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A Strategy for Sperm Cryopreservation of Atlantic Salmon, *Salmo salar*, for Remote Commercial-scale High-throughput Processing

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Abstract

Sperm cryopreservation is an essential tool for long-term storage of genetic resources for aquaculture fishes. The goal of this study was to develop an efficient and streamlined protocol for high-throughput processing for sperm cryopreservation in Atlantic salmon, *Salmo salar*. The objectives were to evaluate: (1) osmolality of blood serum for determining extender osmolality, (2) effects of extenders for fresh sperm dilution and refrigerated storage, (3) effects of methanol and dimethyl sulfoxide (DMSO) on fresh sperm motility, and (4) motility and fertility after thawing. In this study, sperm samples were collected at a hatchery site in Canada and shipped to a freezing site located 2200 miles (3550 km) away in the USA. Evaluation of three extenders indicated that Mounib solution was suitable for diluting dry sperm for sample processing. Ten percent of methanol or DMSO was less toxic to sperm cells than was 15% within 30 min. Further testing with methanol at 5, 10, and 15%, and sperm solution : extender dilutions (v:v) of 1:1, 1:3, and 1:19 (at concentrations of 5×10^7, 3×10^8, and 1×10^9 cells/mL) indicated that methanol at 5 and 10% showed less toxicity to fresh sperm within 1 h at sperm:extender dilutions of 1:1 and 1:3. Post-thaw motility of sperm cryopreserved with 10% methanol was significantly higher than that with 10% DMSO, and fertility reflected those results (0–1% in DMSO vs. 38–55% in methanol). Further evaluation of sperm cryopreservation with 10 and 15% methanol at sperm dilution ratios of 1:1, 1:3, and 1:19 indicated that post-thaw motility in 10% methanol was significantly higher than that in 15% methanol, and post-thaw fertility in 10% methanol at 1:1 and 1:3 dilution ratios had fertilization rates similar to that of fresh sperm controls. Sperm samples from 12 males cryopreserved with 10% methanol showed male-to-male variation in post-thaw motility (0–36%). Overall, a simplified standard protocol was established for cryopreservation of shipped sperm of Atlantic salmon using extender without egg yolk and yielded satisfactory post-thaw motility and fertilization rates. This procedure can be readily adopted by aquaculture facilities to take advantage of high-throughput cryopreservation

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capabilities at remote service centers. Most importantly, this approach lays the groundwork for an alternative commercial model for commercial-scale production, quality control, and development of industrial standards. Control of male variability and sperm quality remain important considerations for future work.

KEYWORDS

Atlantic salmon, *Salmo salar*, commercial high-throughput, sperm cryopreservation

As high-value aquaculture species, fishes from the family Salmonidae have been studied intensively in various aspects including basic biology (Gross 1998), physiology (WaagbØ 1994), genetics and genomics (Moen et al. 2008; Davidson et al. 2010), and aquaculture production in seawater and freshwater systems (Melberg and Davidrajuh 2009). Atlantic salmon, *Salmo salar*, is found naturally in the northern Atlantic Ocean and is the major aquaculture species in many countries in this area, especially Norway. Selective breeding programs have been developed for establishment of culture strains to sustain aquaculture of this species (Thodesen and Gjedrem 2006). Owing to the long-generation intervals (ca. 4 yr) and large body size, maintenance of broodstock quantity and diversity is a substantial challenge for commercial facilities. Therefore, reliable and high-throughput cryopreservation technology for germplasm preservation is needed to preserve genetic resources, reduce maintenance costs, and provide operational efficiency in commercial hatcheries for artificial fertilization.

Sperm cryopreservation has been studied in more than 200 fish species, including more than 30 marine species (Magnotti et al. 2016) and has been applied for hybridization, creation of mutant lines, and other purposes. Sperm cryopreservation in salmonids has been reported with varying results. Although fertilization rates above 65% have been reported in rainbow trout (Baynes and Scott 1987; Cabrita et al. 2001), certain critical factors such as extender selection, equilibration times, cooling rates, and thawing process need to be quantified and optimized for individual target species (Glogowski et al. 1999). For Atlantic salmon, studies have been reported for sperm vitrification (Figueroa et al. 2015) and cryopreservation including various sample containers such as straws (Zell 1978) and ampules (Hoyle and Idler 1968; Truscott et al. 1968; Mounib 1978; Stoss and Refstie 1983). Assessment of sperm viability indicated that penetrating cryoprotectants (10% methanol or dimethyl sulfoxide [DMSO]) yielded better protection on post-thaw sperm motility than did non-penetrating cryoprotectants (0.3 M glucose or 0.6 M sucrose), and freezing in 0.25-mL straws gave better results than freezing in 0.1-mL pellets (Dziewulska et al. 2011). A recent study on Atlantic salmon showed that sperm cryopreserved with DMSO, glucose, and bovine serum albumin (BSA) yielded a post-thaw fertilization of 82% (first cleavage at 16 h) and that post-thaw fertilization was correlated with curved/straight line sperm velocity (Figueroa et al. 2016). Overall, these studies were performed to address laboratory-scale efforts and not address commercial-scale applications with high-throughput procedures.

Aquaculture venues for most fishes including the Atlantic salmon are usually located in remote locations, and necessary equipment is not readily available for commercial cryopreservation. Therefore, shipping of sperm samples to a service center for cryopreservation would often be a necessary operation. Off-site freezing hinders a cryopreservation program because fresh samples require suitable methods to maintain motility capacity under refrigerated storage for at least 24–48 h (assuming priority overnight shipping will be applied). At present, shipping effects on fish sperm have not often been considered, and few examples of long-distance transportation for cryopreservation have been reported. To our knowledge, shipping of sperm from wild common snook, *Centropomus undecimalis*, from Florida to Louisiana for cryopreservation offered some information in this respect

(Tiersch et al. 2004). In the current study, sperm samples were shipped between a commercial hatchery in Canada and a research center with freezing capabilities in Louisiana, a 3550-km distance.

Automated high-throughput cryopreservation and commercial quality standards have been used in the dairy industry for decades (Pickett and Berndtson 1974). However, such systems have not yet been commercially adopted for aquatic species. With expansion of aquaculture, selective breeding and biotechnologies have been increasingly employed to improve production (Rothschild and Ruvinsky 2007), and other genetic approaches such as genomic-assisted breeding (Rise et al. 2007) and transgenics (Hulata 2001) have also been applied to improve production and commercial value. Consequently, distinct fish lines and strains with defined phenotypes and significant economic values have been and will be developed. Cryopreservation, especially with potential for high-throughput processing and standardized quality control, is the most economical and effective method for preservation, maintenance, and distribution of these valuable germplasm resources.

