Cryopreservation of masu salmon sperm using glucose-methanol extender and seminal plasma biomarkers related to post-thaw sperm motility[1]

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[1] **Abbreviations:** ALH, amplitude of lateral head displacement; BCF, beat cross frequency; DMSO, dimethyl sulfoxide; LIN, linearity; MOT, percentage of motile sperm; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity.

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Abstract

The aim of this study was to evaluate the cryopreservation of masu salmon (*Oncorhynchus masou)* semen, using 0.18 M glucose in 9% methanol as an extender, and to characterize semen parameters (sperm motility; sperm concentration; and seminal plasma parameters such as pH, electric conductivity, DNA concentration, and protein concentration), with respect to post-thaw sperm motility. Semen samples were collected from fishes in the middle of the spawning season, at Toya Lake station, Hokkaido University, and the end of the spawning season, at the Nanae freshwater station, Hokkaido University. The semen was diluted with the extender at a 1:5 ratio (semen:extender) and then equilibrated for 15 min. The final concentration was 0.15 M and 7.5% for glucose and methanol, respectively. Sperm motility was assessed for fresh, fresh-diluted, and post-thaw semen using computer-assisted sperm analysis. The post-thaw sperm showed lower motility, compared with those of fresh and fresh-diluted sperm, which ranged from 33.9% to 82.9% and 1.5% to 27.1% in the semen collected in the middle and end the spawning season, respectively. Sperm concentration, pH, and electric conductivity of the semen collected at the end of the spawning season were lower than those of semen collected in the middle of the spawning season. Moreover, remarkably high DNA concentrations were detected in the semen at the end of the spawning season, although no difference was observed in protein concentration. Finally, semen cryopreserved using the aforementioned method could be used for artificial fertilization to

produce offspring. These results indicate that the glucose-methanol extender is useful for sperm cryopreservation in masu salmon and that the suitability of semen for cryopreservation is affected by the condition of the parental fish.

Keywords: Salmonids, Spermatozoa, Sperm motility, Genebanking

1. Introduction

Sperm cryopreservation is the most frequently used germplasm preservation method established in many fish species because sperm is an easily collectable, simple cellular structure with high chilling resistance, which can be used for the reconstruction of individuals by artificial fertilization (Asturiano et al., 2017). Cryopreserved sperm are very useful not only for germplasm preservation but also for hybridization between different fish species that have different spawning seasons and cross-fertilization between individuals in remote areas. However, in general, cryopreserved sperm are of low quality, which means that sperm motility is low and 10 times the amount of cryopreserved sperm is required for achieving fertilization success comparable to that of fresh sperm (Billard, 1992). Therefore, sperm cryopreservation techniques have been improved to increase post-thaw motility and fertilization success.

During cryopreservation, spermatozoa are damaged due to freezing and thawing, which is known as freezing injury. To avoid damage, sperms are diluted using extenders containing several permeable cryoprotectants, such as methanol, dimethyl sulfoxide (DMSO), dimethyl acetamide, and glycerol and non-permeable cryoprotectants such as sugars, amino acids, and BSA (Magnotti et al., 2018). Recently, an effective cryopreservation procedure using a glucose-methanol extender, which was devised for rainbow trout semen (Ciereszko et al., 2014), was reported to be applied to other salmonid species in Europe (Ciereszko et al., 2015; Dietrich et al., 2016; Nynca et

43 al., 2014; Nynca et al., 2015a; Nynca et al., 2015b; Nynca et al., 2016). Cryopreserved sperm
44 produced by this procedure showed high post-thaw sperm motility, and thus, made it possible to
45 reduce the amount of cryopreserved sperm used for artificial fertilization.

46 Masu salmon, *Oncorhynchus masou*, are genetically diverse and distributed throughout far
47 east Asia (Yu et al., 2010), from the Amur River area and western Kamchatka as the northern limit
48 to western Honshu, Japan, and southeastern Korea (Kato, 1991). Masu salmon in Japan includes
49 the following subspecies: *O. masou ishikawae* (amago salmon) distributed along the Pacific coast
50 of southwestern Honshu Island and the northern part of Kyushu Island, and *O. masou* subsp.
51 (Biwa salmon), which is endemic to Lake Biwa (Kato, 1991). Biwa salmon is genetically distinct
52 from the masu salmon population in mitochondrial DNA genealogy and nuclear DNA genetic
53 structure, and the populations of amago salmon distributed in southern Japan are genetically
54 differentiated based on nuclear DNA genetic structure (Yamamoto et al., 2020). Furthermore,
55 Formosan landlocked salmon, *O. masou formosanus*, which is a subspecies of masu salmon
56 distributed in Taiwan, is an endangered species (Burridge, 2019). Thus, indigenous populations
57 are important genetic resources for conservation and genetic breeding of masu salmon in
58 aquaculture. Sperm cryopreservation was conducted using the pellet method to preserve the
59 germplasm of masu salmon (Yamano et al., 1990). However, sperm cryopreservation using straw
60 and the glucose-methanol extender developed by Polish researchers is yet to be attempted.

Sperm motility parameters and biochemical parameters of rainbow trout seminal plasma change throughout the spawning season (Munkittrick and Moccia, 1987; Nynca et al., 2012). These seasonal changes in semen might be effective for the success of sperm cryopreservation. Thus, biomarkers indicating good sperm quality, specifically a high tolerance to freezing, are required for successful sperm cryopreservation. Several biomarkers, such as the pH and osmolality of seminal plasma, have been reported to evaluate sperm quality in rainbow trout (Lahnsteiner et al., 1998). However, the freezing tolerance of sperm during cryopreservation, based on seminal plasma parameters, has not been reported. An easy method without expensive apparatus will be useful for practical evaluation of tolerance during cryopreservation at hatcheries.

The goals of this study were to cryopreserve masu salmon sperm using glucose-methanol extender, and to identify post-thaw sperm motility biomarkers for evaluating sperm quality, with respect to freezing tolerance. We also evaluated the correlation between sperm quality based on post-thaw sperm motility and other biomarkers such as sperm concentration of semen, pH, electric conductivity, DNA concentration, and protein concentration of seminal plasma.

2. Material and methods

79

80 2.1. Semen and egg collection

81 Semen samples were collected from 10 masu salmons at Toya Lake Station, Hokkaido
82 University and three masu salmons from Nanae freshwater station, Hokkaido University, in 2016.
83 The semen samples from the Toya lake station were collected from native fish during the middle
84 of the spawning season in October 2016 (termed “middle spawning samples”), while those at the
85 Nanae freshwater station were collected from brood stock at the end of the spawning season in
86 November 2016 (termed “end spawning samples”). By this time, all females had finished
87 spawning, and some males could release small amounts of semen, although we could not obtain
88 semen from most of the males.

89 To verify the fertilization ability of cryopreserved semen, semen and eggs collected from
90 three males and five females, respectively, were reared at the Nanae freshwater station in
91 September 2020.

92 Fish were anesthetized with 0.5% 2-phenoxyethanol before semen and egg collection. The
93 semen was collected by gentle abdominal massage with special care to avoid contamination of
94 urine, feces, or blood. The collected semen samples were individually kept in a plastic cup on ice
95 until cryopreservation. Eggs with ovarian fluid were also collected into a bowl by gentle
96 abdominal massage. The collected eggs were pooled in a single container and stored at 4 °C until

use.

