Theriogenology 151 (2020) 58–65

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Viability, motility, ATP content and fertilizing potential of sperm from Atlantic salmon (*Salmo salar* L.) in milt stored before cryopreservation

Elisabeth Kommisrud [a, *], Frøydis D. Myromslien [a], Else-Berit Stenseth [a], Teklu T. Zeremichael [a], Nadine Hofman [a], Inger Grevle [b], Jan Sunde [c]

[a] Department of Biotechnology, Inland Norway University of Applied Sciences, Hamar, Norway
[b] Cryogenetics AS, Hamar, Norway
[c] Møreforsking Ålesund AS, Ålesund, Norway

ARTICLE INFO

Article history:
Received 19 December 2019
Received in revised form
7 April 2020
Accepted 7 April 2020
Available online 11 April 2020

Keywords:
Preservation milt
Viability
Adenosine triphosphate
Computer-assisted sperm analysis
Fertilization

ABSTRACT

Artificial fertilization is increasingly used in aquaculture, mostly applying short-term cold stored milt. Large scale cryopreservation of milt could be valuable for increased flexibility and acceleration of breeding progress. The aim of this study was to assess viability, motility and ATP content of sperm from Atlantic salmon as a function of storage time, before and after cryopreservation. The objective was also to investigate whether *in vitro* parameters were associated with sperm fertilizing ability after cryopreservation. Milt from six mature Atlantic salmon males were collected twice, one week apart. The milt was stored undiluted at 5 °C in cell culture flasks for six days. Samples were taken on days 1, 3 and 6 of storage for cryopreservation. In total, 36 batches were diluted to a standardized sperm concentration of 2×10^9 spermatozoa/mL, filled into 0.5 mL French medium straws and cryopreserved. *In vitro* analyses were assessed on the same sample for the 72 combinations of male, collection week, days of storage and cold stored or frozen-thawed. Fertilization trials with cryopreserved milt were carried out for all 36 batches in triplicate for each combination of male, collection week, storage time and sperm:egg ratios of either 2 or 4×10^6 sperm per egg, respectively, totally 218 experimental units, including two egg controls. There was a significant influence of storage and collection week on sperm quality parameters, both cold stored and cryopreserved, and cryopreservation had a significant effect on all tested sperm quality parameters. High correlations for cold stored vs cryopreserved samples was demonstrated for ATP content ($p < 0.00001$), motility and velocity parameters ($p < 0.001$), but not for viability, straightness and linearity. The overall percentage of fertilization achieved was 73.9 ± 1.7%. Sperm collected in week 2 showed significantly lower fertility when cryopreserved after six days of storage than after 1 or 3 days for sperm to egg ratios of 2×10^6 ($p < 0.005$), while there was no such effect for milt collected in week 1. Several post-thaw sperm parameters were correlated to fertilization rates, while curvilinear velocity best explained variations in fertilization by modelling. Our results suggest that cryopreservation of Atlantic salmon milt should be performed soon after milt collection to maximize the cryopreserved sperm quality. Fertilization results seems not to be compromised by storage for three days before cryopreservation.

1. Introduction

Fresh, liquid stored, or cryopreserved semen is used for artificial insemination (AI) worldwide and is the most valuable tool for breeding and genetic improvement in agriculture [1], and also increasingly used for artificial fertilization in aquaculture. Cryopreservation is preferable when compatible with the sperm characteristics of the species and has become the dominant method e.g. in the cattle AI industry after the discovery by Polge et al., in 1949 [2] of the sperm protective effect of glycerol at low temperatures. Soon thereafter, Blaxter demonstrated an important advantage using cryopreserved sperm in aquaculture [3], namely the possibility to apply cross-fertilization in fish spawning at different times or in different geographical locations. Cryopreservation of fish sperm could thus be a particularly valuable tool for acceleration of breeding

* Corresponding author.
E-mail address: elisabeth.kommisrud@inn.no (E. Kommisrud).

https://doi.org/10.1016/j.theriogenology.2020.04.008

progress [4]. Unfortunately, the large number of fish species and significant differences between species has partly limited the interest in cryopreservation of milt to research [5]. There are, however, several opportunities for application of cryopreserved sperm in aquaculture [6], with both challenges and perspectives [4,7]. The aquaculture industry has adopted short term refrigerated storage of milt, a method that offers limited flexibility compared to cryopreservation. This paper explores if cold storage of milt for a short period can be followed by cryopreservation without compromising post-thaw sperm quality and fertilizing potential. This combination would increase the flexibility and applicability of cryopreservation. Commercial extenders developed for cryopreservation of fish milt are offered from e.g. IMV Technologies, France. However, to our knowledge the service of large-scale commercial cryopreservation of fish sperm is currently offered by a single commercial provider (Cryogenetics AS, Norway).

Successful cryopreservation of milt depends on a range of factors, e.g. extender composition, cryoprotectants, freezing and thawing protocols as well as type of semen containers [4,8]. A range of sperm cryopreservation protocols developed by researchers have been published for several freshwater and anadromous fish species, including salmonids [6]. Concerning salmonids, research on cryopreservation of sperm has been performed mostly on trout species (*Oncorhynchus mykiss, Salmo trutta, Salvelinus fontinalis*) [7,9–13], as well as on Atlantic salmon (*Salmo salar* L.) [10,14,15]. Most protocol development is concerned with dilution ratios of milt and extender resulting in various final sperm concentrations as well as cryoprotectant concentrations relative to sperm number [6]. However, recently the importance of standardization of sperm concentration has been demonstrated crucial for comparison and optimal sperm survival [16,17].

The quality of cryopreserved milt can ultimately only be verified by the ability to fertilize eggs. It is, however, desirable to perform *in vitro* quality analyses of the sperm cells to objectively compare and assess different preservation protocols and their efficiency. In bovines, a relationship between fertility and sperm viability [18], motility [19] and adenosine triphosphate (ATP) content [20] has been demonstrated. Viability (as membrane integrity) is a prerequisite for the sperm to deliver the paternal genome, irrespective of species. As sperm from most teleost fish are immotile upon ejaculation or milt collection and gain a very brief period of motility in contact with water, it is assumed that motility of cryopreserved semen is crucial for fertilization [21]. Computer-assisted sperm analyses (CASA) has been used for motility analyses of fresh and cryopreserved sperm from aquatic species [21–24], and may predict the ability of the sperm cells to fertilize eggs [21], and has also demonstrated the appearance and importance of post-thaw subpopulations [25]. Flagellar motion is generated by ATP, fish sperm exhibiting vigorous motility when activated resulting in rapid consumption of intracellular energy [26,27]. As sperm from teleost fish, in particular salmonids, gain a very short period of motility, we have speculated that initial ATP content could be relevant for fertilizing potential.

The aim of the present study was to assess viability, motility and ATP content of sperm from Atlantic salmon as a function of repeated collection and storage time, and secondly asses the effect of storage on sperm quality after cryopreservation. Finally, the objective was to investigate whether *in vitro* parameters were associated with sperm fertilizing ability after cryopreservation applying two different sperm to egg ratios of cryopreserved milt after different time of storage before cryopreservation.