Cryopreservation is a process involving a series of interconnected steps, and errors at any particular step can cause failure of the whole process. For Atlantic salmon, various cryoprotectants have been investigated, such as methanol with egg yolk (Jodun et al. 2007) and different combinations of methanol, DMSO, glucose, and sucrose with the addition of BSA (Figueroa et al. 2015, 2016). For extenders, different recipes, often including inexplicit ingredients, such as "fresh hen egg yolk," soybean, or BSA, were used (Phillips and Lardy 1940; Layek et al. 2016) despite the fact that the utility of such ingredients has not been fully tested. Overall, for commercial-scale production by using commercially available automated equipment, cryopreservation protocols need to be simplified, standardized, and integrated. A commercial-scale automated straw processing system has been used for sperm cryopreservation in blue catfish, *Ictalurus furcatus*, for use in commercial-scale hybridization by crossing with channel catfish females (Hu et al. 2011).

The current study focused on developing a standardized, reliable, and effective cryopreservation protocol for shipped sperm of Atlantic salmon that can be applied for high-throughput commercial-scale processing by using commercially available automated equipment for filling, sealing, and labeling of containers. This study involved sample shipping between two countries that can provide an example for remote commercial hatcheries to process large volumes of sample for cryopreservation at a service center to obtain quality control, inventory tracking, secure storage, and subsequent transport by taking advantage of well-established methodologies. The goal of this study was to develop an efficient and streamlined protocol with high-throughput capacity for sperm cryopreservation in Atlantic salmon. The objectives were to evaluate: (1) osmolality of blood serum for determining extender osmolality, (2) effects of extenders for fresh sperm dilution and refrigerated storage, (3) effects of methanol and DMSO on fresh sperm motility, and (4) motility and fertility after thawing. Overall, this strategic approach can be readily adopted by aquaculture facilities as a commercial model for commercial-scale processing of cryopreserved sperm.

Materials and Methods

Fishes

The Atlantic salmon used in this study were from the St. John River strain at AquaBounty Technologies (https://aquabounty.com), a research and development hatchery located in Bay Fortune, Prince Edward Island, Canada. Sexually mature fish (4 yr old, 8435 ± 1686 g, $n = 16$) were maintained year-round in tanks with photoperiod controlled by a ramping system to mimic natural sunrise and sunset times at this specific location. Fish were fed with a specific broodstock diet (Skretting vitalis 9, Skretting, Stavanger, Norway. http://www.skretting.com) with high levels of digestible protein, a balance of lipids and essential fatty acids, astaxanthin, and enhanced levels of vitamins E and C for at least 6–12 mo prior to spawning. Before sperm collection,

broodstock were maintained at 6–11 C for at least 2 wk, and feeding was suspended for at least 2 d.

Sperm Sample Collection

Mature males were anesthetized with tricaine methanesulfonate (MS-222) (GMG Fish Services, St. George, NB, Canada) at a final concentration of 0.1 g/L and sodium bicarbonate (Fisher Scientific, Waltham, MA, USA) at a final concentration of 0.20 g/L. After anesthesia, fish were rinsed and blotted clean using dry towels, and individually identified by scanning for passive integrated transponder tags. Sperm samples were collected in 50-mL centrifuge tubes by gentle abdominal pressure to avoid contamination with urine, blood, or feces. After collection, sample tubes were immediately covered (but with air supply allowed through perforated lids), labeled, and stored at 4 C until assessment of motility within 1 h (described below). Stripped fish were returned to recovery tanks.

Determination of Sperm Concentration

Sperm concentration was determined using a hemocytometer (Bright-line hemocytometer, Sigma-Aldrich, Buffalo, NY, USA). Before counting, fresh sperm solution was diluted at a ratio of 1:2000 with salmon Ringer's solution (NaCl 110.00 mM, KCl 3.41 mM, $NaHCO_3$ 2.40 mM, $CaCl_2 \cdot 2H_2O$ 2.70 mM, at an osmolality of 215 mOsmol/kg, pH = 7.3). The diluted sperm solution was mixed with an equal volume of 0.4% trypan blue (w/v) in phosphate buffer saline (NaCl 136.89 mM, KCl 2.68 mM, Na_2HPO_4 8.10 mM, KH_2PO_4 1.47 mM, pH = 7.4) to facilitate cell visualization and counting. Cells were counted in five squares within the large middle square and cell concentration was calculated by following the manufacture's instruction.

Motility Estimation

Sperm motility was estimated by visual observation using a dark-phase microscope (Nikon Eclipse TS100, Melville, NY.) at 200× magnification. To activate sperm motility, salmon Ringer's solution was used at 50:1 ratio for fresh sperm because motility activation of salmon sperm requires a reduction in potassium ion concentration (Morisawa and Suzuki 1980), and solution D532 (NaCl 125 mM, Tris 20 mM, glycine 30 mM, at an osmolality of 282 mOsmol/kg, pH = 9.0) was used (50:1 ratio) for thawed sperm (Billard 1992). Sperm motility was observed immediately after mixing 1 μL of sample and 50 μL of Ringer's solution or D532 solution on a glass slide. Motility estimations were based on observation of two sperm samples per male and three different fields per sample. Motility was expressed as the percentage of sperm cells in each field that actively swam forward (not in circular motion). Samples with an initial motility >75% were used in this study.

Shipping of Sperm Samples for Cryopreservation

Fresh sperm samples (10–40 mL) were packaged in sealable bags (Ziploc® double guard quart, S.C. Johnson, Racine, WI, USA), filled with pure oxygen from an oxygen cylinder and sealed for overnight shipping (24–48 h) in a styrofoam box along with ice packs for cold storage from Prince Edward Island, Canada, to Baton Rouge, LA, USA. Upon arrival, the bags were placed at 4 C in a refrigerator for use in studies within 24 h. When sample shipping took longer than 24 h, sample bags were immediately opened to provide an air supply.

Automated Sample Packaging, Sealing, and Labeling for Commercial-scale Processing and Freezing

For commercial-scale sample processing and freezing, an automated high-throughput system (MAPI, Cryo Bio System, IMV Technologies, L'Aigle, France) controlled by a proprietary computer program (SIDE, Cryo Bio System, IMV Technologies) was used to fill, seal, and label 0.5-mL cryo-biosecurity (CBS) straws designed for the MAPI system.

Before freezing, sperm were sampled for motility estimation and mixed with double-strength cryoprotectants (methanol or DMSO) in the same extender as used for the sperm samples to yield the desired final

cryoprotectant concentrations (5 and 10%) and sperm concentrations (ranging from 1×10^8 to 1.7×10^9 sperm/mL) depending on experimental design. Immediately after mixing with cryoprotectant, sperm samples were placed in the sample cradle of the MAPI system for packaging. Samples were drawn into 0.5-mL high-CBS straws by vacuum applied from the cotton end of the straw, and the straws were continuously transferred to the sealing platform to seal both ends by heat clamps at 158 C. The sealed straws were labeled with alphanumeric information on the identification jacket with an ink printer (A400, Domino, Cambridge, UK). The packaged straws were collected from the MAPI system and sorted for freezing.

