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[Communication]

Cryopreservation and Methanol Effects on Burbot Sperm Motility and Egg Fertilization

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Abstract.—Cryopreservation of semen from North American burbot *Lota lota maculosa* was investigated and optimal methanol concentrations were determined for a conservation breeding program. Methods were modified from those reported for Eurasian burbot *L. lota lota*. The permeable cryoprotectant (methanol) concentration in the semen extender was varied to provide final methanol concentrations of 5, 10, and 20%. Semen motility was evaluated at 80 and 363 d postfreeze (dpf). Fertilization was determined at 340 and 367 dpf. Methanol concentration in the extender significantly ($P < 0.05$) affected sperm motility and egg fertilization percentages. Motility and fertilization were lowest when 5% methanol was used. Motility of semen at 80 dpf was not significantly different between 10% and 20% methanol, but semen at 363 dpf had significantly higher motility when stored in 20% methanol than in 10% methanol. Egg fertilization was highest when semen was stored in extenders containing 10% or 20% methanol. Results suggest that good motility and fertilization can be achieved by cryopreserving burbot semen with 10% or 20% methanol in the extender instead of 5% methanol. This study demonstrates the potential to utilize cryopreserved burbot semen in the development of germplasm repositories for imperiled fish stocks.

The burbot *Lota lota* is the only freshwater member of the cod family Gadidae (Lahnsteiner et al. 1994; McPhail and Paragamian 2000; Van Houdt et al. 2003), and there are only two genetically distinct subspecies (Van Houdt et al. 2003). Localized populations of both the North American subspecies (*L. lota maculosa*) and European subspecies (*L. lota lota*) are in peril because of anthropogenic influences (Paragamian 2000; Paragamian et al. 2000; Lahnsteiner et al. 2002, 2004; Van Houdt et al. 2003). This study stems from efforts to develop a recovery strategy for a remnant population of burbot within the Kootenai River drainage, a Columbia River headwater located in northern Idaho and southeastern British Columbia. This metapopulation formerly supported commercial, recreational, and sustenance harvests before habitat alterations and hydroelectric dam operations (Paragamian 2000). Natural recruitment has not been documented over the past decade, and this metapopulation is therefore considered to be near extirpation in Idaho (Paragamian 2000). Recovery strategies are being developed to revitalize local burbot stocks and include conservation breeding program development. As part of the program, semen cryopreservation was investigated should germplasm repositories become necessary to preserve genetic diversity.

Cryopreservation of fish semen is commonly used to enhance breeding programs (Suquet et al. 2000; Chao and Liao 2001; Billard et al. 2004; Urbányi et al. 2006). Positive aspects of semen cryopreservation include the capability to synchronize artificial inseminations, transport ease, storage ease, reduction in the need to maintain large numbers of captive adults, and potential economic benefits given a reliable gamete

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TABLE 1.—Composition of semen-freezing solutions (per 100 mL).

Agent	Methanol concentration (%)		
	5	10	20
Methanol (mL)	5	10	20
SMIS (mL)[a]	88	83	73
Egg yolk (mL)	7	7	7
Glucose (g)	1.5	1.5	1.5

[a] Sperm motility-inhibiting solution; pH, 7.8, relative mmol/L concentrations per component: 100 NaCl, 0.02 KCl, 1.00 $CaCl_2$, 1.00 $MgSO_4$, and 20.0 hepes.

supply (Lahnsteiner et al. 1997, 2002; Lahnsteiner 2000; Suquet et al. 2000; Chao and Liao 2001; Billard et al. 2004; Rideout et al. 2004). However, cryopreservation success varies by species, handling procedures, and the formulation of cryoprotectants (extender) used in freezing solutions (Lahnsteiner et al. 1994, 1996; Piironen 1994; Chao and Liao 2001; Glogowski et al. 2002). The objective of this study was to evaluate sperm motility and fertilization through use of different concentrations of a permeable cryoprotectant (methanol). Thus, three methanol concentrations (5, 10, and 20%) in the extender were evaluated based on postthaw motility and fertilization up to 1 year postfreezing.

Methods

Chemicals and solutions.—All solutions excluding egg yolk were prepared 24 h before use and stored at 4°C. Egg yolks were not centrifuged. All chemicals used were available commercially (Sigma-Aldrich Co., St. Louis, Missouri). A sperm motility-inhibiting solution (Table 1) was used in the semen-freezing solution. The final semen-freezing solutions contained 1.5% glucose and 7.0% hen egg yolk, which was added to the extender just before mixing with semen (Lahnsteiner et al. 2002).

