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Publisher: Taylor & Francis

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North American Journal of Aquaculture

Publication details, including instructions for authors and subscription information:

<http://www.tandfonline.com/loi/unaj20>

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Version of record first published: 09 Jan 2011.

To cite this article: Nabil Mansour, Gavin F. Richardson & Mary A. McNiven (2008): Effect of Seminal Plasma Protein on Postthaw Viability and Fertility of Arctic Char Spermatozoa, North American Journal of Aquaculture, 70:1, 92-97

To link to this article: <http://dx.doi.org/10.1577/A06-094.1>

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Effect of Seminal Plasma Protein on Postthaw Viability and Fertility of Arctic Char Spermatozoa

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Abstract.—Seminal plasma protein of Arctic char *Salvelinus alpinus* was characterized using sodium dodecyl sulfate (SDS) gel electrophoresis. Twelve protein bands with molecular weights of 7.2, 12.4, 15.3, 20.0, 20.4, 22.6, 39.4, 66.3, 74.0, 92.0, 94.5, and 130.1 kilodaltons (kDa) were detected. The effect of total seminal plasma protein and protein fractions of three categories (<50, 50–100, and >100 kDa) on postthaw sperm motility, viability, and fertility was tested. Incorporation of total seminal plasma protein, the fraction greater than 100 kDa, or the fraction less than 50 kDa into the semen extender (300 mmol of glucose/L of water, plus 10% methanol) had a deleterious effect on postthaw sperm motility, viability, and fertility in comparison with spermatozoa frozen in the semen extender only. However, adding the 50–100-kDa fraction of seminal plasma protein to the semen extender did not affect the postthaw sperm motility and fertility relative to spermatozoa frozen in the extender only. Further experiments are needed to test the effect of different concentrations of seminal plasma proteins alone or in a combination with other seminal plasma constituents on sperm physiology and viability during short-term storage and cryopreservation.

In salmonids, some functions of seminal plasma in sperm physiology have been identified. Seminal plasma supplies the sperm with energy resources (Lahnsteiner et al. 1993), contains proteolytic enzymes that help to eliminate the aged spermatozoa from the spermatic duct (Loir et al. 1990), and inhibits the activation of sperm motility during its storage in the spermatic duct (Morisawa and Suzuki 1980; Cosson et al. 1999). Moreover, dietary supplementation with antioxidants increases the level of antioxidants in the seminal plasma and inhibits the process of sperm membrane lipid peroxidation (Mansour et al. 2006a).

In rainbow trout *Oncorhynchus mykiss*, seminal plasma protein has been fractionated and characterized (Loir et al. 1990; Lahnsteiner et al. 2004; Lahnsteiner 2006), and the addition of seminal plasma protein to a

motility-inhibiting saline solution stabilized sperm viability (activated motility rate and swimming velocity) for a period of 48 h at 5°C (Lahnsteiner et al. 2004). Additionally, incubation of spermatozoa in seminal plasma has a more potent stabilizing effect on viability than incubation in a saline solution containing total or fractionated seminal plasma protein (Lahnsteiner 2006). The effect of seminal plasma protein on the success of sperm cryopreservation in fish has not been studied to date. In mammals, addition of seminal plasma protein reversed the effect of cold shock during freezing (Barrios et al. 2000) and the presence of some protein fractions improved semen freezability (Jobim et al. 2004; Zahn et al. 2005). Therefore, this study was conducted to characterize the seminal plasma protein and to test the effect of total protein and several protein fractions on postthaw sperm membrane integrity and fertility in Arctic char *Salvelinus alpinus*.

Methods

Fish and semen collection.—Mature Arctic char (body weight = 1,000–1,500 g) were purchased and held in round, fiberglass tanks (volume = 3.3 m³) at a stocking density of 15 fish/tank. All tanks were supplied with 7°C recirculating water at a rate of 16 L/min. Water was oxygen enriched using a regenerative air blower (Rotron, Fremont, California) and filtered via mechanical and biofilters (Baker Hydro, Canton, Miami). The photoperiod was a cycle of 12 h light : 12 h dark. Fish were fed salmonid pellets (Corey Feed Mills Ltd., Fredericton, New Brunswick) twice daily ad libitum. Fish were acclimatized to these holding conditions for 1 month before the onset of the experiment.

