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FRESH AND FROZEN STORAGE

OF

SALMONID SPERMATOZOA

A Thesis

Submitted to the Graduate Faculty

in Partial Fulfillment of the Requirements

for the Degree of

Master of Science

in the Department of Health Management

Faculty of Veterinary Medicine

University of Prince Edward Island

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ABSTRACT

This study was carried out to evaluate techniques for fresh and frozen storage of salmonid spermatozoa and develop methods to assess sperm viability following storage. Three *in vitro* methods (motility, fluorometry, and enzyme leakage) were evaluated to assess the viability of stored salmonid spermatozoa. All three *in vitro* methods revealed highly significant relationships ($p<0.001$) with various proportions of live/killed spermatozoa. Optical density was found to be better than spermatocrit to determine sperm concentration. Percentage fertility of fresh rainbow trout eggs fertilized with various proportions of live and killed spermatozoa revealed a significant relationship ($p<0.05$) but fertility results were variable. Fluorometry and motility were simpler to perform than enzyme leakage and gave reliable results. Several techniques were assessed to store rainbow trout semen in the fresh state. Semen stored above the oxygenated fluorocarbon layer in the moisture-saturated air atmosphere at 4°C expressed a fertility of 81.2 % after 37 days of storage. Motility and fluorometry measurements indicated that semen stored above the oxygenated fluorocarbon layer exhibited significantly ($p<0.05$) longer duration of motility and significantly ($p<0.05$) lower proportions of dead spermatozoa after 37 days of storage. Measurement of lactate dehydrogenase leakage did not identify any one treatment as superior for semen storage and was difficult to perform. Fifteen diluents (five extenders x three levels of cryoprotectant) were evaluated to cryopreserve Atlantic salmon semen and two superior diluents as identified by fluorometry were used to cryopreserve spermatozoa from six individual fish at a freezing rate of -30°C per minute to -196°C. There was a significant difference ($p<0.05$) detected by fluorometry in the post-thaw viability of spermatozoa from the six fish. Motility and LDH leakage indicated non-significant relationships in the post-thaw viability of spermatozoa from the six fish. A new cryoprotectant, dimethyl acetamide (DMA) was evaluated as a cryoprotective agent for rainbow trout semen. Sixteen diluents (four extenders x two cryoprotectants x two levels) were evaluated to cryopreserve rainbow trout spermatozoa. Extender 1 with 10% DMA performed better than other diluents in the experiment. All three *in vitro* methods significantly ($p<0.05$) predicted fertility. The maximum fertiltiy achieved with frozen-thawed rainbow trout spermatozoa was 66.2% using approximately 6.9 x 10^6 sperm/egg.

A combination of fluorometry and motility are reccommended to assess the viability of stored spermatozoa of salmonids to predict fertility.

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1. GENERAL INTRODUCTION

Finfish aquaculture originated in Asia centuries ago when fish were raised on natural pond water constituents. Aquaculture spread rapidly to Europe and North America where the use of formulated feeds allowed for rapid growth and intensive culturing techniques. Within the last 30 years, finfish aquaculture for food purposes has developed at a tremendous pace. With this development there has been a need to develop larger, faster growing fish with good disease resistance. To accomplish this, broodstocks with desirable qualities have received much attention and efforts have been focused to preserve the genetic material of such species. Techniques for fresh storage of semen and frozen storage or cryopreservation are essential to breeding programs to preserve genetic material.

1.1 Life Cycle of Salmonids

Salmonid species, such as the Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*) have yearly reproductive cycles with most individuals spawning in late October or November. The female digs a depression or redd in the stream bottom gravel and deposits several thousand eggs. As the female spawns, the male deposits semen containing billions of spermatozoa over the eggs. Fertilization of the egg occurs when a single spermatozoon gains access to the inside of the egg through a narrow opening called the micropyle. The rate of embryological development is water temperature dependent and proceeds slowly with the onset of winter. With the appearance of eyes in the embryo, development nears completion and the embryo is ready to hatch. Young fish hatch in the spring, and after 2-3 years of feeding in the river, migrate downstream to the ocean to continue feeding and undergo reproductive development.

1.2 Broodstock Considerations

Many aquaculture operations maintain large numbers of broodstock in captivity for commercial purposes and it has become necessary to control reproductive performance to enhance

productivity. Salmonids do not spawn naturally in captivity. Therefore, the hatchery operator must determine when his broodstock fish are ripe or ready for egg and semen collection. It is common for all broodstock to reach maturity at about the same time. Because the maturity period is very brief, especially for female fish, fertilization must take place in a brief period. Otherwise the eggs are reabsorbed. The hatchery operator must coordinate his/her efforts to carry out a successful spawning season. New techniques for fresh storage of semen and cryopreservation will allow an extension of the breeding period for the hatchery.

1.3 Storage of Spermatozoa

New techniques for fresh and frozen spermatozoa storage have become increasingly popular in the aquaculture industry. For example, semen is collected, the quality evaluated by motility, and stored fresh for short periods of time prior to egg collection and fertilization. Semen is also frozen or cryopreserved to maintain viable spermatozoa for longer periods of time. However, fresh and frozen storage techniques do not give consistent results under field conditions.

Whether semen is stored in the fresh state for several days or cryopreserved for extended periods of time, researchers have cited several advantages for establishing reliable methods to store salmonid spermatozoa. These methods would:

1) reduce the numbers of male brood fish thus reducing carrying costs
2) ensure against lack of male broodstock resulting from disease, natural disaster or precocious maturation.
3) allow genetic material to be shipped across boundaries.
4) ensure that spermatozoa of fish with desirable qualities be available for years after the death of the parent.
5) conserve a broad-based gene pool for the future.

1.4 Sperm Cell Morphology, Concentration and Viability

The salmonid spermatozoon consists an ovate flattened head, a mitochondrial collar or neck and an elongated flagellum. The head contains an anteriorally located nucleus and no acrosome.

The absence of the acrosome, which is typical on spermatozoa of terrestrial animals, is thought to relate to the presence of a micropyle in the egg allowing the sperm head direct access to the cellular membrane of the egg. The spermatozoon of rainbow trout is approximately 32 μm long. The head is elongated with a length of 3.1 μm and a maximum diameter of 2.2 μm. A mitochondrial collar adjoins the base of the head and averages 0.8 μm in length. The collar surrounds the base of the flagellum and contains numerous mitochondria. The flagellum ranges in length from 26 μm to 31 μm and consists of a long principal piece that tapers posteriorally into a short end piece (1).

The concentration of spermatozoa in salmonids ranges from 9 to 18 billion cells per millilitre of semen (2). During fertilization, the concentration of sperm may drastically affect the success rate. The minimal concentration of sperm for optimal fertilization of rainbow trout eggs has been reported to be 200,000 sperm per egg (3).

Several methods have been developed for fish and non-fish species to determine sperm concentration. They include optical density, packed sperm cell volume (spermatocrit), fluorescence of genetic material (fluorometry), sperm counts using a haemocytometer or electronic counts. Many workers fail to report the concentration of spermatozoa used to fertilize the eggs and this leads to discrepancy in interpretation of results.

New methods to assess the viability of salmonid spermatozoa need to be developed. Evaluation of motility is a common laboratory tool to assess salmonid sperm viability but is not widely used in hatcheries. Hatchery operators tend to over-fertilize eggs by inseminating with semen from two or three males, thus reducing the chance of inseminating with poor quality semen. More advanced hatcheries, involved in genetic selection of fish, require more sophisticated laboratory methods to assess sperm viability. Techniques such as the fluorescence of genetic material of damaged cells and the detection of enzyme leakage from damaged cells into the seminal plasma have been utilized in terrestrial species but have not been adapted for fish. Development

of such techniques for fish will allow rapid evaluation of fresh semen or stored semen so the industry can maximize the utilization of semen.

1.5 Activation of Spermatozoa

Before entering a fresh water river to spawn, adult salmonids contain spermatozoa primarily in the testis. The sperm duct of chum salmon is void of spermatozoa while the fish migrates from the sea into a bay. When the fish travel for several days up stream to the spawning areas, spermatozoa are moved into the sperm duct (4). Spermatozoa in the distal portion of the sperm duct are subjected to a high concentration of potassium ions that are believed responsible for a state of quiescence (5). Upon release into potassium free water, spermatozoa from the sperm duct exhibit vigorous motility.

The life of the spermatozoa is brief in the water column. Spermatozoa at first swim vigorously, then the rate of movement decreases. After approximately 30 seconds, the number of spermatozoa showing forward motion rapidly declines. With the exception of a few spermatozoa that show vibrating motion, all motility stops (6).

Hatcheries now employ the dry method of fertilization. Using this technique, eggs are collected free of water. Semen from male fish is deposited over the eggs. The ovarian fluid of eggs has the ability to initiate motility of spermatozoa and prolong its activity for up to 60 seconds (6). Eggs are then washed free of excess semen and ovarian fluid and water hardened for incubation.

1.6 Fresh Storage of Salmonid Semen

Fresh storage of semen at temperatures close to 0°C does not offer a permanent solution to long term preservation of genetic material. However for periods of several days to several weeks, fresh storage can be beneficial to minimize handling of broodstock and allow desired pair matings to take place. Fish sperm are particularly suited to fresh storage since they are quiescent in seminal plasma and do not utilize energy for motility (7). Semen of salmonid fish has been fresh stored in two forms: diluted and non-diluted. Semen has been diluted with extender in an attempt to prolong viability. An extender is a solution of salts, sometimes including organic compounds,

which help maintain the viability of cells during refrigeration (8). Mann (9) described an ideal diluent as being isotonic with a good buffering capacity and containing nutrients, antioxidants and antibacterial agents. A good extender will allow dilution of cells without their activation (10).

At least four factors may influence storage of salmonid spermatozoa. These include:

(1) ***Temperature*** - Storage capacity is prolonged at temperatures just above 0°C (11, 12).

(2) ***Gaseous exchange*** - Oxygen is most suitable for maintaining cell viability (13).

- Sample height determines gaseous exchange.

(3) ***Sterile conditions*** - Bacterial growth often limits storage to a few days.

- Addition of antibiotic and antimycotic prolongs storage (14).

(4) ***Prevention of desiccation*** - A moisture saturated atmosphere is superior to non-saturated atmosphere (13).

Other types of fresh storage have been evaluated, including supercooling of semen and postmortem storage of parent fish. Supercooling, or the holding of gametes just below the freezing point with addition of cryoprotectants, may have potential; however, the cost of controlled freezers may not justify the method compared to storage above 0°C. Postmortem storage is limited by the temperature-dependent physiological breakdown of the fish leaving spermatozoa unviable after several hours to a one or two days (15).

