased from VWR, Bie & Berntsen, Denmark.
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143 *2.2. Gamete collection*

144 The majority of milt samples were obtained from fish produced from wild broodstock at Nesvík

145 Marine Centre, Faroe Islands in 2013 (n = 12 samples from five fish). Other samples were

146 obtained from wild fish captured near the shore by divers 8-10 days prior to this experiment (n =

147 4 samples from two fish) (supplementary table 3). Fish were held in 3 metre diameter cylindrical

148 tanks, water depth 1 m with flow through filtered and UV treated sea water (35‰). Fish were fed

149 ad lib with 3 mm standard commercial fish feed, Atlantic salmon type: "Margæti" from

150 Havsbrún, Faroe Islands. The feed contained 48% and 27% crude protein and fat respectively.

151

152 Males with a reddish appearance were placed in a tank along with females (one male/three

153 females) at ambient temperature (9-10° C) for a few days prior to stripping in order to enhance

154 gamete production (Klokseth & Øiestad, 1999). A pre-stripping check was done by light

155 pressure on the sides and abdomen of the males. If milt was released, fish were placed in a 20

156 litre container with sea water along with 20 ml of Benzocaine (anaesthetic). Once fish were

157 unconscious, the milt was collected into 5 ml syringes, and placed on ice immediately. The fish

158 were placed back into the tank with continually flowing sea water to recover.

159

160   Eggs were obtained from wild females (n=10) captured 8-10 days prior to our trials near Faroese

161   coastlines by local fishing vessels. Females with large bulging abdomens were easily identified

162   as carrying roe and swelling around the urogenital opening signalled imminent spawning. Before

163   stripping began, females were anaesthetized in same procedures as with the males mentioned

164   earlier. Eggs were stripped by placing 1-2 fingers into the urogenital opening to release the roe

165   and applying mild pressure to the abdomen, allowing the roe to drain into cleaned measuring

166   containers. The amount of stripped eggs ranged from 100 ml to 400 ml per fish. Stripped eggs

167   were immediately stored at 4° C until the fertilisation procedure was initiated, which was usually

168   within an hour.

169

170   Spermatozoa were counted under a microscope (Leica DM1000 led) using a hemocytometer

171   (thoma 0.1 mm) using standard counting protocols: milt was diluted 1 to 1000 in a non activating

172   medium (NAM) previously used in Fauvel et al. (1998) prior to counting. 10 µl of the dilution

173   was loaded into both counting chambers of the hemocytometer and allowed to settle for 10

174   minutes before counting the spermatozoa. Cells were counted in five squares each in two

175   chambers on the thoma cell counting chamber (not counting cells on the bottom and right edges)

176   and multiplied up to a cell count per ml. The osmolality of milt was measured using a Gonotec

177   Osmomat 030-D cryoscopic osmometer. The pH value of stripped milt was measured using a

178   PHM 62 standard pH meter.

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181   *2.3. Motility measurements*

182   Triplicates of fresh milt samples were examined within 30 minutes after stripping. The milt was

183   diluted 1 to 200 in an activating medium (AM) made of 50% filtered sea water (SW), and 50%

184   bovine serum albumin (BSA) (VWR, Bie & Berntsen, Denmark), prepared in distilled water (10

185   mg/ml) beforehand, to avoid the cells from sticking to the microscope slide. Immediately after

186   milt was added to the AM, the dilution was cautiously mixed with the pipette tip to distribute the

187   cells evenly, and then 6 µl of the dilution was quickly transferred into one chamber of a Leja 2

188   chamber CASA microscope slide (SC-20-01-02-B) (Leja Products B.V.).