During the spawning season, semen was stripped from fish by abdominal massage and collected in clean, dry plastic cups. Care was taken to avoid any contamination with urine, mucus, blood, or feces. Sperm cell concentration of the pooled semen sample was determined spectrophotometrically according to methods of Ciereszko and Dabrowski (1993). To

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Received December 25, 2006; accepted May 21, 2007
Published online January 14, 2008

collect the seminal plasma, semen samples were centrifuged at $1,500 \times$ gravity (g) for 15 min at 5°C and seminal plasma was separated. Seminal plasma was re-centrifuged at $5,000 \times g$ for 10 min at 5°C to separate the remaining spermatozoa, and the supernatant seminal plasma was frozen at -80°C until use.

Fractionation and dilution of seminal plasma protein in the semen extender.—Seminal plasma collected from several males was pooled and used for separation of seminal plasma protein. Total seminal plasma protein was separated from other seminal plasma components by dialyzing pure seminal plasma for 12 h at 5°C against a glucose solution of 300 mmol/L of water using disposable cellulose ester dialyzers (Sigma Co.). Additionally, three fractions of seminal plasma protein (molecular weights: <50 , 50 – 100 , and >100 kilodaltons [kDa]) were obtained using Amicon Ultra centrifugal filters (Millipore Corporation, Bedford, Massachusetts) in two consecutive filtering steps. In the first step, seminal plasma was filtered via a 100-kDa filter by centrifugation at $3,000 \times g$ for 20 min at 5°C and the fraction greater than 100 kDa was washed from the filter using 300-mmol/L glucose. In the second step, the filtrate was filtered through a 50-kDa filter and the 50–100-kDa fraction was washed from the filter using 300-mmol/L glucose. The filtrate was collected to obtain the fraction less than 50 kDa. The three fractions were dialyzed against 300-mmol/L glucose as described previously to separate the total seminal plasma protein. Protein concentration in the dialyzed samples of total seminal plasma and the three fractions were measured using the method of Lowry et al. (1951). The samples were then further diluted with 300-mmol/L glucose to a final diluent concentration of 150 mg protein/100 mL glucose (similar to the concentration of seminal plasma protein in salmonids; Lahnsteiner et al. 1998). Methanol, an established cryoprotectant when added to simple glucose diluent for Arctic char sperm (Mansour et al. 2006b), was added at a concentration of 10% to the glucose diluent containing the total or fractionated seminal plasma protein. A solution of 0.3 M glucose plus 10% methanol without seminal plasma protein was used as a control.

Protein analysis by gel electrophoresis.—Whole seminal plasma, total seminal plasma protein, and fractionated proteins were analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) according to the method of Bollag et al. (1996). Sub-samples were resuspended in 2-mercaptoethanol at 714.5 mmol/L, tris(hydroxymethyl)aminomethane (Tris) at 62.5 mmol/L, 2% SDS, 0.005% bromophenol blue, and 10% glycerol at pH 6.8 and were mixed well. Electrophoresis was performed in a BIO-RAD Mini Protean 3 Cell using a gel buffer (0.1%

SDS and 375-mmol/L Tris at pH 8.8), a running buffer (120-mmol/L Tris, 960-mmol/L glycine, and 0.5% SDS), and 12% and 15% bis-acrylamide gels (0.75 mm thick). Proteins were stained with Coomassie Brilliant Blue. A protein range of 10–250 kDa (Precision Plus Protein Standards, BIO-RAD, California) was used as a standard (see Figure 2a, b). Gels were scanned and protein molecular weights were determined using Scion Imaging Software (Scion Corporation, Frederick, Maryland).