2.2 Sperm cryopreservation and thawing

Cryopreservation was performed according to a previously described method (Ciereszko et al., 2014). Semen samples were diluted with extender (0.18 M glucose in 9% methanol) at a ratio of 1:5 (semen:extender), loaded into 0.25 mL plastic straws (Minitube, EcoStraw clear, 0.25 mL, Germany), and sealed using sealing powder (Fujihira, Japan). The straws were placed on a floating rack set at a height of 3 cm (Minitube, Germany) and equilibrated on ice for 15 min. After equilibration, the floating rack with the straws was floated on liquid nitrogen for 5 min for cooling. The straws were then directly thrown into liquid nitrogen and stored in liquid nitrogen until subsequent sperm analysis.

2.3 Analysis of sperm motility

Fresh semen, diluted and equilibrated semen, and cryopreserved and thawed semen were used for the analysis of sperm motility using a sperm motility analysis system (SMAS, Ditect, Ver. 3.19, Japan), which recorded motion images (triplicates per sample) using a microscope (Nikon E200, Japan) with a 10× phase contrast objective and a digital high-speed camera (Ditect, HAS-L2, Japan) at 60 frames per second. Cryopreserved semen was thawed in a water bath at

115 40 °C for 5 s. Thawed semen was immediately transferred to a 1.5 mL tube and mixed well.
116 Sperm was activated using an activating solution [D532 buffer (pH 9.0): 1 mM $CaCl_2$, 20 mM
117 Tris, 30 mM glycine, and 125 mM NaCl, according to Billard (1992)] supplemented with 1%
118 bovine serum albumin, at a ratio of 1:1000 (semen:activating solution). Six microliters of diluted
119 semen were dropped on a glass slide printed with hydrophobic black ink (TF0215; Matsunami
120 Glass Ind., Ltd., Japan), and then, a cover glass (C018181; Matsunami Glass Ind., Ltd., Japan)
121 was placed on the dropped semen to create a sperm monolayer between the glass layers. The
122 following sperm motility parameters were assessed: percentage of motile sperm (MOT),
123 straight-line velocity (VSL), curvilinear velocity (VCL), average path velocity (VAP), linearity
124 (LIN, calculated from VSL/VCL), amplitude of lateral head displacement (ALH), and beat cross
125 frequency (BCF) measured for 1 s at 15 s after activation.
126
127 2.4 Measurement of sperm concentration
128 Sperm concentration was determined by measuring absorbance at 505 nm using a
129 spectrophotometer (GE healthcare, Gene Quant 100 classic, USA) and a semi-micro cuvette of 10
130 mm path length (Sarstedt, 67.742, Germany) according to a previous study (Ciereszko and
131 Dabrowski, 1993). Semen was diluted 1000 times with 0.7% NaCl. The regression formula
132 between the sperm concentration measured by Makler counting chamber (Sefi Medical

133 Instruments Ltd., Israel) and the absorbance of sperm samples was calculated using 10 semen
134 samples (Fig. 1). The regression formula, with a coefficient of determination of 0.918, was as
135 follows:
136 Sperm concentration ($\times 10^9$ sperm mL^{-1}) = 24.256 × (absorbance at 505 nm) – 6.289
137
138 2.5 Seminal plasma analysis
139 Seminal plasma was obtained by centrifuging semen for 5 min at 10000 × *g* at 4 °C. Electric
140 conductivity, pH, DNA concentration, and protein concentration were measured using an electric
141 conductivity meter (Horiba, B-771, Japan), pH meter (Horiba, B-71X, Japan), and Qubit 3.0
142 fluorometer (Thermo Fisher Scientific, USA) with a DNA assay kit (Thermo Fisher Scientific,
143 Qubit™ dsDNA BR Assay Kit, USA) and protein assay kit (Thermo Fisher Scientific, Qubit™
144 Protein Assay Kit, USA), respectively.
145
146 2.6 Fertilizing ability of cryopreserved semen and developmental ability of fertilized eggs
147 Semen samples were collected from three males. The semen of each male was cryopreserved
148 as described in section 2.2 using 0.25 mL straws and stored in liquid nitrogen until use. The
149 remaining 5 mL of fresh semen was stored on ice for 7 h using a 70 mL tissue/cell culture flask.
150 The eggs pooled from five females were divided into 18 batches to include 100 ± 10 eggs (14 g) in

each batch. The divided eggs were used for fertilization with fresh or thawed semen. Ten straws of cryopreserved semen were thawed and pooled for each male. Sperm motility of fresh and thawed semen was measured 5 min before fertilization trials, according to the method mentioned in section 2.3. For fertilization trials, 40, 80, and 160 μL of fresh semen and 240, 480, and 960 μL of diluted-cryopreserved semen were used. According to the mean value of sperm concentration of semen in the middle of the spawning season, the calculated sperm-to-egg ratios in 40, 80, and 160 μL of fresh semen or 240, 480, and 960 μL of diluted-cryopreserved semen were 10,000,000:1, 20,000,000:1, and 40,000,000:1 in the fertilization environment, respectively. Each volume of semen and 10 mL of D532 buffer were respectively added to the eggs simultaneously and mixed well by swirling for 30 s. After 2 min, the eggs were rinsed with hatchery water and incubated for 5 min. The eggs were then incubated in an upwelling incubation tray. To calculate the fertilization rates, 22–26 eggs at 16 h post fertilization were fixed with Bouin's fixative for one day and then stored in 70% ethanol. Fertilization success was determined by the presence of cleavage furrows. Fertilization rates were calculated using the following formula: fertilization rate = number of eggs with cleavage furrows/total number of eggs observed. The developmental and hatching rates were measured at the eyed-embryo (25 days post-fertilization) and hatching (44 days post-fertilization) stages, respectively. The percentage of eyed-embryos and hatched larvae was calculated using the initial number of eggs.

169

170 2.7 Statistical analyses

171 All results are presented as the mean ± standard deviation (SD). All analyses were performed
172 using Statcel4 (The publisher OMS Ltd., Tokyo, Japan) at a significance level of 0.05. The
173 percentage data were normalized using the arcsine square root transformation. For sperm motility
174 analysis and fertilization ability of cryopreserved sperm, the data were analyzed using one-way
175 ANOVA followed by Scheffe's post hoc test. Sperm concentration and seminal plasma
176 parameters (pH, electric conductivity, and protein concentration) were analyzed using Student's
177 *t*-test, and DNA concentration was analyzed using the Mann-Whitney *U*-test between the middle
178 and end spawning samples. Simple linear regression analyses were performed to determine the
179 relationship between post-thaw sperm motility and other factors.

180

181

182 **3. Results**

183

184 3.1. Sperm motility of fresh, fresh-diluted, and cryopreserved semen

185 The percentages of sperm motility of fresh, fresh-diluted, and cryopreserved semen in the
186 middle and end spawning samples are shown in Fig. 2. In fresh semen, the sperm motility in the

187 middle and end spawning samples ranged from 95.7% to 99.3% (97.6 ± 1.3%) and 32.9% to
188 83.6% (65.6 ± 28.3%), respectively. After dilution, the sperm motility in the middle and end
189 spawning samples ranged from 64.8% to 99.4% (94.0 ± 10.7%) and 60.1% to 76.1% (66.5 ±
190 8.4%), respectively. Some semen samples were affected by equilibration. The percentage of
191 sperm motility of cryopreserved semen ranged from 33.9% to 82.9% (64.0 ± 16.1%) and 1.5% to
192 27.1% (10.1 ± 14.7%) in the middle and end spawning samples, respectively. The average
193 motilities of cryopreserved semen were significantly lower than those of fresh and fresh-diluted
194 semen.