2. Materials and methods

2.1. Sample collection and preservation

Milt from six mature Atlantic salmon males kept at a broodstock facility in the Southwestern part of Norway were collected twice, one week apart, during spawning season. Sperm collection was performed according to the routines at the facility, where each male was stripped at least twice before culling. The males came into spawning naturally, without any hormonal, photoperiod or temperature manipulation. Milt was collected by gentle massage of the belly, without urine and faecal contamination, and stored in sealed plastic Ziploc® bags filled with oxygen. Samples were shipped overnight to the laboratory, kept cold on paper on top of crushed ice and had a temperature of 5 °C on arrival at the laboratory.

The milt was stored undiluted at 5 °C in TC-treated cell culture flasks (40 mL, 25 cm^2 growth area) with filter screw caps (VWR International, Radnor, PA, USA) for six days with gentle agitation (SSM4 see-saw rocker, Stuart Instruments, Staffordshire, UK). Samples were taken on days 1, 3 and 6 of storage and analyzed for viability, motility and ATP content. Correspondingly, aliquots of milt were taken for cryopreservation. Sperm concentration was estimated after 1:100 dilution in 0.9% NaCl (10 μL: 990 μL) using an SDM6 photometer (Minitüb GmbH, Tiefenbach, Germany) at 546 nm using a pre-determined calibration curve for Atlantic salmon sperm. Sperm concentration varied between 4.3–9.7 $\times$ 10^9 sperm/mL. Based on the concentration estimate, the milt was diluted using a proprietary fish sperm extender (Cryogenetics AS, Hamar, Norway) to a standardized sperm concentration of 2 $\times$ 10^9 spermatozoa/mL before cryopreservation. Milt was filled into preprinted 0.5 mL French medium straws (IMV Technologies, L'Aigle, France), sealed with ultrasound using an MPP Uno automated filling and sealing machine (Minitüb GmbH, Tiefenbach, Germany) and cryopreserved according to a proprietary protocol (Cryogenetics AS, Hamar, Norway) before transfer to liquid N_2 for storage. A total of 36 batches were cryopreserved. The preprint ensured correct identification of male, collection date and sample date.

2.2. Experimental setup in vitro analyses

Both fresh (cold stored) and frozen-thawed milt samples were treated identically according to the following procedure. Three cryopreserved milt doses per batch were thawed in a water bath (25 °C, 30 s) and pooled before analysis. For each combination of male, collection week, treatment (cold stored or frozen-thawed) and sample date (one, three or six days of storage), analyses for viability, motility and ATP content were carried out on the same sample. Samples were analyzed in triplicate for viability and ATP content, and in duplicate for motility assessment. All chemicals were purchased from Sigma-Aldrich (Saint Louise, MO, USA) unless otherwise noted.

2.3. Viability – plasma membrane integrity

Sperm membrane integrity (spermatozoa viability) was assessed using propidium iodide (PI, L-7011, LIVE/DEAD®Sperm Viability Kit, Molecular Probes, ThermoFisher Scientific, Waltham, MA USA), to discriminate between live (PI negative) and dead (PI positive) spermatozoa. Prior to flow cytometry analysis, the spermatozoa were stained in dark (4 °C, 10 min) in a PBS (137 mM NaCl, 2.7 mM KCl, 1.76 mM KH_2PO_4, 8.1 mM Na_2HPO_4 x $2H_2O$, pH 7.4) staining solution with a final concentration of ≈1.5 $\times$ 10^6 spermatozoa/mL and 0.48 μM PI. The staining protocol was validated by assessing membrane integrity of four ratios of fresh and heat-treated (60 °C, 10 min) sperm cells (100:0, 75:25, 50:50 and 25:75). Upon staining, analysis of the spermatozoa was performed using a Cell Lab Quanta ™ SC MPL flow cytometer (Beckman Coulter, Fullerton, CA, USA). The instrument was equipped with a 22 mW argon laser with an excitation wavelength of 488 nm. Data

was analyzed using Cell Lab Quanta SC MPL Analysis software program (Software Version 1.0, Beckman Coulter, Fullerton, CA, USA). Side scatter (SS) signals were used for identification of spermatozoa (Fig. 1A). The reliability of using SS to identify the spermatozoa was confirmed in control samples upon staining of the spermatozoa with SYBR-14 (L-7011, LIVE/DEAD®Sperm Viability Kit, Molecular Probes, ThermoFisher Scientific, Waltham, MA USA) or Mito-Tracker Orange CMTMRos (M7510, Invitrogen, Carlsbad, CA, USA). PI fluorescence in SS gated spermatozoa was detected using a 670 nm long pass (LP) filter (FL3) (Fig. 1B).

2.4. Motility analyses

Motility analysis was performed using a Sperm Vision® CASA system (Minitüb GmbH, Tiefenbach, Germany) consisting of an Axio Lab.A1 trinocular microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) outfitted with a negative phase-contrast objective (20x) and an automated microscope stage (Minitüb GmbH, Tiefenbach, Germany). Images were captured by a Basler aviator avA1000-120km (1 MP, 120 fps) Camera Link camera (Basler Vision Technologies AG, Ahrensburg, Germany) connected through a IEE 1394 interface to a PC running Microsoft® Windows® 98 and the Sperm Vision® software. All materials and solutions used, including samples and chambers, were kept on ice before and during analysis. Samples were diluted to a working sperm concentration of 0.087×10^9 cells/mL in AquaBoost® Dilutor (Cryogenetics AS, Hamar, Norway) prior to analysis. Sperm activation was initiated by addition of AquaBoost® Activator (Cryogenetics AS, Hamar, Norway) simultaneously with sample application to the microscope slide. Using a Picus (0.2–10) μL electronic 1-channel pipette (Sartorius Stedim Biotech GmbH, Göttingen, Germany) in Multi-Aspirating Mode, aspiration was done sequentially in the following order: 1.2 μL diluted milt - air - 1.2 μL AquaBoost® Activator. For each CASA measurement, the aspiration volume was dispensed into a single chamber on a Leja 20 μm 4 chamber microscope slide (Leja, Nieuw-Vennep, the Netherlands). Image analysis was performed on four consecutive fields within approximately 8 s after activation, with a total of at least 400 cells analyzed per sample. Total motility (TM, %), progressive motility (PM, %), straight line velocity (VSL, μm/s), velocity average path (VAP, μm/s), curvilinear velocity (VCL, μm/s), straightness (STR, ratio VSL/VAP) and linearity (LIN, ratio VSL/VCL) were registered based on the following data capture criteria: head area between 15 and 100 μm^2 for sperm detection, 60 Hz frame rate, 30 frames captured per object. Immotile cells were defined as having VAP <10 μm/s and VCL <25 μm/s, locally motile cells were defined as cells with path distance covered along the straight line (DSL) < 10 μm, otherwise cells were classified as progressively motile.

