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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 (L-Glu), the basic sucrose and potassium bicarbonate medium without L-Glu, 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 L-Glu had a post-thaw motility score of 8.64 percentage points (pp) higher than to that with added L-Glu, and an addition of EY to the Mounib's medium lowered the post-thaw motility score by 15.07 pp. The difference in motility between both freezing rates was 5.23 pp, and samples cryopreserved on a 4.8 cm high tray resulted in a better post-thaw motility score. Cryopreserved milt had a 23.76 pp lower post-thaw motility score when compared with 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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1. Introduction

Cryopreservation is an effective method for long-term storage of viable spermatozoa in fish (Blaxter 1953). This technique offers several benefits, including artificial fertilisation, which allows for efficient use of milt. This especially concerns fish species from which milt samples are difficult to obtain (Ohta & Izawa 1996), or when limited volumes are available when stripping (Clearwater & Crim 1995). Other advantages of the technique include the option to preserve declining stocks (Tian et al. 2008), and to retain genetic variability in broodstocks (Suquet et al. 2001).

Milt cryopreservation has been well established for some freshwater fish species, belonging to families of salmonids (Billard 1992), cyprinids (Billard et al. 1995) and siluroids (Legendre et al. 1996). Cryopreservation efforts for marine fish species were ongoing already in the 90's, and several successful cryopreservation protocols have been defined (Suquet et al. 2001), for e.g. haddock (Rideout et al. 2004), flounder (Lanes et al. 2008; Zhang et al. 2003), and Atlantic halibut (Ding et al. 2011). However, it is a challenging task to determine an optimal protocol for cryopreservation of milt for a particular fish species as diluent and cryoprotectant selection, dilution ratio, as well as freezing and thawing rates are parameters that interact with one another, and have all been found to vary greatly between species (Rideout et al. 2004; Suquet et al. 2001).

Recent research has shown that lumpfish (*Cyclopterus lumpus*), a pelagic fish species naturally found in the North Atlantic Ocean, is an effective cleaner fish in combating infestation with sea lice (*Lepeophtheirus salmonis* and *Caligus elongatues*) among farmed salmon (Imsland et al. 2014), which is a growing problem in that industry (Costello 2009a). Commercial production of lumpfish juveniles is ongoing in Norway (Schaer & Vestvik 2012; Willumsen 2001), and in the Faroe Islands, the aim is to establish a year-round production of lumpfish juveniles.

The lumpfish breeding season is considered to range from March to August (Kennedy et al. 2014), but in Faroese waters, mature lumpfish have been caught as late as in November. During this period brood fish are obtained in shallow and in deeper waters, and are primarily captured by local fishing vessels using gillnets as the fish travel from deep waters towards the shore for spawning. Males specifically, are found around the shores during the breeding season, as they prepare their territory and wait for females to arrive, so spawning can take place (Kennedy et al. 2014). Females lay their eggs in a nest site and then leave the spawning grounds for the males to stay and protect their offspring from predators (Kennedy et al. 2014). It has been reported that lumpfish males have a breeding period that lasts for 6 – 10 weeks (Davenport 1985). It can be quite challenging to obtain gametes from wild males as they may be captured during various stages in their breeding period, i.e. early and late.

Reddish colouration is an indication that males have reached sexual maturity. Our observation is however, that this is not always a guarantee that stripping may be successful. Stripping most often results in small volumes of milt: in our trials volume ranged from 0.08 to 3.2 ml (mean ± SD; 0.95 ± 0.84, n= 24). This becomes a limiting factor when a large number of eggs from females are to be fertilized at the same time. Delaying stripping or females is unadvisable as they may release their roe prematurely.

Cryopreservation of lumpfish milt could solve the issues mentioned above, and ensure a year-round supply of male gametes for a sustainable juvenile production. Additionally, storing of milt from individuals with certain genetic qualities may be important if breeding programs are to be initiated for this species.

Therefore, an effective protocol has been established based on earlier cryopreservation protocols for other marine fish species (Ding et al. 2011; Rideout et al. 2004; Suquet et al. 2001; Zhang et al. 2003).