195 Among the parameters of sperm motility in middle spawning samples, VSL (fresh semen:
196 50.0 ± 12.9 μm s^{-1}, fresh-diluted semen: 28.2 ± 7.7 μm s^{-1}, cryopreserved semen: 25.7 ± 5.5 μm
197 s^{-1}), ALH (fresh semen: 2.1 ± 0.3 μm, fresh-diluted semen: 1.8 ± 0.3 μm, cryopreserved semen:
198 1.2 ± 0.3 μm), and BCF (fresh semen: 13.3 ± 1.9 Hz, fresh-diluted semen: 6.5 ± 2.5 Hz,
199 cryopreserved semen: 4.6 ± 1.2 Hz) declined due to cryopreservation, while VCL (fresh semen:
200 130.5 ± 10.3 μm s^{-1}, fresh-diluted semen: 134.1 ± 10.3 μm s^{-1}, cryopreserved semen: 111.8 ± 8.9
201 μm s^{-1}), VAP (fresh semen: 117.6 ± 9.8 μm s^{-1}, fresh-diluted semen: 121.3 ± 10.7 μm s^{-1},
202 cryopreserved semen: 106.2 ± 7.9 μm s^{-1}), and LIN (fresh semen: 0.38 ± 0.10, fresh-diluted
203 semen: 0.21 ± 0.06 μm s^{-1}, cryopreserved semen: 0.26 ± 0.06) were not affected (Fig. 3). On the
204 other hand, the parameters of sperm motility in end spawning samples showed different

205 tendencies in VSL (fresh semen: 44.6 ± 6.4 μm s^{-1}, fresh-diluted semen: 56.8 ± 6.6 μm s^{-1},
206 cryopreserved semen: 43.3 ± 22.2 μm s^{-1}), VCL (fresh semen: 119.2 ± 17.3 μm s^{-1}, fresh-diluted
207 semen: 110.0 ± 6.3 μm s^{-1}, cryopreserved semen: 68.7 ± 34.8 μm s^{-1}), VAP (fresh semen: 113.7 ±
208 15.5 μm s^{-1}, fresh-diluted semen: 108.2 ± 6.0 μm s^{-1}, cryopreserved semen: 62.1 ± 39.2 μm s^{-1}),
209 ALH (fresh semen: 1.1 ± 0.2 μm, fresh-diluted semen: 0.8 ± 0.1 μm, cryopreserved semen: 0.9 ±
210 0.2 μm), and BCF (fresh semen: 8.7 ± 1.4 Hz, fresh-diluted semen: 10.4 ± 0.3 Hz, cryopreserved
211 semen: 9.0 ± 4.0 Hz) than those of middle spawning samples; specifically, in cryopreserved sperm,
212 VCL and VAP declined, while VSL, ALH, and BCF remained unaffected (Fig. 3). LIN of end
213 spawning samples (fresh semen: 0.42 ± 0.12, fresh-diluted semen: 0.52 ± 0.04, cryopreserved
214 semen: 0.50 ± 0.23) was also not affected by equilibration and freezing.
215
216 3.2. Sperm concentration and parameters of seminal plasma
217 Sperm concentration and the pH, electric conductivity, and DNA concentration of seminal
218 plasma differed significantly between the middle and end spawning samples (Fig. 4). Sperm
219 concentration (24.0 ± 3.5 × 10^9 cells mL^{-1}), pH (8.45 ± 0.06), and electric conductivity (15.50 ±
220 0.34 mS cm^{-1}) of semen in middle spawning samples were significantly higher than those of end
221 spawning samples (sperm concentration: 8.9 ± 4.7 × 10^9 cells mL^{-1}, pH: 8.00 ± 0.10, electric
222 conductivity 13.63 ± 0.24 mS cm^{-1}). The DNA concentration in the seminal plasma of end

223 spawning samples (2190.67 ± 922.38 μg mL^{-1}) was extremely high compared to those of the
224 middle spawning samples (5.12 ± 1.30 μg mL^{-1}). No significant difference was observed in
225 protein concentration between the middle and end spawning samples (0.44 ± 0.02 mg mL^{-1}).
226
227 3.3. Regression parameters and coefficient of determination between post-thaw sperm motility
228 and other factors
229 The correlation between sperm motility of cryopreserved semen and other values measured
230 in this study was analyzed using regression analysis (Table 1). A coefficient of determination
231 (*r*-squared value) of more than 0.7 was noted between pH and DNA concentration of seminal
232 plasma. A positive correlation was noted between pH and sperm motility, while a negative
233 correlation was noted between DNA concentration and sperm motility, in cryopreserved semen.
234 Sperm concentration and electric conductivity of seminal plasma also showed a positive
235 correlation with post-thaw sperm motility with an *r*-squared value of 0.52 and 0.54, respectively.
236 Among the parameters of sperm motility, the motility of fresh sperm showed an *r*-squared value
237 of 0.53 with post-thaw sperm motility, indicating a positive correlation. The motility of
238 fresh-diluted sperm, ALH, and BCF were also noted to have a positive correlation with *r*-squared
239 values of 0.45, 0.49, and 0.45, respectively. The other parameters did not show a significant
240 correlation with post-thaw sperm motility.

241

242 3.4. Fertilizing ability of cryopreserved semen and developmental ability of fertilized eggs

243 Sperm motilities of the three males were 98.2%, 97.9%, and 96.7% in fresh semen and
244 47.2%, 54.1%, and 23.6% in cryopreserved semen, respectively. Fertilization rates, measured by
245 the presence of cleavage furrows, were high for both fresh and cryopreserved semen (fresh
246 semen: 97.4–100%, cryopreserved semen: 90.8–100%) and were not significantly different
247 among the different sperm-to-egg ratios (Fig. 5A). Mortality was observed with the progress of
248 developmental stages, regardless of semen type and sperm-to-egg ratio. There was no statistically
249 significant difference, with respect to the percentage of eyed embryos and the hatching rates,
250 between fresh and cryopreserved semen, as well as between the different sperm-to-egg ratios (Fig.
251 5B, C).

252

253

254 **4. Discussion**

255 In this study, we applied a glucose-methanol extender developed by Ciereszko et al. (2014)
256 for sperm cryopreservation of masu salmon. The efficacy of the glucose-methanol extender for
257 achieving high post-thaw sperm motility in sperm cryopreservation has been reported by Polish
258 researchers in several salmonid species and other fish species such as rainbow trout (Ciereszko et

al., 2014), brown trout (Nynca et al., 2014), European huchen and grayling (Nynca et al., 2015b), brook trout (Nynca et al., 2015a), whitefish and northern pike (Dietrich et al., 2016), and European perch (Judycka et al., 2019). In these previous studies, post-thaw sperm motility using the glucose-methanol extender was relatively high (more than 40%) compared with other cryopreservation procedures (Ciereszko et al., 2014). However, a decrease in the percentage of motile sperm in cryopreserved semen was commonly observed, except for the northern pike (Dietrich et al., 2016). In this study, the post-thaw sperm motility obtained during the middle spawning season was almost equal to that reported in previous studies, wherein the glucose-methanol extenders were used in salmonid fish, although a decrease in sperm motility was observed in cryopreserved semen (Ciereszko et al., 2014; Nynca et al., 2014; Nynca et al., 2015a; Nynca et al., 2015b). Thus, the glucose-methanol extender is useful for sperm cryopreservation in masu salmon.