Cryopreservation and thawing technique.—Seven semen samples with a motility rate of greater than 60% (estimated under a light microscope) were pooled and used in this experiment. In a 5°C cold room, semen was diluted 1:3 with the aforementioned extender with total or fractionated seminal plasma protein. As a control, semen was diluted 1:3 in extender containing no protein. After dilution, extended semen was aspirated into 0.5-mL straws (Minitube, Ingersoll, Ontario). The straws were frozen within 2 min on an adjustable rack above the surface of liquid nitrogen in an insulated box (Figure 1a, b). The movable rack can hold up to fifty 0.5-mL straws and was adjusted to 6 cm (freezing rate of $40 \pm 8^{\circ}\text{C}/\text{min}$) above the surface of liquid nitrogen (Figure 1b, c). After 10 min, the movable rack loaded with the straws was plunged into the liquid nitrogen. The straws were removed from liquid nitrogen and immersed in a 25°C water bath for 30 s to thaw. The straws were then wiped dry, and the thawed semen was collected in 3-mL test tubes.

Measurement of postthaw sperm viability.—The percentage of dead sperm cells (membrane integrity) was determined using fluorometry (Bilgili and Renden 1984; McNiven et al. 1992). Each treatment was assessed in triplicate. For each fertility trial, eggs from three females were pooled. In each experiment, there were three replicates for each treatment. Three replicates of freshly collected semen were used as a control. Eggs were fertilized with 5×10^6 sperm/egg. For each replicate, an aliquot of 5.3 g of eggs (100 ± 2 eggs) was weighed into a 50-mL, dry beaker and the semen was added and mixed well. Gametes were activated with 3.0 mL of 60-mmol/L NaHCO_3 plus 50-mmol/L Tris at pH 9.0 (Lahnsteiner 2000) and mixed again. After 1 min, the fertilized eggs were rinsed twice with 7°C hatchery water; poured into round, polyvinyl chloride (PVC) incubators (internal diameter = 7.7 cm, external diameter = 9.0 cm, PVC screen was fixed to the base; Figure 1d); and placed randomly into Heath incubator trays. Eggs were incubated for 42 d in $7 \pm 1^{\circ}\text{C}$ hatchery water until they reached the eyed stage. During incubation, opaque eggs were removed weekly and placed in a clearing solution (47.4 mL of 37% [weight per volume] formaldehyde, 37.9 mL of glacial