1.7 Cryopreservation of Salmonid Semen

Cryopreservation has been employed in animal science since the 18th century. Interest in the development of techniques to preserve spermatozoa by low temperature storage dates back to the work of Spallanzani in 1776. The realization of benefits that could accrue to the livestock industry came into focus after 1949, when Polge *et al.* (16) discovered that fowl spermatozoa retained full motility after freezing and thawing in glycerol. It was not until the late 1960" and

early 1970" that cryopreservation of fish sperm was addressed by scientists interested in the future of aquaculture.

Spermatozoa of salmonid fish cannot be cryopreserved in an undiluted state. Scientists have shown that additives to the semen, such as extenders and cryoprotectants, enhance the freezability of cells. As with fresh storage, the extender dilutes cells without activating them. A cryoprotectant may protect cell membranes during the freezing process by preventing electrolytes from concentrating in and around cells during freezing or by exhibiting a protective effect on the cell surface. Various cryoprotectants have been used for salmonid spermatozoa, but dimethylsulfoxide (DMSO) has proven to be the most successful (17, 18, 19, 20). Dimethylsulfoxide has a low toxicity to spermatozoa and penetrates the plasma membrane quickly to protect against damage by freezing (21).

During the freezing process, the external medium freezes before the cell contents. Protective solutes depress the freezing point to below zero. Between -5°C and - 15°C ice forms in the external medium, but the cell contents remain unfrozen and supercooled because the plasma membrane, solutes, cryoprotectant and added protein block the growth of ice crystals in the cell. Supercooled water inside the cell has a higher vapour pressure than ice and a lower solute concentration than that of water outside the cell; therefore, water flows out of the cell and freezes externally. The rate of freezing below -15°C determines damage to the cell (22). If the cooling velocity is above the optimum, large ice crystals may form inside the cell causing rupture of cell organelles or the plasma membrane. If the cooling velocity is below the optimum, the cell may dehydrate excessively and high concentrations of solutes in the cell may alter cell equilibrium (23).

In summary, a significant volume of work has been reported on the physiology and storage of salmonid spermatozoa; however, results are variable and many questions remain.

The objectives of this study were to develop and evaluate repeatable methods to assess the viability of salmonid spermatozoa after storage and develop techniques for fresh storage of rainbow trout semen and cryopreservation of rainbow trout and Atlantic salmon sperm.

Three methods (motility, fluorometry and enzyme leakage) to assess the viability of stored salmonid spermatozoa are evaluated in Chapter 2. These *in vitro* methods are used to assess the degree of damage to spermatozoa inflicted during storage and predict the fertility of spermatozoa with eggs *in vivo*.

Several treatments for fresh storage of rainbow trout semen including storage undiluted, with various extenders, and with an oxygen loaded medium in a moisture saturated atmosphere and a non-moisture saturated atmosphere are evaluated in Chapter 3. Treatments are evaluated by *in vitro* methods and fertility.

Several extenders and cryoprotectant levels are used to cryopreserve Atlantic salmon spermatozoa in Chapter 4. Freezing success is evaluated by fluorometry. This chapter studies freezing variation between fish by evaluating thawed sperm by the *in vitro* methods.

Several extenders and cryoprotectants used to cryopreserve spermatozoa of rainbow trout are assessed in Chapter 5 by the *in vitro* methods and fertility.

2. *IN VITRO* METHODS TO ASSESS SALMONID SPERMATOZOA VIABILITY AFTER STORAGE.

2.1 Introduction

A variety of methods have been developed to assess spermatozoa viability. They include spermatozoa motility, proportion of sperm with intact membranes, enzyme leakage and fertility. To date, motility has been the most common method used to assess the viability of salmonid spermatozoa. Criticisms of using motility to predict fertility have been based on the subjectiveness of the method and short activity period (15-30 seconds) on which to base motility ratings.

Fluorometric techniques have been used to evaluate the viability of chicken, bull and human spermatozoa (24, 25, 26). Fluorometry is based on the principle that the dye, ethidium bromide (EtBr), binds to the double stranded, undenatured nucleic acids of spermatozoa and induces an intense fluorescence (24). Normally the movement of EtBr is restricted by intact cell membranes (26). Such membrane systems may be made permeable to EtBr by the addition of a digitonin solution or by damage during fresh storage or cryopreservation. The fluorescence from damaged sperm and the total fluorescence following digitonin treatment reflect the proportion of sperm with damaged membranes and thus, the proportion of sperm with intact membranes (25).

Measurement of enzyme leakage from the cytoplasm of damaged cells has also been used to assess spermatozoa viability. At least two enzymes have been used to indicate membrane damage, namely aspartate aminotransferase (GOT or AST) and lactate dehydrogenase (LDH). AST activity has been found in most tissues and catalyses the transamination of L-aspartate and alpha ketoglutarate to oxaloacetate and glutamate. The release of AST into the extra-cellular medium following storage has been used to indicate membrane integrity and spermatozoa viability in the bull, boar, human, turkey and chicken (27, 28, 29, 30, 31, 32).

LDH activity has also been isolated in all tissues and catalyses the reversible reaction of

L-lactate to pyruvate. LDH activity in seminal plasma as compared to total LDH has been monitored to assess the degree of damage during cryopreservation of semen from fowl (33).

Although fertility is the ultimate indicator of spermatozoa viability, fertility trials in fish are expensive due to the cost of eggs, water pumping, incubation equipment and the time taken for eggs to reach the eyed stage. The availability of good eggs is seasonal and may last only a few weeks.

The objectives of this study were: (1) to evaluate the relationships between the proportion of live/dead sperm to motility, fluorometry, LDH leakage, fertility and the production of eyed eggs, (2) to assess the validity of using *in vitro* methods to predict fertility, (3) to evaluate the repeatability and precision of *in vitro* methods and fertility and, (4) to assess optical density and spermatocrit to predict sperm concentration.

2.2 Materials and Methods

2.2.1 Spermatozoa Collection

Semen was collected from mature adult rainbow trout held in outdoor circular tanks supplied with well water at approximately 4°C. Prior to semen collection, fish were anesthesized with tricaine methane sulfonate (MS-222) at a concentration of 100 mg/L, rinsed in fresh water and wiped dry. Abdominal massage was used to express semen into glass jars which were stored on crushed ice. Semen samples were observed for motility by activation with 0.12 M NaCl. One small drop of semen placed on a glass slide and two drops of saline solution were mixed with a wooden applicator stick and observed using a light microscope (40x). Semen samples were rated using the scale in Table I and samples with a 9 or 10 rating were pooled and used in the experiment.

2.2.2 Preparation of Killed Spermatozoa

A 10 ml sample of pooled semen was sonicated for 90 seconds at a setting of 60 percent with an intermediate tip of a Sonic Dismembranator (Fisher Scientific). The container holding the sample was surrounded with crushed ice to prevent heat damage to the spermatozoa during sonication.

Table I. Criteria used to assess spermatozoa motility for salmonids and corresponding motility rating.[a]

Criteria	Rating
No cells progressively motile	0
Most cells immotile with an occasional one slightly vibrating	0.5
Most cells immotile with a few (<5%) slightly vibrating and an occasional one in slow progressive motion	1
Most cells immotile with very few slightly vibrating and few with slow progressive motion	2
Most cells immotile with very few vibrating and a few with rapid progressive motility	2.5
Most cells immotile with 5-10% vibrating and some (<10%) with progressive motility	3
Most cells immotile with some vibrating and 10-24% exhibiting rapid progressive motility	4
Most cells immotile with 25-50% vibrating or exhibiting progressive motility	5
Most cells (>50%) vibrating *in loco* with a few (<5%) exhibiting progressive motility	6
Most cells vibrating *in loco* with 10-50% exhibiting progressive motility	7
Most cells (>50%) exhibiting progressive motility with others vibrating *in loco*	8
Most spermatozoa progressively motile (>90%) with vigorous motility and some exhibiting strong vibrations *in loco*	9
All spermatozoa progressively motile	10

[a] Modified from the table appearing in Guest *et al.* (69)

2.2.3 Live/Killed Proportions

Live or killed semen was pipetted into tubes as follows to prepare semen with different proportions of live and killed spermatozoa:

Tube	Killed Sperm (%)	Live Semen Volume (ml)	Killed Semen Volume (ml)
1	0	3.00	0.00
2	25	2.25	0.75
3	50	1.50	1.50
4	75	0.75	2.25
5	100	0.00	3.00

Tubes were mixed gently by inversion following assay of live and killed semen, randomized by an individual not involved in the experiments, and stored at a 4°C.

2.2.4 *In vitro* Tests for Spermatozoa Viability

Three *in vitro* methods were used to assess salmonid spermatozoal viability: motility, fluorometry and lactate dehydrogenase leakage. Motility ratings were performed as per sample evaluation of semen collected in the hatchery and rated on the scale in Table I.

Fluorescence measurements were performed in duplicate on a Hewlett Packard MPF-66 Fluorescence Spectrophotometer. Excitatory light was set at 360 nm and emission was measured above 575 nm. Cuvettes were filled with 3.990 ml of phosphate buffered saline (NaH_2 PO4.H_20, 1.9 mM; Na_2 HPO_4, 8.1 mM; NaCl, 128.2 mM; KCL, 29.2 mM; 330 m0sm/kg H_20; pH 7.2) containing 12.5 mg EtBr/ml. A 10 μl sample of semen was mixed with the EtBr solution by inversion for 60 seconds and the resulting fluorescence was recorded (first reading). The remaining cells were made permeable to EtBr by addition of 25 μl of digitonin solution (5 μg digitonin/ml of

absolute ethanol), inverted for 60 seconds and a fluorescence intensity recorded (second reading). The estimated percentage of damaged or dead spermatozoa was equal to the ratio of the first and second fluorescence readings multiplied by 100 (25).

A 400 µl semen sample was centrifuged at 500g for 5 minutes at 4°C. The top fraction of seminal plasma was collected and diluted 1 to 6 with 3.2 M $(NH_4)_2SO_4$. LDH activity was measured spectrophotometrically at 340 nm (Coulter Electronics, Inc Hialeah FL.) and expressed in units per litre of seminal plasma. One unit is equal to 1 µmole of substrate catalyzed per minute.