189

190   A two-step dilution procedure is often performed for measuring motility in milt (Dreanno et al.,

191   1997; Fauvel et al., 1999; Groison et al., 2010). This procedure involves first diluting milt in

192   NAM, an isotonic medium similar to the chemical composition of milt (Fauvel et al., 1998), in

193   order to keep spermatozoa quiescent. Subsequently, the milt dilution is transferred to a

194   microscope slide, where an activating agent (usually sea water) is added before monitoring the

195   spermatozoa. In our trials, this procedure resulted in an uneven distribution of spermatozoa when

196   the AM was added to the milt dilution within the chamber of the microscope slide. As lumpfish

197   spermatozoa may be motile up to several minutes (unpublished data), we chose to do a one-step

198   procedure and diluted all samples in the AM directly, which allowed us to get an even

199   distribution of cells for observation and recording video for the CASA system.

200

201   All samples were observed with phase contrast (PH2) under a Leica DM1000 led microscope

202   (object lens: 20x). A digital camera (Leica DFC 295) was attached to the microscope and

203   coupled to a computer, and with the included Leica application suit (LAS) software, a clear live

204   video feed of spermatozoa was obtained. Settings on the LAS software were adjusted to: 44.5 ms

205   exposure, 1.4 x gain and gamma 1.34, and image set to greyscale. Recording was achieved using

206   a Blueberry software (BB flashback Pro 4) player. Two minutes of each sample was recorded,

207   and the first 20 seconds of each movie were always excluded to avoid measurements of

208   spermatozoa moving due to flow and avoiding the lag period caused by mixing milt and AM,

209   and loading into the chamber of the slide. A total of 46 video frames were extracted from each

210   video and saved as AVI files. Image J (http://rsb.info.nih.gov/ij) (Rasband) open source

211   software, including a CASA plug-in that allows measuring the motility percentage of fish milt

212   (Wilson-Leedy & Ingermann, 2007), was used for video analysis. To get accurate measurements

213   of motility percentage of lumpfish spermatozoa using the CASA plug-in in ImageJ, the image of

214   the imported AVI file threshold was adjusted to 57. In the CASA plug-in sperm tracker fields we

215   only adjusted a few settings to get accurate measurements, these included, the maximum sperm

216   size to 99 pixels, the minimum track length to 10 frames, the maximum sperm velocity between

217   frames to 50 pixels, and the frame rate to 10 frames per seconds (the video was recorded at

218   10fps).

219

220   *2.4. Cryopreservation and thawing.*

221 "Mounib" and "Mounib no GSH" diluents were prepared within a week before the

222 cryopreservation experiments, and stored at 4 º C along with hen's egg yolk. DMSO was stored

223 at room temperature. Milt samples in syringes were stored on ice not more than an hour before

224 the cryopreservation set-up was prepared. The cryopreservation set-up was prepared on ice; first

225 by adding diluent stock into cooled eppendorf tubes, thereafter DMSO. In cryosolution "Mounib

226 plus EY", egg yolk was added after the diluent, then the cryoprotectant. Finally, milt was added

227 to all tubes, and ingredients cautiously mixed by aspiration with a pipette, samples were allowed

228 to stand for a 10 minute equilibration time. All samples were cryopreserved in 250 µl cryo-

229 straws (Cryo Bio System). Samples were drawn into the straws manually and sealed. They were

230 then attached on to the top of a floating tray that was either 4.8 cm in height, or 6.4 cm in height,

231 representing two freezing rates. Floating trays were placed within a Styrofoam box (inside

232 dimensions H x L x W= 21cm x 35.5cm x 23cm) filled with liquid nitrogen (-196º C) with an

233 approximate depth of 10cm to allow straws to cool in the nitrogen gas layer for 10 minutes.

234 Thereafter, the trays were turned over and samples plunged directly into the liquid nitrogen and

235 left for at least 15 minutes before the thawing process was initiated. Straws were taken directly

236 from liquid nitrogen into a water bath at 37 º C for a duration of 7 seconds to be thawed. The

237 ends of straws were cut off with scissors to allow the samples to drain into fresh cooled

238 eppendorf tubes. The examination of the cryopreserved milt was done in the same way as with

239 fresh milt samples, only difference being that cryopreserved samples, having already been

240 diluted in cryosolution, were diluted only 1 to 30 in the AM.