2.5. Sperm ATP content

The ATP content in milt samples was determined using the CellTiter-Glo® Luminescent Cell Viability Assay (G7571, Promega, Madison, WI, USA) [28]. Prior to analysis, milt samples were diluted to 5×10^6 spermatozoa/mL in PBS, and 50 μL of each sample was transferred to wells in a white 96-well microtiter plate (NUNC™, Roskilde, Denmark). Subsequently, 50 μL CellTiter-Glo® Reagent was added to each well and the mixture was gently shaken for 2 min in a rotary shaker (IKA® MS 3 digital, IKA®-Werke GmbH & Co, Staufen, Germany) to induce cell lysis. Bioluminescence measurement was performed after further 10 min incubation at room temperature using a FLUOstar OPTIMA multiwell plate reader (BMG Labtech GmbH, Offenburg, Germany) with MARS data analysis software (Version 1.10, BMG Labtech GmbH, Offenburg, Germany). For generation of a standard curve, ATP standard solutions were prepared from ATP disodium salt hydrate (A7699-1G). The bioluminescence value for each sample, measured in relative luminescence units (RLU), was converted to the corresponding ATP concentrations (nM) using the standard curve.

2.6. Fertilization trial

The fertilization trial was performed on site at the broodstock facilities using cryopreserved sperm from the storage trials (2.1). The incoming water temperature on the day of fertilization was 7 °C, and all solutions used were refrigerated to this temperature before use by immersion in water.

Eggs were obtained from mature Atlantic salmon (*Salmo salar* L.) females euthanized with an overdose of benzocaine (Benzoak vet., ACD Pharmaceuticals AS, Leknes, Norway), and were removed from the abdominal cavity by excision. Once removed, eggs were held in their ovarian fluid until fertilization. Fertilization batches were

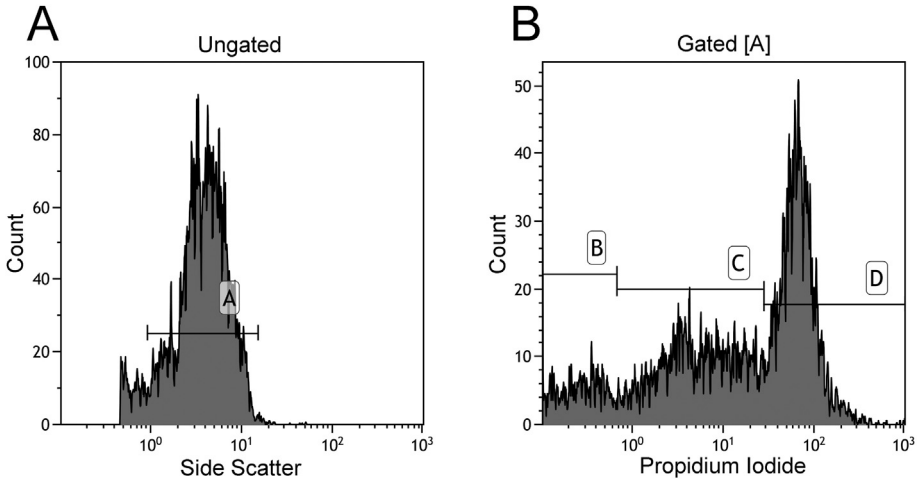

Fig. 1. Typical histograms of frozen thawed Atlantic salmon (*Salmo salar* L.) spermatozoa generated by Cell Lab Quanta TM SC MPL flow cytometer upon staining with propidium iodide (PI). The figure illustrates how regions were included for assessment of membrane intact (viable, PI negative) spermatozoa. A) Side scatter histogram where spermatozoa are gated (region A) for further analyses. B) PI fluorescence (FL3 fluorescence) based on side scatter gated events where region B, C and D represent viable, dying and dead spermatozoa, respectively.

prepared by mixing eggs from a total of five females. To minimize storage time and prevent eggs from drying out, preparation of batches was performed in two rounds, first using eggs from three females and later in the day by mixing eggs from two females. All egg batches were visually inspected by experienced on site personnel and considered to be of good quality. Equal volumes of eggs from each female were mixed to make fertilization units of approximately 150 eggs, the volume needed was estimated in advance by counting and weighing 150 eggs (average 18 g) from each female in triplicate. Egg quality controls were prepared by fertilization with fresh milt collected from a mature male on the day of the trial. Milt quality was assessed by microscopic evaluation of motility after activation with a drop of ovarian fluid, as per standard operating procedures at the facility.

Fertilization units were prepared in disposable plastic cups immediately before fertilization. All fertilizations were carried out in triplicate for each combination of male, collection week, storage time and sperm:egg ratio. Two cryopreserved straws were thawed at a time (25 °C, 30 s) in an 18 L heated circulating plastic water bath (Grant T100, Grant Instruments, Cambridge, UK), before being pooled and gently mixed. Immediately afterwards, a volume of either 150 or 300 μL was added to the fertilization unit, corresponding to sperm:egg ratios of either 2 or 4 × 10^6 sperm per egg, respectively. After addition of milt and gentle mixing for 30 s, 7.5 mL AquaBoost® Activator (Cryogenetics AS, Hamar, Norway) was added to each unit, the cup gently swirled and left to incubate for 5 min. Afterwards, eggs were rinsed with physiological saline (0.9% NaCl) solution and transferred to circular PVC incubator trays, each containing 10 or 12 compartments. Trays were kept in physiological saline (0.9% NaCl) to prevent water hardening of the eggs until each tray was filled. Each full tray was disinfected with 1% Buffodine® (Fish Tech AS, Vestby, Norway) in 0.9% NaCl for a minimum of 10 min. Finally, incubator trays were transferred to larger circular hatchery incubators (each holding 5 trays) and supplied with ambient flow-through freshwater (7 °C). Altogether, 218 experimental units, including two egg controls, were included in the trial.

After approximately 115 day-degrees (range 100–120) of incubation, fertilization percentages were calculated for all fertilization batches. Each batch was treated with acetic acid (10%) for 5 min and each egg visually inspected. Eggs were considered fertilized if a fully formed spinal cord was visible, indicating normal embryo development.

2.7. Statistical analyses

All reported values are given as means and standard error of mean (SEM) unless otherwise stated. Statistical analyses were performed using R version 3.6.1 [29]. All sperm parameters were tested for normality and heteroskedasticity using Kolmogorov-Smirnov's and Bartlett's tests, respectively, and transformed to approach a normal distribution, either using logarithmic or square root transformation. Changes in sperm quality parameters during cold storage were modelled using the *lmer* function with days of storage and collection week as fixed effects and male id as random effect. *Post hoc* comparison between days and weeks were performed between days within week using the *emmeans* function with a Tukey multiple comparison correction. Pearson correlation coefficients were calculated between cold stored and cryopreserved sperm parameters, and between cryopreserved sperm parameters and fertility on the same samples using the *rcorr* function. For other comparisons, paired t-tests were used after transformation of variables that were not normally distributed, or, in cases where normal distribution was not achieved, the non-parametric paired Wilcoxon test was applied. Finally, fertilization outcome was modelled using a linear mixed model, where *in vitro* parameters from cryopreserved samples was modelled against fertilization outcome either from a sperm:egg ratio of 2 or 4 × 10^6, week and day as fixed effects and male id as random effect. Models were compared and evaluated based on normality of residuals and parameters' significant contribution to the model. The significance level in all statistical tests was set to 0.05.

3. Results

Sperm concentration was significantly different for the two collection weeks, 8.05 ± 0.53 × 10^9 and 6.27 ± 0.87 × 10^9 sperm/mL ($p < 0.05$), for week 1 and 2, respectively. The volume of the milt was similar for both weeks, with average 71.7 ± 4.6 mL.

