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[Article]

Methanol and Egg Yolk as Cryoprotectants for Atlantic Salmon Spermatozoa

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Abstract.—The effects of four extenders on the fertilization rates of eggs fertilized with cryopreserved sperm of Atlantic salmon *Salmo salar* were tested. We used (1) glucose extender (54.04 g glucose/L and 1.7 g KCl/L) with 5% DMSO, (2) glucose extender with 5% DMSO supplemented with 13.3% egg yolk, (3) glucose extender with 10% methanol, and (4) glucose extender with 10% methanol supplemented with 13.3% egg yolk. Fertilization rates, expressed as the percentage of eyed embryos, ranged from 52.7% to 83.5%. Sperm cryopreserved with the glucose extender and 10% methanol supplemented with 13.3% egg yolk yielded significantly higher fertilization rates (83.5%) than did sperm cryopreserved with the other three extenders. Our fertilization rates compare favorably with those observed for eggs from the same year-class fertilized with fresh milt (81.4%) and reared at the White River National Fish Hatchery. The presence of egg yolk in extenders incorporating 10% methanol provided additional protection to salmonid sperm during the freezing and thawing processes and resulted in an increase in survival from 72.9% to 83.5%. However, the cryoprotective effect of egg yolk may be specific to the individual formulation of extenders. In our trials, glucose and 5% DMSO without egg yolk yielded a 66.9% fertilization rate, while glucose and 5% DMSO supplemented with 13.3% egg yolk produced only 52.7% fertilization after cryopreservation.

Despite decades of stocking, sea-run returns of Atlantic salmon *Salmo salar* continue to be at very low levels (U.S. Atlantic Salmon Assessment Committee 2002). In 2002, returns of endangered sea-run Atlantic salmon on the Pleasant, Dennys, and Narraguagus rivers in Maine numbered fewer than 10 fish. Connecticut and Merrimack River returns numbered less than 60 fish (U.S. Atlantic Salmon Committee 2002) and the returning female-to-male ratio on the Connecticut River was skewed approximately 2:1 (J. Rowan, U.S. Fish and Wildlife Service [USFWS], personal communication). The continued decline of Atlantic salmon populations across the entire northeastern United States and the sparsity of males in some river systems have led the USFWS to take a strong interest in the potential of cryopreservation as a tool to preserve fish germ plasm for extended periods and to form sperm banks or repositories at fish production facilities where a shortage of male fish exists.

Cryopreservation of salmonid sperm has been reported for several species (Scott and Baynes 1980; Scheerer and Thorgaard 1989; Cloud et al. 1990; Billard 1992; Lahnsteiner et al. 1996a, 1996b). However, the literature indicates that cryopreservation protocols contain critical factors, such as composition of the extender, equilibration time, and rates of freezing and thawing, that need to be optimized for individual species (Glogowski et al. 1999).

Several methods have been used with varying results to cryopreserve Atlantic salmon spermatozoa. Early attempts to freeze sperm in straws (Zell 1978) and in 1-mL ampoules (Hoyle and Idler 1968; Truscott et al. 1968) were unsuccessful. In contrast, a pellet technique in which ampoules were frozen produced high fertilization rates (80%) in Atlantic salmon (Mounib 1978). However, these results could not be duplicated and, in later studies, variable results were obtained with the pellet technique (Stoss and Refstie 1983). Preliminary trials (W. A. Jodun, unpublished) conducted at the Northeast Fishery Center, Lamar, Pennsylvania, tested the effect of various extenders on the cryopreservation of Atlantic salmon semen in straws. In those investigations, glucose extender (54.04 g glucose/L and 1.7 g KCl/L) with 5% DMSO yielded significantly higher fertilization rates (26.0%) than sperm cryopreserved with the other more complex extenders (J. W. Fletcher, USFWS, personal communication).

Methanol has been used for semen cryopreservation

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in sharptooth catfish *Clarias gariepinus* (Steyn and Van Vuren 1987), Mozambique tilapia *Oreochromis mossambicus* (Harvey 1983; Rana and McAndrew 1989), and barramundi *Lates calcarifer* (Leung 1987). Additionally, Lahnsteiner et al. (1997) demonstrated the suitability of extenders supplemented with 10% methanol to cryopreserve the semen of rainbow trout *Oncorhynchus mykiss*, lake trout *Salvelinus namaycush*, and Arctic char *S. alpinus*. However, extenders supplemented with methanol have yet to be tested on Atlantic salmon.

In this study, our objectives were to test the effect of glucose extender with or without egg yolk supplementation and containing either methanol or DMSO on the fertilizing ability of cryopreserved Atlantic salmon semen.