The cryopreserved sperm of masu salmon showed an average post-thaw motility of 64% in semen samples obtained in the middle of the spawning season. This post-thaw motility is relatively high compared with that observed in closely related species, amago salmon [18.5%, Ohta et al. (1995)] and Formosan landlocked salmon [14.5%, Gwo et al. (1999)], in which semen cryopreservation was conducted via the dry ice pellet method using an extender consisting of 300 mM glucose (90%) and DMSO (10%). Since the glucose-methanol extender could be utilized for

277 sperm cryopreservation in various salmonid species as mentioned above, the cryopreservation
278 procedure reported in the present study could be applicable to the conservation of closely related
279 species of *O. masou*, such as *O. masou rhodurus* and *O. masou formosanus,* which are indigenous
280 species in Biwa Lake, Japan and Taiwan, respectively. Furthermore, the procedure can be
281 implemented in the hatchery production of masu salmon for aquaculture.

282 Sperm motility of fresh and cryopreserved semen was significantly different between the
283 middle and end spawning samples. A decrease in sperm motility in fresh semen at the end of the
284 spawning season has been reported in rainbow trout (Nynca et al., 2012; Munkittrick and Moccia,
285 1987). The reduced sperm motility was considered to be due to the disturbance of composition in
286 seminal plasma, which provides an optimal environment for the storage of sperm (Ciereszko,
287 2008). In masu salmon, low sperm motility in fresh semen at the end of the spawning season
288 might be caused due to the aging of semen. Semen cryopreserved at the end of the spawning
289 season did not preserve motile sperm at a higher ratio, compared to semen cryopreserved in the
290 middle of the spawning season, owing to a decrease in freezing tolerance. This result suggests that
291 changes in seminal plasma during the spawning season affect sperm quality parameters related to
292 the susceptibility to cryopreservation.

293 Changes in the motility parameters of fresh, fresh-diluted, and cryopreserved semen were
294 noted between the middle and end spawning samples. In other salmonid fish species, motility

295 parameters decreased due to the effects of cryopreservation (Ciereszko et al., 2014; Nynca et al.,
296 2014; Nynca et al., 2015a; Nynca et al., 2015b). Masu salmon sperm also showed a decrease in
297 several parameters, owing to cryopreservation. However, the patterns of changes in some
298 parameters (VSL, VCL, VAP, ALH, and BCF) differed between samples collected in the middle
299 and at the end of the spawning season. Sperm collected in the middle of the spawning season
300 showed a decrease in VSL, ALH, and BCF due to cryopreservation. This means that sperm
301 motility changed from a straight-line to a circular movement and the sperm head lost its active
302 movement due to cryopreservation (Supplementary Fig. S1). However, average values of velocity
303 (VSL) and trajectory (LIN and BCF) parameters of sperm collected at the end of the spawning
304 season and cryopreserved were higher than those of semen collected in the middle of the
305 spawning season. These contradictory results might be explained by the calculation procedure of
306 SMAS, which calculates those values using only the motile sperm detected in the field for
307 measurement.

308 Biochemical parameters of seminal plasma also change during the spawning season in
309 teleosts (Alavi et al., 2008; Ciereszko et al., 1996; Nynca et al., 2012). Antitrypsin activity and
310 lactate dehydrogenase activity in seminal plasma have been suggested as indicators of semen
311 quality because the anti-trypsin activity is related to the maintenance of sperm (Ciereszko et al.,
312 1996; Dabrowski and Ciereszko, 1994) and the lactate dehydrogenase is derived from the leakage

313 of injured sperm in rainbow trout (Ciereszko et al., 2004). Lahnsteiner et al. (1998) used
314 regression models to determine semen quality of rainbow trout based on sperm motility, seminal
315 plasma composition, and sperm metabolism as biomarkers. In this study, we found significant
316 differences in sperm concentration, pH, electric conductivity, and DNA concentration of seminal
317 plasma between the middle and end spawning seasons. Among these, pH might be a useful
318 parameter to select semen that show better post-thaw sperm motility, prior to cryopreservation,
319 because the *r*-squared value between pH and post-thaw sperm motility was the highest in this
320 study. The high DNA concentration in the seminal plasma of the end spawning season was
321 explained by leakage of DNA from sperm. The sperm either degenerates or gets phagocytized in
322 the sperm duct during the post-spawning period in rainbow trout (Billard and Takashima, 1983).
323 Masu salmon are commonly semelparous, whereas rainbow trout are iteroparous. In contrast,
324 masu salmon from the Nanae freshwater station used in this study were inbred strains that can
325 survive after spawning. Thus, high DNA concentration caused by the degradation of sperm and
326 lower sperm concentration might be detected in masu salmon. DNA concentration in the seminal
327 plasma is a simple parameter for evaluating sperm quality in iteroparous salmonid fish, such as
328 rainbow trout and brown trout. However, it is necessary to verify the reproducibility of these
329 biomarkers by using a sufficient number of native fish and other strains of masu salmon and more
330 sampling points, because it is possible that these findings occur only in a specific strain of masu

331 salmon or might be influenced by the rearing conditions.

332 We successfully obtained hatched larvae using cryopreserved sperm in the fertilization
333 trials in this study. The hatching rate at the minimum sperm-to-egg ratio was similar to that of
334 the control group. However, the minimum sperm-to-egg ratio in this study was relatively high
335 compared to that in the previous study performed using brook trout in which the sperm-to-egg
336 ratio was 300,000:1 (Nynca et al., 2015a). Billard (1992) recommended using 10 or 100 times
337 more cryopreserved sperm than fresh sperm, assuming that 300,000 spermatozoa from fresh
338 semen per egg is recommended for fertilization in rainbow trout. Thus, the minimum
339 sperm-to-egg ratio required for successful fertilization using cryopreserved sperm in masu
340 salmon need to be clarified in order to use the sperm effectively, given the limitations of
341 cryopreserved semen.

342 In conclusion, the present study demonstrated remarkably high motility in cryopreserved
343 sperm of masu salmon and successfully obtained hatching larvae from eggs fertilized with
344 cryopreserved sperm and fresh semen. Further studies are required to optimize sperm
345 concentration in straws and to examine the utility of different sizes of straw (0.25 mL and 0.5 mL),
346 to increase cryopreservation success and improve hatchery use. Moreover, lower sperm-to-egg
347 ratios for fertilization should be evaluated, to increase the efficacy of cryopreserved semen.

348

Declaration of competing interest

The authors declare that they have no conflict of interests.

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References

Alavi, S.M.H., Psenicka, M., Rodina, M., Policar, T., Linhart, O., 2008. Changes of sperm morphology, volume, density and motility and seminal plasma composition in *Barbus* (Teleostei: Cyprinidae) during the reproductive season. Aquat. Living Resour. 21, 75–80.

Asturiano, J.F., Cabrita, E., Horváth, Á, 2017. Progress, challenges and perspectives on fish gamete cryopreservation: A mini-review. Gen. Comp. Endocrinol. 245, 69–76.

Billard, R., 1992. Reproduction in rainbow trout: Sex differentiation, dynamics of gametogenesis, biology and preservation of gametes. Aquaculture 100, 263–298.

Billard, R., Takashima, F., 1983. Resorption of spermatozoa in the sperm duct of rainbow trout during the post-spawning period. Nippon Suisan Gakkaishi 49, 387–392.

Burridge, C.P., 2019. Politics and pride: Maintaining genetic novelty may be detrimental for the conservation of Formosa landlocked salmon *Oncorhynchus formosanus*. Aquatic Conserv: Mar. Freshw. Ecosyst. 29, 840–847.

Ciereszko, A., 2008. Chemical composition of seminal plasma and its physiological relationship with sperm motility, fertilizing capacity and cryopreservation success in fish, in: Alavi, S.M.H., Cosson, J.J., Howard, K., Rafiee, G. (Eds.), Fish Spermatology. Alpha Science International Ltd., Oxford, pp. 215–240.