For freezing, the straws were arrayed on horizontal racks (40 straws per rack) and placed in a commercial-scale programmable freezer (Micro Digitcool, IMV Technologies). The cooling program was initiated at 30 min after mixing of cryoprotectant and sperm (i.e., equilibration time). The cooling rate was set at 40 C/min from 4 to −80 C based on chamber temperature, and samples were held for 5 min after reaching −80 C and removed from the freezer and plunged into liquid nitrogen. Frozen samples were sorted under liquid nitrogen into storage containers (12-compartment daisy goblets, reference number: 015144, Cryo Bio System, IMV Technologies) for long-term storage. Before return shipping to AquaBounty Technologies, Canada, for fertilization testing, frozen samples were held in liquid nitrogen in storage dewars at the Louisiana State University Agricultural Center (LSUAC) for at least 48 h.

In Vitro *Fertilization of Cryopreserved Sperm Samples*

Oocytes were collected by stripping through applying gentle pressure to the abdomen and stroking toward the vent. During handling, care was taken to avoid contamination of the oocytes by urine or feces. After collection, oocytes were immediately moved to a dark and cool (3–11 C) location in a covered container. After stripping, females were returned to a recovery tank. Oocyte quality was visually assessed within 15 min after stripping by observing color, size, and shape. Oocytes of small sizes (<2 mm) or sticking to each other (incomplete ovulation) or overripe (flaccid and loss of spherical shape) were not used. Total oocyte number collected from each female was estimated by counting a subsample of oocytes in a known volume of ovarian fluid (i.e., ca. 50 mL). Batches of 100 or 250 oocytes were placed in individual containers for fertilization testing.

For fertilization, frozen samples were thawed by immersing straws in a water bath at 40 C for 12 sec directly from the storage dewar. Thawed sperm were released and mixed immediately with oocytes after drying of the straw surface (to avoid sperm activation) and cutting the two sealed ends. To activate gametes for fertilization, 25 mL of activating solution D532 was added and mixed well by gently swirling the container. After 5 min in the dark, the fertilized eggs were rinsed with hatchery water and transferred to individual baskets in a tray in a Heath Incubator (Higgins Equipment Ltd., Fredericton, NB, Canada).

During hatching, constant water flow (16 ± 2 L/min) was maintained through the trays and baskets in the Heath Incubator. Dissolved oxygen was maintained at approximately 12 mg/L (ranging from 10 to 18 mg/mL), and total gas pressure of the water supply were monitored at approximately 102%. Incubators were covered with black plastic to minimize light exposure. Mortalities were not removed during the incubation period.

For all *in vitro* fertilizations, one aliquot of oocytes from each cross was fertilized with fresh sperm (same number as that of cryopreserved sperm) from one or two males used as a control to test oocyte quality.

Assessment of Fertilization Rate

To determine percent fertilization, embryos were observed at approximately 14 d after fertilization at 7.5–8.5 C (i.e., the streak stage at neurulation or organogenesis). A subsample of 5–10 embryos was examined to confirm developmental stage before counting the entire batch. Water was drained from the hatching tray completely and embryos were fixed in Stockard's solution (5% formalin, 4% glacial acetic acid, 6%

glycerine, and 85% distilled water), a standard fixative for embryo observation (Costello et al. 1957; Galat 1972). After about 5 min in fixative, the embryos were examined in a petri dish visually with each recorded as fertilized, unfertilized, or decomposing.

Experiment I. Measurement of Blood Plasma Osmolality

Blood plasma from six males was collected and transferred to 1.5-mL microcentrifuge tubes for separation of serum plasma by centrifugation on a tabletop centrifuge at 500 *g* for 5–10 min. A volume of 10 µL of the supernatant was sampled for measurement of osmolality using a vapor pressure osmometer (Vapro 5520, Wescor Inc., Logan, UT, USA).

Experiment II. Evaluation of Extenders to Maintain Sperm Motility Capacity

Three extenders were tested in this study:

1. Extender B (Lahnsteiner 2000, 2011): NaCl 600 mg, KCl 315 mg, $CaCl_2 \cdot 2H_2O$ 15 mg, $MgSO_4 \cdot 7H_2O$ 20 mg, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 470 mg, pH 7.8. Methanol 10 mL, sucrose 0.5 g, BSA 1.5 g, dissolved in 100 mL ddH_2O with a final osmolality of 305 mOsmol/kg;
2. Mounib solution (Mounib 1978): Sucrose 43 g, reduced glutathione 2 g, $KHCO_3$ 10 g, dissolved in 1 L ddH_2O with a final osmolality of 315 mOsmol/kg; and
3. Hanks' balanced salt solution (HBSS) (Hanks 1975): NaCl 4.0 g, KCl 0.20 g, KH_2PO_4 0.027 g, $NaHCO_3$ 0.176 g, $Na_2HPO_4 \cdot 7H_2O$ 0.021 g, D-glucose $C_6H_{12}O_6$ 0.50 g, $CaCl_2$ 0.096 g, $MgCl_2$ 0.111 g, H_2O 500 mL, pH = 7.1, with a final osmolality of 320 mOsmol/kg.

To ensure that the three extenders did not activate sperm motility, 10 µL of fresh dry sperm (no dilution) were sampled from five males and mixed with 100 µL of each extender for immediate motility observation (preactivation motility). In addition, 2 µL of sperm suspension in each extender were activated by adding 20 µL of salmon Ringer's solution for motility assessment.

To test the capacity of these three extenders for maintenance of sperm motility, fresh sperm samples (300 µL) were mixed with each extender (900 µL) in 1-mL microcentrifuge tubes with the lid punctured for aeration and stored in a refrigerator. Sperm motility was assessed immediately and after 24 h of storage.

Experiment III: Acute Toxicity of Cryoprotectants on Fresh Sperm Motility

Two trials were performed for the evaluation of cryoprotectant toxicity. In the first trial, dry sperm were diluted in three extender buffers (Extender B, Mounib, and HBSS320) and were exposed to DMSO and methanol at final concentrations of 10 and 15%, and sperm motility was estimated at 1, 10, 20, and 30 min at room temperature. Owing to the large number of treatments, this trial was conducted once, and data were treated as repeated measures.

In the second trial, fresh sperm were diluted with Mounib solution at ratios of 1:19, 1:3, and 1:1 (fresh sperm : extender) yielding concentrations of 5×10^7, 3×10^8, and 1×10^9 cells/mL, and sperm at each concentration were exposed to methanol at final concentrations of 5, 10, and 15% for motility estimation after exposure for 1, 10, 20, 30, 40, 50, 60 min, and 2 h at room temperature. Three replicates were produced by using sperm from different males.