Adult handling.—Adult burbot were captured from Duncan Reservoir, British Columbia, with baited cod traps (Neufeld and Spence 2004). After capture, adults were transported to the Kootenai Tribal Fish Hatchery (Bonners Ferry, Idaho) and were acclimated to captivity for approximately 2 months in a 5,400-L tank that received filtered Kootenai River water. Adults were then transported to the Aquaculture Research Institute (University of Idaho, Moscow) and maintained in 1,200-L tanks in a closed recirculation system. Average stocking density was 10 kg/m^3, and fish ranged in size from 65 to 71 cm in total length and from 1.0 to 2.1 kg in weight. During these experiments, adults were exposed to an electronically regulated photoperiod and temperature regime. All adults were held at 4°C during the experimental periods. Adults were fed live prey ad libitum (rainbow trout *Oncorhynchus mykiss*: 5–50-g average body weight). Burbot were held captive for approximately 18 months before the study was initiated.

All semen and ova were collected from adults anesthetized with tricaine methanesulfonate (MS-222; Argent Chemical Laboratories, Inc., Redmond, Washington) at 0.51 mmol of MS-222/L of water. The gametes were collected in 500-mL, dry plastic bags, and care was taken to prevent mucus or urine contamination. The bags were then sealed to allow maximum air space and were stored at 4°C immediately after collection. Thereafter, adult fish were returned to their respective rearing tanks for recovery. Seven males were used to establish two separate sets of semen samples on 6 and 11 April 2005.

Within 1 h of semen collections, subjective motility observations were made to ensure that samples were viable and uncontaminated. As described by Liley et al. (2002) for rainbow trout, samples with less than 80% motility or those found to be contaminated were excluded from the experiments. Acceptable samples were cryopreserved by use of methods similar to those outlined by Lahnsteiner et al. (2002). Semen was diluted with extender (1:5) and chilled for 3 min at 2°C before being loaded into 0.5-mL French storage straws (IMV International Corp., Maple Grove, Minnesota). Loaded straws were then placed in goblets on canes (5 straws/goblet, 2 goblets/cane; IMV International). Samples were frozen by suspending them 1 cm above liquid nitrogen for 10 min before submersion of canes into liquid nitrogen for storage.

Experiment 1: sperm motility.—Seven males were used to evaluate postthaw motility at 80 and 363 d postfreeze (dpf) with two 0.5-mL straws per male in each of the three extenders. Straws were thawed for 20 s in a thermostat-regulated water bath (Thelco Corporation, Englewood, Colorado; Model 82) maintained at 25°C. Immediately after thawing, straws were drained into 1.5-mL containers, and sperm motility was assessed postdilution (1:100) with sperm-activating solution (NaCl = 25 mmol/L; Tris = 20 mmol/L; pH = 8.5) using the methods of Zuccarelli et al. (2007). The percentage of motile sperm in the sample was assessed based on two sequential images captured 1 s apart using Motic Images software (Motic Instruments, Inc., Richmond, British Columbia) in conjunction with a digital microscope (National Optical and Scientific Instruments, Inc., San Antonio, Texas; Model DC3–163). Sperm were considered motile if overlain sequential images showed cell position changes; based on this method, vibrating sperm were not counted as

motile if they did not change position. All motility measurements were performed at 20°C.

Experiment 2: fertilization.—To assess postthaw fertilization, three males were randomly selected at 340 and 367 dpf (trials 1 and 2, respectively). Trials 1 and 2 varied based on thaw times of 20 or 25 s at 25°C and the use of different females. Duplicate samples from each male in each of the three extenders were thawed and applied to duplicate groups of ova. Fresh semen (1 mL) from the same males was also applied to duplicate groups of ova.

For fertilization trials, female burbot were anesthetized and injected with one gonadotropin-releasing hormone analog pellet (GnRHa; GnRH-D-Arg^{6}-Pro^{9}-Net) in the dorsal white muscle (approximately 1 cm laterally between the two dorsal fins) with a 25-mL syringe equipped with a fixed plunger rod and an 8-gauge needle. The pellets (Ovaplant; Syndel Laboratories, Ltd., Vancouver, British Columbia) are designed for advanced maturation and synchronization of salmonids and contained 75 μg of GnRHa in an inert biodegradable vehicle. Implanting of pellets occurred 22 d before ovulation for females used in fertilization trial 1 and 38 d before ovulation for females used in trial 2. Ova (with ovarian fluid) were stored at 4°C immediately after collection for periods of up to 2 h and then were kept on ice during experimental procedures, which took nearly 1 h to complete. For fertilization trials, 2 mL of ova with ovarian fluid were measured into the incubators. Simultaneously, the required semen sample was thawed and added directly to the premeasured ova. After combining thawed semen and ova, 48 mL of activating solution was immediately added. Gamete mixtures were gently stirred for 10 s postactivation as chilled water (3–5°C) was introduced into each incubator. Water inflow rates ranged from 40 to 50 mL/min. Each incubator remained uncovered for approximately 30 min while eggs were rinsed. Incubators were continuously monitored to ensure that eggs were not lost during rinsing. Each incubator was then covered with 500-μm-mesh screening until fertilization was evaluated at 48 h postactivation.