Ciereszko, A., Dabrowski, K., 1993. Estimation of sperm concentration of rainbow trout, whitefish and yellow perch using a spectrophotometric technique. Aquaculture 109, 367–373.

Ciereszko, A., Dietrich, G.J., Nynca, J., Dobosz, S., Krom, J., 2015. Maturation of spermatozoa from rainbow trout (*Oncorhynchus mykiss*) sex-reversed females using artificial seminal plasma or glucose-methanol extender. Theriogenology 83, 1213–1218.

Ciereszko, A., Dietrich, G.J., Nynca, J., Dobosz, S., Zalewski, T., 2014. Cryopreservation of rainbow trout semen using a glucose-methanol extender. Aquaculture 420–421, 275–281.

Ciereszko, A., Liu, L., Dabrowski, K., 1996. Effects of season and dietary ascorbic acid on some biochemical characteristics of rainbow trout (*Oncorhynchus mykiss*) semen. Fish Physiol.

385 Biochem. 15, 1–10.

386 Ciereszko, A., Wlasow, T., Dobosz, S., Goryczko, K., Glogowski, J., 2004. Blood cells in rainbow trout

387 *Oncorhynchus mykiss* milt: Relation to milt collection method and sampling period.

388 Theriogenology 62, 1353–1364.

389 Dabrowski, K., Ciereszko, A., 1994. Proteinase inhibitor(s) in seminal plasma of teleost fish. J. Fish Biol.

390 45, 801–809.

391 Dietrich, G.J., Nynca, J., Szczepkowski, M., Dobosz, S., Szczepkowska, B., Ciereszko, A., 2016. The effect

392 of cryopreservation of semen from whitefish (*Coregonus lavaretus*) and northern pike (*Esox*

393 *lucius*) using a glucose-methanol extender on sperm motility parameters and fertilizing ability.

394 Aquaculture 464, 60–64.

395 Gwo, J.C., Ohta, H., Okuzawa, K., Wu, H.C., 1999. Cryopreservation of sperm from the endangered

396 formosan landlocked salmon (*Oncorhynchus masou formosanus*). Theriogenology 51, 569–582.

397 Judycka, S., Żarski, D., Dietrich, M.A., Palińska-Żarska, K., Karol, H., Ciereszko, A., 2019. Standardized

398 cryopreservation protocol of European perch (*Perca fluviatilis*) semen allows to obtain high

399 fertilization rates with the use of frozen/thawed semen. Aquaculture 498, 208–216.

400 Kato, F., 1991. Life histories of masu and amago salmon (*Oncorhynchus masou* and *Oncorhynchus*

401 *rhodurus*), in: Groot, C., Margolis, L. (Eds.), Pacific slmon life histories. UBC Press, Vancouver,

402 British Columbia, Canada, pp. 448–520.

403 Lahnsteiner, F., Berger, B., Weismann, T., Patzner, R.A., 1998. Determination of semen quality of the
404 rainbow trout, *Oncorhynchus mykiss*, by sperm motility, seminal plasma parameters, and
405 spermatozoal metabolism. Aquaculture 163, 163–181.
406 Magnotti, C., Cerqueira, V., Lee-Estevez, M., Farias, J.G., Valdebenito, I., Figueroa, E., 2018.
407 Cryopreservation and vitrification of fish semen: A review with special emphasis on marine
408 species. Rev. Aquacult. 10, 15–25.
409 Munkittrick, K.R., Moccia, R.D., 1987. Seasonal changes in the quality of rainbow trout (*Salmo gairdneri*)
410 semen: Effect of a delay in stripping on spermatocrit, motility, volume and seminal plasma
411 constituents. Aquaculture 64, 147–156.
412 Nynca, J., Dietrich, G.J., Dobosz, S., Grudniewska, J., Ciereszko, A., 2014. Effect of cryopreservation on
413 sperm motility parameters and fertilizing ability of brown trout semen. Aquaculture 433, 62–65.
414 Nynca, J., Dietrich, G.J., Dobosz, S., Zalewski, T., Ciereszko, A., 2015a. Effect of postthaw storage time
415 and sperm-to-egg ratio on fertility of cryopreserved brook trout sperm. Theriogenology 83, 253–
416 256.
417 Nynca, J., Dietrich, G.J., Grudniewska, J., Dobosz, S., Liszewska, E., Krzyś, M., Różyński, R., Ciereszko,
418 A., 2015b. Efficient method for cryopreservation of European huchen (*Hucho* L.) and grayling
419 (*Thymallus thymallus* L.) semen. Aquaculture 435, 146–151.
420 Nynca, J., Judycka, S., Liszewska, E., Dobosz, S., Arai, K., Fujimoto, T., Grudniewska, J., Ciereszko, A.,

2016. Usefulness of different sugars for cryopreservation of salmon (*Salmo salar*) semen. Anim. Reprod. Sci. 169, 105.

Nynca, J., Kuźmiński, H., Dietrich, G.J., Hliwa, P., Dobosz, S., Liszewska, E., Karol, H., Ciereszko, A., 2012. Changes in sperm parameters of sex-reversed female rainbow trout during spawning season in relation to sperm parameters of normal males. Theriogenology 77, 1381–1389.

Ohta, H., Shimma, H., Hirose, K., 1995. Relationship between fertility and motility of cryopreserved spermatozoa of the amago salmon *Oncorhynchus masou ishikawae*. Fish. Sci. 61, 886–887.

Yamamoto, S., Morita, K., Kikko, T., Kawamura, K., Sato, S., Gwo, J.C., 2020. Phylogeography of a salmonid fish, masu salmon *Oncorhynchus masou* subspecies-complex, with disjunct distributions across the temperate northern Pacific. Freshw. Biol. 65, 698–715.

Yamano, K., Kasahara, N., Yamaha, E., Yamazaki, F., 1990. Cryopreservation of masu salmon sperm by the pellet method. Bull. Fish. Sci. Hokkaido Univ. 41, 149–154.

Yu, J.N., Azuma, N., Yoon, M., Brykov, V., Urawa, S., Nagata, M., Jin, D.H., Abe, S., 2010. Population genetic structure and phylogeography of masu salmon (*Oncorhynchus masou masou*) inferred from mitochondrial and microsatellite DNA analyses. Zool. Sci. 27, 375–385.

438 Figure captions

439

440 Fig. 1. Correlation between absorbance (measured at 505 nm) and sperm concentration of masu
441 salmon semen.

442

443 Fig. 2. Percentage of motile sperm in fresh, fresh-diluted, and cryopreserved semen samples of masu
444 salmon. Circles indicate the percentage of motile sperm in individuals, and squares indicate mean ±
445 standard deviation values. Solid and open symbols express fish in the middle (n = 10) and at the end of
446 spawning season (n = 3), respectively. Values with the same superscript are not significantly different
447 ($P < 0.05$).

448

449 Fig. 3. Motility parameters of fresh, fresh-diluted, and cryopreserved semen of masu salmon. Results
450 are expressed as mean ± standard deviation. Solid and open circles indicate sperm motility in the
451 middle (n = 10) and at the end of spawning season (n = 3), respectively. Values with the same
452 superscript are not significantly different ($P < 0.05$). VSL, straight-line velocity; VCL, curvilinear
453 velocity; VAP, average path velocity; LIN, linearity; ALH, amplitude of lateral head displacement;
454 BCF, beat cross frequency.

455

Fig. 4. Sperm concentration and parameters of seminal plasma (pH, electric conductivity, DNA concentration, and protein concentration) of semen from masu salmon. Circles and squares indicate individual data and mean ± standard deviation values, respectively. Solid and open symbols indicate fish in the middle (n = 10) and at the end of spawning season (n = 3), respectively. Asterisks indicate significant differences between samples in the middle and at the end of spawning season ($P < 0.05$).