Experiment IV: Sperm Motility and Fertility After Thawing

To evaluate the effect of cryoprotectants on sperm cryopreservation, motility of fresh sperm after dilution in extenders, after equilibration (the time after mixing with cryoprotectant until initiation of cooling process), and after thawing was recorded for experiments in each trial. Three trials were performed for sperm cryopreservation, and post-thaw sperm fertility was tested in two trials.

In the first trial, Mounib solution was used as extender to dilute fresh sperm samples at a ratio of 1:3, and DMSO and methanol at final concentrations of 10% were tested for freezing

at a cooling rate of 40 C/min (chamber temperature). Fresh sperm motility, equilibration sperm motility after incubating with cryoprotectants at 1 min and 30 min, and post-thaw motility were recorded. Three replicates were produced by using sperm from three males. In this trial, samples were shipped back to Canada after storage in liquid nitrogen for 11 d for fertilization testing. Three females were used for oocyte collection, and 2×10^5 sperm per oocyte were used for *in vitro* fertilization. Additionally, frozen samples from two males (of three) were used for further fertilization with oocytes collected from another three females at a double number of post-thaw sperm (4×10^5 sperm per oocyte).

In the second trial, sperm samples from three males were diluted at ratios of 1:19, 1:3, or 1:1 in Mounib solution and cryopreserved with methanol at final concentrations of 10 or 15% at a cooling rate of 40 C/min after an equilibration time of 15 min. The frozen samples cryopreserved with 10% methanol at concentrations of 1.5×10^8 (1:3 dilution ratio) and 5×10^8 cells/mL (1:1 dilution ratio) were used for *in vitro* fertilization after storage in liquid nitrogen for 2 wk. Three females were used for oocyte collection, and two sperm-to-oocyte ratios (1×10^6 and 2×10^6 sperm per oocyte) were used for fertilization trials.

In the third trial, sperm samples from 12 males were cryopreserved with 10% methanol at a cooling rate of 40 C/min after 15 min of equilibration. Sperm concentration was standardized as 3×10^8 cells/mL in Mounib solution. No post-thaw fertilization was conducted in this trial.

Data Collection and Analysis

Data in this study were analyzed using JMP® Pro (12.0.1, SAS, Cary, NC, USA). Percentage data were arcsine square root transformed before analysis. Differences were tested by two-sample *t* test or ANOVA with the accepted significance as $P < 0.050$, and pairwise comparisons among the treatments were analyzed with Tukey's post hoc test.

Results

Protocol development for commercial-scale sperm cryopreservation was performed through systematic estimation of major factors in the freezing procedure. The schematic workflow was illustrated in Figure 1.

Measurement of Blood Plasma Osmolality

The osmolality of blood plasma was 327 ± 4 mOsmol/kg ($n = 6$). Based on this measurement, the osmolalities of extenders used for sperm maintenance and dilution were prepared at 305–320 mOsmol/kg.

Evaluation of Three Extenders to Maintain Sperm Motility Capacity

All of the three extenders (Extender B, Mounib solution, and HBSS320) tested for maintenance of fresh sperm motility caused some preactivation of sperm motility after diluting (empty

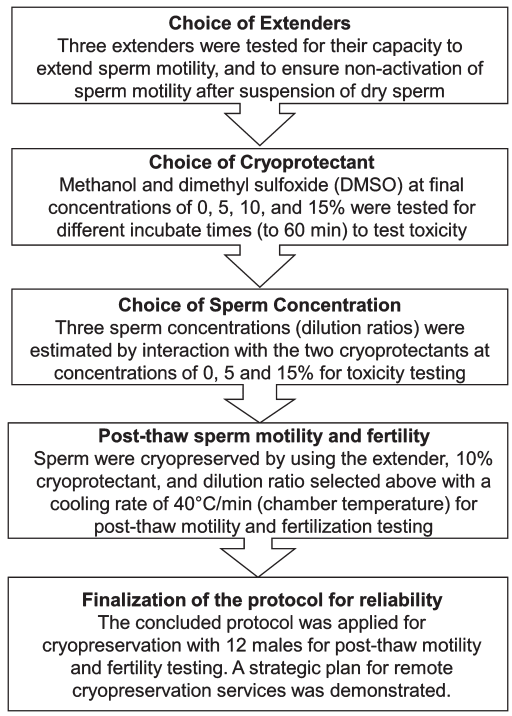

FIGURE 1. *Workflow of experimental designation in this study for protocol development of commercial-scale sperm cryopreservation in Atlantic salmon,* Salmo salar.

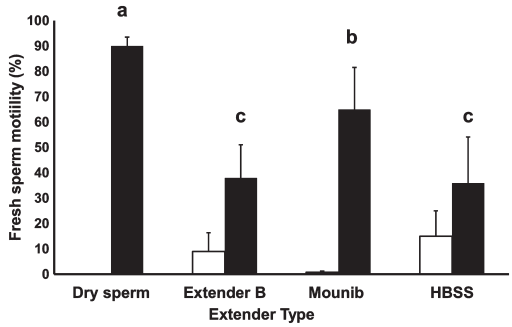

FIGURE 2. *Motility of fresh sperm of Atlantic salmon,* Salmo salar *(mean ± SD,* n = *5), diluted with Extender B, Mounib solution, and HBSS320 (Hanks' balanced salt solution 320) before (empty bars) and after activation (filled bars) by salmon Ringer's solution. Shared letters represent no significant difference in sperm motility after activation.*

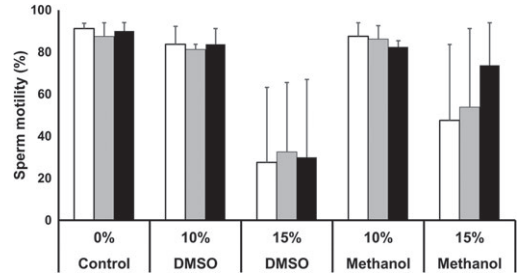

FIGURE 3. *Motility evaluation of shipped (pre-freeze) sperm of Atlantic salmon,* Salmo salar*, after incubating with dimethyl sulfoxide and methanol (10 and 15%) in three extenders: Extender B (white), Mounib solution (gray), and HBSS320 (Hanks' balanced salt solution 320) (black).*

bars, Fig. 2). The lowest level of sperm preactivation (1 ± 0%) occurred in Mounib solution, which was not different from that of fresh dry sperm (0%, $P = 0.680$), whereas the levels of sperm preactivation in Extender B (9 ± 7%) and HBSS320 (15 ± 10%) were significantly higher than those in fresh dry sperm ($P \leq 0.009$). After being activated with salmon Ringer's solution, sperm diluted in the three buffers showed significantly lower motility than that of fresh sperm ($P \leq 0.025$; Fig. 2). However, among the three extenders, Mounib solution showed the least reduction in fresh sperm motility compared with Extender B and HBSS320 ($P \leq 0.040$).