2.2.5 Spermatozoa Concentration of Rainbow Trout Semen

To determine the concentration of spermatozoa, two relationships were evaluated: optical density vs sperm cell counts and packed sperm cell volume (spermatocrit) vs sperm cell counts. Semen was collected and pooled from several adult rainbow trout. Into each of twenty tubes 0.1 ml of semen was pipetted and diluted with 0-0.66 ml of 1.1 M sodium citrate to give a range of sperm concentrations and mixed gently. Two 25 µl samples were withdrawn from each tube and suspended in 4.975 ml of 1.1 M sodium citrate. Each tube was inverted slowly 10 times and an optical density (in absorbance units) read in a Spectronic 20 spectrophotometer at a wavelength of 550 nm. A triplicate sperm cell count was performed on a Neubauer haemacytometer. Single linear regression was used to predict spermatozoal concentration (billions of sperm per ml of semen) from the optical density. Similarly, the technique of using spermatocrit to determine sperm concentration was evaluated by filling non-heparinized microhematocrit tubes (75 mm in length and 1.2 mm internal diameter) with diluted semen and centrifuging in a Canlab Haemofuge for 15 minutes at 17000 rpm. Results were expressed as follows: Spermatocrit(%) = (height of sperm column / height of sperm + fluid column) x 100. A regression equation was developed to predict sperm concentration by spermatocrit. The correlation coefficients of the two regressions were compared to determine the superior method to predict sperm concentration.

2.2.6 Ova Collection

Eggs were collected from mature adult rainbow trout held in indoor circular tanks supplied

with well water at 10°C. Prior to egg collection, fish were anesthesized with MS-222, rinsed and wiped dry. Abdominal massage was used to strip eggs into several containers. Eggs from several fish were pooled and mixed. A sealed container of pooled eggs was suspended in hatchery water for a short time prior to fertilization to maintain eggs at constant temperature.

2.2.7 Fertility Trials

The superior method to predict sperm concentration was used to determine the concentration of spermatozoa in each treatment tube. The volume of semen from each treatment that was required to fertilize lots of 300 eggs with a ratio of 200 000 sperm per egg was calculated. Appendix A contains a sample calculation.

One lot of eggs was fertilized for each treatment by the dry method. Semen remained on the eggs for 4-5 minutes, then a small quantity of water was added and left for 2-3 minutes. The eggs were rinsed three times in hatchery water to remove any excess semen or ovarian fluid and allowed to water harden for 30 minutes in fertilization dishes. Lots of eggs were placed randomly into small compartments of Heath Incubators and incubated at 10°C with well water.

2.2.8 Repeatability and Precision of Tests

The *in vitro* determinations of spermatozoa viability and fertility were repeated three times for the live/killed proportions. A second and third 10 ml sample of semen was then sonicated and two sets of live/killed proportions were prepared. Tubes were randomized and the *in vitro* methods were used to estimate the viability of spermatozoa in each treatment.

2.2.9 Determination of Fertilized and Eyed Eggs

Dead eggs that turned opaque were removed periodically and cleared in a solution of methanol, acetic acid and distilled water (1:1:1) (34). The presence of an embryo upon clearing indicated the egg had been fertilized. Likewise, the absence of an embryo upon clearing indicated the egg was not fertilized. A dead egg with an eyed embryo was recorded as fertilized and eyed. Before hatching live eyed eggs were counted for each treatment so that the proportion of eggs fertilized and reaching the eyed stage could be determined for each treatment.

2.2.10 Statistical Analysis

Simple linear regression was used to study the relationships between *in vitro* methods used to assess sperm viability and proportions of killed sperm. Percentage values for fluorometry, fertility and eyed eggs were transformed by the arcsin transformation for the regression analysis. Indicator variables were created to analyze variation within experiments and between experiments. Simple linear regression was used to check the validity of using the *in vitro* methods to predict fertility. The coefficient of variation (CV) was used to measure the precision of the *in vitro* methods and fertility.

2.3 Results

2.3.1 *In vitro* Methods to Assess Proportion of Killed Spermatozoa

Simple regression analysis of motility, fluorometry and LDH leakage on percentage killed spermatozoa using indicator variables for experiment and set revealed highly significant trends ($p<0.001$) for the *in vitro* methods and non-significant trends for the replications. Table II presents the mean (SEM) for motility rating, percentage dead sperm by fluorometry, LDH activity of the seminal plasma, percentage fertility and percentage eyed eggs for the various proportions of live/killed sperm expressed as percentage killed sperm.

Figure 1 is a plot of motility and percentage killed sperm. The regression analysis of motility on percentage killed sperm indicated a highly significant trend ($p<0.001$) with a coefficient of determination (r^2) of 0.937. There was no significant variation between the two experiments or within experiments.

Figure 2 is a plot of the percentage of dead sperm determined by fluorometry for proportions of killed sperm. The regression of fluorometry on percentage killed sperm indicated a highly significant trend ($p<0.001$) and an r^2 of 0.981. There was no significant difference between experiments or within experiments.

Figure 3 is a plot of LDH concentration in the seminal plasma for percentages of killed sperm. The regression of LDH concentration on percentage killed sperm revealed a highly

TABLE II. **Estimation of Sperm Viability Using *In Vitro* Methods, Fertility and Eyed Eggs for Live/Killed Proportions of Rainbow Trout Semen (SEM for n = 6)**

Killed Sperm %	*Motility 0 - 10*	*Fluorometry % Dead*	*LDH (U/L)*	*Fertility %*	*Eyed Stage %*
0	9.0 (.00)	1.7 (.18)	1.3 (.02)	20.5 (3.69)	20.5 (3.69)
25	8.3 (.21)	32.6 (.42)	74.8 (.49)	52.7 (5.10)	2.7 (5.10)
50	4.8 (.17)	56.6 (.38)	142.2 (1.40)	54.6 (4.77)	54.6 (4.77)
75	4.0 (.00)	78.6 (.32)	204.3 (1.66)	43.8 (3.82)	43.8 (3.82)
100	0 (.00)	97.9 (.38)	263.3 (5.39)	0.1 (.18)	0.1 (.18)

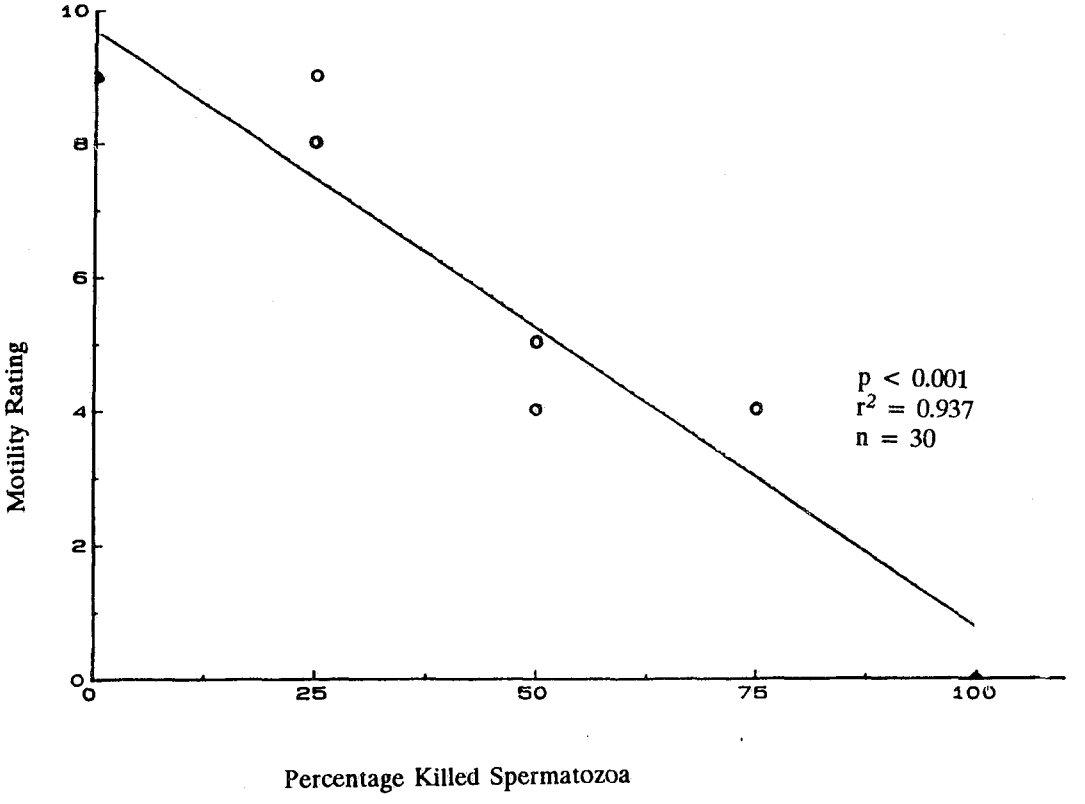

Figure 1. Linear regression line for the motility rating for different proportions of live/killed rainbow trout spermatozoa.

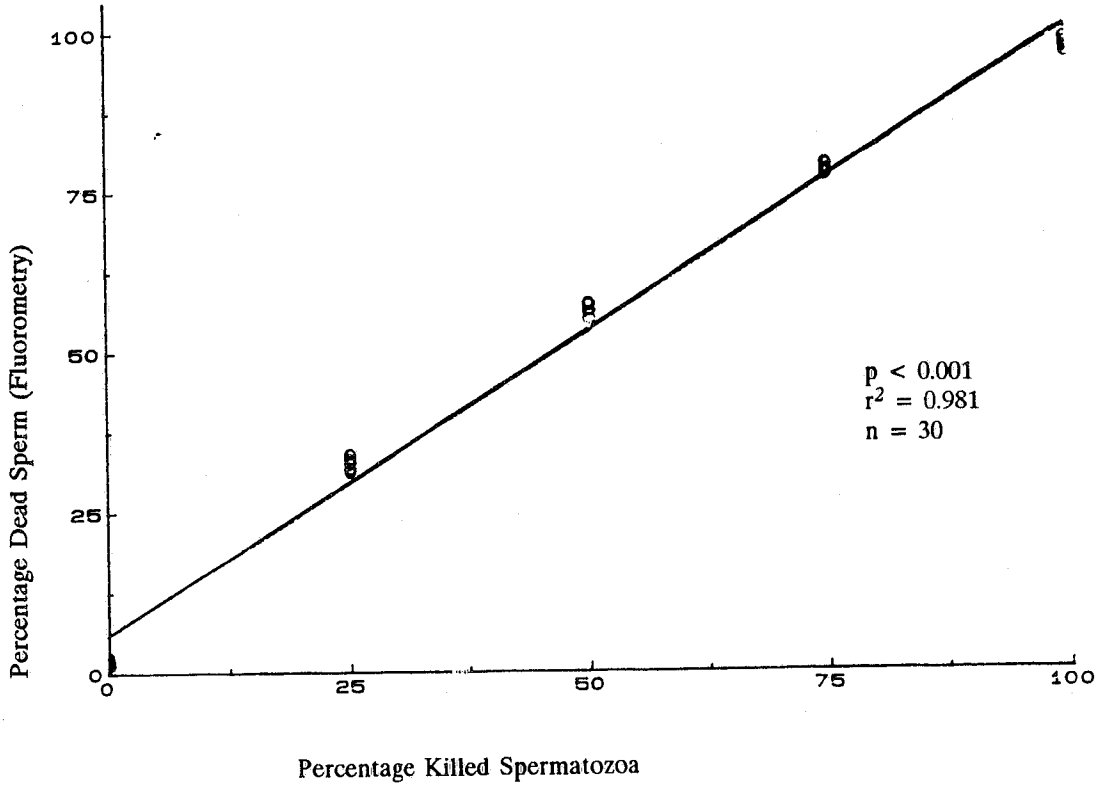

Figure 2. Linear regression line of the percentage of dead sperm as determined by fluorometry for different proportions of live/killed spermatozoa of rainbow trout.