3.1. Sperm viability

Initial sperm viability (day 1) for cold stored milt measured as membrane intact cells (% PI negative sperm, Fig. 1, region B) was similar between the two collection weeks and on average 98.0 ± 0.1%. Viability decreased slightly with days of storage and was significantly lower than initial values after 6 days of storage for both collection weeks (Table 1). A more marked decrease was observed for sperm collected in week 2, with a significant reduction in viability already on day 3, leading to a significantly lower viability on day 6 for week 2 samples compared to week 1 ($p < 0.05$).

There was a significant overall effect of cryopreservation on all sperm quality parameters ($p < 0.002$). Cryopreserved samples showed a significantly lower viability than cold stored samples ($p < 0.0001$). Storage for six days before cryopreservation led to a significantly lower post-thaw viability than cryopreservation at day 1 after collection. Week 1 samples showed significant higher viability compared to week 2 samples ($p < 0.05$) on both day 1 (34.7% vs 31.9%) and day 6 (32.3% vs 28.0%).

3.2. Sperm motility parameters

Initial sperm motility measured both as TM and PM was significantly higher for milt collected in week 2 (56.3% and 49.0%, respectively) compared to milt collected in week 1 (40.4% and 25.0%, respectively) (Table 1). In addition, motility for sperm collected in week 2 was significantly higher than for week 1 throughout the duration of the storage ($p < 0.05$), although both collections showed a decrease in both parameters, with TM and PM being significantly higher on day 1 than subsequent days. During cold storage, the average velocity of sperm in the motile fraction either increased (week 2) or remained stable throughout the storage period (week 1). Average velocity parameters VSL, VAP and VCL on day 1, being not different between collection weeks, were 81.7 μm/s, 93.4 μm/s and 106.4 μm/s, respectively. All parameters increased during storage in week 2 samples, resulting in significantly higher values on day 6 of storage ($p < 0.005$). The parameters STR and LIN, measuring the discrepancy of the sperm path from a straight line, were both significantly higher initially (day 1) for week 1 collections compared to week 2 ($p < 0.05$), but due to a slight reduction in week 1 samples the values for sample stored for three and six days were not different.

Cryopreserved samples showed lower TM ($p < 0.0001$) and PM ($p < 0.0001$) than cold stored samples. Motility of cryopreserved samples from week 1 was influenced by days of storage before cryopreservation, day 1 samples having significantly higher TM and PM (9.9% and 7.5%, respectively) than both day 3 (4.2% and 2.5%) and day 6 (5.2% and 4.0%). TM and PM for day 1 samples in week 2 were significantly lower than corresponding samples in week 1

Table 1
In vitro sperm parameters of milt from six Atlantic salmon (*Salmo salar* L.) males collected in two separate weeks are presented as mean ± SEM for cold stored and cryopreserved milt. Milt was stored cold for one, three and six days, respectively, with consecutive cryopreservation. Different superscripts denote significant differences ($p < 0.05$) between days within collection week.

	Collection week 1			Collection week 2		
In vitro parameters	Day 1	Day 3	Day 6	Day 1	Day 3	Day 6
Cold stored						
Viability (%)	97.9 ± 0.1^{a}	97.5 ± 0.2^{ab}	97.2 ± 0.2^{b}	98.1 ± 0.2^{a}	97.2 ± 0.2^{b}	96.3 ± 0.3^{c}
TM (%)	40.4 ± 8.1^{a}	29.0 ± 6.8^{b}	25.6 ± 3.9^{b}	56.3 ± 5.5^{a}	38.7 ± 5.4^{b}	41.5 ± 6.4^{b}
PM (%)	25.0 ± 5.4^{a}	22.9 ± 5.8^{b}	20.9 ± 3.2^{ab}	49.0 ± 5.9^{a}	33.2 ± 5.1^{b}	37.6 ± 6.0^{ab}
VSL (μm/s)	83.8 ± 3.8	87.3 ± 7.0	79.3 ± 4.9	79.5 ± 3.9^{a}	93.6 ± 4.1^{b}	100.3 ± 3.4^{b}
VAP (μm/s)	92.3 ± 4.7	100.6 ± 7.8	92.6 ± 6.1	94.6 ± 5.2^{a}	100.3 ± 5.7^{b}	118.8 ± 3.7^{b}
VCL (μm/s)	104.1 ± 4.5	112.2 ± 8.1	104.3 ± 6.2	108.7 ± 5.3^{a}	122.1 ± 5.8^{ab}	129.5 ± 3.8^{b}
STR (%)	90.6 ± 1.2^{a}	82.5 ± 4.2^{b}	85.2 ± 1.3^{ab}	83.9 ± 1.5	84.9 ± 1.7	84.0 ± 1.6
LIN (%)	79.7 ± 0.9	73.9 ± 4.3	75.3 ± 1.5	72.6 ± 1.4	76.5 ± 1.7	77.1 ± 2.0
ATP (nM)	190.9 ± 20.9^{a}	135.6 ± 15.2^{b}	124.3 ± 12.7^{b}	256.3 ± 18.8^{a}	222.4 ± 24.0^{b}	223.6 ± 26.8^{b}
Cryopreserved						
Viability (%)	34.7 ± 1.0^{a}	31.2 ± 1.0^{b}	32.3 ± 0.6^{ab}	31.9 ± 1.0^{a}	32.3 ± 1.0^{a}	28.0 ± 0.8^{b}
TM (%)	9.9 ± 1.4^{a}	4.2 ± 0.8^{b}	5.2 ± 0.5^{b}	5.8 ± 0.0	5.6 ± 0.8	4.4 ± 0.8
PM (%)	7.5 ± 1.3^{a}	2.5 ± 0.7^{b}	4.0 ± 0.4^{b}	5.7 ± 0.9	4.9 ± 0.8	3.9 ± 0.8
VSL (μm/s)	62.9 ± 3.7	59.9 ± 5.3	68.9 ± 3.6	76.6 ± 5.2^{a}	77.6 ± 5.2^{a}	61.3 ± 4.6^{b}
VAP (μm/s)	68.9 ± 3.9	67.5 ± 6.0	77.0 ± 4.1	87.0 ± 6.0^{ab}	91.1 ± 6.0^{a}	72.7 ± 5.8^{b}
VCL (μm/s)	77.1 ± 4.1	73.9 ± 6.6	83.1 ± 4.2	94.2 ± 6.4^{ab}	97.3 ± 6.4^{a}	77.7 ± 6.2^{b}
STR (%)	83.4 ± 3.3^{a}	65.6 ± 4.9^{b}	78.6 ± 3.4^{a}	77.6 ± 4.1	72.5 ± 3.9	65.9 ± 3.9
LIN (%)	74.0 ± 3.0^{a}	59.5 ± 4.4^{b}	72.2 ± 3.3^{a}	71.1 ± 3.8	67.8 ± 3.7	61.4 ± 3.6
ATP (nM)	53.5 ± 5.8^{a}	39.1 ± 4.5^{b}	25.4 ± 2.0^{b}	78.3 ± 7.5^{a}	67.3 ± 10.7^{b}	52.6 ± 6.6^{b}

($p < 0.05$), while there was no significant reduction of motility due to further storage before cryopreservation. Average velocity parameters VSL, VAP, VCL, and STR and LIN, in cryopreserved samples from week 1 was significantly reduced ($p < 0.00001$) compared to cold stored samples. Post-thaw values of VSL, VAP and VCL was not influenced by storage before cryopreservation. Interestingly, week 2 samples displayed a less pronounced reduction in velocity parameters compared to cold stored ($p < 0.01$) but was significantly influenced by storage before cryopreservation (Table 1).