In the second trial, sperm suspended in the three extenders did not show preactivation motility, and after refrigerated storage for 24 h, the motilities after activation were 53 ± 4 (Extender B), 50 ± 14 (Mounib), and 33 ± 4% (HBSS320), and no differences were found ($P > 0.194$).

Evaluation of Cryoprotectant Toxicity

In the first trial, shipped sperm motility was affected significantly by cryoprotectant ($P = 0.027$) and cryoprotectant concentration ($P < 0.001$; Fig. 3), but was not affected by the extender type ($P = 0.782$) and the interaction of extender and cryoprotectant ($P = 0.884$; Fig. 3). Among the different cryoprotectant concentrations, 10% methanol or DMSO did not cause significant motility decrease compared with control group (without cryoprotectants) within the duration time tested (30 min) ($P \geq 0.904$), but 15% methanol and DMSO did affect the sperm motility significantly compared with control group ($P < 0.002$) and 10% methanol or DMSO ($P < 0.043$; Fig. 3).

The second trial evaluated the toxicity of methanol at concentrations of 5, 10, and 15% on shipped sperm diluted in Mounib solution at three dilutions (e.g., three cell concentrations) for as long as 2 h (Fig. 4). Overall, analysis showed that methanol concentration ($P < 0.0001$) and sperm dilution ($P < 0.0001$) significantly affected sperm motility after exposure to methanol, but no interactions were identified ($P = 0.299$). Analysis of exposure time showed that it as a significant factor ($P < 0.0001$) affecting sperm motility, and significantly interacted with methanol concentration ($P = 0.001$), but there was no interaction with sperm dilution ($P = 0.139$). For sperm samples at a 1:1 dilution, motility in 5 and 10% methanol did not show significant changes during the exposure time (compared with the control) ($P \geq 0.072$), but sperm motility in 15% methanol showed a significant decrease after 50 min of exposure ($P \leq 0.029$). For sperm samples at a 1:3 dilution, motility in 5 and 10% methanol did not show significant changes during the exposure (compared with the control) ($P \geq 0.114$), but

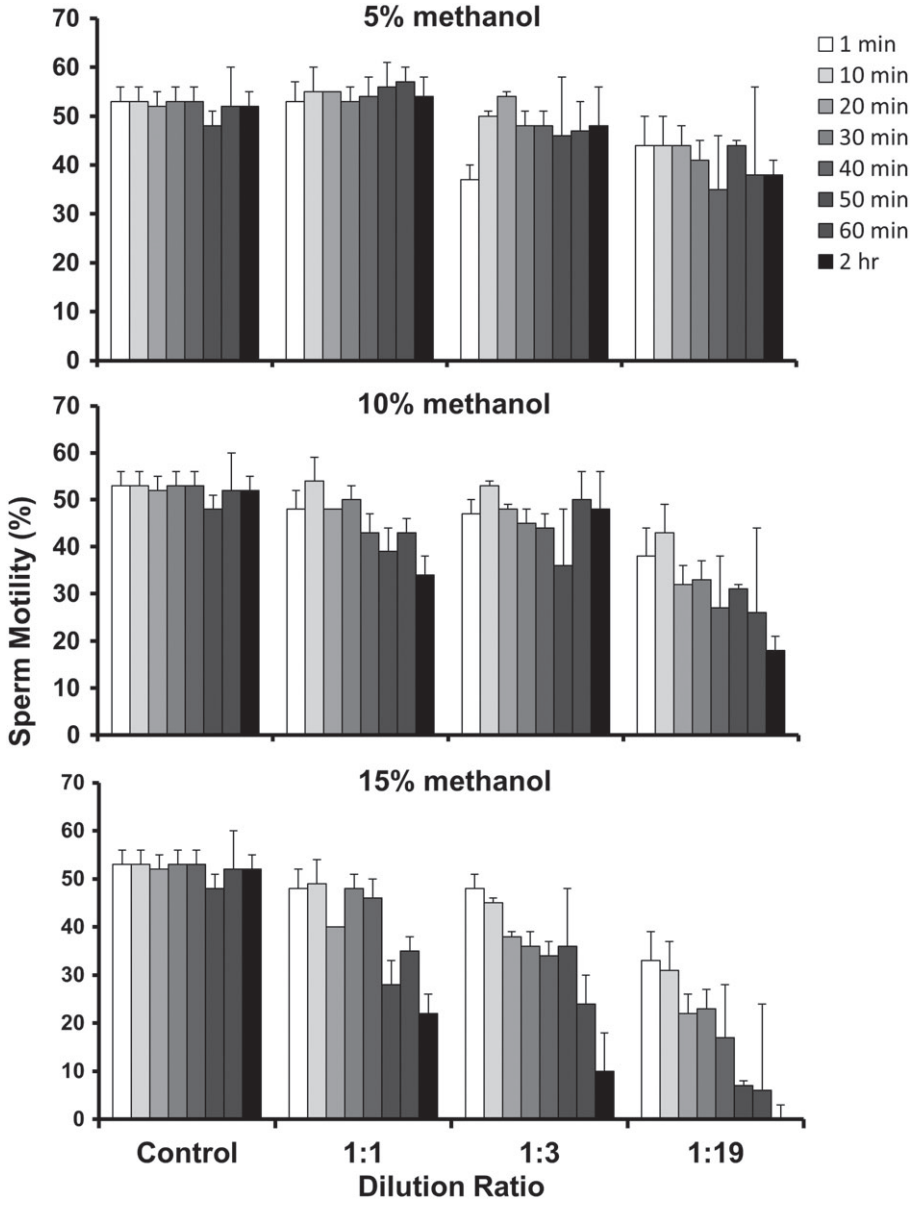

FIGURE 4. *Motility of shipped (pre-freeze) sperm of Atlantic salmon,* Salmo salar*, over 120 min (mean ± SD) after mixing with methanol at final concentrations of 5, 10, and 15%. Sperm was extended in Mounib solution at ratios of 1:1, 1:3, or 1:19 yielding sperm concentrations of* 1×10^9, 3×10^8, *and* 5×10^7 *cells/mL.*

motility in 15% methanol decreased significantly after 30 min of exposure ($P \leq 0.004$). For sperm samples at a 1:19 dilution, motility in 5% methanol did not show significant changes during exposure ($P \geq 0.107$), but motility in 10% methanol significantly decreased after 2 h ($P = 0.020$), and motility in 15% methanol decreased significantly immediately after exposure ($P \leq 0.001$).