241

242 *2.5. Fertilization*

243 Lumpfish eggs become sticky and attach to each other on contact with sea water and form dense

244 lumps within a few minutes (Davenport, 1985). This property can make it difficult to obtain

245 good estimates of fertilisation success as visually inspecting roe on the inside of such lumps is

246 impractical. Ballan Wrasse (*Labrus bergylta*) roe have a similar biochemical reaction when

247 exposed to sea water, but mixing their roe with subtilisin, an enzyme also referred to as

248 'alcalase', along with Ringer's solution removes the egg stickiness and allows eggs to remain

249 unattached (Lein et al. 2013). This method was tested in our own laboratory using lumpfish eggs

250 with successful results (unpublished data) and was therefore incorporated into the fertilization

251 procedure of this study. Before fertilization was initiated, the Ringer's solution found in Young

252 (1933) was combined with 10% Alcalase® 2.4 L FG (Novozymes A/S). Each sample (20 ml;

253 about 2.300 eggs) was fertilised with milt at an approximately 1:30.000 egg to motile

254 spermatozoa ratio. After adding the milt sample to the eggs, approximately 10 ml of sea water

255 was stirred in to activate the spermatozoa. The compounds were mixed by gently stirring for at

256 least 30 sec, and then allowed to stand for 2 minutes before 10 ml of the Ringer's + alcalase

257 solution was added, and again gently stirred into to the sample for a few seconds to obtain an

258 even distribution. The mixture was allowed to stand for 5 min before being poured into a fresh

259 petri-dish containing SW (35‰), and gently stirred for a few seconds to dissolve the "glue" away

260 from the eggs. Fertilized eggs were then placed in round incubators (15 L) with eight

261 compartments separated by netting (1 mm mesh size) Water flow was set to $9L \cdot min^{-1}$ to allow

262 enough water change in all compartments. Water temperature was measured daily and ranged

263 between 11.1 - 11.3° C. After 78.4 – 89.7 day degrees (dd), eggs were inspected under a

264 dissection microscope to ascertain fertilisation. Approximately 50-200 eggs were inspected in

265 each sample.

266

267 *2.6. Statistical analysis*

268 Analysis was carried out using R version 3.0.3 (R Core Team 2014) and Excel (MS Office).

269 While motility and fertilisation data are measured in percentages, the residuals in the chosen

270 linear mixed effects models were sufficiently normal (Shapiro-Wilks tests for normality: Effect

271 of cryopreservation on motility; W = 0.98, P = 0.41, Effect of tray height and diluent type on

272 motility; W = 0.97, P = 0.36, Effect of cryopreservation on fertility; W = 0.96, P = 0.34), so

273 using generalized linear models or transforming the data was not justified in this case.

274

275 Effect of cryopreservation and the effects of diluent type and tray height on motility were

276 analysed using similar models. In both cases, a linear mixed effects model, "lmer" in the R

277 package lme4 (Bates et al., 2014) was used with the aforementioned dependent and independent

278 variables as well as "batch" as a random effect. Batch refers to an individual stripping of milt and

279 any one male may produce several batches of milt. We chose "batch" as the random effect

280 because males did not differ significantly in motility scores (Anova; $F_{6,9} = 2.06$, P = 0.16), which

281 can be explained by high within-male variation in motility (supplementary fig. 1). P values were

282 extracted using a type II Wald Chi squared test included in the R package "car" (Fox &

283   Weisberg, 2011). Effect of cryopreservation of milt on fertilisation success was analysed using a

284   simple linear model.

285

286   Differences between treatments are expressed in percentage points (pp) and means are provided

287   with Standard Deviations.

288

289   *2.6. Ethical statement*

290   As there is no animal experimentation legislation on the Faroe Islands, the local "animal

291   protection act" was adhered to (Vinnumálaráðið, 1990) throughout this study. A fish veterinarian

292   advised on best practice in relation to stripping to ensure no undue suffering caused by the

293   procedure. Our impact on wild populations was limited, as we used predominately captive bred

294   fish, which were bred for the purpose of producing a domestic lumpfish stock. There were no

295   fish mortalities caused by our study and effort was put into providing optimal care and welfare

296   for all fish involved.