3.3. Sperm ATP content

Sperm ATP content for milt collected in both weeks was significantly reduced from day 1 of cold storage to day 3, but not further reduced to day 6. Initial ATP content on day 1 was significantly higher ($p < 0.05$) in week 2 samples (256.3 nM) compared to week 1 samples (190.9 nM) and the difference between collection weeks remained significant throughout the period of storage.

Sperm ATP content was significantly reduced in cryopreserved samples compared to cold stored samples ($p < 0.0001$). Week 2 samples showed significantly higher levels of ATP post-cryopreservation in comparison with week 1 samples throughout the storage period ($p < 0.001$). For both weeks day 1 samples had significantly higher sperm ATP content than day 6 samples, 78.3 nM vs 52.6 nM and 53.5 nM vs 25.4 nM in week 2 and 1, respectively.

Correlations (Pearson correlation) between sperm quality parameters in cold stored vs cryopreserved samples are shown in Fig. 2. The highest correlation was found for ATP content ($p < 0.00001$), while TM, PM, VSL, VAP and VCL were also significantly correlated ($p < 0.001$). Viability, STR and LIN did however, not show correlations between pre- and post-cryopreservation values.

3.4. Fertilization

The overall percentage of fertilization achieved using cryopreserved milt was 73.9 ± 1.7%, compared to 81.1 ± 1.2% for fresh milt controls. There was a significant influence of milt collection week, days of cold storage before cryopreservation and sperm:egg ratio (Fig. 3). With milt collected in week 1 using 4 and 2 × 10^6 sperm per egg the overall fertilization was 78.5% and 72.2%, respectively, the difference being close to significant ($p = 0.056$). Overall results for milt collected in week 2 with 4 and 2 × 10^6 sperm per egg was 75.3% and 67.3, respectively ($p < 0.05$). For both sperm:egg ratios, milt stored for six days showed inferior fertilization rate to day 3 ($p < 0.05$) (Fig. 3).

Majority of the post-thaw *in vitro* parameters were correlated (Pearson correlation) to fertilization rates using 2 × 10^6 sperm per egg. The highest correlations were found for the kinematic parameters VCL ($r = 0.79$, $p < 0.00001$), VAP ($r = 0.78$, $p < 0.00001$), LIN ($r = 0.78$, $p < 0.00001$) and STR ($r = 0.77$, $p < 0.0001$). All correlations were weaker for fertilizations with a sperm:egg ratio of 4 × 10^6.

Of the post-thaw *in vitro* parameters assessed, VCL was the only parameter that made a significant contribution to the linear mixed model where fertility rates with 2 × 10^6 sperm per egg was the response variable ($p < 0.0001$). When fertility rates for 4 × 10^6 sperm per egg was the outcome, none of the measured *in vitro* parameters increased the accuracy of the model.

4. Discussion

The results of our study show that milt from Atlantic salmon preserve fertilizing capacity during cold storage and subsequent cryopreservation, even though *in vitro* quality parameters were significantly negatively influenced by both storage and cryopreservation. Although a change in *in vitro* parameters have been reported during cold storage of Atlantic salmon sperm [14,30], to our knowledge fertility of milt cold stored prior to cryopreservation has not previously been reported.

Fertilization was not affected by storage prior to cryopreservation when applying milt collected the first week of the experiment, which could be consistent with a report of maintained fertility of milt stored for 10 days at 0 °C [14]. However, milt collected the second week showed a significant decline in fertility when using sperm stored for 6 days before cryopreservation. This might be a

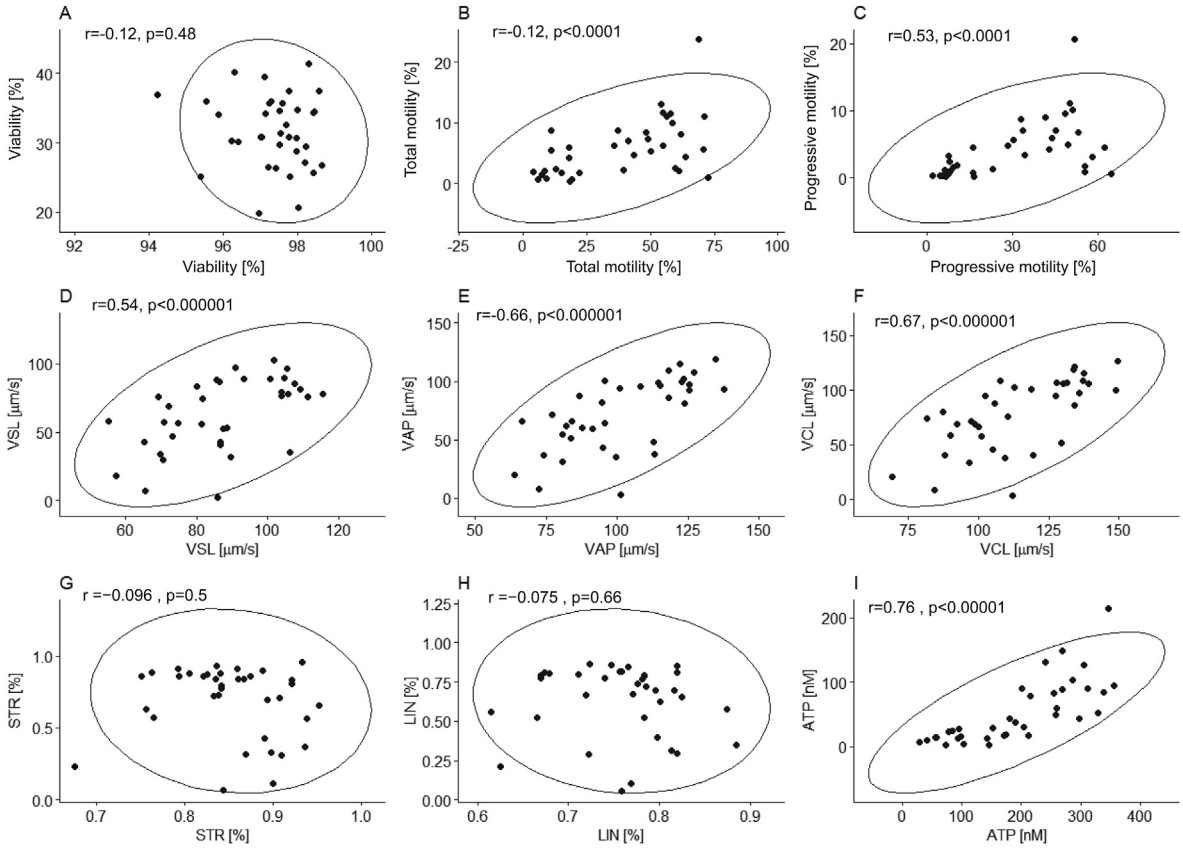

Fig. 2. Correlation between pre- (x-axis) and post- (y-axis) cryopreservation values of sperm viability (A), total motility (B), progressive motility (C), straight line velocity, VSL (D), average path velocity, VAP (E), curvilinear velocity, VCL (F), straightness, STR (G), linearity, LIN (H) and ATP content (I). Correlations indicated as Pearson correlation coefficients (r) with associated p-values.