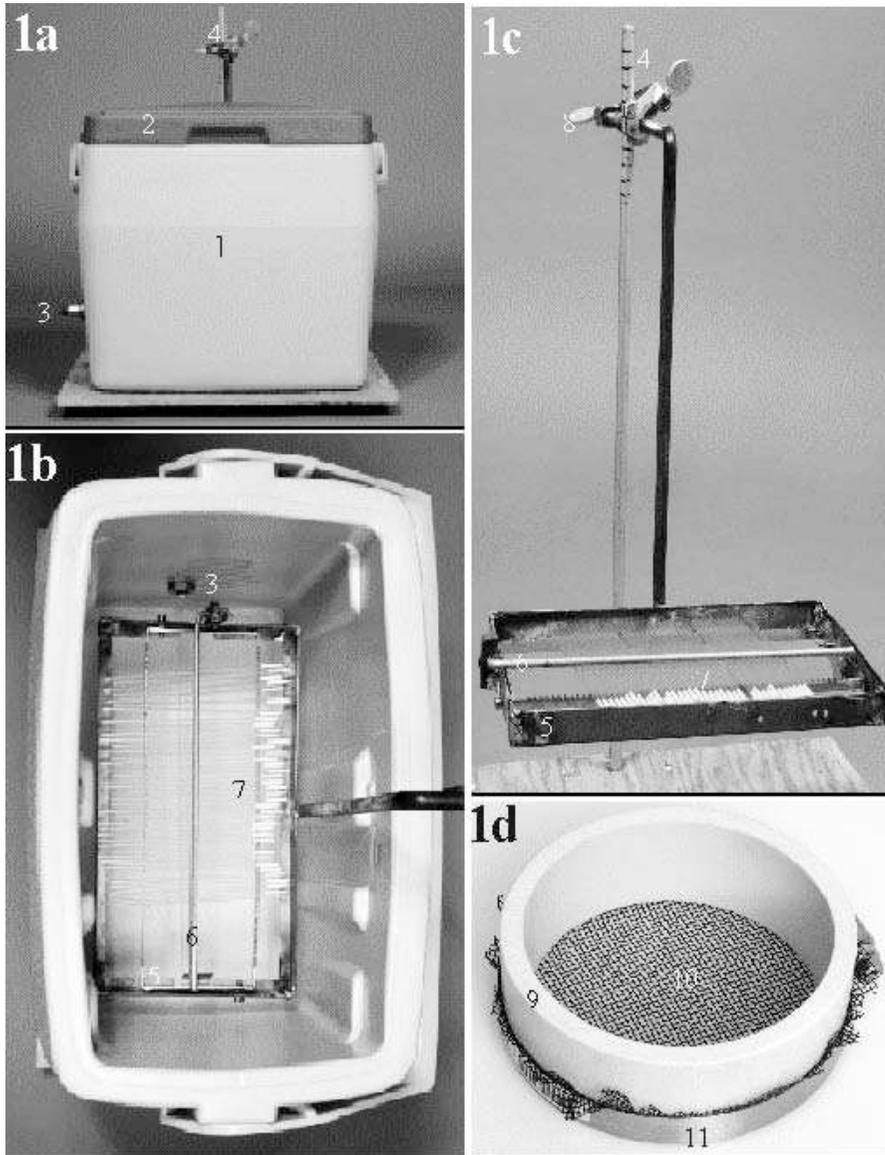


FIGURE 1.—Photographs depicting equipment used to study the effect of added seminal protein on the viability and fertility of cryopreserved Arctic char sperm: (a) freezing box used for cryopreservation; (b) open freezing box, showing the freezing rack loaded with 0.5-mL straws; (c) freezing rack (with straws), which can be moved up and down on a marked scale; and (d) PVC incubator used for small numbers of eggs. Labeled equipment components are (1) freezing box; (2) cover of freezing box; (3) liquid nitrogen drainage tap; (4) marked scale; (5) freezing rack; (6) metal bar used to prevent straws from floating when plunged into liquid nitrogen; (7) 0.5-mL straws; (8) screw clamp for changing the level of the freezing rack above the surface of liquid nitrogen; (9) wall of PVC incubator; (10) PVC screen; and (11) clamp.

acetic acid, 56.8 mL of glycerol, and 804.4 mL of distilled water) and the fertilized eggs were counted and recorded. At the end of the fertilization trial, eyed eggs were counted for each treatment. The proportions of fertilized and eyed eggs from each treatment were calculated from total number of eggs.

Statistical analysis.—To compare the effects of total seminal plasma protein and protein fractions on Arctic char sperm cryopreservation, the fertility (% fertilization and % eyed eggs) and fluorometry data were arcsine transformed and tested for normality. Analysis of variance (ANOVA) with subsequent Tukey's *b*-test