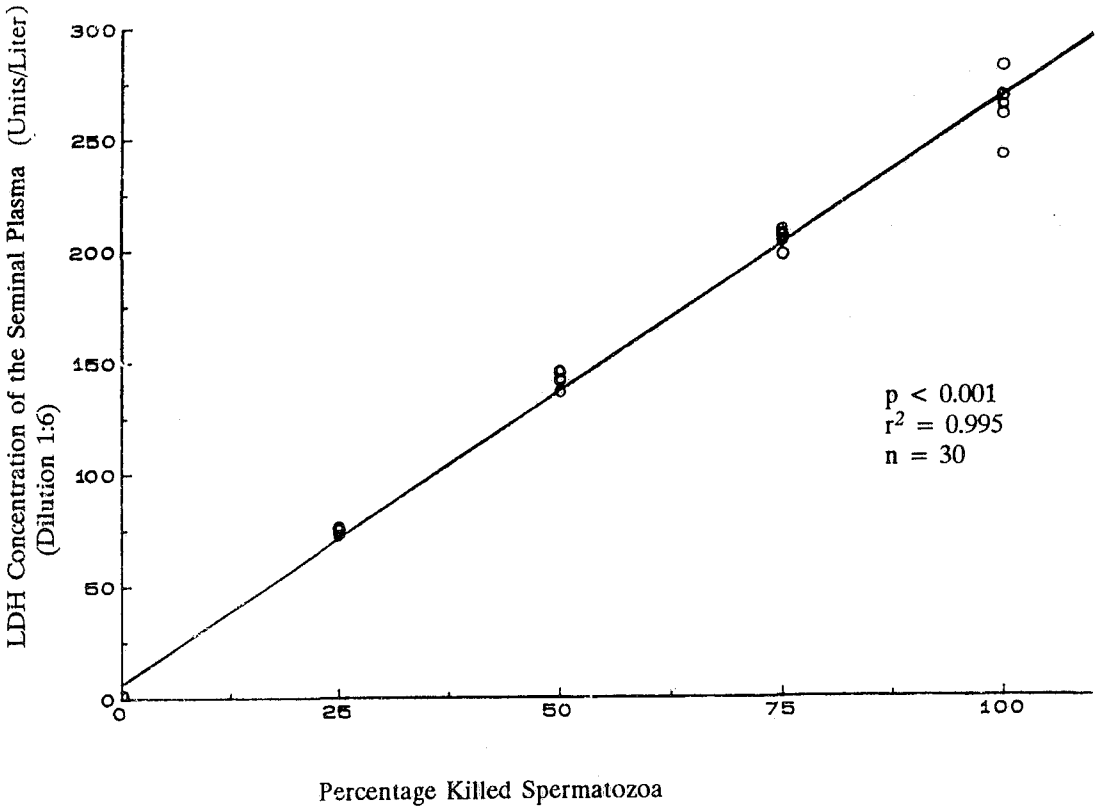

Figure 3. Linear regression line for the Lactate Dehydrogenase concentration of seminal plasma for different proportions of live/killed spermatozoa of rainbow trout.

significant trend ($p<0.001$) and an r^2 of 0.995. There was no significant difference between or within experiments.

2.3.2 Sperm Concentration Predicted by Optical Density and Spermatocrit

The regression of sperm concentration, expressed in 10^9 spermatozoa/ ml of semen, on optical density indicated a highly significant relationship ($p<0.001$) with an r^2 of 0.982. Figure 4 is a plot of sperm concentration and optical density with a least squares regression line for a sample of 40.

The regression of sperm concentration on packed sperm cell volume (spermatocrit) indicated a highly significant trend ($p<0.001$) with an r^2 of 0.988. Figure 5 is a plot of sperm concentration and spermatocrit with a least squares regression line for a sample size of 13. Comparison of the correlation coefficients for optical density and spermatocrit on sperm concentration by Fisher Z transformation revealed that the optical density method was superior to the spermatocrit method to determine sperm concentration ($z = 2.598$; $p<0.05$).

2.3.3 Fertilized and Eyed Eggs to Predict the Proportion of Killed Sperm

The regression of fertility on percentage killed spermatozoa revealed a significant trend ($p<0.05$) with an r^2 of 0.096. However, fertility data was quite variable. There was no significant variation between experiments or within experiments. Figure 6 presents the data for fertility and increased proportions of killed spermatozoa. The percentage of eggs reaching the eyed stage was similar to the percentage of eggs fertilized, therefore a figure was not presented.

2.3.4 Predicting Fertility

Single linear regression of motility, fluorometry and LDH concentration of the seminal plasma to predict fertility each revealed significant results ($p<0.05$). There was no significant variation between or within experiments.

2.3.5 Precision of Methods to Assess Sperm Viability

Table III presents the coefficients of variation of the *in vitro* methods, fertility and eyed eggs for the percentages of killed sperm. The average coefficients of variation for motility, fluorometry, and LDH concentration, and fertility were 3.0%, 1.8%, 3.0%, and 27.7%, respectively.

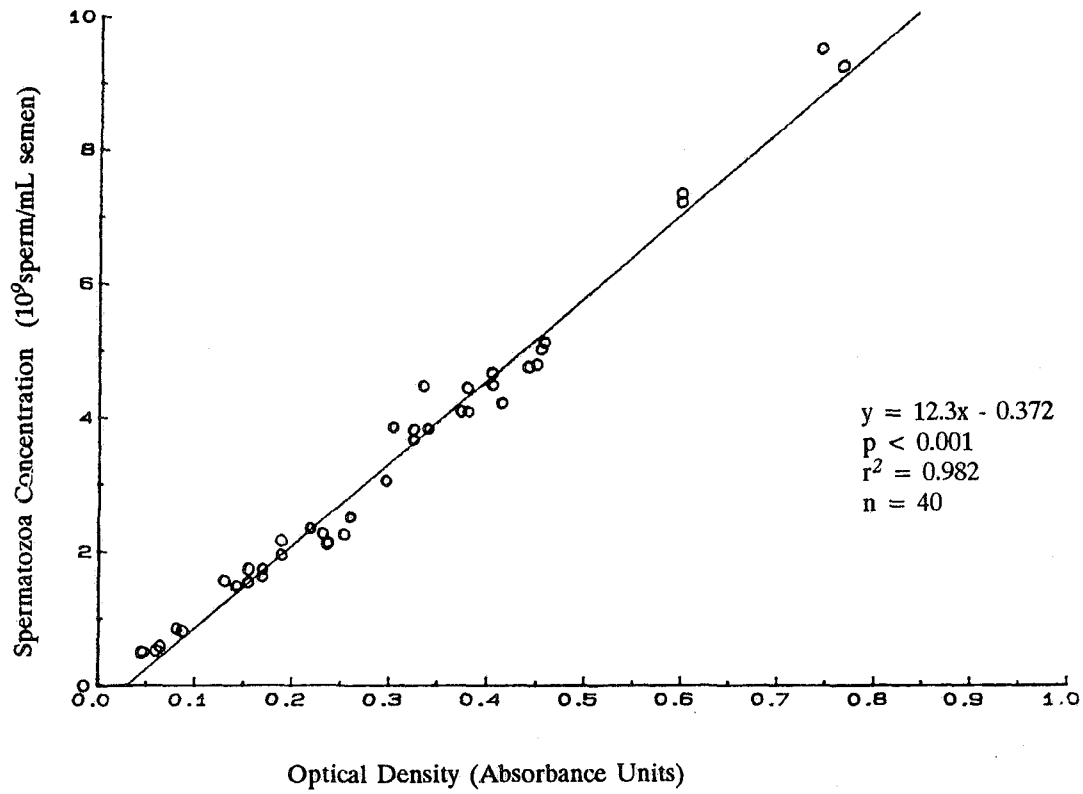

Figure 4. Linear regression line to predict spermatozoa concentration by optical density at 550 nm for rainbow trout semen.