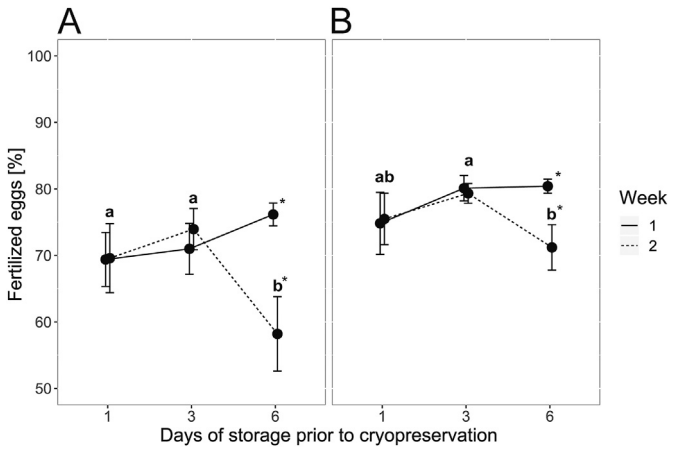

Fig. 3. Percentage fertilized eggs (mean ± SEM) after fertilization with cryopreserved sperm stored cold (5 °C) for one, three and six days before cryopreservation. Fertilizations were carried out in triplicate with sperm-to-egg ratios of 2 (A) or 4 × 10^6 (B). Different letters and asterisks denote significant differences ($p < 0.05$) between storage days and collection week within the same sperm:egg ratio, respectively.

seasonal effect with changes in physical properties and content of the seminal plasma [31] and declining quality passing the optimum time of season, our results being consistent with a report on halibut [32]. Another possibility is an adverse effect of the relatively short period between the two collections. The negative effect of six days storage in the second week was even more pronounced when the sperm:egg ratio was reduced from 4 to 2 × 10^6 sperm per egg, while there was no significant difference in fertilization between the two different sperm:egg ratios for milt collected in week 1. The fact that sperm concentration but not the milt volume was reduced in week 2, suggests an effect of season more than collection frequency, being consistent with the reduced fertilization for milt stored for six days.

Viability of cold stored milt has been found to decline [33]. Interestingly, in our study there seems to be a small positive effect of storage on fertilization even though the viability was reduced. Storage of milt for several days might not compromise fertility if the storage conditions are optimal and milt properties are good [34]. On the other hand, our experiment might have shown a distinct decrease in fertility upon different storage times if the sperm number per egg had been reduced further, as it has been demonstrated that a sperm number of 0.6 × 10^6 sperm per egg does not compromise fertility in rainbow trout [35].

Percentage of viable sperm measured as membrane intact spermatozoa were high during cold storage for both collection weeks. There was a significant decrease in week 2 at both three and six days of storage, however the decrease was only around 2% indicating no biological effect. Storage capacity might be influenced by membrane stabilizing elements from the seminal plasma [36], and it is likely that seminal components were different in the two weeks of collection. Not surprisingly, viability was significantly reduced in frozen-thawed samples which is congruent with reports on cryopreserved sperm from mammals [37,38] as well as from fish [13,30,39]. Interestingly, the variation between collection weeks and storage times were small though significant, which might indicate that the potential of sperm cryo-survival to a limited

extent is influenced by storage before cryopreservation.

Our results on total motility and progressive motility are significantly lower for cold stored milt than otherwise reported [40,41], but comparable for frozen-thawed milt [15]. Motility results could be influenced by use of different CASA systems [42], temperature during analysis [43,44] and by activation procedures. For cold stored milt there was a reduction of both TM and PM from one to three days of storage, and no reduction thereafter. Cryopreserved sperm cells showed pronounced reduced motility, also reported by others [15,45–47]. Taken into account that fertilization results were superior for milt stored for six days in week 1, in spite of reduced motility compared to day 1 samples, arises the question how important motility is for fertilization and which role it plays. In many fish species, including salmonids, sperm cells must enter the narrow micropyle to fertilize the egg. As described by Yanagimachi et al. [48,49] the outer opening of the micropyle in some species possesses glycoproteins being attractants to sperm. It might be possible that salmon eggs also attract sperm cells to the micropyle, that a sperm cluster is established more or less passively around the opening, and that a short period of motility is important only for entering the micropyle, a hypothesis that could explain our good fertility results with cryopreserved milt apparently having poor motility. Sperm velocity parameters could be of importance for the short period of motility [50]. For cold stored milt VSL, VAP and VCL increased significantly throughout the period for samples collected in week 2, but not for week 1. It might be possible that storage affects batches differently when it comes to protection and possible influence on the ability to be activated *in vitro*. For cryopreserved samples the velocity pattern related to storage disappeared, even though there were significant differences at some time points. STR and LIN was to a limited extent influenced by cryopreservation, which could indicate that the overall swimming pattern is maintained.

The current motility results are obtained from a CASA system with 60 Hz frame rate which could be too low for accurate analysis of salmonid sperm [44]. Further, our settings for immotile sperm could have been too restrictive, possibly resulting in underestimation of TM and PM. Concerning activation there is an interesting question how optimal activation of the sperm cells should be, and if the same procedure should be used for undiluted, diluted and cryopreserved milt. Our procedure did not include e.g. BSA or other surfactants which could have been beneficial to avoid stickiness of the sperm cells [51]. Further, the three-dimensional movement of sperm might be of specific interest in fish, and the analysis by CASA has been questioned for this reason [52]. However, the correlation analysis showed that TM, PM and velocity parameters are correlated both between pre- and post-cryopreserved samples and also to fertility obtained, which indicates that the motility values for the different samples are comparable relative to each other.

Sperm motility is generated by ATP however, the production and metabolism thereof are not fully understood and varies between species. Some authors have reported correlation with fertility in different bull breeds [20] and with motility in sterlet [53], while other reports on mammals find no correlation between fertility and sperm ATP content [28,54], but with motility parameters [28]. In the present study cold stored milt from week 2 had significantly higher ATP level than week 1 samples throughout the storage period, with a marked drop from day 1–3. This corresponds with and is correlated with the TM and PM during storage, consistent with a report on sterlet [53]. ATP content was reduced significantly in frozen-thawed samples, as was the motility. ATP in cryopreserved samples were correlated to TM and PM post-thaw. Storage induced a gradual reduction of ATP post-thaw, but again week 2 samples displayed significant higher ATP content than week 1 samples. ATP levels reflects the ATP content in inactive sperm cells [55] and represents the motility potential and duration when activated. Our results show that frozen-thawed sperm have both reduced ATP content and motility compared to cold stored sperm.

Significant positive correlations were found for most sperm quality parameters pre- and post-cryopreservation, except for viability, STR and LIN. This suggests that these parameters in fresh milt are poor indicators of post-thaw sperm quality. Most of the post-thaw *in vitro* parameters were correlated to fertilization rates for 2×10^6 sperm per egg. The highest correlations were found for the kinematic parameters VCL, VAP, LIN and STR, being in compliance with results obtained by others [15,56].