In a pilot study to cryopreserve lumpfish milt we tested three cryosolutions (see table 1) previously found successful in the cryopreservation of sperm from other marine fish species, including haddock and cod (Rideout et al. 2004), flounder (Zhang et al. 2003) and halibut (Ding et al. 2011). We found that out of the three, only spermatozoa cryopreserved in the modified Mounib's medium used by Ding et al. (2011), resulted in motile sperm. The original Mounib's medium also includes 6.5mM reduced L-Gluthation (Mounib 1978), which is known to prevent

free radicals from stealing electrons from the lipid bilayer in the cell membrane, and thus prevents cell degradation. The role of sucrose in the medium is known to stabilise the liposomal membrane of spermatozoa during cryopreservation (Gwo 2000; Quinn 1985).

Freezing rate has an effect on motility post-cryopreservation (Suquet et al. 2001). The height of the tray used in the cryopreservation process has a great effect on the rate. Rideout et al (2004) found that a 3 cm high tray resulted in samples reaching -90º C in 90 seconds whereas it took 12 minutes on a tray 5.5 cm high. They also found better survival in the spermatozoa when the freezing rate was slow. Our pilot study indicated better motility post cryopreservation when samples were cryopreserved on a 4.8 cm tray than on a 2.5 cm tray.

Thawing temperature may also affect post-cryopreservation motility. For marine fish species, applied thawing temperatures typically vary between 10 and 40º C (Suquet et al. 2001), these are lower than temperatures used for milt samples from freshwater fish species, which vary between 30 and 80º C (Suquet et al. 2001). These findings correspond well to our pilot study where two thawing rates were tested; 37 and 52º C and results showed clearly that lumpfish milt thawed in 37 º C resulted in higher motility scores.

In this present study, we test the efficacy of cryopreserving milt in three different diluents: 1) Mounib's medium minus L-Glutathione (Ding et al; 2011) "Mounib - L-Glu", 2) Mounib's original medium (Mounib, 1987) "Mounib", and 3) Mounib's medium as modified by Ding et al (2011) plus egg yolk, which is a non-penetrating cryoprotectant that is commonly used in cryopreservation (Jamieson, 1991) "Mounib + EY". Additionally, we test two tray heights: 1) the previously successful 4.8 cm tray and, 2) a higher tray of 6.4 cm to ascertain whether an even slower freezing rate may further increase motility.

Table 1

Cryosolutions tested in a pilot study. The chemical composition of the diluents and cryoprotectants are shown in the table along with the dilution factor (milt:diluent) used.

Cryo-solution	Source	Diluent	Dilution (milt:diluent)	Cryoprotectant (v/v)
1	(Rideout et al. 2004)	0.137 M NaCl, 0.011 M KCl, 0.004 M $(Na_2HPO_4)7H_2O$	1:3	10% Propylene glycol

2	(Ding et al. 2011)	0.1 M $KHCO_3$ and 0.125 M sucrose	1:3	10% DMSO
3	(Zhang et al. 2003)	423 mM NaCl, 8.99 mM KCl, 9.25 µM $CaCl_2.2H_2O$, 22.92 µM $MgCl_2$-$6H_2O$, 25.52 µM $MgSO_4.7H_2O$, and 2.14 µM $NaHCO_3$	1:2	12% Glycerol

2. Materials & Methods

2.1. Experimental design

Stripped milt from six lumpfish males (weight: mean ± SD; 434.93 ± 341.58 g, and length: mean ± SD; 23 ± 7.92 cm) was used in this experiment (n=15 due to that some males were stripped more than once). Motility percentage, pH (mean ± SD; 6.55 ± 0.21), osmolality (mean ± SD; 0.463 ± 0.06 mOsmol kg^{-1}), and milt concentration (mean ± SD; 29.72 x 10^9 ± 8.83 x 10^9 cells ml^{-1}) were analysed for each fish. Triplicates of milt samples from all six fish were cryopreserved in three different diluents all based on Mounib's medium (table 2) with or without reduced l-glutathione and hen's egg yolk. The cryoprotectant used was DMSO for all samples. All milt samples were tested at two freezing rates except "Mounib", which only was tested at one of these due to insufficient volumes of milt.