297

298   3. Results

299   Cryopreserved milt had a 24.1 pp lower motility than fresh milt (type II Wald test; Chi squared =

300   41.88, df = 1, P < 0.001) with an average motility of fresh milt being 72.82 ± 13.60 %, and that

301   of cryopreserved milt 48.73 ± 19.01 %. On average, milt samples treated with Mounib no GSH

302   (tray height: 4.8 cm) had the best motility recovery of all treatments, which was 27.4 pp higher

303   than milt samples cryopreserved with Mounib plus EY (tray height 6.4 cm), which had the

304   lowest motility recovery.

305

306   Tray height had a significant effect on post-thawing motility with the lower tray (4.8cm)

307   producing a 13.0 pp higher motility than the higher one (Chi squared = 14.05, df = 1, P < 0.001;

308   figure 1). Using different cryosolutions also affected the motility recovery; adding 'EY' to the

309   modified Mounib's medium resulted in a 19.3 pp lower motility while the modified Mounib's

310   medium had 6.4 pp higher motility than the original Mounib's medium (Chi squared = 44.05, df

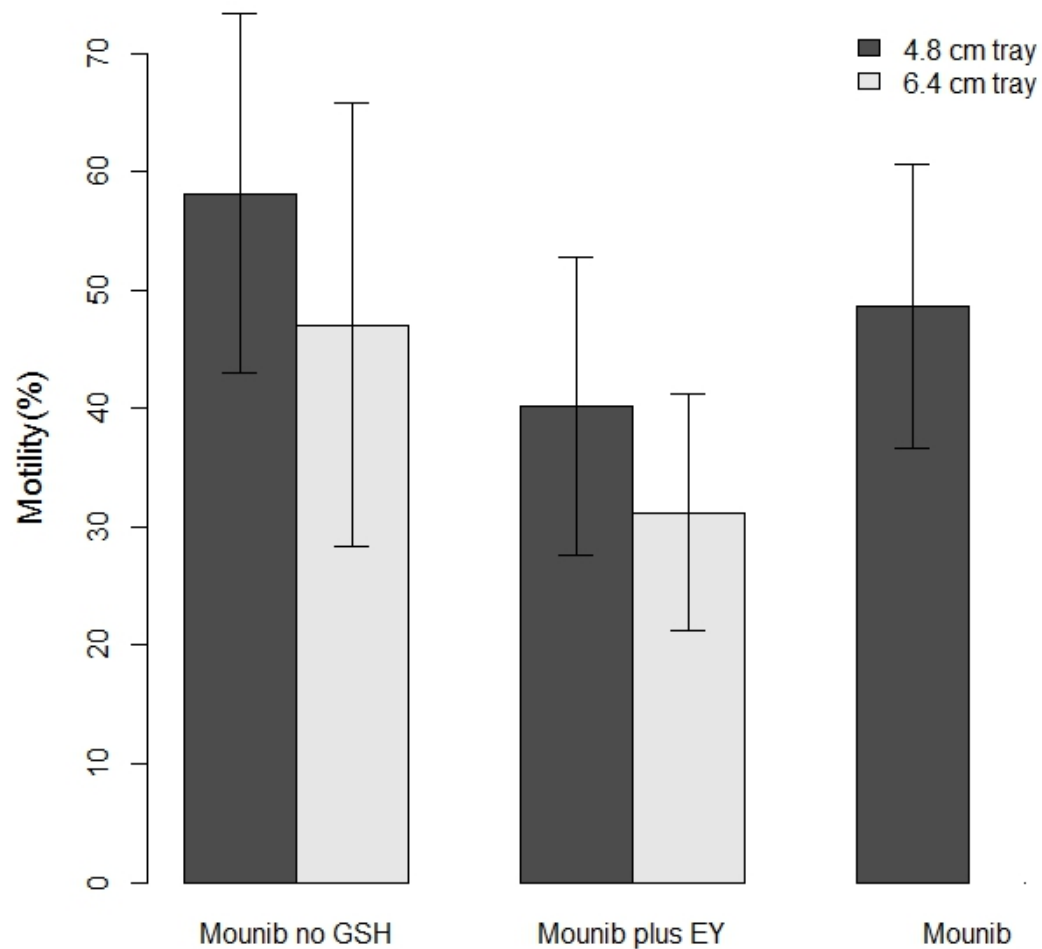
311   = 2, P < 0.001) (figure 1).