Evaluation of Post-thaw Motility and Fertility

In the first cryopreservation trial, equilibration motility after incubation with 10% methanol

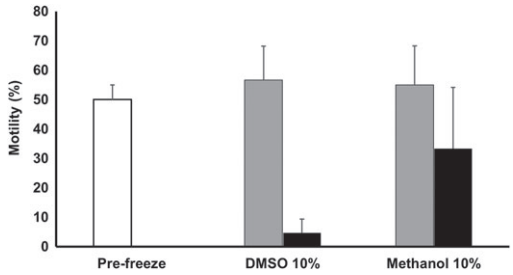

FIGURE 5. *Shipped (pre-freeze) sperm motility (white bar), equilibration motility (gray bars: after 60 min exposure to cryoprotectant at room temperature), and post-thaw motility (black bar) of Atlantic salmon,* Salmo salar *(n = 3). Sperm samples were extended in Mounib solution and cryopreserved with 10% dimethyl sulfoxide or 10% methanol.*

or DMSO for 60 min was not different from pre-freeze motility (Fig. 5). Post-thaw motility in the 10% methanol group ($33 \pm 21\%$) was higher than that in the 10% DMSO group ($5 \pm 5\%$) ($P = 0.047$).

On average, post-thaw sperm from three males in 10% DMSO resulted in fertilization rates of 0–1% (Table 1), and post-thaw sperm in 10% methanol yielded fertilization rates from 32 to 70% (Table 1), which were significantly higher than those in 10% DMSO ($P \leq 0.017$). In control groups (oocyte quality test), fertilization of the same oocytes with fresh sperm was $95 \pm 3\%$, ranging from 93 to 95% (Table 1). In addition, fertilization performed by doubling the amount of post-thaw sperm in 10% methanol (i.e., 4×10^5 cells/oocyte) from two males showed higher fertilization rates (significant for Male 1, $P = 0.021$, but not significant for Male 3, $P = 0.256$) (Table 1), which were comparable with that of control groups ($75 \pm 10\%$, $P \geq 0.224$).

In the second trial, after incubating with 10 or 15% methanol, equilibration motility of sperm samples diluted at ratios of 1:19, 1:3, or 1:1 in Mounib solution was significantly affected by methanol concentration, sperm dilution, and their interaction (Fig. 6). Equilibration motility in 10% methanol with sperm dilution ratios of 1:1 and 1:3 was significantly higher than that in 10% methanol with a dilution of 1:19 and that in 15% methanol regardless of dilution ratios ($P \leq 0.007$). However, the post-thaw motility after cryopreservation was not significantly affected by methanol concentration ($P = 0.058$), dilution ratio ($P = 0.686$), or their interaction ($P = 0.922$), although the post-thaw motility in 10% methanol ($16 \pm 21\%$ for 1:1 dilution, $20 \pm 18\%$ for 1:3 dilution, and $18 \pm 14\%$ for 1:19 dilution) was higher than that in 15% methanol ($1 \pm 1\%$ for 1:1 dilution, $12 \pm 21\%$ for 1:3 dilution, and $9 \pm 16\%$ for 1:19 dilution). Compared with pre-freeze

TABLE 1. *Fertilization by post-thaw sperm from three males cryopreserved with DMSO (10%) or methanol (10%) at a cooling rate of 40 C/min.*

			Fertilization rate (%)				
Male	Cryoprotectant	Sperm : oocyte ratio	Female 1	Female 2	Female 3	Mean	SD
Fresh sperm			93	94	99	95	3
Male 1	DMSO 10%	2×10^5 sperm per oocyte	1	2	0	1	1
Male 2			0	0	0	0	0
Male 3			0	0	0	0	0
Male 1	Methanol 10%		32	41	42	38	6
Male 2			47	49	70	55	13
Male 3			43	61	61	55	10
			Female 4	Female 5	Female 6	Mean	SD
Fresh sperm		4×10^5 sperm per oocyte	64	80	82	75	10
Male 1	Methanol 10%		58	79	66	68	11
Male 3			96	85	59	80	19

DMSO = dimethyl sulfoxide.

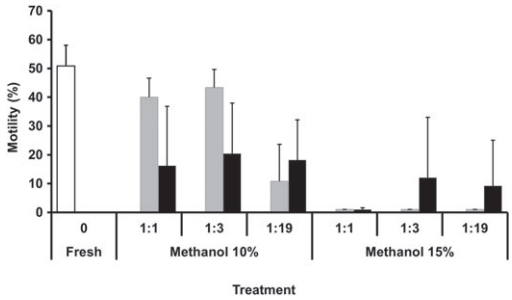

FIGURE 6. *Motility of shipped (pre-freeze) sperm (white bar), after 15 min equilibration (gray bars), and after thawing (black bars) for sperm cryopreserved with 10 and 15% methanol after diluted in Mounib solution at 1:1, 1:3, and 1:19 ratios in Atlantic salmon,* Salmo salar *(*n = *3).*

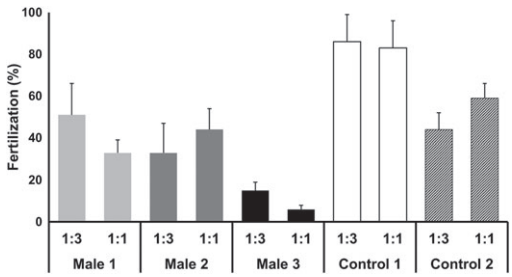

FIGURE 7. *Fertilization rates of post-thaw sperm from three male Atlantic salmon with a pool of oocytes from three females (mean* ± *SD,* n = *3). Sperm was cryopreserved with Mounib solution and 10% methanol at a sperm dilution ratio of 1:1 (*1×10^9 *cells/mL) and 1:3 (*3×10^8 *cells/mL). Fertilization in the control groups was made by using fresh sperm to fertilize oocytes from the same batches used for post-thaw sperm.*

motility, equilibration motility decreased significantly in treated groups ($P < 0.046$) except in 10% methanol with dilutions of 1:1 and 1:3 ($P \geq 0.721$), and post-thaw motility in all treatment groups were significantly reduced ($P < 0.0001$).

In vitro fertilization of thawed sperm in 10% methanol had fertilization rates of 33 ± 18% at sperm dilution ratios of 1:1 and 28 ± 20% at dilution ratio of 1:3 (Fig. 7). Meanwhile, between the two sperm dilution ratios, no differences were found in the fertilization rates in control groups ($P = 0.431$) or the post-thaw sperm ($P = 0.644$).