Table 2 The chemical composition of three cryosolutions testes in this experiment. All are based on Mounib's basic medium with and without reduced l-glutathione and hen's egg yolk. Mounib no L-Glu and Mounib + EY were tested on two freezing trays (height 4.8 and 6.4 cm), and Mounib was only tested on tray 4.8 cm. The concentration in every sample was: milt and 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-Gluthation (reduced)	1:2	10% DMSO
Mounib - L-Glu	100 mM KHCO3 and 125 mM Sucrose	1:2	10% DMSO
Mounib + EY	100 mM KHCO3, 125 mM Sucrose and 10% hen egg yolk	1:2	10% DMSO

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

2.2. Gamete collection

The majority of milt samples were obtained from fish produced from wild broodstock at Nesvík Marine Centre, Faroe Islands in 2013. Other samples were obtained from wild fish captured near the shore by divers 8-10 days prior to this experiment. Fish were held in 3 metre diameter cylindrical tanks, water dept 1 m with flow through filtered and UV treated sea water. Fish were fed ad lib with 3mm commercial fish feed.

Males with a reddish appearance were placed in a tank along with females (one male/three females) for a few days prior to stripping in order to enhance gamete production (Klokseth & Øiestad 1999). A pre-stripping check was done by lightly stroking the sides and abdomen of the males. If milt was released, fish were placed in a 20 litre container with sea water along with 20 ml of Benzocaine (anaesthetic). Once fish were unconscious, the milt was stripped by lightly stroking and pressing the abdomen of the fish and collected into 5 ml syringes, and placed on ice immediately. The fish were placed back into the tank with continually flowing sea water to recover.

Sperm were counted under a microscope (Leica DM1000 led) using a hemacytometer (thoma 0.1 mm) using standard counting protocols. Milt was diluted 1 to 1000 in a non activating medium (NAM) previously used in Fauvel et al., (1998) prior to counting. The osmolality of milt was measured using a Gonotec Osmomat 030-D cryoscopic osmometer. The pH value of stripped milt was measured using a PHM 62 standard pH meter.

2.3. Motility measurements

Triplicates of fresh milt samples were examined within 30 minutes after stripping. The milt was diluted 1 to 200 in an activating medium (AM) made of 50% filtered sea water (SW), and 50% bovine serum albumin (BSA) (VWR, Bie & Berntsen, Denmark), prepared in distilled water (10 mg/ml) beforehand, to avoid the cells from sticking to the microscope slide. Immediately after milt was added to the AM, the dilution was cautiously mixed with the pipette tip to distribute the cells evenly, and then 6 µl of the dilution was quickly transferred into one chamber of a Leja 2 chamber CASA microscope slide (SC-20-01-02-B) (Leja Products B.V.).

A two-step dilution procedure is often performed for measuring motility in milt (Dreanno et al. 1997; Fauvel et al. 1999; Groison et al. 2010). This procedure involves first diluting milt in NAM, an isotonic medium similar to the chemical composition of semen (Fauvel et al. 1998), in order to keep spermatozoa quiescent. Subsequently, the milt dilution is transferred to a microscope slide, where an activating agent (usually SW) is added before monitoring the spermatozoa. In our trials, this procedure resulted in an uneven distribution of spermatozoa when the AM was added to the milt dilution within the chamber of the Leja microscope slide. As lumpfish spermatozoa may be motile up to several minutes (own unpublished data), so we chose to do a one-step procedure and diluted all samples in the AM directly, which allowed us to get an even distribution of cells for observation and recording video for the CASA system.

All samples were observed with phase contrast (PH2) under a Leica DM1000 led microscope (object lens: 20x). A digital camera (Leica DFC 295) was attached to the microscope and coupled to a computer, and with the included Leica application suit (LAS) software, a clear live video feed of spermatozoa was obtained. Settings on the LAS software were adjusted to: 44.5 ms exposure, 1.4 x gain and gamma 1.34, and image set to greyscale. Recording was achieved using a Blueberry software (BB flashback Pro 4) player. Two minutes of each sample was recorded, and the first 20 seconds of each movie were always excluded to avoid measurements of spermatozoa moving due to flow and avoiding the lag period caused by mixing milt and AM, and loading into the chamber of the slide. With the blueberry software a total of 46 video frames were extracted from each video and saved as AVI files. Image J (http://rsb.info.nih.gov/ij) (Rasband 1997-2014.) open source software, including a CASA plug-in that allows measuring the motility percentage of fish milt (Wilson-Leedy & Ingermann 2007), was used for video analysis. To get accurate measurements of motility percentage of lumpfish spermatozoa using the CASA plug-in in ImageJ, the image of the imported AVI file threshold was adjusted to 57. In the CASA plug-in sperm tracker fields we only adjusted a few settings to get accurate measurements, these included, the maximum sperm size to 99 pixels, the minimum track length to 10 frames, the maximum sperm velocity between frames to 50 pixels, and the frame rate to 10 frames per seconds.