312

313   The fertilisation success of eggs fertilised with fresh milt was not significantly higher than those

314   fertilised with cryopreserved milt (30.81 ± 16.87 % and 27.30 ± 17.02 % respectively; $F_{1,24} =$

315   0.91, P = 0.35) (supplementary table 4).

316

317

318   **Figure 1.** Mean motility of milt preserved in three different mediums on two different tray heights. Error bars
319   represent 1 standard deviation.

320

321   4. Discussion

322

323   This paper details a successful method for cryopreserving lumpfish milt. To our knowledge no

324   such methodology has previously been published on this species, and this information may be

325   beneficial in future development of lumpfish as an aquaculture species. Our results indicate a

326   motility loss of less than 14.2 pp when using our most optimal cryopreservation protocol. This is

327   comparable to cryopreservation of e.g. cod, halibut, ocean pout, sea bream, striped trumpeter and

328   turbot reviewed in Suquet et al. (2000), in which post-frozen motility recovery, expressed as a

329   percentage of fresh sperm motility rate, ranged from 39 – 85 % compared to our result, which

330   was 80.92% at optimal tray height and cryosolution (67.8% on average).

331

332   Based on previous findings in our pilot study, we tested three variants of Mounib's medium. Our

333   results indicate that the best diluent is Mounib's no GSH. This suggests that the penetrating

334   cryoprotectant DMSO is sufficient to protect the spermatozoa from cell damages that occur

335   during the freezing and thawing process. One would expect that the addition of GSH would have

336   a positive effect on motility recovery as it has been shown in other animal species that the

337   freezing process leads to a significant reduction of GSH content in spermatozoa (Bilodeau et al.,

338   2000; Gadea et al., 2004, 2005). In 2011, a study was carried out to test the antioxidant effect in

339   cryopreservation of fish spermatozoa by adding different antioxidants to cryosolutions including

340   GSH. The outcome was that the addition of GSH and any other antioxidant compound had no

341   significant effect on motility or fertility, and in some instances it had a slightly negative effect

342   (Lahnsteiner, Mansour & Kunz, 2011). This is similar to our findings, but a more recent study

343   also using milt from rainbow trout (*Oncorhynchus mykiss*) revealed that addition of GSH (using

344   the same concentration as the study from 2011) to the cryosolutions actually did have an

345   significant positive effect on motility but no effect on fertility (Kutluyer et al., 2014). It is

346   possible that lumpfish milt benefits from the antioxidant property of GSH during a longer storage

347   period comparable to that in Mounib's study (1973).

348

349   Our pilot study indicated that a tray height of 2.5 cm was too low, and findings in this study

350   point to that 6.4 is too high with 13.04 pp lower motility than the lower tray (4.8 cm).

351   Interestingly, Rideout et al's 5.5 cm tray was better than their 3 cm tray. This suggests that there

352   may be an optimal tray height yet to be found or that optimal tray height is species dependent.

353   Perhaps the optimal height for lumpfish milt lies somewhere between 4.8 and 5.5 cm.

354

355   In Suquet et al. (2000) the optimal thawing temperature for marine fish species is said to vary

356   from 10 – 40° C. We have only tested two thawing temperatures (37° C and 50 ° C) in our pilot

357   study, which showed that 37° C is useful and 50 ° C is not (supplementary table 2). The duration

358   time was 1 min or 1.5 min in that case, as samples were cryopreserved in vials, which need a

359   longer thawing period, compared with straws as they are thinner and have a larger surface area.