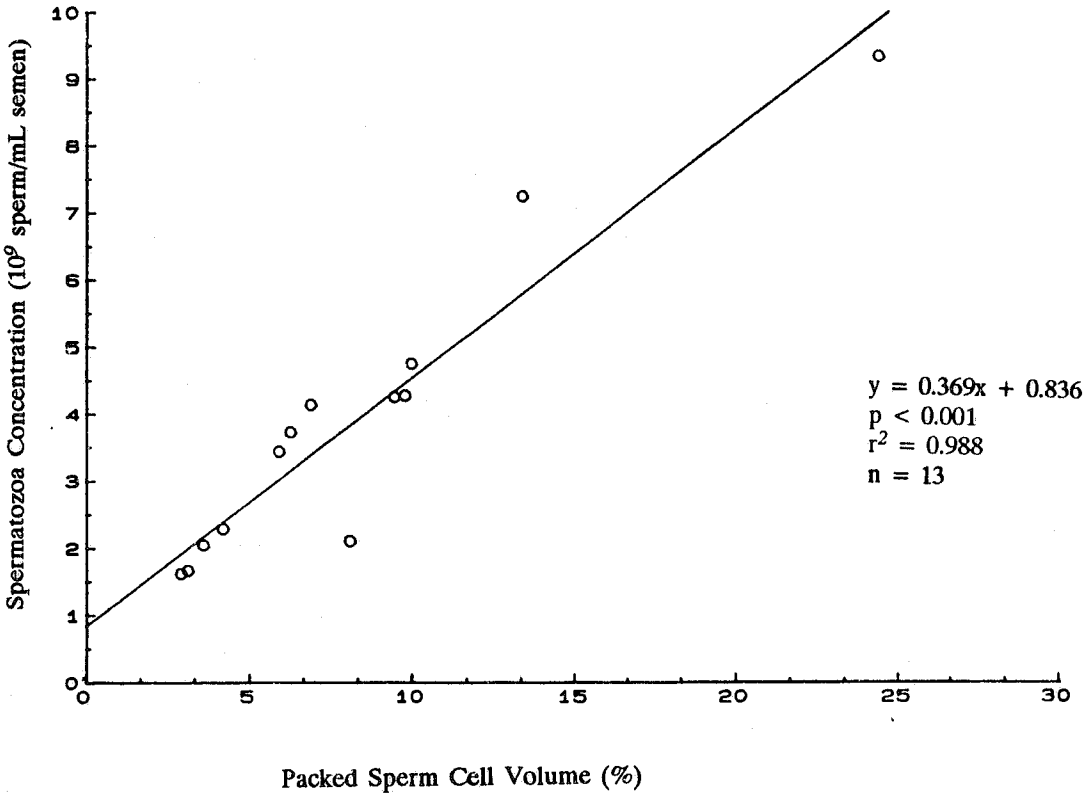

Figure 5. Linear regression line to predict spermatozoa concentration by packed sperm cell volume for rainbow trout.

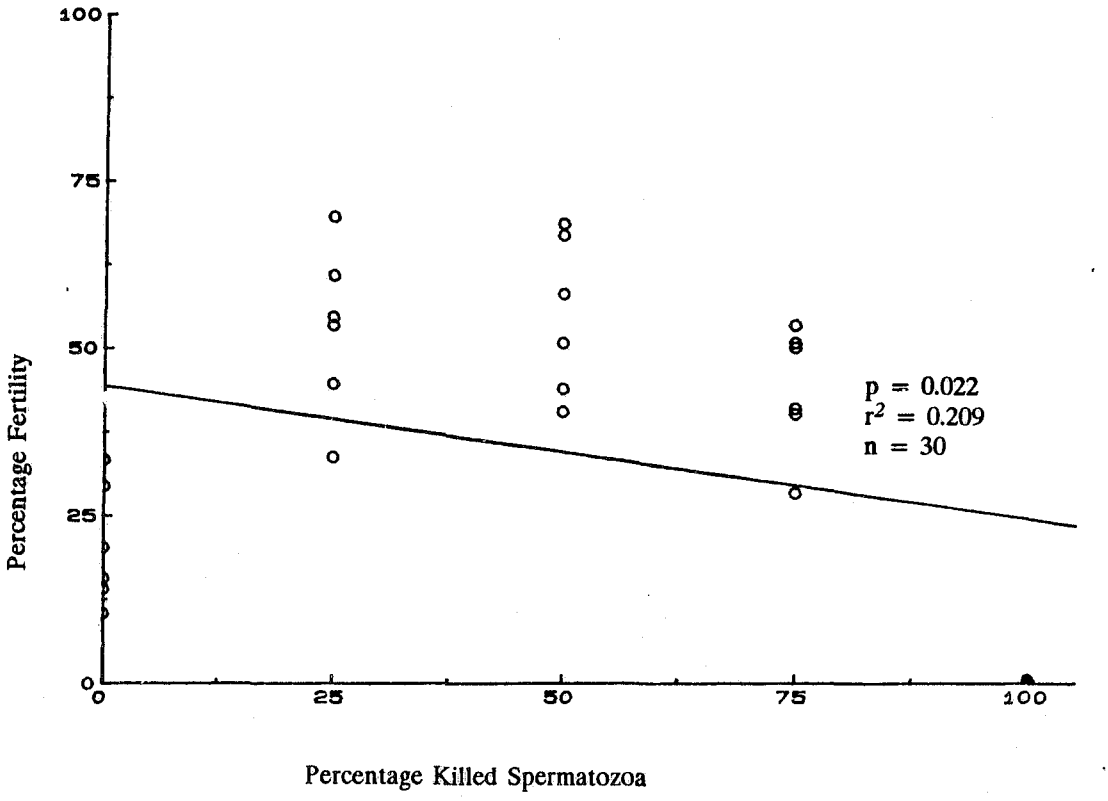

Figure 6. Linear regression line of the percentage of eggs fertilized for different proportions of live/killed spermatozoa of rainbow trout.

TABLE III. **Coefficients of Variation for Motility, Fluorometry, LDH Leakage, Fertility and Eyed Eggs for Proportions of Live/Killed Rainbow Trout Spermatozoa (n = 6)**

Killed Sperm %	*Motility CV*	*Fluorometry CV*	*LDH Leakage CV*	*Fertility CV*	*Eyed Eggs CV*
0	0	3.7	3.8	44.1	44.1
25	6.2	1.6	1.6	23.7	23.7
50	8.7	1.6	2.4	21.4	21.4
75	0	1.0	2.0	21.4	21.4
100	a	1.0	5.0	b	b
Average	3.0	1.8	3.0	27.7	27.7

a Average motility 0 causing CV in the formula CV = (S/Y) x 100 to be undefined

b Average fertility and eyed eggs approximately 0 causing CV to be very large.

2.4 Discussion

Fluorometry gives a good indication of the percentage of dead cells in a sample. The technique is straight forward and rapid. The method does not contain the subjectiveness of motility ratings and, unlike LDH determinations, requires only a small volume of semen. Halangk and Bohnensack (25) used the fluorescence of EtBr bound to the DNA of bull sperm to estimate and intactness of cells. Bilgili *et al.* (32) compared fluorometry to eosin-nigrosin differential staining for chicken sperm and found a positive correlation between the two techniques ($r=0.99$; $p<0.001$). Differential staining has not been reported for fish sperm. This technique was attempted with limited success at this laboratory. In our study, a highly positive correlation was observed between the proportion of live/killed sperm and the percentage of dead sperm measured by fluorometry, indicating the accuracy of this technique. The small CV for fluorometry supports this concept. Our findings support the results of Bilgili *et al.* (32) who observed a high accuracy in estimating the predicted percentages of dead chicken sperm by fluorometry in prepared mixtures.

Lactate dehydrogenase leakage is a common diagnostic tool for *in vitro* diagnosis in medicine. Highly elevated LDH levels in the serum are an aid in diagnosing myocardial infarction, renal infarction or trauma, acute hepatitis, disseminated malignancy and pernicious anemia. LDH can be found in five isomeric forms with different combinations in tissues. Zinkman *et al.* (35) isolated a sixth isoenzyme from the testes and spermatozoa of human and rabbit accounting for 80 to 100 percent of the LDH activity in these tissues. LDH isoenzymes have not been isolated from fish semen. The highly significant relationship between LDH concentration and the percentage of killed sperm suggests this method might be a valuable aid to assess salmonid sperm viability. Our findings support the results of Bilgili and Renden (24) who reported a significant relationship between AST leakage and percentage of killed chicken sperm. Wischart and Palmer (33) cryopreserved fowl and turkey sperm and used LDH concentration to evaluate freezing success. They expressed LDH activity in seminal plasma as a percentage of maximal LDH collected after sonication to standardize LDH concentration for the two species investigated. Our study measured

the activity of LDH leaking into the seminal plasma. The semen used in the experiment was a pooled sample thus eliminating possible variation of LDH content that might be experienced when working with individual fish or different species.

The LDH method does have limitations. The volume of semen required to determine LDH concentration is substantally greater than the volume required for fluorometry. The collection of seminal plasma takes time and there may be damaging effects during centrifugation although this was not studied during this experiment. Also, LDH activity may vary from fish to fish and during the reproductive cycle.

Motility is a quick and accurate parameter to assess the viability of sperm cells but requires considerable experience and may vary between individuals. The conditions under which motility ratings are made may vary between laboratories. The present motility ratings were carried out by dilution with 0.12 M NaCl as reported by Terner and Korsh (36). The motility of sperm diluted by salt solutions remains longer than sperm diluted by water, thus giving added time to perform the motility rating. Sperm diluted by water are likely killed by osmotic shock within 30 seconds of activation (37). Motility ratings were performed at 4°C. This constant temperature assured against temperature shock to sperm cells by keeping materials in contact with the cells at the same temperature and reducing the heat of the microscope bulb. The temperature of activation can have a drastic effect on motility. Lindroth (38) studied motility of brown trout spermatozoa and found that sperm activated at 20°C, 10°C and 5°C had motility duration of 35, 60 and 90 seconds respectively.

Ovarian fluid is the ultimate activator of sperm during fertilization. However collection of ovarian fluid daily for experimental purposes is not practical and may lead to variable motility results depending on fluid composition. Ovarian fluid from a source containing dead or broken eggs may cause sperm clumping and thus difficulty in obtaining a motility rating.

Based on the coefficient of determination, the more accurate technique to determine rainbow trout sperm concentration appeared to be the optical density measurements compared to the

spermatocrit. Others have looked at the coefficient of variation and determined that estimating chicken sperm concentration by optical density (CV = 2.24%; (39)) was superior to estimating sperm concentration by spermatocrit (CV = 10.2%; (40)). These authors have looked at replicates on the same subsample expressing a CV for sets of replicates. This experiment evaluated optical density and spermatocrit using a range of dilutions with one or two observations per dilution.

Bouck and Jacobson (2) performed sperm counts and spermatocrits for coho salmon and steelhead trout. They pooled data for the two species and observed a r^2 of 0.578 ($p<0.05$) and concluded spermatocrit was a useful field technique to estimate fish sperm concentration, however recommended future investigators establish new standard curves increasing centrifugation time from 10 to 15 minutes.

Brillard and McDaniel (39) reported correlation coefficients for optical density and haemacytometer counts ranging from 0.78 - 0.93 for undiluted and diluted samples of chicken semen. Others have found a high correlation between optical density and haemacytometer techniques for other types of fowl (41, 42).

Similarly, high correlations were found between spermatocrit and haemacytometer techniques ($r = 0.83$ and $r = 0.77$) with chicken semen undiluted and diluted 1 to 1 with Tyrodes diluent (39) which was in agreement with work reported by Shaffner and Andrews (43), Taneja and Gowe (42) and Arscott and Kuhns (44).