Fig. 5. Developmental ability of embryos fertilized with fresh and cryopreserved sperm: (A) percentage of fertilized eggs, (B) eyed embryos, and (C) hatched larvae in different sperm-to-egg ratios (n = 3). Values are expressed as mean ± standard deviation. Values with the same superscript are not significantly different ($P < 0.05$).

468 Table 1. Regression parameters (Y = aX + b) and the coefficient of determination

469 (*r*-squared) between post-thaw sperm motility of cryopreserved semen and other

470 parameters.

471

	r-squared	a	b
Sperm motility (fresh)	0.53	1.12	-49.50
Sperm motility (fresh-diluted)	0.45	1.21	-54.54
Straight-line velocity (VSL)	2.28E-05	-0.011	52.15
Curvilinear velocity (VCL)	0.16	0.87	-59.59
Average path velocity (VAP)	0.033	0.47	-3.42
Linearity (LIN)	0.11	-88.69	86.30
Amplitude of lateral head displacement (ALH)	0.49	37.50	-17.56
Beat-cross frequency (BCF)	0.45	7.08	-35.09
Sperm Concentration	0.52	2.69	-3.55
pH	0.76	117.31	-927.51
Electric conductivity	0.54	23.44	-301.73
DNA Concentration	0.74	-0.024	63.58
Protein Concentration	0.026	-320.90	192.29

472

473

474

475

Fig. 1.

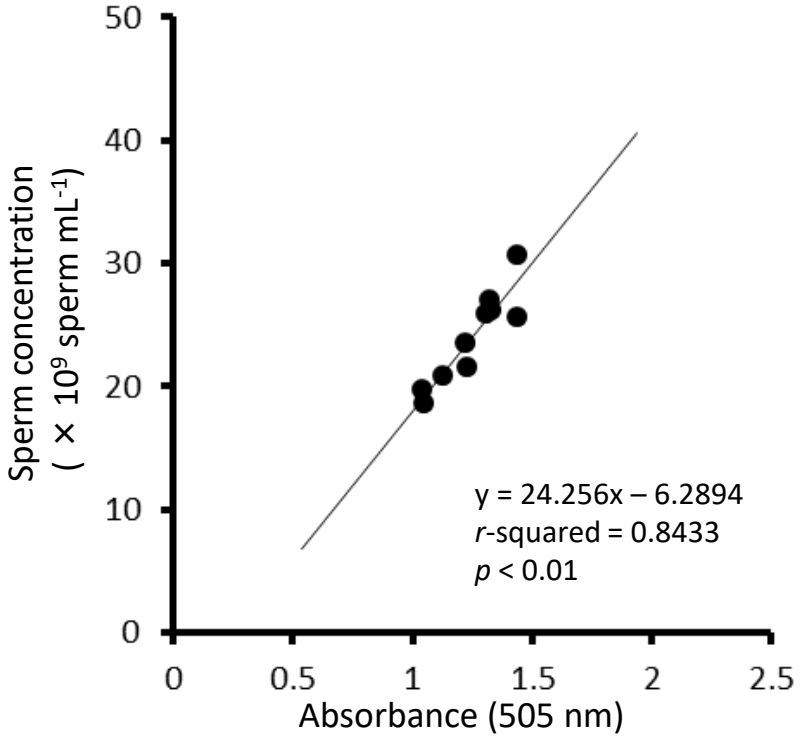

Sperm concentration
(× 10^9 sperm mL^-1)
50
40
30
20
10
0
0
0.5
1
1.5
2
2.5
Absorbance (505 nm)
y = 24.256x – 6.2894
r-squared = 0.8433
p < 0.01

Fig. 2.

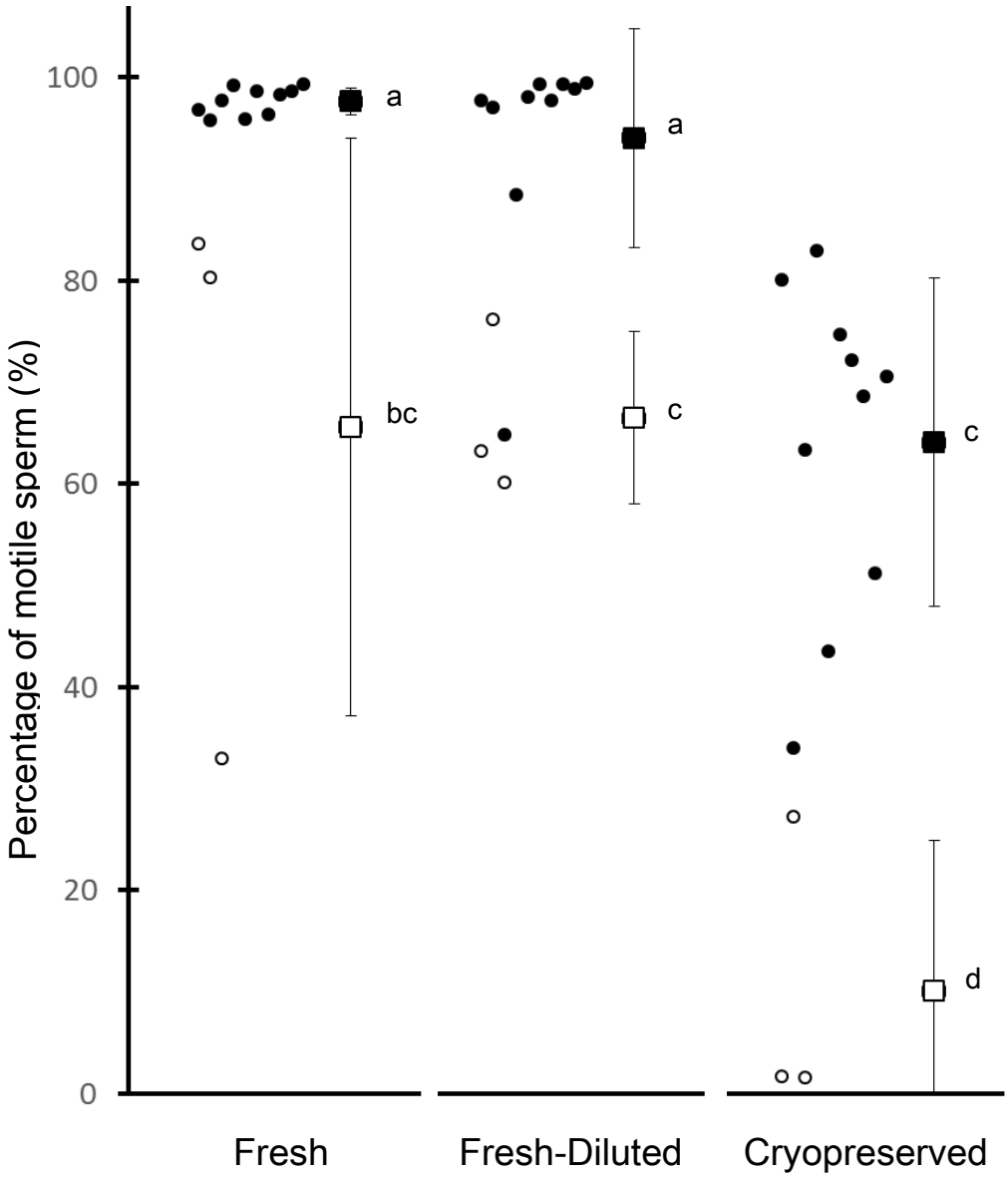

Percentage of motile sperm (%)
100
80
60
40
20
0
a
bc
a
c
c
d
Fresh
Fresh-Diluted
Cryopreserved