360   Therefore 37° C was used in this study and the thawing time (7 seconds) was estimated to end

361   when bubbles appeared within the straw - indicating that samples were thawed. It could therefore

362   be interesting to test if an even lower thawing temperature and using a longer thawing time

363   would optimize the motility recovery of lumpfish milt. It is also possible that tray height and

364   thawing temperature interact, in which case a tray height resulting in low motility recovery at

365   one temperature may result in better recovery at a different temperature.

366

367   This study showed that cryopreserved lumpfish milt can be used to fertilise eggs. Using our

368   cryopreservation method the fertilisation success was only 3.51 pp lower using cryopreserved

369   milt compared to fresh milt. The two groups were not significantly different, but our estimates

370   are confounded by a number of factors that are not easily accounted for in this study. Female egg

371   quality may differ drastically and thus fertilisation success (own observations), so variation

372   seems to be large. Additionally, there are concerns regarding the ability to detect fertilised eggs

373   early on (78-89 dd) as our own observations indicate a variability in speed of development.

374   Because of this, some eggs that were in fact fertilised may have been classified as not fertilised.

375   That being said, this kind of variation applies to both eggs fertilised with cryopreserved milt and

376   fresh milt, so there was no systematic difference between the groups other than the milt used. In

377   order to get good estimates of fertilisation success we recommend obtaining a much larger

378   sample size from one single population of fish.

379

380   Establishing a captive lumpfish broodstock for a production of cleanerfishes with certain genetic

381   qualities may become essential in the future. Being able to cryopreserve lumpfish milt is a

382   necessary part of this process and we believe we have developed a reliable method, which can be

383   put into use in most laboratories. It is also important to note, that cryopreservation of lumpfish

384   milt according to this protocol can be achieved without use of expensive equipment. These

385 findings and this protocol will contribute to a reliable year-round production of lumpfish

386 juveniles, and improve the utilization of the limited amounts of lumpfish milt available.

387 Ultimately, this could allow for the generation of more in-depth knowledge and use of this fish

388 species as a biological solution to sea-lice problems, without overfishing of the wild lumpfish

389 population.

390

391 In conclusion: cryopreservation of lumpfish milt prepared in modified Mounib's medium

392 (Mounib no GSH) in a 1:2 (milt:diluents) dilution along with 10% DMSO is currently in use at

393 our own hatchery, using the lower freezing rate (trey height: 4,8 cm) and thawing procedure: 37°

394 C for 7 sec. This study finds this to be the best combination of parameters, but the authors

395 acknowledge that the method may be even further improved upon in further studies.