Brillard and McDaniel (39) observed a non-significant correlation between optical density and spermatocrit for semen diluted 1:3. Our study utilized a range of dilutions of various concentrations and observed significant trends for optical density and spermatocrit with correlation coefficients of 0.991 and 0.944 respectively. Using the Fisher Z transformation, we determined spectrophotometry to be superior method. Therefore, optical density was employed to determine sperm concentration of the live/killed treatments before fertilizing eggs.

The fertility results are more variable than motility, fluorometry and LDH determinations. A critical ratio of 200,000 sperm/egg proposed by Billard *et al.* (3) was used by Moccia and

Munkittrick (45) to maintain high fertility of rainbow trout eggs. The average number of eggs per lot was under-estimated in this experiment (400 eggs) thus reducing the ratio to 150,000 sperm/egg. Limiting the number of sperm to a minimum served to increase the sensitivity of the trial (46, 32) but may have added variation to the results. Using minute volumes of semen to fertilize eggs made it difficult to distribute semen evenly over all eggs. A sperm diluent might be beneficial in the fertilization process. The linear relationship of fertility and percentage dead in this experiment supports the results of Bilgili *et al.* (32) who reported a linear model ($p<0.001$) with an r^2 of 0.30 for percentage fertility and percentage of killed sperm for chicken inseminated with mixtures of live/killed semen.

Motility, fluorometry and LDH leakage show good potential to predict fertility of rainbow trout sperm. However, the fertility results presented in this experiment warrant further investigation. The value of using motility to predict fertility is limited by the subjectiveness of the method although many researchers have based success during fresh storage or cryopreservation solely on motility. Enzyme leakage is variable and raises questions of standardizing enzyme concentrations when using new pools of semen and semen from different fish or species. Fluorometry has excellent repeatability and gives a good indication of damage during storage or in this experiment damage by sonication.

3. FRESH STORAGE OF RAINBOW TROUT SEMEN

3.1 Introduction

Techniques for short-term preservation of salmonid semen may be valuable to hatchery operations for the following reasons:

1) male salmonids frequently mature before females, thus necessitating collection and short-term storage of semen before fertilization of eggs

2) freshly collected and stored semen can be shipped short distances for fertilization or to centres for cryopreservation

3) collection, evaluation and storage of semen for one to several days prior to collection and fertilization of eggs allows the hatchery to use the highest quality semen to carry out the desired pair matings.

It is generally accepted that sperm kept at about 0°C will survive for several days (47, 37). Recent research has identified new conditions to store fresh semen for longer periods of time. Buyukhatipoglu and Holtz (13) found that when undiluted semen was stored at 4°C under an oxygen atmosphere, fertilizing capacity was retained for up to 15 days. The addition of antibiotic and antimycotic agents helped prolong storage for longer than three weeks (12). Billard (48) found that an oxygen atmosphere was superior to air for sperm storage. Sperm are dependent on oxygen for survival and, as a result of respiration, a reduction of oxygen in the medium may limit storage time. At high sperm concentrations, rapid exhaustion of oxygen may occur during short-term storage. Attempts to dilute semen with an extender to decrease sperm concentration for storage proved unsuccessful (49). Stoss *et al.* (50) concluded that when chilled semen was stored in open vials under oxygen, sample depth should be limited to 5-6 mm and agitation should be reduced to a minimum. Stoss and Holtz (51) found that a moisture-saturated oxygen atmosphere, created by passing oxygen through water, allowed sperm to retain its full fertilizing capacity for 34 days. However, they used 2 ml of stored semen for batches of approximately 250 eggs. These large volumes of semen likely enhanced their fertility results by increasing the live sperm to egg ratio.

Transfer of oxygen to living cells through the development of artificial blood substitutes such as fluorocarbon compounds has shown promise in human medicine (52). Fluorocarbons (FC) are liquids that are synthesized by replacing the hydrogen atoms of hydrocarbons with fluorine atoms. Fluorocarbons have a great capacity to hold oxygen. The solubility of oxygen in fluorocarbon is 16 times greater than water (53). Fluorocarbons also have excellent carbon dioxide solubility, a necessary property for sperm storage. An accumulation of carbon dioxide in the seminal plasma of stored semen may lower the pH and harm the spermatozoa. Fluorocarbons are chemically inert and non-toxic (52). Fluorocarbons are non-polar compounds and must be emulsified with the aid of a surfactant to mix with the extender. Pluronic F-68 is widely accepted as the surfactant for fluorocarbons because it is highly water soluble and non-toxic (54). The use of fluorocarbon emulsions to store poultry semen above 0°C has been investigated by Rogoff (55). There are no reports of storage of fish semen in fluorocarbon emulsions or storage of semen with non-emulsified fluorocarbon compound.

The objective of this study was to evaluate the storage of rainbow trout semen at 4°C undiluted as well as diluted with several media such as fluorocarbon, fluorocarbon emulsions and extender under an atmosphere of moisture-saturated air or an atmosphere of non-saturated air. Experiments were carried out with simplified equipment adaptable to hatchery design. Sperm viability was evaluated over time by the *in vitro* methods of motility, fluorometry and LDH leakage (Chapter 2) and also by fertility trials.

3.2 Materials and Methods

Fresh semen was collected from 30 mature adult rainbow trout held in outdoor circular cement ponds supplied with river water at 2°C. Fish were anesthesized in MS-222 at a concentration of 100mg/L of water, rinsed in fresh water, and wiped dry. Semen was collected by abdominal massage into clean glass jars and placed on crushed ice. Motility ratings were made on the semen samples using the procedure outlined in Chapter 2 and samples with ratings of 9 or 10 were pooled. Lyophilized antibiotic/antimycotic (penicillin 10,000 U/ml, amphotericin-B 25 μg/ml and streptomycin 10,000 μg/ml) was rehydrated with fresh seminal plasma collected from pooled

semen by centrifugation at 500g for five minutes, and 500 U (based on penicillin) of the mixture were added per milliliter of pooled semen and mixed by inversion. An optical density reading was taken on the pooled semen as outlined in Chapter 2 and the regression equation developed in Chapter 2 (sperm concentration = 12.3 x absorbance - 0.372) was used to determine the concentration of sperm in the pooled semen.

Fresh rainbow trout semen was stored using five treatments in two gas atmospheres to identify the superior storage conditions. Treatments evaluated included: 1) semen undiluted; 2) semen diluted 1:1 with fluorocarbon; 3) semen diluted 1:1 with a 1:1 fluorocarbon-extender emulsion; 4) semen diluted 1:1 with a 2:1 fluorocarbon-extender emulsion and 5) semen diluted 1:1 with extender. Duplicates of each storage treatment were stored under one of two atmospheres: a moisture-saturated air atmosphere or a non-saturated air atmosphere. The moisture-saturated air atmosphere was set up similar to Stoss and Holtz (51) except that an aquarium aerator was used as an air supply and aeration stones were placed in the first aeration container and at the bottom of the desiccator to assure full saturation of the air. The aquarium aerator pumped air through the system throughout the duration of the experiment. The non-saturated air atmosphere was created by covering storage vessels with parafilm. Fluorocarbon was distilled and collected at 100-105°C, mixed with 20% KOH and 1g of activated charcoal per 200 ml, shaken for 10 minutes and filtered over a column of activated alumina to remove acid impurities detrimental to sperm survival. The extender used in treatments 3, 4 and 5 was a modified extender of Horton and Ott (56) and contained: NaCl, 0.137M; KCl, 0.011M; $Na_2HPO_4.7H_2O$, 0.004M; and l-α-lecithin, 7.5g/L; (237 mOsm/kg H_20). The 1:1 fluorocarbon:extender emulsion was prepared by homogenizing 15 ml of FC, 15 ml of extender and 1.2g of F-68 Pluronic Prill (BASF Co., Montreal, Que.) for 1.5 minutes on a Virtus Homogenizer set at 60 per cent. The emulsion was centrifuged at 500g for 5 minutes and the foam aspirate removed. The emulsion was resuspended by vigorous agitation. The 2:1 FC:extender emulsion was prepared in the same way except that 20 ml of FC and 1.6 g of Pluronic Prill were added to 10 ml of extender.

The distilled fluorocarbon for treatment 2, the FC emulsions for treatments 3 and 4 and the extender for treatment 5 were oxygenated for 20 minutes with compressed oxygen fed by small diffusion stones. All diluents were cooled to 4°C before addition of semen.

Twenty 50 ml beakers were cooled to 4°C to be used as storage vessels for the semen. The final stored volume in each beaker was 10 ml corresponding to approximately 4 mm of storage depth as reported by Stoss *et al.* (50). The contents of all storage vessels were swirled gently to mix semen and diluent.

3.2.1 Protocol to Evaluate Sperm Viability Over Time

The viability of semen stored under the five treatments and the two air atmospheres was estimated weekly by the *in vitro* methods: motility, fluorometry and LDH leakage and by fertility trials during week 1, week 3 and week 5 of storage. Two times each week, motility ratings and the proportions of dead sperm (fluorometry) were estimated on samples of stored sperm from each treatment. Samples were collected by swirling the contents of each storage vessel and removing a small sample by pipet. The procedures for motility and fluorometry were the same as used in Chapter 2. An LDH measurement was taken weekly on the aqueous phase (seminal plasma and extender) of stored semen from the sample collected for motility and fluorometry. The procedure used was the same as outlined in Chapter 2 except that seminal plasma was diluted at a ratio of 1:1 with ammonium sulfate instead of 1:6.

LDH activity was adjusted for some treatments to account for the differences in sperm concentration in the different storage treatments. LDH measurements for treatment 1 were not adjusted because semen was not diluted in these vessels. LDH measurements for treatment 2 were taken only on the semen layer that floated on top of the fluorocarbon layer so no adjustment was required. The fluorocarbon was analyzed for the presence of LDH activity but none was found. LDH measurements for treatment 3 were multiplied by a factor of 1.5 to correct for dilution with extender. LDH measurements for treatment 4 were multiplied by 1.25 to account for dilution with extender and LDH measurements for treatment 5 were multiplied by 2.0 to account for dilution of semen by extender.