Methods

Source of milt and eggs.—In November 2003, 20 ripe males were anesthetized with tricaine methanesulfonate (MS-222). After anesthetization, fish were held head down with the ventral surface exposed and wiped dry. A catheter was gently inserted about 3 cm into the gonoduct. The first few drops of milt were discarded to avoid contamination from urine, which could be present in the urogenital papilla. Approximately 5 mL of sperm was then withdrawn into a 10-mL syringe attached to the catheter. Individual milt specimens were maintained in separate Whirl-Pak plastic bags (NASCO, Fort Atkinson, Wisconsin) and held on ice until they could be examined microscopically for viability. Motility of each of the 20 lots of milt was determined by placing a small amount of sperm in contact with ovarian fluid on a slide and examining it microscopically. Only high-quality samples (at least 90% initial motility) were used and were initially maintained as separate collections.

Eggs were collected from five ripe females. Eggs were pooled and stored in a loosely covered bowl in a water bath at 8.0°C before fertilization. All fish used for the study were Connecticut River F_1-generation Atlantic salmon born in February 2000 and reared in captivity at White River National Fish Hatchery, Bethel, Vermont.

Freezing of semen.—The following extenders were used: (1) glucose extender with 5% DMSO (GD), (2) glucose extender with 5% DMSO supplemented with 13.3% egg yolk (GDE), (3) glucose extender with 10% methanol (GM), and (4) glucose extender with 10% methanol supplemented with 13.3% egg yolk (GME). All cryoprotectants were reagent grade (Sigma-Aldrich Corporation, St. Louis, Missouri). Sixteen sperm samples with motility of at least 90% were used for the investigation. Both semen and extenders were stored on ice during preparation for cryopreservation. To freeze sperm, 1-mL aliquots of milt from each of the 16 males were collected, pooled, and then mixed with each of the four extender combinations at a dilution ratio of 1:4 (semen : extender). Diluted semen was immediately drawn into 0.5-mL French straws (IMV International Corporation, Maple Grove, Minnesota). Lots of five straws were loaded into plastic goblets (10 mm diameter, 12 cm long; IMV International), attached to the bottom position on 20-cm aluminum canes (Southland Cryogenics, Inc., Carrollton, Texas), and frozen at a rate of −22°C/min in the bottom of nitrogen-vapor shipping dewars (Taylor-Wharton, Theodore, Alabama; Model CP-35). Close attention was paid to equilibration times, and all straws were filled and placed in the liquid nitrogen vapor within 2 min, 45 s. After 30 min, samples were removed from the nitrogen-vapor shipping dewars and plunged into dewars containing liquid nitrogen (−196°C) where they were stored submerged for 48 h.

Thawing of semen and fertilization.—Frozen semen was thawed at 40°C in a water bath for 7 s and immediately placed on eggs. A single 0.5-mL straw of extended semen was used to fertilize approximately 225 eggs. After the addition of sperm, eggs were gently mixed for 2 min, and after fertilization, eggs were water-hardened in 50 ppm (mg/L) iodophore solution for 30 min. Eggs were then gently placed in randomly assigned compartments within Heath trays (Flex-a-lite Consolidated, Milton, Washington) and placed in the incubator.

Incubation.—During incubation, freshwater was introduced at a rate of 15 L/min. All eggs received a 15-min flow-through treatment of 1,500 mg Paracide F/L (Argent Laboratories, Redmond, Washington) every other day beginning on the second day of incubation and continuing until eggs were eyed. Water temperature (range, 0.5–8.3°C; mean temperature = 3.3°C) was recorded daily. Upon attaining eye-up in February 2004, all egg lots were mechanically shocked, after which dead and unfertilized eggs (those that appeared opaque because of the rupture of the egg membrane) were removed manually with a bulb and pipette and enumerated.

Statistical analyses.—Postshock fertilization rates were calculated as the proportion of total eggs per egg lot that remained viable (eyed) at the conclusion of the trial. All trials were replicated five times. Computations were performed with SigmaStat version 2.0, (Jandel Scientific, Chicago). Data were checked for normality (Kolmogorov–Smirnov test) and equality of variance (Levene median test) and then analyzed with a one-way analysis of variance (ANOVA). Tukey's honestly significant difference was used for all posthoc

comparisons. All statistical analyses were performed at an α level of 0.05.