In this study, we found weak associations between fertilization rates and post-thaw sperm viability, ATP content, TM and PM. However, this can be explained by the relatively high number of sperm cells per egg, and that these parameters are considered to be compensable traits. Of all kinematic parameters that showed strong correlations with fertilization rates, VCL was the only parameter that made a significant contribution to the model of fertility rates with 2×10^6 sperm per egg.

Our results suggest that cryopreservation of Atlantic salmon sperm should be performed as soon as possible after sperm collection to maximize the cryopreserved sperm quality. Both viability, TM, PM and ATP content were highest for sperm cryopreserved within one day of collection. On the other hand, there was no reduction in fertilization rates applying cryopreserved sperm stored for three days before cryopreservation. Even with sperm:egg ratios of 2×10^6, results were comparable to values achieved with fresh sperm. In this study, eggs were considered fertilized when a fully formed spinal cord was observed. However, normal embryo development at this stage does not guarantee hatching and a viable alevin.

In conclusion, fertilization rates with milt stored for three days before cryopreservation was not different from results obtained for one day of storage. Motility and velocity parameters, especially VCL could be predictive of sperm fertilizing potential after cryopreservation.

Funding

Financial support was received from Regional Research Fund Inland, Norway (grant number 224814).

Declaration of competing interest

The authors declare that they have no competing interests.

CRediT authorship contribution statement

Elisabeth Kommisrud: Conceptualization, Data curation, Writing - original draft. **Frøydis D. Myromslien:** Data curation, Writing - original draft. **Else-Berit Stenseth:** Data curation, Writing - original draft, Formal analysis. **Teklu T. Zeremichael:** Formal analysis, Data curation, Writing - original draft. **Nadine Hofman:** Formal analysis, Data curation, Writing - original draft. **Inger Grevle:** Conceptualization, Data curation, Writing - original draft. **Jan Sunde:** Conceptualization, Data curation, Writing - original draft.

References

[1] Hafez B, Hafez ESE. Reproduction in farm animals. seventh ed. Blackwell Publishing; 2008.
[2] Polge C, Smith AU, Parkes AS. Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature 1949;164:666.
[3] Blaxter JHS. Sperm storage and cross-fertilization of spring and autumn spawning herring. Nature 1953;172:1189–90.