Fertility measurements were conducted during week 1, week 3 and week 5. Eggs for each fertility trial were collected from mature adult rainbow trout held in hatchery water at 9-10°C. Eggs from individual fish were collected by abdominal massage and clean samples (lots containing few egg cases or blood) were pooled. Eggs were divided into lots of approximately 300 eggs. Stored semen was transported to the hatchery in capped vials immersed in hatchery water. Semen was allowed to acclimate to the temperature of the eggs before fertilization. One lot of eggs was fertilized per sample of stored semen (5 treatments x 2 replicates x 2 atmospheres) so that 20 lots of 300 eggs were fertilized during each fertility trial. Eggs were fertilized with approximately 1.4 x 10^6 sperm per treatment, the number of sperm corresponding to 50 μl of semen. A small volume of hatchery water was added to the eggs 4-5 minutes after fertilization and left for 2-3 minutes. The eggs were then rinsed three times to remove any excess ovarian fluid and semen and allowed to water harden for approximately 30 minutes in their fertilization dishes. Egg lots were then placed randomly in small compartments of Heath Tray incubators. During each fertility trial semen was collected from several adult male fish to serve as control semen. Motility ratings were made on the semen samples and good quality samples were pooled to serve as control semen. Optical density readings were taken on the pooled sample to determine the concentration of sperm in the semen and three lots of 300 eggs were fertilized with 50 μl samples from the pool.

Eggs that turned opaque were picked periodically and cleared in a solution of methanol, acetic acid and water (1:1:1) to check for the presence of an embryo (indication of fertilization). When experimental eggs reached the eyed stage the number of eyed eggs was counted and used to calculate the percentage of eggs fertilized and the percentage of eggs reaching the eyed stage. These values were expressed as as percentage of control values.

3.2.2 Statistical Analysis

Regression analysis and analysis of variance were used to determine the superior storage conditions by the *in vitro* methods and fertility. Motility data over the 37 days for each treatment and atmosphere of fresh storage were subjected to linear regression analysis. Motility data were included in the regression until the rating fell below 0.5. For each regression equation, the number

of days for semen in the treatment to reach 0 motility was predicted. Predicted values for day of 0 motility were compared by two-way analysis of variance to determine if there was any significant difference between treatments and between atmospheres. The Student-Newman-Keuls (SNK) test was then used to determine which treatment and atmosphere prolonged motility for a greater number of days. Similarly for fluorometry and LDH leakage, data were regressed on storage time by regression analysis and the number of days for semen in each treatment to reach 95.0% dead sperm and 700 U/L LDH activity were predicted. These predicted values were compared by two-way analysis of variance and the SNK test to determine the superior storage conditions by fluorometry and LDH leakage.

Fertility data were analyzed by analysis of variance with a 5 x 2 factorial design. Data for each of the three fertility trials were analyzed by analysis of variance and SNK tests to determine which combination of treatment and atmosphere gave the greatest number of days of viable sperm.

3.3 Results

The regression equations describing the relationships between motility, proportion of dead sperm and LDH concentration, and time for each treatment and atmosphere were significant ($p<0.05$). Appendix A contains the regression equations and the p values for the estimations of sperm viability for each treatment and atmosphere. Table IV presents the predicted number of days for 0 motility, 95% dead sperm by fluorometry, and 700 U/L LDH activity for each treatment and atmosphere. Analysis of variance for the motility data revealed there was a significant difference between treatments ($p<0.05$) and a significant difference between atmospheres ($p<0.05$). SNK testing indicated that treatment 2, semen stored above the fluorocarbon layer, exhibited significantly longer duration of viable sperm ($p<0.05$). The moisture-saturated air atmosphere (MSA) gave significantly longer duration of viable sperm than the non-saturated air atmosphere (NSA). Figure 7 displays a comparison of regression lines for semen stored above the oxygenated fluorocarbon layer and semen stored undiluted in the MSA. Semen stored above the oxygenated fluorocarbon layer gave motility ratings of 5.0 on day 37 of the experiment while semen stored undiluted did not exhibit motility after 9 days.

Table IV. **Predicted number of days for semen stored under five treatments and two atmospheres at 4°C to reach 0 motility, 95% dead sperm and 700 Units/Liter of LDH activity in the seminal plasma.[a]**

Semen Treatment	Atmosphere	Days of Storage (motility, y=0)	Days of Storage (fluorometry,y=95%)	Days of Storage (LDH,y=700U/L)
Non-diluted	MSA[b]	9.15	26.35	16.33
	NSA[c]	7.87	18.78	18.98
Dilute 1:1 with FC	MSA	83.76	68.04	62.47
	NSA	77.09	59.69	56.92
Dilute 1:1 with FC-extender emulsion	MSA	43.55	38.04	51.84
	NSA	38.95	42.16	80.58
Diluted 1:1 with 2:1 FC-extender emulsion	MSA	46.28	34.67	53.30
	NSA	35.70	37.16	57.73
Diluted 1:1 with extender	MSA	33.67	29.24	35.48
	NSA	18.61	32.07	52.47

[a] Predicted from regression equations for each treatment and atmosphere represented in Appendix B.

[b] Moisture - saturated air atmosphere

[c] Non-saturated air saturated atmosphere

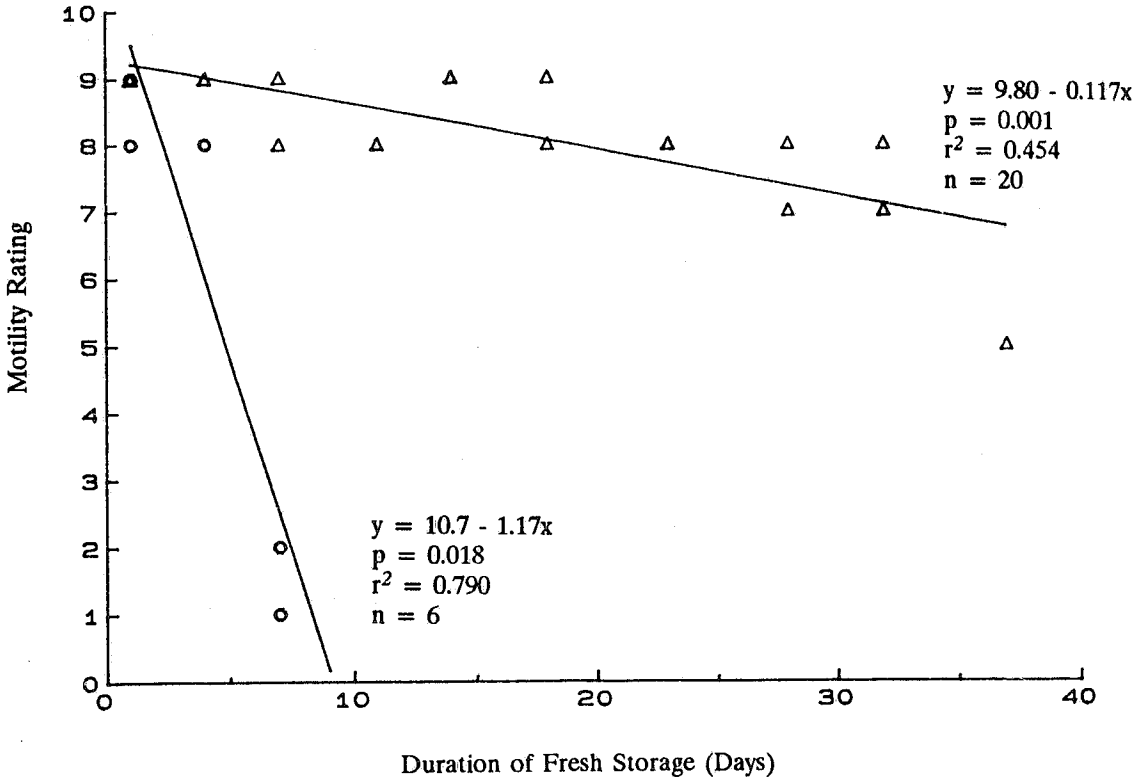

Figure 7. Regression lines comparing motility ratings over 37 days for semen stored on top of an oxygenated fluorocarbon layer (Δ) and semen stored undiluted (o), both in a moisture saturated air atmosphere.

Analysis of variance of the number of days of viable sperm for fluorometry values in Table IV revealed a significant difference between treatments ($p<0.05$). The SNK test indicated that the fluorometry values of percentage dead sperm for semen stored under treatment 2 were significantly lower than semen stored under all other treatments. There was no significant difference detected by fluorometry for the two atmospheres. Figure 8 shows the regression lines for semen stored above the oxygenated fluorocarbon layer and stored undiluted. Semen stored above the oxygenated fluorocarbon layer contained less than 50% dead sperm after 37 days while semen stored under treatment 1 did not contain viable sperm after 20 days.

Analysis of variance of the predicted LDH values revealed a significant difference between treatments ($p<0.05$) and no significant difference between atmospheres. The SNK test indicated there was no significant difference in the time for treatments 2, 3, 4, and 5 to reach the maximum LDH value but semen stored undiluted in treatment 1 deteriorated at a much faster rate than semen stored under the other treatments.

Table V presents the percentages of eggs fertilized for the treatments and atmospheres on day 3, day 21 and day 37. Analysis of the fertility data from day 3 revealed no significant difference between fertility values for the treatments. Semen stored under the moisture-saturated atmosphere fertilized significantly more eggs than semen stored in the non-saturated atmosphere on day 3 ($p<0.05$).

Analysis of fertility data from day 21 revealed a significant difference between treatments ($p<0.05$) but no significant difference between atmospheres. The SNK test revealed that semen stored above the fluorocarbon layer in treatment 2 in the moisture-saturated atmosphere fertilized significantly more eggs than semen stored under all other treatments and atmospheres ($p<0.05$). Semen stored in replicate 1 of treatment 2 in the moisture-saturated atmosphere, deteriorated quickly after day 21, likely due to activation and subsequent death of sperm by moisture falling into the storage vessel.