396
397
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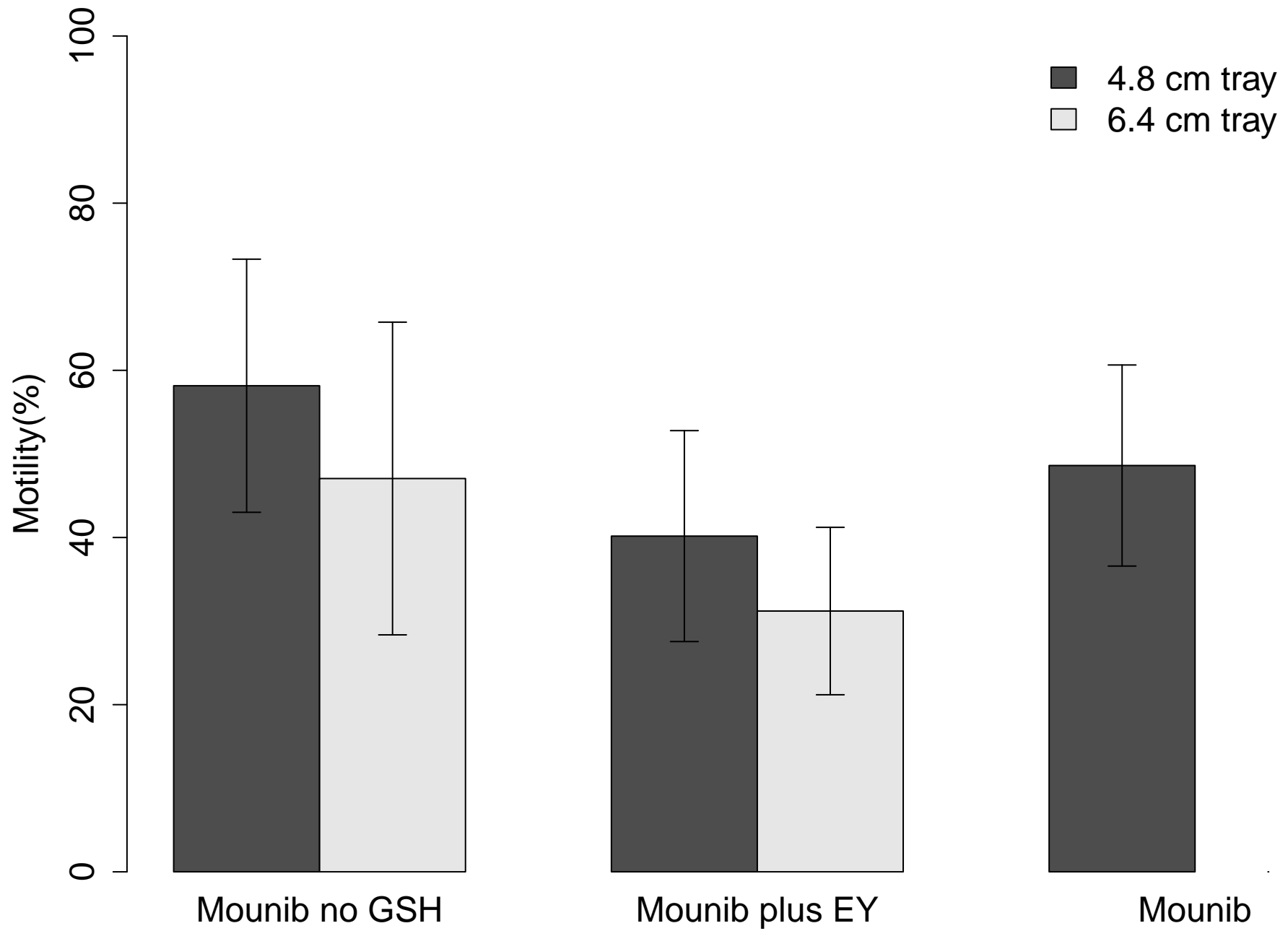
531

Motility of cryopreserved sperm

Mean motility of milt preserved in three different mediums on two different tray heights.
Error bars represent 1 standard deviation.

Components included in cryopreserved samples

The chemical composition of the three cryosolutions tested in this experiment. All are based on Mounib's basic medium with and without reduced l-glutathione and hen's egg yolk. Mounib no GSH and Mounib plus EY were tested on two freezing trays (height 4.8 and 6.4 cm), and Mounib was only tested on one tray (height 4.8 cm). The concentration in every sample was: milt to diluent (1:2) and 10% of the diluent volume was DMSO.

Cryosolution:	Diluent:	Dilution:	Cryoprotectant
Mounib	100 mM KHCO3, 125 mM Sucrose and 6.5 mM L-Gluthatione (reduced)	1:2	10% DMSO
Mounib no GSH	100 mM KHCO3 and 125 mM Sucrose	1:2	10% DMSO
Mounib plus EY	100 mM KHCO3, 125 mM Sucrose and 10% hen egg yolk	1:2	10% DMSO

3   All reagents in this table are purchased from VWR, Bie & Berntsen, Denmark.