2.4. Cryopreservation and thawing.

Mounib and Mounib - L-Glu diluents were prepared within a week before the cryopreservation experiments, and stored at 4 º C along with hen's egg yolk. DMSO was stored at room temperature. Milt samples in syringes were stored on ice not more than an hour before the cryopreservation set-up was prepared. The cryopreservation set-up was prepared on ice; first by adding diluent stock into cooled eppendorf tubes, thereafter DMSO. In cryosolution Mounib+EY, egg yolk was added after the diluent, then the cryoprotectant. Lastly milt was loaded to all samples, and ingredients cautiously mixed by aspiration with a pipette, samples were allowed to stand for a 10 minute equilibration time. All samples were cryopreserved in 250 µl cryo-straws (Cryo Bio System). Samples were drawn into the straws manually and sealed. They were then attached on to the top of a floating tray that was either 4.8 cm in height, or 6.4 cm in height, representing two freezing rates. Floating trays were placed within a Styrofoam box (inside dimensions H x L x W= 21cm x 35.5cm x 23cm) filled with liquid nitrogen (-196º C) with an approximately depth of 10cm to allow straws to cool in nitrogen gas for 10 minutes. Thereafter the trays were turned over and samples plunged directly into the liquid nitrogen and left for at least 15 minutes before the thawing process was initiated. Straws were taken directly from liquid nitrogen into a water bath at 37 ºC for a duration time of 7 seconds to be thawed. The ends of straws were cut off with scissors to allow the samples to drain into fresh cooled eppendorf tubes. The examination of the cryopreserved milt was done in the same way as with fresh milt samples, only difference being that cryopreserved samples, having already been diluted in cryosolution, were diluted only 1 to 30 in the AM.

2.5. Statistical analysis

Analysis was carried out using R (R version 3.0.3) (R Core Team 2014) and Excel (MS Office). Differences between motility in fresh and cryopreserved milt were analysed using a linear mixed effects model, "lmer" in the R package lme4 (Bates et al. 2014) and P values were extracted using a type II Wald Chi squared test included in the R package "car" (Fox & Weisberg 2011). While motility data are measured in percentages, the residuals in the chosen model were sufficiently normal, so using a generalized linear model or transforming the data was not justified in this case. The differences between diluent types and tray types were analysed using a similar model. In both models, "batch" was used as a random effect. This, because while some males produced several batches of milt, each batch differed sufficiently that "male" was not a useful random