Results

Mean fertilization rates (±SD), expressed as the percentage of eyed embryos for each of the four extender combinations tested, were as follows: 83.5 ± 5.2% (GME), 72.9 ± 4.5% (GM), 66.9 ± 5.3% (GD), and 52.7 ± 5.5% (GDE). Overall, sperm cryopreserved with methanol as a cryoprotectant yielded significantly higher ($P < 0.001$) fertilization rates (78.3%) than did sperm cryopreserved with DMSO (59.9%). Sperm cryopreserved with GME yielded significantly higher ($F_{3,19} = 31.4$; $P < 0.001$) fertilization rates than did sperm cryopreserved with the other three extenders. No significant difference in fertilization rate was observed between sperm treated with the GM and GD extender combinations. However, the fertilization rate of sperm cryopreserved with GDE was significantly lower than either the GM or GD formulations.

Discussion

Mean fertilization rate at the eyed egg stage for semen preserved with GME was 83.5 ± 5.2%. This is in close agreement with the findings of Lahnsteiner et al. (1997), who observed eye-up rates of between 75.3 and 89.2% for rainbow trout when using 10% methanol as a cryoprotectant. Our fertilization rates also compare favorably with those observed for Atlantic salmon eggs of the same year-class fertilized with fresh milt and reared at the White River National Fish Hatchery (81.4%; S. Frost, USFWS, personal communication). Sperm concentrations were not determined during the current study and, therefore, sperm-to-egg ratios could not be calculated. However, during typical spawning operations, 2–4 mL of fresh sperm are used to fertilize 5,000–8,000 eggs, which is a ratio of 200–250 eggs per 0.1 mL of fresh sperm. In the current study, 0.1 mL of cryopreserved sperm (0.5-mL straw of diluted sperm) was used to fertilize 225 eggs, which is comparable with that used in typical hatchery operations.

In previous studies on cryopreservation of Atlantic salmon sperm, DMSO has been primarily used as the cryoprotectant (Truscott et al. 1968; Truscott and Idler 1969; Mounib 1978; Stoss and Refstie 1983; Alderson and Macneil 1984; Gallant et al. 1993). In this study, methanol provided a higher level of cryoprotection than did DMSO based on the fertilization results. Sperm cryopreserved with methanol has produced higher motility and fertilization rates then sperm cryopreserved with DMSO for other salmonid species (Lahnsteiner et al. 1996b, 1997). Both chemicals function as intracellular cryoprotectants and are believed to achieve their cryoprotective abilities by decreasing the water content within the cells (McGann 1978). However, DMSO is generally more toxic to fish sperm than methanol at similar concentrations (Tiersch et al. 2004a, 2004b; Bates et al. 2005).

In this study, the addition of egg yolk to extenders incorporating 10% methanol provided additional protection to Atlantic salmon sperm during the freezing and thawing processes and resulted in increased fertilization rates. Egg yolk has been reported to coat the cell membrane wall, thereby reducing lysis during the freezing process (Scott and Baynes 1980). Although the specific action of egg yolk is unknown, it has been theorized that a low-density lipoprotein fraction loosely interacts with the sperm plasma membrane (Quinn et al. 1980) or that exogenous lipids are used to repair the plasma membrane damaged during cooling (De Leeuw et al. 1993). However, the beneficial effects of egg yolk supplementation appear to be species specific, as increased fertilization rates have been observed in Atlantic salmon (Alderson and Macneil 1984) and rainbow trout (Legendre and Billard 1980; Baynes and Scott 1982), but equal or decreased fertilization rates have been reported in Arctic char (Piironen 1993) and asp *Aspius aspius* (Babiak et al. 1998). Additionally, the cryoprotective effect of egg yolk may also be specific to the individual formulation of extenders. In our trials, sperm cryopreserved with GDE yielded a lower fertilization rate than did sperm cryopreserved with GD. A similar result was found with northern pike *Esox lucius*. The addition of egg yolk to Stoss and Holtz's extender (Stoss and Holtz 1981) reduced postthaw motility and fertilization rates compared with the same extender without egg yolk; however, the addition of egg yolk to Erdahl and Graham's extender (Erdahl and Graham 1980) improved postthaw motility and fertilization rates (Babiak et al. 1995). A more recent study of the addition of egg yolk or egg yolk proteins to an extender of sucrose and 15% DMSO produced no significant differences in hatch rates of northern pike (Babiak et al. 1999). This illustrates the need to assess external cryoprotectants on a species-by-species basis and in regards to a particular extender–cryoprotectant combination.

Cryopreserved sperm has the potential to be used as a tool for Atlantic salmon management. When frozen in liquid nitrogen, the viability of spermatozoa does not change appreciably with increasing storage time (Ashwood-Smith 1980; Whittingham 1980). Therefore, the main advantages inherent to the use of sperm cryopreservation technology include conservation of rare or specific genes, alleviation of the frequent problem of disproportionate male-to-female ratios, greater control for selective breeding programs, and

a convenient means of providing disease-free sperm for spawning.

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