To assess fertilization, eggs were subsampled at 48 h postactivation and counted to determine the number of fertilized eggs (blastomere cleavage evident) and unfertilized eggs. Eggs were subsampled and volumetrically measured three times from each incubator with a 1-mL tuberculin syringe.

Statistics.—Statistical Analysis System software (Cary, North Carolina) was used to determine significant differences between treatments and time of storage for mean motility and fertilization. Due to sperm density and volume differences, fresh semen and cryopreserved samples were not analyzed statistically.

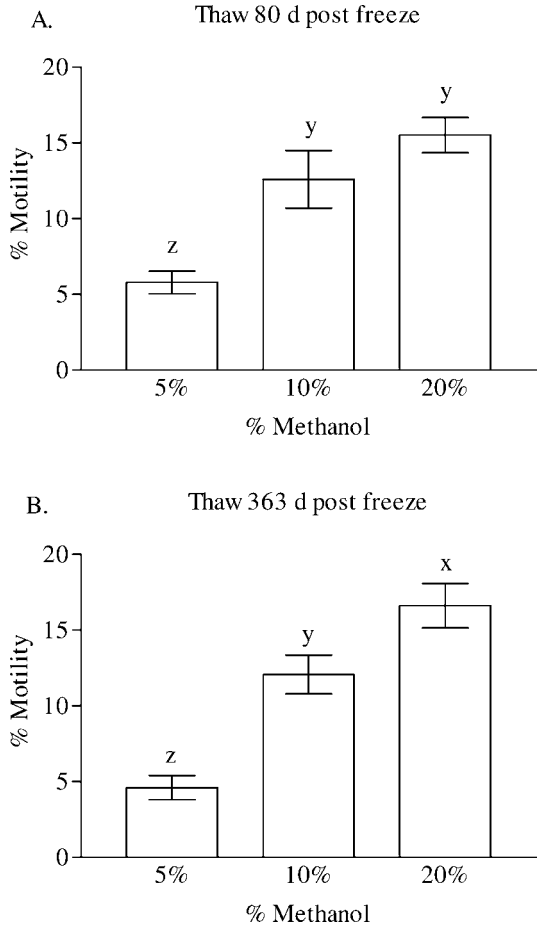

FIGURE 1.—Percent motility (mean ± SE) of Duncan Reservoir (British Columbia) burbot sperm cryopreserved in three extenders (5, 10, and 20% methanol) and stored for **(A)** 80 or **(B)** 363 d postfreeze. Duplicate samples from seven males were used in storage duration analysis. Different letters above bars indicate significant differences between extenders ($P < 0.05$).

All motility data were arcsine–square-root transformed (Snedecor and Cochran 1980) before one-way analysis of variance. Tukey's post hoc pairwise comparisons were used to determine significance between treatment means ($P \leq 0.05$).

Results

For motility evaluations, significant differences ($P \leq 0.05$) between males and the percent of motile sperm by extender were found (Figure 1). Motility and fertilization were lowest at 80 and 363 dpf when the 5% methanol extender was used. Motility of semen at 80 dpf was not significantly different between 10% and 20% methanol (Figure 1A), but 363-dpf semen stored