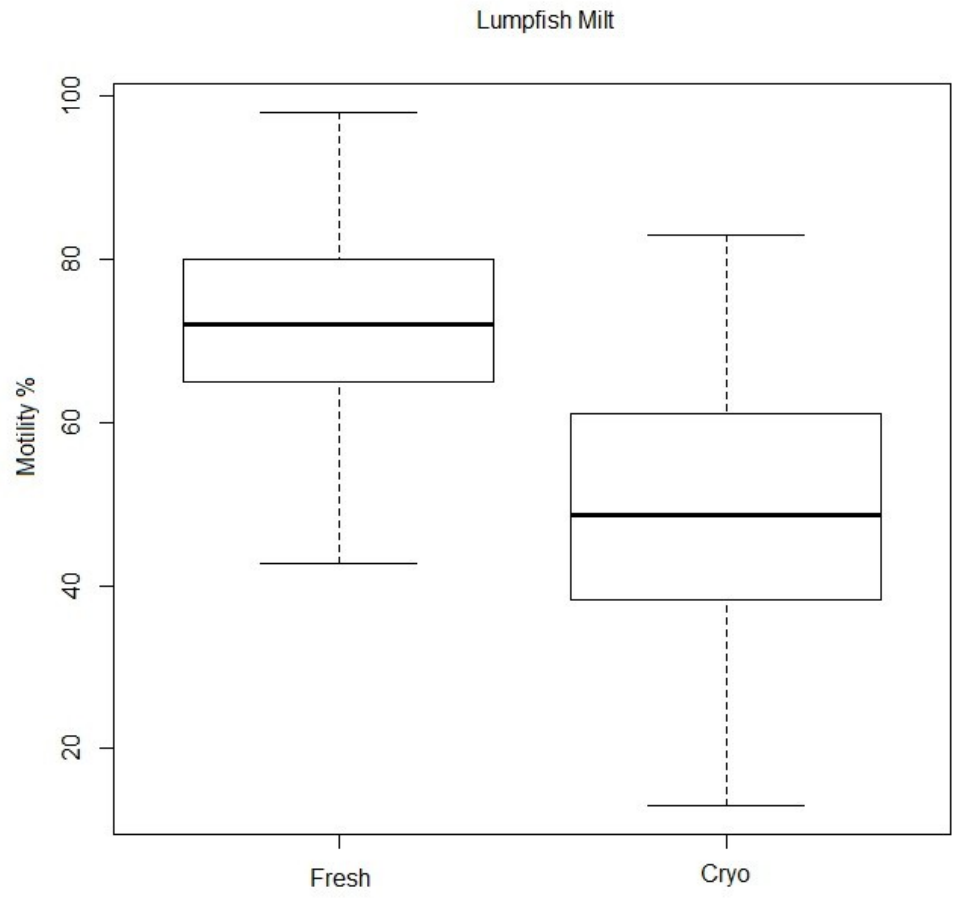
control. Differences found are expressed as percentage point (pp) estimates from the relevant models adjusting for "batch" and not as true percentages.

2.6. Ethical statement

As there is no animal experimentation legislation on the Faroe Islands, the local "animal protection act" was adhered to (Løgtingslóg um vernd av dýrum, 2007) throughout this study. A fish veterinarian advised on best practice in relation to stripping to ensure no undue suffering caused by the procedure. Our impact on wild populations was limited, as we used predominately captive bred fish, which were bred for the purpose of producing a domestic lumpfish stock. There were no fish mortalities caused by our study and effort was put into providing optimal care and welfare for all fish involved.

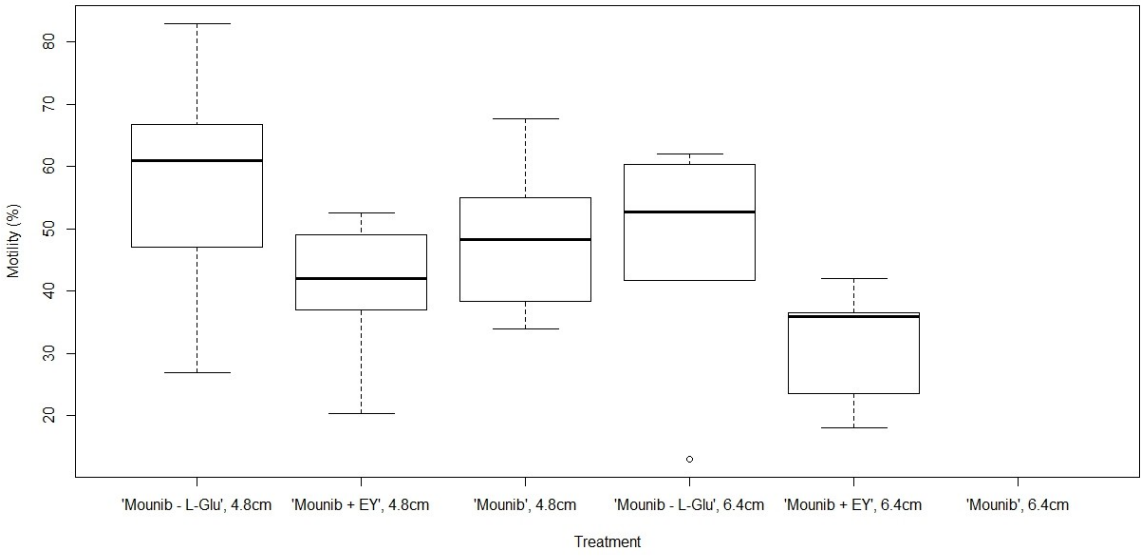
3. Results

Cryopreserved milt had a 23.76 pp lower motility than fresh milt (type II Wald test; Chi squared = 35.42, df = 1, $P < 0.001$) with an average motility of fresh milt being 71.87 % and of cryproserved milt 48.10 % on average (figure 1).