In the third trial, the equilibration motility decreased to 25 ± 13% ($P = 0.005$), ranging from 1 to 50% for sperm samples from 12

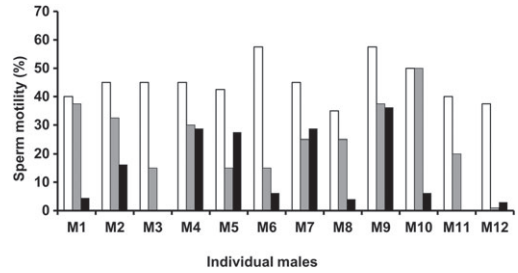

FIGURE 8. *Motility of fresh sperm (white bars), after 15 min equilibration (gray bars), and post-thaw sperm (black bars) from 12 individual males of Atlantic salmon,* Salmo salar. *Sperm samples were diluted in Mounib solution and cryopreserved with 10% methanol at a cooling rate of 40 C/min from 4 to −80 C.*

individual males at the same sperm concentration (2×10^8 cells/mL) with an average fresh motility of 45 ± 7% (ranging from 35 to 60%) after incubating with 10% methanol for 30 min (Fig. 8). After cryopreservation, post-thaw motility averaged 13 ± 14%, ranging from 0 to 36%, which varied significantly ($P < 0.0001$) from male to male compared with fresh motility and equilibration motility.

Discussion

In aquaculture, genetic improvement and maintenance of commercially important strains mostly rely on husbandry of live animals. Therefore, gamete cryopreservation is in high demand because it can preserve germplasm in perpetuity for in-season and out-of-season use many years beyond the lifespan of broodstock and can also save time, space, and labor while reducing risks for maintenance of live animals. So far, commercial-scale sperm processing and cryopreservation have been studied in blue catfish used for producing hybrids through crossing with channel catfish (Hu et al. 2011), and an automated system was used for straw filling, sealing, and labeling in a high-CBS straws originally designed and manufactured for mammals. Automated processing systems such as this provide the advantages of rapid sample handling assuring sperm quality, commercial-scale processing, minimizing sample variability, and improving process standardization and quality control. In the current

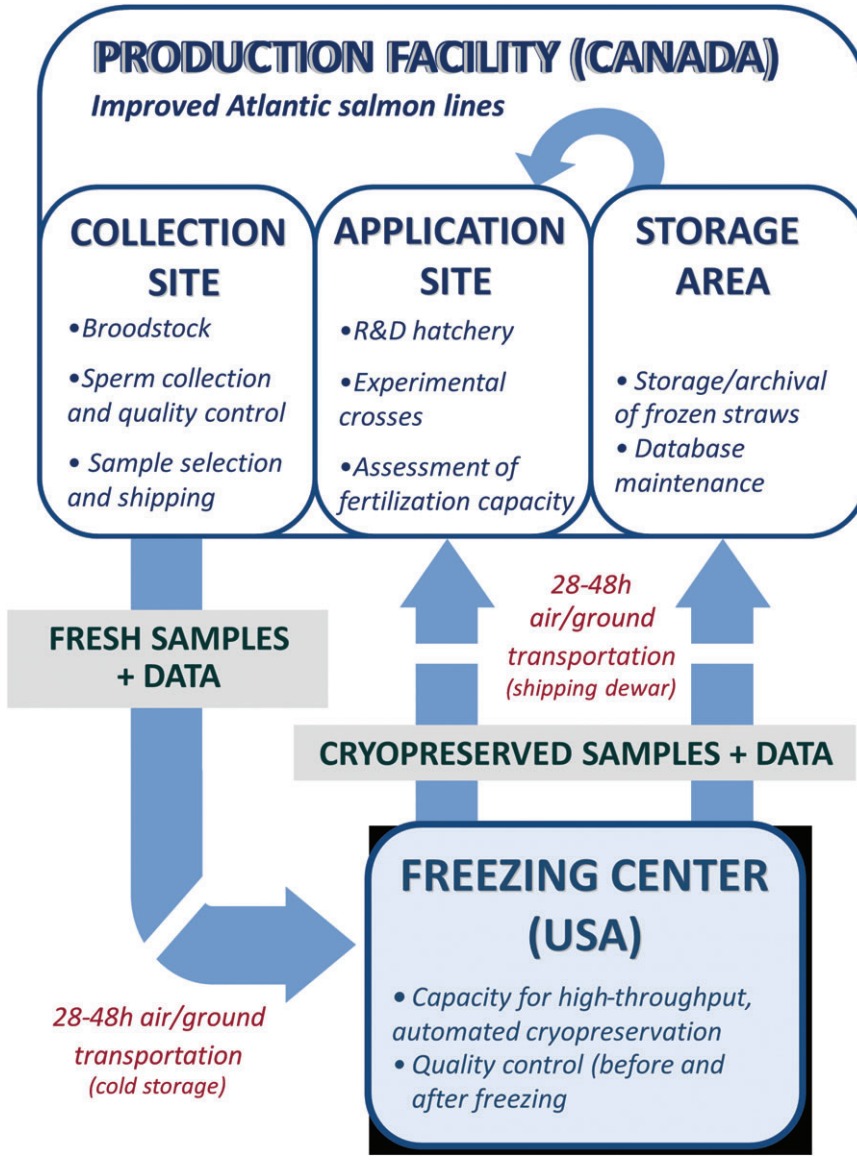

FIGURE 9. *Overview of the strategy for high-throughput cryopreservation of sperm from Atlantic salmon between two distant locations.*

study, the same automated system was used to process sperm samples from Atlantic salmon for commercial-scale cryopreservation through remote services by overnight sample shipping, and a reliable cryopreservation protocol was developed by evaluation of the basic factors in the collection, handling, shipping (round trip), processing, freezing, storage, and post-thaw processes (Fig. 9).

Remote Service for Large-scale Commercial Cryopreservation

Aquaculture farms and hatcheries are often located in remote locations and lack capabilities for handing sperm samples for cryopreservation. Cold storage of sperm for as long as 48 h with oxygen enrichment provides a solution for overnight shipment of samples to service centers equipped with necessary facilities for processing

and freezing. The current research demonstrated these concepts by shipping of sperm samples from Canada to the southern USA (3550 km); processing shipped samples with an automated system for filling, sealing, and labeling; freezing the packaged samples; and shipping frozen samples back to Canada for fertilization (Fig. 9). Refrigerated storage of sperm samples has been investigated in other fish species such as Atlantic Cod (Babiak et al. 2012) for artificial fertilization or other hatchery operations. Although shipping places additional demands on the sample and can reduce quality, these effects are offset by enabling access to automated procedures which provide high-throughput processing of samples in a commercial setting.

Choice of Extender for Refrigerated Storage and Cryoprotectant for Cryopreservation

For Atlantic salmon, glucose, sucrose, and egg yolk with or without addition of BSA have been used as extenders for sperm cryopreservation without systematic evaluation of their function (Jodun et al. 2007; Dziewulska et al. 2011; Effer et al. 2013; Figueroa et al. 2015, 2016). In this study, three different buffers previously used as fish sperm extenders were tested for refrigerated storage and cryopreservation in Atlantic salmon. Differences were found among the three extenders for sperm motility activation and after 24 h of refrigerated storage during shipping. Mounib solution was identified as a suitable extender for Atlantic salmon sperm under these conditions and was used for cryopreservation in this study.