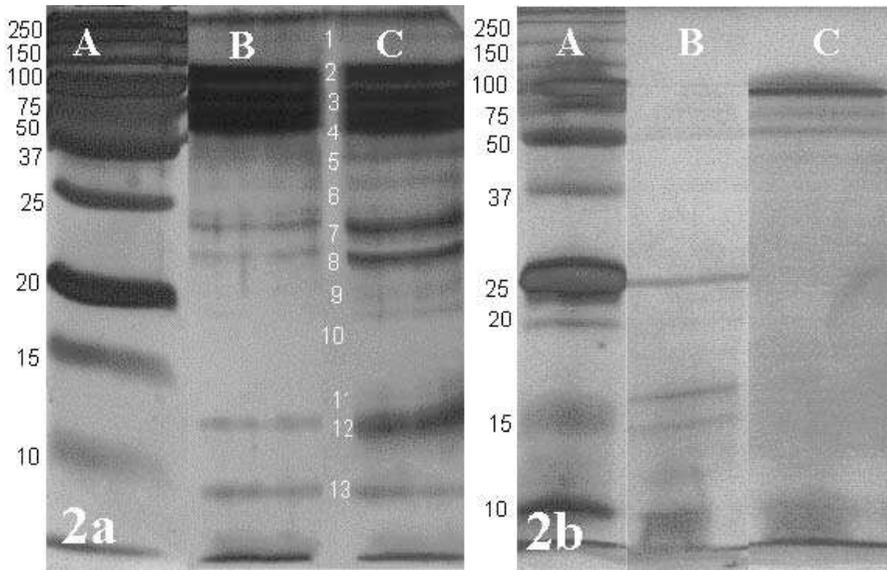


FIGURE 2.—Characterization of Arctic char seminal plasma protein as revealed by sodium dodecyl sulfate polyacrylamide gel electrophoresis: (a) 15% bis-acrylamide gel (A = protein standard [molecular weights expressed as kDa]; B = seminal plasma sample; C = total seminal plasma protein component); (b) 12% bis-acrylamide gel (A = protein standard; B = <50-kDa protein fraction; C = 50–100-kDa fraction).

was used for comparison of mean values of the various treatments and results are represented as mean \pm SE. Differences were considered significant at P -values less than 0.05.

Results

Identification and Characterization of Seminal Plasma Protein

Seminal plasma samples and the total protein component contained 12 proteins with molecular weights of 7.2, 12.4, 15.3, 20.0, 20.4, 22.6, 39.4, 66.3, 74.0, 92.0, 94.5, and 130.1 kDa (15% bis-acrylamide gel; Figure 2a). The seminal plasma protein fraction less than 50 kDa contained seven proteins with molecular weights of 7.2, 12.4, 15.3, 20.0, 20.4, 22.6, and 39.4 kDa (12% gel; Figure 2b). The 50–100-kDa fraction contained four proteins: 66.3, 74.0, 92.0, and

94.5 kDa (12% bis-acrylamide gel; Figure 2b). The fraction greater than 100 kDa contained only one protein (130.1 kDa).

Effect of Seminal Plasma Protein on Postthaw Sperm Viability and Fertility

Fresh semen (control) produced higher percentages of fertilized and eyed eggs than did frozen semen in the extender with or without addition of seminal plasma protein (Table 1). Semen frozen in the extender containing total seminal plasma protein, the fraction greater than 100 kDa, or the fraction less than 50 kDa had a higher percentage of dead cells and lower fertility than semen frozen in the extender only (Table 1). Within each treatment, there was no significant difference between the percentages of fertilized and eyed eggs. Relative to semen frozen in extender only,

TABLE 1.—Effect of seminal plasma protein (total and some fractions) on postthaw sperm viability (% dead cells) and percentages of fertilized and eyed eggs in Arctic char. Means \pm SE within a column followed by different letters are significantly different ($P < 0.05$).

Treatment	Dead cells (%)	Fertilized eggs (%)	Eyed eggs (%)
Fresh semen (control)	—	81.1 \pm 7.6 z	80.1 \pm 5.9 z
No seminal plasma protein	17.5 \pm 2.1 x	65.0 \pm 5.9 y	62.7 \pm 6.1 y
With seminal plasma protein			
Total protein	43.7 \pm 2.4 z	2.1 \pm 1.3 x	2.0 \pm 0.9 x
>100 kDa fraction	39.9 \pm 1.0 yz	11.9 \pm 1.4 x	11.5 \pm 1.3 x
50–100 kDa fraction	32.9 \pm 2.1 y	67.6 \pm 4.0 y	62.9 \pm 4.2 y
<50 kDa fraction	36.2 \pm 1.1 yz	1.4 \pm 0.4 x	1.3 \pm 0.4 x

incorporation of the 50–100-kDa seminal plasma protein in the semen extender produced similar postthaw fertilization and eyed eggs percentages and a higher percentage of dead sperm cells (Table 1).