[4] Judycka S, Nynca J, Ciereszko A. Opportunities and challenges related to the implementation of sperm cryopreservation into breeding of salmonid fishes. Theriogenology 2019;132:12–21.
[5] Tiersch TR, Yang H, Jenkins JA, Dong Q. Sperm cryopreservation in fish and shellfish. Soc Reprod Fertil Suppl 2007;65:493–508.
[6] Cabrita E, Sarasquete C, Martínez-Páramo S, Robles V, Beirão J, Pérez-Cerezales S, et al. Cryopreservation of fish sperm: applications and perspectives. J Appl Ichthyol 2010;26:623–35.
[7] Asturiano JF, Cabrita E, Horváth Á. Progress, challenges and perspectives on fish gamete cryopreservation: a mini-review. Gen Comp Endocrinol 2017;245:69–76.
[8] Betsy CJ, Kumar JSS, Jawahar KTP. Effect of different extenders and dilution ratios on the spermatological and fertilization parameters of Cyprinus carpio during cryopreservation. Biopreserv Biobanking 2019;17:157–62.
[9] Perez-Cerezales S, Martinez-Paramo S, Beirao J, Herraez MP. Evaluation of DNA damage as a quality marker for rainbow trout sperm cryopreservation and use of LDL as cryoprotectant. Theriogenology 2010;74:282–9.
[10] Nynca J, Judycka S, Liszewska E, Dobosz S, Grudniewska J, Arai K, et al. Utility of different sugar extenders for cryopreservation and post-thaw storage of sperm from Salmonidae species. Aquaculture 2016;464:340–8.
[11] Lahnsteiner F, Berger B, Weismann T, Patzner R. The influence of various cryoprotectants on semen quality of the rainbow trout (Oncorhynchus mykiss) before and after cryopreservation. J Appl Ichthyol 1996;12:99–106.
[12] Cabrita E, Robles V, Rebordinos L, Sarasquete C, Herraez MP. Evaluation of DNA damage in rainbow trout (Oncorhynchus mykiss) and gilthead sea bream (Sparus aurata) cryopreserved sperm. Cryobiology 2005;50:144–53.
[13] Cabrita E, Alvarez R, Anel L, Rana KJ, Herraez MP. Sublethal damage during cryopreservation of rainbow trout sperm. Cryobiology 1998;37:245–53.
[14] Stoss J, Refstie T. Short-term storage and cryopreservation of milt from Atlantic salmon and sea trout. Aquaculture 1983;30:229–36.
[15] Dziewulska K, Rzemieniecki A, Czerniawski R, Domagala J. Post-thawed motility and fertility from Atlantic salmon (Salmo salar L.) sperm frozen with four cryodiluents in straws or pellets. Theriogenology 2011;76:300–11.
[16] Judycka S, Nynca J, Liszewska E, Dobosz S, Grudniewska J, Ciereszko A. Optimal sperm concentration in straws and final glucose concentration in extender are crucial for improving the cryopreservation protocol of salmonid spermatozoa. Aquaculture 2018;486:90–7.
[17] Nynca J, Judycka S, Liszewska E, Dobosz S, Ciereszko A. Standardization of spermatozoa concentration for cryopreservation of rainbow trout semen using a glucose-methanol extender. Aquaculture 2017;477:23–7.
[18] Christensen P, Labouriau R, Birck A, Boe-Hansen GB, Pedersen J, Borchersen S. Relationship among seminal quality measures and field fertility of young dairy bulls using low-dose inseminations. J Dairy Sci 2011;94:1744–54.
[19] Farrell PB, Presicce GA, Brockett CC, Foote RH. Quantification of bull sperm characteristics measured by computer-assisted sperm analysis (CASA) and the relationship to fertility. Theriogenology 1998;49:871–9.
[20] Garrett LJA, Revell SG, Leese HJ. Adenosine triphosphate production by bovine spermatozoa and its relationship to semen fertilizing ability. J Androl 2008;29:449–58.
[21] Kime DE, Van Look KJW, McAllister BG, Huyskens G, Rurangwa E, Ollevier F. Computer-assisted sperm analysis (CASA) as a tool for monitoring sperm quality in fish. Comp Biochem Physiol C Toxicol Pharmacol 2001;130:425–33.
[22] Alavi SMH, Cosson J. Sperm motility in fishes. (II) Effects of ions and osmolality: a review. Cell Biol Int 2006;30:1–14.
[23] Judycka S, Cejko BI, Dryl K, Dobosz S, Grudniewska J, Kowalski RK. The effect of supplementation of a trehalose-based extender with KCl on rainbow trout (Oncorhynchus mykiss) sperm freezability and post-thaw motility. Aquaculture 2016;465:303–10.
[24] Judycka S, Nynca J, Liszewska E, Dobosz S, Zalewski T, Ciereszko A. Potassium ions in extender differentially influence the post-thaw sperm motility of salmonid fish. Cryobiology 2016;73:248–56.
[25] Beirao J, Cabrita E, Perez-Cerezales S, Martinez-Paramo S, Herraez MP. Effect of cryopreservation on fish sperm subpopulations. Cryobiology 2011;62:22–31.
[26] Cosson J, Demoy-Schneider M, Le Pennec M, Hui B. Traits of sperm motility in marine fish: activation, regulation and precocious arrest. In: Proc 11th Pac Sci Inter Congress Papeete; 2009. ISBN N°978-2-11-098964-2.
[27] Dzyuba B, Bondarenko O, Fedorov P, Gazo I, Prokopchuk G, Cosson J. Energetics of fish spermatozoa: the proven and the possible. Aquaculture 2017;472:60–72.
[28] Tremoen NH, Gaustad AH, Andersen-Ranberg I, van Son M, Zeremichael TT, Frydenlund K, et al. Relationship between sperm motility characteristics and ATP concentrations, and association with fertility in two different pig breeds. Anim Reprod Sci 2018;193:226–34.
[29] Team RCR. A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing.; 2019.
[30] Diaz R, Lee-Estevez M, Quinones J, Dumorne K, Short S, Ulloa-Rodriguez P, et al. Changes in Atlantic salmon (Salmo salar) sperm morphology and membrane lipid composition related to cold storage and cryopreservation. Anim Reprod Sci 2019;204:50–9.
[31] Nynca J, Kuzminski H, Dietrich GJ, Hliwa P, Dobosz S, Liszewska E, et al. Changes in sperm parameters of sex-reversed female rainbow trout during spawning season in relation to sperm parameters of normal males. Theriogenology 2012;77:1381–9.
[32] Babiak I, Ottesen O, Rudolfsen G, Johnsen S. Quantitative characteristics of Atlantic halibut, Hippoglossus hippoglossus L., semen throughout the reproductive season. Theriogenology 2006;65:1587–604.
[33] Trigo P, Merino O, Figueroa E, Valdebenito I, Sánchez R, Risopatrón J. Effect of short-term semen storage in salmon (Oncorhynchus mykiss) on sperm functional parameters evaluated by flow cytometry. Andrologia 2015;47:407–11.
[34] Scott AP, Baynes SM. A review of the biology, handling and storage of salmonid spermatozoa. J Fish Biol 1980;17:707–39.
[35] Ciereszko A, Dietrich GJ, Nynca J, Dobosz S, Zalewski T. Cryopreservation of rainbow trout semen using a glucose-methanol extender. Aquaculture 2014;420–421:275–81.
[36] Lahnsteiner F. Characterization of seminal plasma proteins stabilizing the sperm viability in rainbow trout (Oncorhynchus mykiss). Anim Reprod Sci 2007;97:151–64.
[37] Watson PF. The causes of reduced fertility with cryopreserved semen. Anim Reprod Sci 2000;60–61:481–92.
[38] Gurler H, Malama E, Heppelmann M, Calisici O, Leiding C, Kastelic JP, et al. Effects of cryopreservation on sperm viability, synthesis of reactive oxygen species, and DNA damage of bovine sperm. Theriogenology 2016;86:562–71.
[39] Linhart O, Rodina M, Flajshans M, Gela D, Kocour M. Cryopreservation of European catfish Silurus glanis sperm: sperm motility, viability, and hatching success of embryos. Cryobiology 2005;51:250–61.
[40] Parodi J, Guerra G, Cuevas M, Ramirez-Reveco A, Romero F. Effects of storage time on the motility, mortality and calcium levels of Atlantic salmon Salmo salar spermatozoa. J Fish Biol 2017;90:1506–16.
[41] Dziewulska K, Rzemieniecki A, Domagała J. Motility and energetic status of Atlantic salmon (Salmo salar L.) sperm after refrigerated storage. J Appl Ichthyol 2010;26:668–73.
[42] Boryshpolets S, Kowalski RK, Dietrich GJ, Dzyuba B, Ciereszko A. Different computer-assisted sperm analysis (CASA) systems highly influence sperm motility parameters. Theriogenology 2013;80:758–65.
[43] Vladič T, Jätrvi T. Sperm motility and fertilization time span in Atlantic salmon and brown trout—the effect of water temperature. J Fish Biol 1997;50:1088–93.
[44] Caldeira C, Garcia-Molina A, Valverde A, Bompart D, Hassane M, Martin P, et al. Comparison of sperm motility subpopulation structure among wild anadromous and farmed male Atlantic salmon (Salmo salar) parr using a CASA system. Reprod Fertil Dev 2018;30:897–906.
[45] Lahnsteiner F, Berger B, Weismann T, Patzner R. Changes in morphology, physiology, metabolism, and fertilization capacity of rainbow trout semen following cryopreservation. Progress Fish Cult 1996;58:149–59.
[46] Cabrita E, Anel L, Herraez MP. Effect of external cryoprotectants as membrane stabilizers on cryopreserved rainbow trout sperm. Theriogenology 2001;56:623–35.
[47] Gwo JC, Ohta H, Okuzawa K, Wu HC. Cryopreservation of sperm from the endangered formosan landlocked salmon (Oncorhynchus masou formosanus). Theriogenology 1999;51:569–82.
[48] Yanagimachi R, Cherr G, Matsubara T, Andoh T, Harumi T, Vines C, et al. Sperm attractant in the micropyle region of fish and insect Eggs1. Biol Reprod 2013;88.
[49] Yanagimachi R, Harumi T, Matsubara H, Yan W, Yuan S, Hirohashi N, et al. Chemical and physical guidance of fish spermatozoa into the egg through the micropyle†. Biol Reprod 2017;96:780–99.
[50] Gallego V, Asturiano JF. Sperm motility in fish: technical applications and perspectives through CASA-Mot systems. Reprod Fertil Dev 2018;30:820–32.
[51] Figueroa E, Lee-Estevez M, Valdebenito I, Watanabe I, Oliveira RPS, Romero J, et al. Effects of cryopreservation on mitochondrial function and sperm quality in fish. Aquaculture 2019;511. 634190.
[52] Boryshpolets S, Kholodnyy V, Cosson J, Dzyuba B. Fish sperm motility analysis: the central role of the flagellum. Reprod Fertil Dev 2018;30:833–41.
[53] Hulak M, Gazo I, Shaliutina A, Linhartova P. In vitro effects of bisphenol A on the quality parameters, oxidative stress, DNA integrity and adenosine triphosphate content in sterlet (Acipenser ruthenus) spermatozoa. Comp Biochem Physiol, C 2013;158:64–71.
[54] Berg HF, Kommisrud E, Bai G, Gaustad ER, Klinkenberg G, Standerholen FB, et al. Comparison of sperm adenosine triphosphate content, motility and fertility of immobilized and conventionally cryopreserved Norwegian Red bull semen. Theriogenology 2018;121:181–7.
[55] Ulloa-Rodriguez P, Figueroa E, Diaz R, Lee-Estevez M, Short S, Farias JG. Mitochondria in teleost spermatozoa. Mitochondrion 2017;34:49–55.
[56] Figueroa E, Valdebenito I, Merino O, Ubilla A, Risopatron J, Farias JG. Cryopreservation of Atlantic salmon Salmo salar sperm: effects on sperm physiology. J Fish Biol 2016;89:1537–50.