As for cryoprotectant, the commonly used DMSO and methanol at final concentrations of 5, 10, and 15% were tested in different trials for their effects on shipped (pre-freeze) sperm motility, and post-thaw motility and fertility. Overall, 15% concentrations of DMSO and methanol were found to significantly reduce the motility of sperm compared with that after exposure to 5 and 10% DMSO or methanol. Post-thaw motility and fertility tests indicated that methanol functioned better than DMSO. The same results were reported elsewhere for sperm cryopreservation in Atlantic salmon (Jodun et al. 2007; Dziewulska et al. 2011). Thus, 10% methanol was used as the cryoprotectant of choice in this study. However, choice of cryoprotectants can interact with cooling rates, container type, and container size; therefore, DMSO has also been used as cryoprotectant for Atlantic salmon (Figueroa et al. 2015, 2016).

Male-to-Male Variation in Post-thaw Viability and Fertility

Male-to-male variability in post-thaw motility and fertilization has been reported as a common phenomenon for most fish species such as channel catfish (Christensen and Tiersch 2005); zebrafish (Yang et al. 2007); and the platyfish, *Xiphophorus couchianus* (Yang et al. 2009). In the current study, although no significant male variation was detected in the fresh sperm motility in the 12 males tested, post-thaw motility was significantly different. In fact, identification of sperm parameters that are related to post-thawing viability and fertility is always an important topic for cryopreservation in any species. Sperm velocity and beat frequency estimated using computer-assisted sperm analysis have been used in several species (Yang and Tiersch 2011), and in Atlantic salmon sperm curved- and straight-line velocity showed correlation with post-thaw sperm motility and fertility (Figueroa et al. 2016). In addition, other cellular-level analyses, such as plasma membrane integrity, mitochondria membrane integrity, and acrosome integrity assessed by flow cytometry (Daly and Tiersch 2011), and molecular level assays such as DNA integrity (Gandini et al. 2006) and DNA markers (Payan-Carreira et al. 2013), were also investigated for correlation analysis with post-thaw sperm viability.

Importantly, post-thaw fertility testing involves not only sperm quality, but also sperm-to-egg ratios, the total mixture volume of oocytes and sperm, and oocyte quality. Therefore, additional standardized fertilization assays are needed. In this study, post-thaw fertilization was performed from several post-thaw samples of the 12 males, and the oocyte quality for

fertilization tests was examined by using fresh sperm.

Improvements and Future Considerations for Sperm Cryopreservation of Atlantic Salmon

To apply high-throughput sperm cryopreservation for commercial-scale industry use, quality control is essential. Although fertility is evidently the most important criteria for evaluation of sperm quality, this characteristic solely belongs to the end products. Thus, it is not practical to only apply this single criterion especially during multistep procedures such as sperm cryopreservation. Currently, motility is a direct and convenient index to evaluate quality and viability of fresh and cryopreserved sperm (Hu and Tiersch 2011), and analyses of sperm cell characteristics (membrane integrity and mitochondria integrity) by flow cytometry provides another approach for evaluation of quality (Ogier De Baulny et al. 1997). More approaches are needed, especially rapid and objective flow cytometry analyses. In addition, effective quality control tools could identify and prescreen poor-quality samples to yield consistent high-quality products for markets (Torres et al. 2016).

Standardization of procedures is another important factor for applying cryopreservation for industry use and also an important aspect for quality control. Cryopreservation involves a series of interconnected steps, and standardization (quantification and qualification) of procedures at each step can ensure consistent and predictable products. For example, sperm concentration in the current study affected sperm viability during storage and cryopreservation and thus should be taken into account throughout the quality control process. A second example is the sperm number used in artificial fertilization for fertility testing. The results in this study indicated that by increasing the sperm-to-egg ratio, the fertilization rates for thawed sperm were increased significantly. Therefore, quantification and standardization of procedures is needed to improve application and achieve optimal efficiency.

Currently, Atlantic salmon aquaculture includes transgenic AquAdvantage salmon (www.fda.gov), polyploid salmon (Buchanan and Barbosa-Solomieu 2011), and different strains and/or lines from selective breeding programs. Therefore, it would be useful to develop sperm cryopreservation protocols for tetraploid and transgenic fish. However, owing to differences in cell size, chromosome number, and genetic backgrounds, sperm physiology and performance could be different compared with sperm from normal diploids, such as was observed for tetraploid Pacific oysters, *Crassostrea gigas* (Dong et al. 2005), indicating differential responses of sperm from tetraploids to cryopreservation with that from diploids.

Technically, further improvements for the protocol include: (1) increasing the cell concentration within the straws to enhance fertilization capacity on a per-unit basis, (2) establishment of optimal quality control and procedure standardization for consistent and reliable products, (3) development of cryopreservation for sperm from tetraploid males, and (4) cryopreservation of sperm from neomales (i.e., fish that are genetically female but produce functional sperm cells) and transgenic males.

Conclusions

Salmon hatcheries and sperm cryopreservation facilities are often geographically separated, presenting a hurdle for efficient commercial-scale gamete cryopreservation for aquaculture production and breeding programs. In the current research, a cryopreservation protocol was developed for Atlantic salmon by shipping samples from a remote location and processing using automated equipment for high-throughput sample filling, sealing and labeling. This research provides a foundation for commercial-scale cryopreservation through use of remote service facilities. “Donor” facilities can provide sperm collection by stripping, initial quality assessment, and shipping of oxygen-enhanced dry (nonextended) sperm under cold storage to the freezing facility. Upon arrival within 24–48 h, samples can be processed for concentration determination, standardized to a specific

cell concentration (e.g., 2×10^8 cells/mL) with Mounib solution (315 mmol/kg), and mixed with an equal volume of 20% methanol in Mounib solution (yielding a final concentration of 10% methanol at a sperm concentration of 1×10^8 cells/mL). During 30 min of equilibration, sperm samples can be packaged in 0.5-mL high-CBS straws by use of an automated system to fill, seal, and label the straws. At 30 min, straws can be cooled in a programmable freezer at 40 C/min (chamber temperature) from 4 to −80 C. The frozen straws can be retrieved by hand, plunged into liquid nitrogen, and sorted into multicompartment goblets designed for storage in liquid nitrogen. At this point, the freezing facility could store the frozen samples or ship them back to the donor site by air or ground transportation in vapor-phase shipping dewars. For artificial fertilization at the production site, frozen samples can be thawed at 40 C for 12 sec in a water bath, mixed with freshly collected eggs at a specific sperm:egg ratio (e.g., 4×10^5 cells/oocyte), activated by adding a set volume of salmon Ringer's solution and adding a set volume of fish water for embryo development. Fertilization rates can be determined by counting the dividing embryos among the total oocytes used for fertilization. A quality assurance program can be instituted by applying quality control checkpoints at relevant steps within the whole process (Torres et al. 2016).

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