199 **Figure 1** Motility of fresh and cryopreserved lumpfish spermatozoa. Results suggest that fresh spermatozoa were on
200 average ~24 pp more motile than cryopreserved spermatozoa.

201 Tray height had a significant effect on post-thawing motility with the lower tray (4.8cm)
202 producing a 5.23 pp higher motility than the higher one (type II Wald test; Chi squared = 14.05,
203 df = 1, $P < 0.001$). Cryosolution also affected motility with an addition of 'EY' to the Mounib's
204 medium resulting in 15.07 pp lower motility and an addition of 'L-Glu' resulting in 8.64 lower
205 motility (type II Wald test; Chi squared = 44.05, df = 2, $P < 0.001$). T values from the summary
206 table for this model in R (summary(model)) were: tray: -3.75, L-Glu: -3.02 and, EY: -6.45,
207 suggesting that all factor levels differed significantly (figure 2).



208 **Figure 2** The difference of motility recovery of lumpfish milt samples cryopreserved in three different cryosolutions
209 Mounib, Mounib no L-Glu and Mounib + EY on a lower freezing tray (4.8 cm) and on a higher freezing tray (6.4
210 cm). No bar appears in Mounib 6.4 because this test was not completed.

211 ## 4. Discussion

212 This paper details a successful method for cryopreserving lumpfish milt. To our knowledge no
213 such methodology has been published before, and may this information be beneficial in future
214 development of lumpfish as an aquaculture species. Our results indicate a motility loss of less
215 than 24pp when using our optimal cryopreservation protocol. This is in concordant compared to
216 cryopreservation of e.g. cod, halibut, ocean pout, sea bream, striped trumpeter and turbot
217 reviewed in Suquet et al. 2000, in which post-frozen motility recovery, expressed as a percentage
218 of fresh sperm motility rate, ranged from 39 – 85 % compared to our result that was (mean ± SD)
219 72.3 % ± 21.6 %.

220 Based on previous findings in our pilot study, we here test three variants of Mounib's medium.
221 Our results indicate that the best diluent is Mounib's minus L-Gluthation. This indicates that the
222 penetrating cryoprotectant DMSO is sufficient to protect the spermatozoa from cell damages that
223 occur during the freezing and thawing process.

Our earlier tests indicated that a tray of 2.5 cm was too low, and findings in this study point to that 6.4 is too high with 5.23 pp lower motility than the lower tray (4.8 cm). Interestingly, Rayling et al's 5.5 cm tray was better than their 3 cm tray. This suggests that there may be an optimal tray height yet to be found. Perhaps the optimal height is somewhere between 4.8 and 5.5 cm.

Aquaculture is an expanding industry, and production of Atlantic salmon is still growing in the North Atlantic (FAO 2014). The need for ecologically and economically sustainable methods of removing sea lice is ever growing as sea lice have a great impact on fish farm economy (Costello 2009a; Johnson et al. 2004), possibly wild salmon populations (Costello 2009b; Skilbrei et al. 2013; Torrissen et al. 2013) and the welfare of farmed fish (Ashley 2007). Using cleaner fish such as lumpfish could be one such method, but in order to avoid affecting wild lumpfish populations, establishing captive broodstock is essential. Being able to cryopreserve lumpfish milt is a necessary part of this process and we believe we have developed a reliable method, which can be put into use in most laboratories.

To our knowledge, this is the first cryopreservation of lumpfish milt method, and it is currently applied at our own hatchery. It is also important to note, that cryopreservation of lumpfish milt according to this protocol can be achieved without use of expensive equipment. These findings and this protocol will contribute to a reliable year-round production of lumpfish juveniles, and improve the utilization of the limited amounts of lumpfish milt available. Ultimately, this could allow for the generation of more in-depth knowledge and use of this fish species as a biological solution to sea-lice problems, without overfishing of the wild lumpfish population.

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