Fig. 3

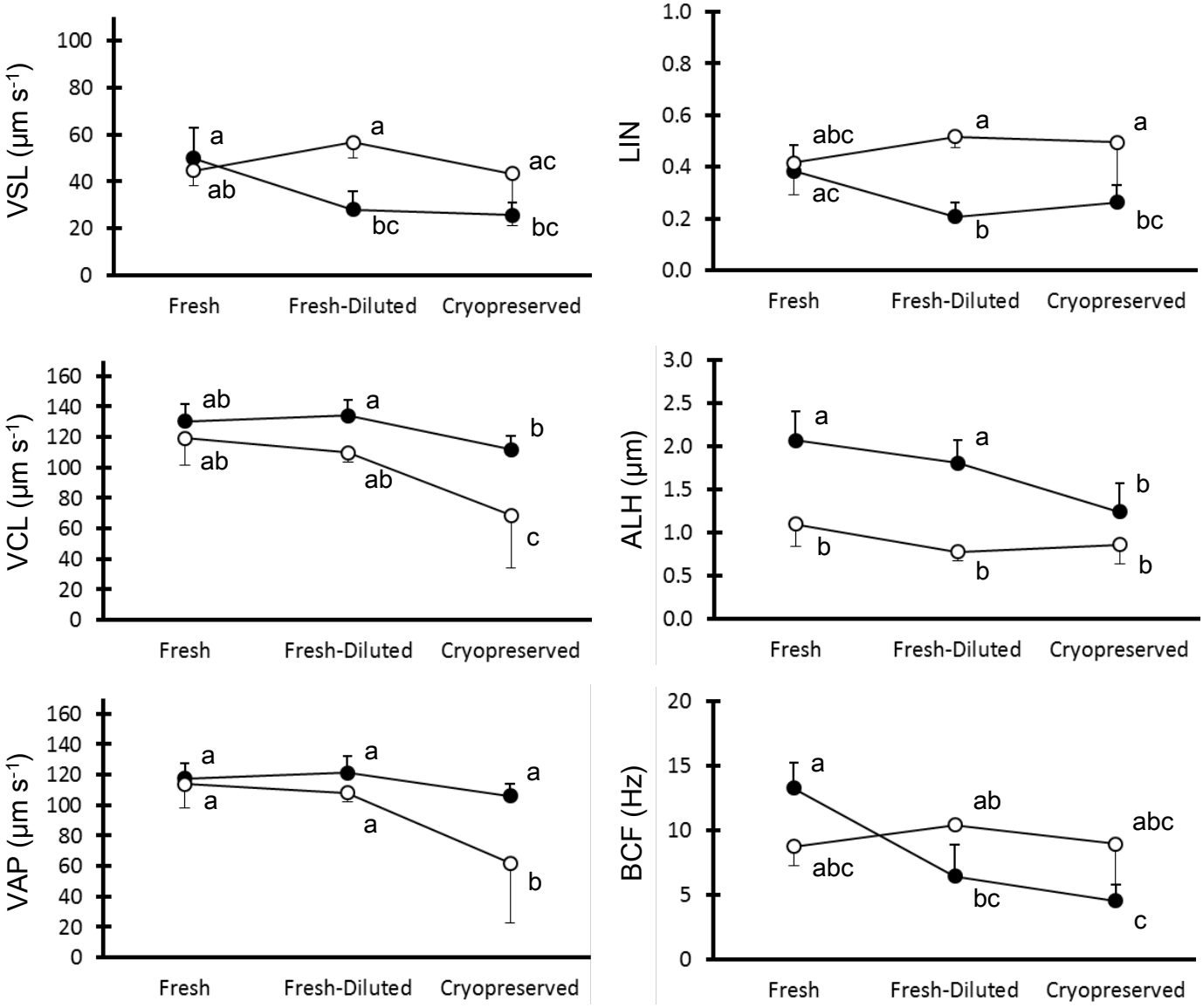

VSL (µm s-1)
100
80
60
40
20
0
a
ab
a
bc
ac
bc
Fresh
Fresh-Diluted
Cryopreserved
LIN
1.0
0.8
0.6
0.4
0.2
0.0
abc
ac
a
b
a
bc
Fresh
Fresh-Diluted
Cryopreserved
VCL (µm s-1)
160
140
120
100
80
60
40
20
0
ab
ab
a
ab
b
c
Fresh
Fresh-Diluted
Cryopreserved
ALH (µm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
b
a
b
b
b
Fresh
Fresh-Diluted
Cryopreserved
VAP (µm s-1)
160
140
120
100
80
60
40
20
0
a
a
a
a
a
b
Fresh
Fresh-Diluted
Cryopreserved
BCF (Hz)
20
15
10
5
0
a
abc
ab
bc
abc
c
Fresh
Fresh-Diluted
Cryopreserved