Discussion

This is the first study testing the effect of total and fractionated seminal plasma protein on viability and fertility of frozen, thawed spermatozoa in fish. Seminal plasma of Arctic char contained 12 protein bands with different molecular weights ranging from 7.2–130.1 kDa. Two previous studies of rainbow trout determined that seminal plasma contained eight protein bands. Molecular weights ranged from 14.5 to 78.0 kDa in the first study (Loir et al. 1990) and from 13.6 to 91.7 kDa in the second (Lahnsteiner et al. 2004). However, a more recent study of rainbow trout seminal plasma (Lahnsteiner 2006) detected 12 types of protein with a molecular weight range of 16–135 kDa, which is similar to that we detected in Arctic char.

Salmonid seminal plasma contains not only protein but also other constituents, such as amino acids, fatty acids, neutral lipids, and monosaccharides (Lahnsteiner et al. 1998; Ciereszko et al. 2000). These constituents could be removed by dialysis at a molecular weight limit of 1 kDa (Lahnsteiner 2006). Therefore, we did not examine the effect of these additional seminal plasma constituents on the sperm freezing and thawing process.

In our study, addition of total seminal plasma protein, the fraction greater than 100 kDa, or the fraction less than 50 kDa to the semen extender had a deleterious effect on postthaw sperm viability and fertility in comparison with spermatozoa frozen in semen extender only. This inhibitory effect could be explained in two ways. First, some catalytic enzymes (especially proteases), which might be included in protein fractions, might have been activated, thus inducing a deleterious effect on the sperm cells. Generally, protease enzymes have been recorded in seminal plasma of salmonids (Lahnsteiner et al. 1998). Second, the added seminal plasma protein might have been damaged during preparation; for example, oxidation of lipoprotein could have been activated after removal of low-molecular-weight antioxidants during dialysis.

From this study, it is clear that addition of seminal plasma protein alone to the semen extender has no stabilizing or protecting effect on Arctic char sperm freezability; perhaps other constituents of seminal plasma work synergistically with seminal plasma protein to produce the stabilizing effect. This is consistent with the results of Lahnsteiner (2006), who found that incubation of spermatozoa for 48 h at 5°C in

seminal plasma had higher sperm motility and swimming velocity than those incubated in a saline solution containing total or fractionated seminal plasma protein. Additionally, we (Mansour et al. 2006a) recently showed that seminal plasma of Arctic char had a low antioxidant effect on spermatozoa during *in vitro* incubation. Lipid peroxidation is one of the most deleterious processes affecting spermatozoal membranes during freezing and thawing (Bilodeau et al. 2000; Brouwers et al. 2005).

Incorporation of the 50–100-kDa protein fraction in the semen extender in our experiment had no effect on postthaw sperm fertility. This is in contrast to mammals, in which some protein fractions improve postthaw sperm motility and fertility and, consequently, semen freezability (Jobim et al. 2004; Zahn et al. 2005). Moreover, seminal plasma protein composition varied between male horses *Equus caballus* with high and low fertility (Brandon et al. 1999).

In our experiment, fluorometry results indicated that sperm membrane damage was higher in semen frozen in extender containing total or fractional seminal plasma protein than in semen frozen in extender only. The exact cause of this phenomenon is not known, but the added protein may have damaged cell membranes or interfered with the fluorescence readings. Further experiments are needed to test the effect of different concentrations of seminal plasma proteins alone or in a combination with other seminal plasma constituents on sperm physiology and viability during short-term storage and cryopreservation.

Acknowledgments

This project was supported by Coastal Zone Research Institute, Inc., Canada.

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