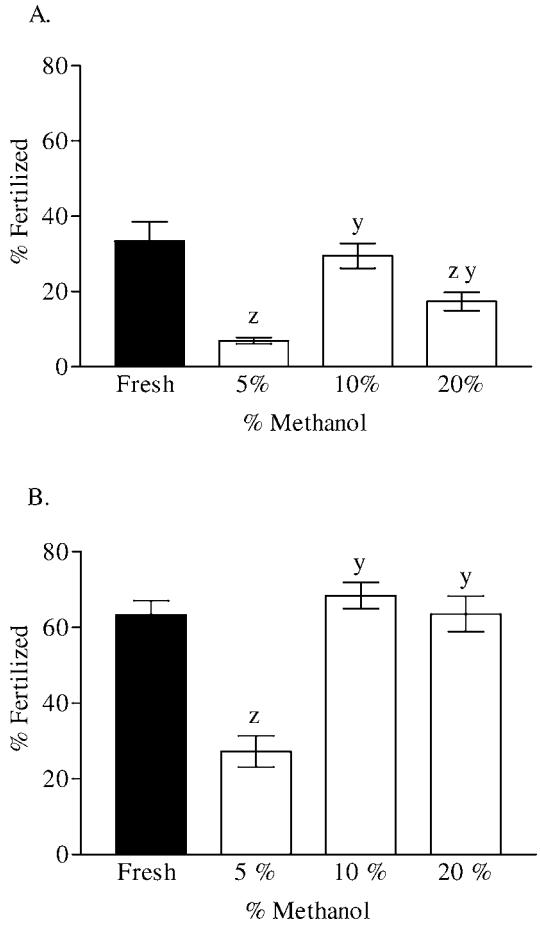

FIGURE 2.—Percent egg fertilization (mean ± SE) achieved by Duncan Reservoir (British Columbia) burbot sperm cryopreserved in three extenders (5, 10, and 20% methanol) during two trials: **(A)** trial 1 (thaw time = 25 s; 340 d postfreeze) and **(B)** trial 2 (thaw time = 20 s; 367 d postfreeze). Duplicate samples from three males per trial were used. Different letters above bars indicate significant differences between extenders ($P < 0.05$). Fertilization rates by fresh semen are included for comparison ($n = 18$).

in 20% methanol had significantly higher motility than any other treatment (Figure 1B).

The mean egg volume after water hardening was 4.5 mL/incubator. The mean number of eggs per milliliter was estimated at 1,362 eggs. In fertilization trial 1 (thaw time = 25 s; 340 dpf), means of 7, 30, and 17% fertilization were achieved with 5, 10, and 20% methanol extenders, respectively. Mean fertilization by fresh semen was 33%. For trial 2 (thaw time = 20 s; 367 dpf), mean fertilization was 27, 69, and 64% with 5, 10, and 20% methanol extenders, respectively (Figure 2). Mean fertilization by fresh semen in trial 2 was 63%. In both trials, 10% or 20% methanol significantly improved fertilization relative to that of 5% methanol. For both trials, fertilization was not significantly different between 10% and 20% methanol (Figure 2).

Discussion

This study demonstrates that cryopreservation of burbot semen is feasible for establishing germplasm repositories for locally imperiled burbot stocks. These findings also support prior studies of burbot and other fish in which optimal postthaw sperm motility and fertilization were achieved using methanol. Salmonid semen has been cryopreserved more effectively with methanol as the permeable cryoprotectant than with dimethyl sulfoxide (DMSO), glycerol, a DMSO–glycerol mix, 1,2-propanediol, or N,N-dimethyl acetamide (Lahnsteiner et al. 1996, 1997, 2002; Lahnsteiner 2000). Similar studies of ictalurid, silurid, and acipenserid semen have also shown improved postthaw motility or fertilization by use of methanol (Tiersch et al. 1994; Horváth and Urbányi 2000; Glogowski et al. 2002). Despite the proven effectiveness of methanol as a permeable cryoprotectant, the optimal concentration (the highest possible concentration without toxicity) for use with burbot semen is yet to be determined. Future research should investigate methanol toxicity to burbot semen, because the present study did not test methanol concentrations above 20%. The results of this study may indicate that 20% methanol is yet approaching the threshold concentration for toxic effects on burbot sperm or ova during fertilization. However, this remains unclear since higher methanol concentrations were not tested.

This study demonstrates that handling techniques and the extender recipe developed by Lahnsteiner et al. (2002) can be successfully applied to burbot semen. This study also expands on preexisting knowledge by demonstrating sperm motility and fertilization success when extender methanol concentration is 10% or 20% instead of 5%. Future applications would also probably improve cryoprotective success by collecting gametes earlier in the spawning season (Chao and Liao 2001; Rideout et al. 2004); in Atlantic cod *Gadus morhua*, a marine relative of burbot, late-season gamete collection can result in decreased gamete viability, thereby decreasing fertilization (Rideout et al. 2004). Nevertheless, our study demonstrates that burbot sperm can be effectively cryopreserved using established methods, and our findings will facilitate future research. To our knowledge, this is the first effort to cryopreserve burbot semen and will be valuable for establishing germplasm repositories for locally endangered burbot populations.

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