Fig. 4

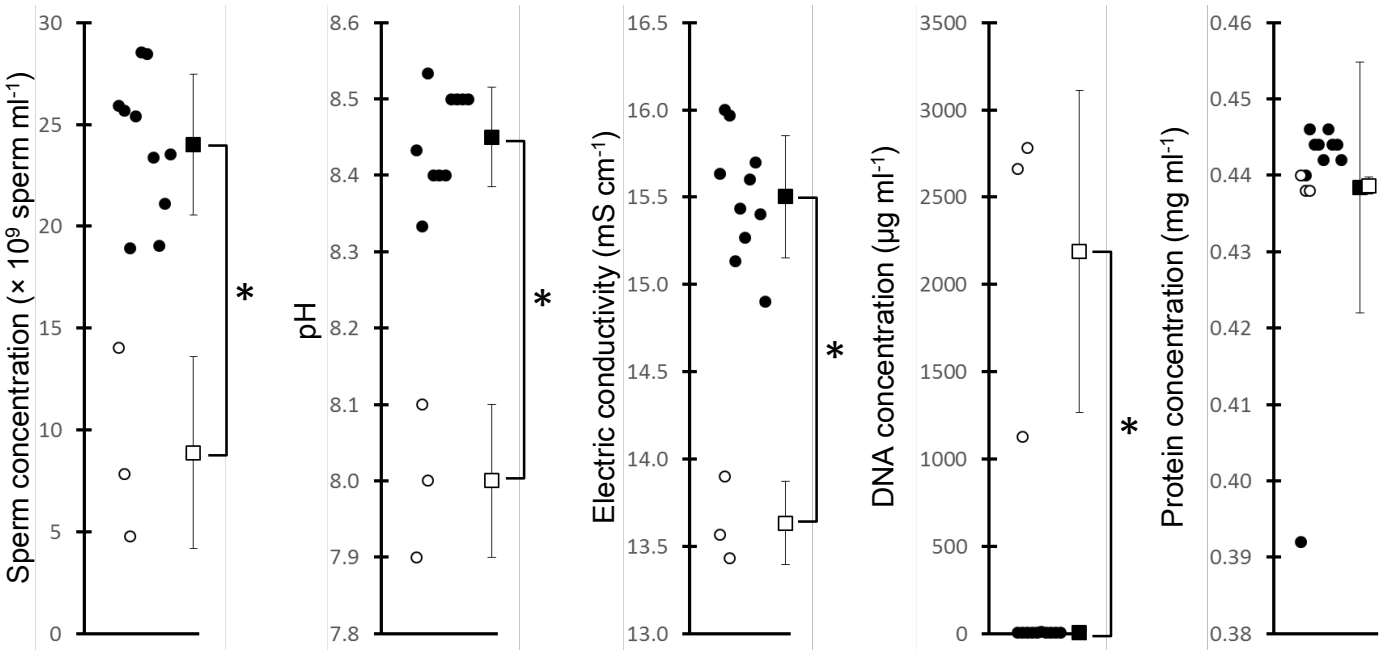
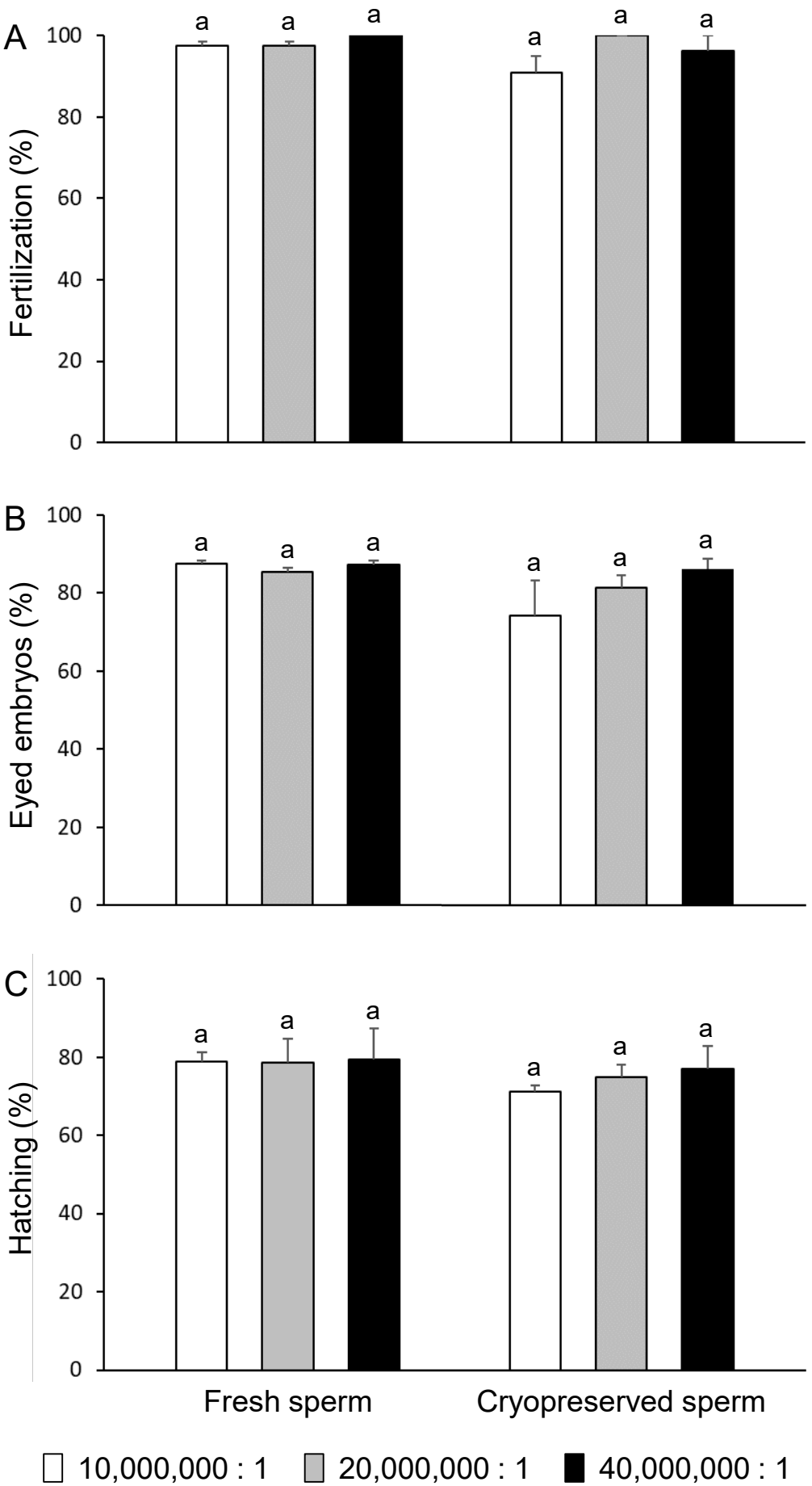

Sperm concentration (× 10^9 sperm ml^-1)
30
25
20
15
10
5
0
*
pH
8.6
8.5
8.4
8.3
8.2
8.1
8.0
7.9
7.8
*
Electric conductivity (mS cm^-1)
16.5
16.0
15.5
15.0
14.5
14.0
13.5
13.0
*
DNA concentration (μg ml^-1)
3500
3000
2500
2000
1500
1000
500
0
*
Protein concentration (mg ml^-1)
0.46
0.45
0.44
0.43
0.42
0.41
0.40
0.39
0.38

Fig. 5


A
Fertilization (%)
100
80
60
40
20
0
a
a
a
a
a
a
B
Eyed embryos (%)
100
80
60
40
20
0
a
a
a
a
a
a
C
Hatching (%)
100
80
60
40
20
0
a
a
a
a
a
a
Fresh sperm
Cryopreserved sperm
10,000,000 : 1
20,000,000 : 1
40,000,000 : 1

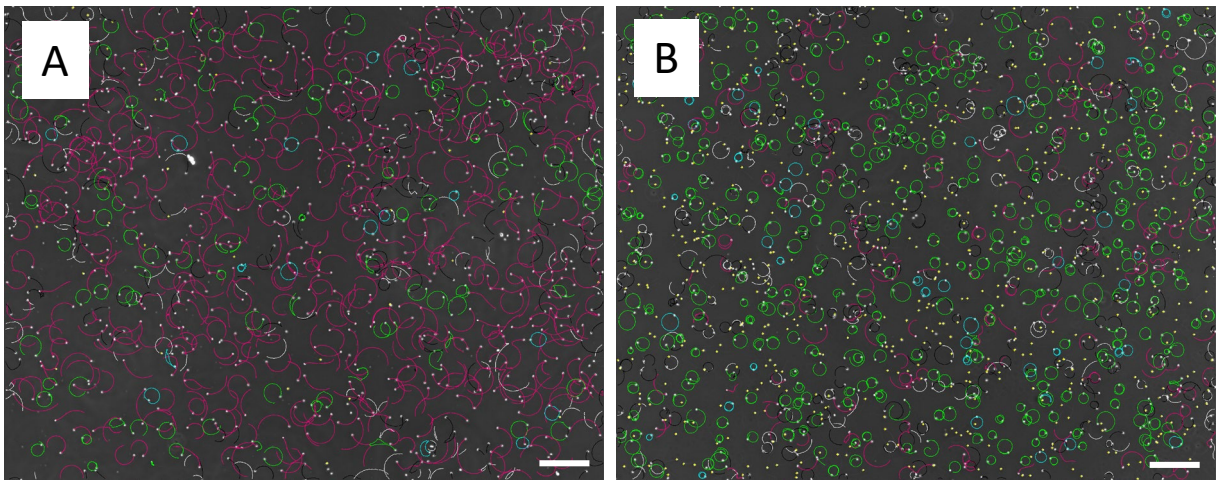


Figure S1. Trajectories of fresh sperm (A) and cryopreserved sperm (B) in masu salmon (*Oncorhynchus masou*). The fresh and cryopreserved sperm were derived from the same individual. Straight-line velocities of >25 µm/s, 5-25 µm/s, <5 µm/s, and no motility in each sperm sample are indicated using magenta, green, blue, and yellow colors respectively. Scale bars indicate 100 µm.