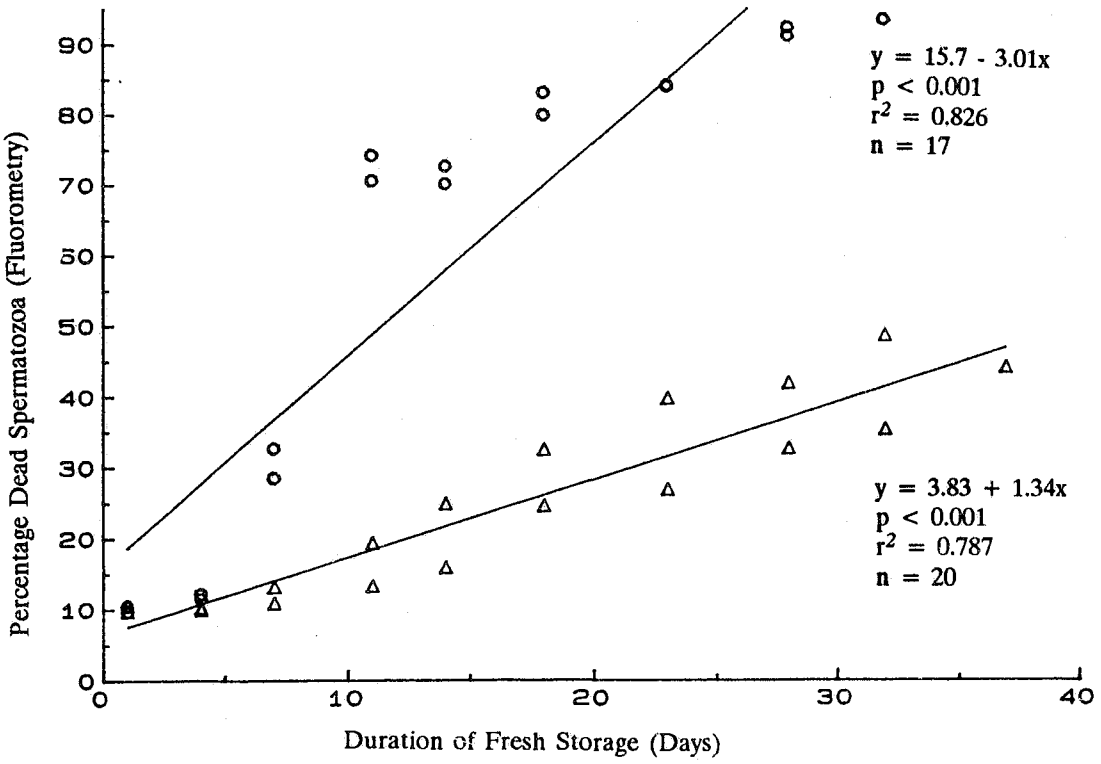

Figure 8. Regression lines comparing percentages of dead spermatozoa estimated by fluorometry over 37 days for semen stored on top of an oxygenated fluorocarbon layer (Δ)and semen stored undiluted (o), both in a moisture saturated air atmosphere.

Table V. **Percentages of eggs fertilized and eyed for semen fresh stored under five treatments and two atmospheres of air at 4°C.**

Semen Treatment	Atmosphere	Replicate	Fertility (% eyed) Day 3	Fertility (% eyed) Day 21	Fertility (% eyed) Day 37
1	MSA	1	85.3(85.2)	0.0(0.0)	0.0(0.0)
1	MSA	2	103.2(103.7)	0.0(0.0)	0.0(0.0)
1	NSA	1	88.3(88.2)	0.0(0.0)	0.0(0.0)
1	NSA	2	9.9(9.9)	0.6(0.6)	0.0(0.0)
2	MSA	1	113.7(113.2)	79.7(79.7)	0.0(0.0)
2	MSA	2	114.2(113.8)	98.0(98.0)	81.2(81.1)
2	NSA	1	106.5(106.5)	22.8(22.8)	8.5(8.5)
2	NSA	2	87.7(87.5)	82.2(82.2)	41.6(41.4)
3	MSA	1	105.4(104.3)	56.8(56.8)	0.0(0.0)
3	MSA	2	99.9(100.4)	18.1(18.1)	0.0(0.0)
3	NSA	1	65.2(65.5)	81.4(81.4)	0.0(0.0)
3	NSA	2	80.3(80.7)	5.2(5.2)	0.0(0.0)
4	MSA	1	102.3(101.7)	40.0(40.0)	0.0(0.0)
4	MSA	2	93.0(93.0)	34.9(34.9)	0.0(0.0)
4	NSA	1	77.6(77.7)	1.5(1.5)	0.0(0.0)
4	NSA	2	72.3(71.7)	6.0(6.0)	0.0(0.0)
5	MSA	1	53.9(53.7)	0.0(0.0)	0.0(0.0)
5	MSA	2	75.9(76.2)	8.4(8.4)	0.0(0.0)
5	NSA	1	77.9(77.0)	9.7(9.7)	0.0(0.0)
5	NSA	2	38.8(38.9)	0.9(0.9)	0.0(0.0)

On day 37 of fresh storage, semen from replicate 2 of treatment 2 in the moisture-saturated atmosphere fertilized 81.1% of the eggs in the lot. This fertility result was significantly greater ($p<0.05$) than fertility results for all other treatments and atmospheres. After 37 days of fresh storage semen stored above the fluorocarbon layer was the only semen to show fertility.

The concentration of pooled semen used in the fresh storage experiment was 8.07×10^9 sperm/ml semen. The concentrations of pooled semen used to fertilize control lots of eggs during each fertility trial were 7.14×10^9 sperm/ml semen on day 3, 8.23×10^9 sperm/ml on day 21, and 8.5×10^9 sperm/ml semen on day 37.

3.4 Discussion

Semen stored above the oxygenated fluorocarbon layer in the moisture-saturated air atmosphere contained viable sperm for 37 days. Viability was not estimated for a longer period of time because weekly sampling used nearly all the semen in the storage vessels. The apparatus was easily set up and adaptable to a hatchery setting. Fluorocarbon acted to provide oxygen to the sperm cells and to remove carbon dioxide from the seminal plasma. Using this technique we were able to store semen for a longer period of time than Stoss and Holtz (51) (37 days vs 34 days) at a higher temperature (4°C vs 0°C) and using less semen (50 μl vs 2 ml) to achieve similar fertility results.

The deterioration of semen stored undiluted in treatment 1 in both atmospheres shortly after the onset of the experiment suggests that an air atmosphere with or without saturation is insufficient for fresh storage of semen. Oxygenation of fluorocarbon emulsions and extender in the other treatments likely prolonged the viability of semen stored under these treatments compared to semen stored undiluted. Rogoff (55) reported significantly better fertilities with air bubbled through turkey semen mixed with fluorocarbon emulsions than semen stored unaerated. The rate of oxygen diffusion into unaerated semen (from air) can not replenish the oxygen utilized by stored semen (57). The fluorocarbon of treatment 2 must have supplied enough oxygen to the sperm cells to maintain viability without a pure oxygen atmosphere. This supports the work of Billard (48) who found that sperm survival was improved when storing with oxygen as compared to air. Perhaps

a combination of oxygenated fluorocarbon such as used in this experiment and a moisture-saturated oxygen atmosphere as proposed by Stoss and Holtz (51) may have prolonged sperm viability for several additional weeks. However, this was not tested in this experiment.

Storage of semen with fluorocarbon emulsions or with an extender did not improve the duration of viable sperm beyond that of the fluorocarbon layer in treatment 2. These findings of the unfavorable effects of extenders on the survival of fish spermatozoa support conclusions of Buyukhatipoglu and Holtz (13) who reported greater motility ratings in semen stored non-diluted. Erdahl and Graham (49) found that high dilution ratios resulted in high fertility values if storage time of diluted semen was less than one hour. For fresh storage of longer periods of time, dilution ratios had to be decreased to maintain fertility. The reason diluted sperm do not remain viable for longer periods of time than sperm stored non-diluted has yet to be determined, although dilution media have proven beneficial to activate sperm prior to fertilization of eggs in order to enhance fertility results when using small quantities of semen (58) or when fertilizing eggs with frozen-thawed semen (59).

The *in vitro* tests used to estimate sperm viability detected differences between treatments. Motility gave a rapid indication of the condition of sperm cells stored over time. Buyukhatipoglu and Holtz (13) used motility as the lone *in vitro* test to estimate sperm viability stored in the liquid state. Motility estimates should not be used as the lone method to assess sperm viability, especially when many dilution rates are tested, because semen samples containing different concentrations of sperm can be difficult to assign a percentage motility under the light microscope.

Fluorometry was very rapid in detecting the percentage of dead sperm in each treatment even when samples were autolytic. The procedure was easily repeatable and did not require adjustment for varying concentration in the different treatments. This observation is supported by the findings in Chapter 2.

LDH leakage was not acceptable as a method to estimate sperm viability of semen stored for long periods of time. The dilutions of sperm with fluorocarbon emulsions and extenders made concentration adjustments difficult because after centrifugation the fluorocarbon came out of

suspension and did not contain LDH activity. In addition, the sampling of semen from treatments that contained large numbers of dead cells was difficult since the semen became gelled and seminal plasma was cloudy after centrifugation, probably because of cell lysis. LDH determination required large volumes of semen for the collection of seminal plasma. This reduced the volume of semen that could be used to fertilize eggs.

Fertility results were supportive of conclusions based on motility and fluorometry results. Treatment 2 was clearly the better storage condition since it was the only treatment on day 37 with fertility values comparable to acceptable hatchery fertilities of 85% (7).

The relative fertility values on day 3 above 100% indicate that the semen used to fertilize control eggs was of poor quality compared to semen stored under some of the treatments. The lower sperm concentration of control semen on day 3 may account for lower fertility in control eggs thus larger relative fertilities in experimental eggs. However, fertility expressed relative to the controls allowed for the comparison of eggs fertilized on different days. The similar results for percentage fertility and percentage of eggs reaching the eyed stage indicate that few eggs died after fertilization. This confirms absence of water chemistry problems or disease during the experiment.

It is noteworthy in this experiment that samples had to be swirled gently two times per week to obtain homogeneous samples to estimate sperm viability by the *in vitro* methods and fertility. It has been the conclusion of others (12, 50) that agitation of sperm cells is detrimental during fresh storage. Additional semen was not available to run a third replicate of each treatment to test the effect of agitation on sperm survival or time. Agitation may have been the cause of some variation in the results of this experiment.

Some investigators believe that semen is best stored by the parent fish. Billard (48) concluded that the fertility of gametes stored *in vivo* remained stable and that semen stored *in vitro* in air or O_2 lost its fertilizing capacity more rapidly. This may not always hold true especially when adult fish undergo stress due to fluctuation in oxygen levels, reduced availability of food or reproductive damage due to male aggression. Techniques of fresh storage are valuable for the collection, evaluation, and storage of viable gametes and are currently available for use to improve

fertility rates and exercise genetic selection. Fluorocarbon compounds saturated with oxygen show much promise for the successful fresh storage of salmonid semen.

4. CRYOPRESERVATION OF ATLANTIC SALMON SPERMATOZOA

4.1 Introduction

Salmon genetics and the preservation of gene pools have been increasingly important to the recent advances in salmon culture in Canada, Norway and Chile. Cryopreservation of salmon sperm is one means to preserve broad-based gene pools for the future. Early investigations into cryopreservation concentrated on the chemical composition of extenders and the choice of cryoprotectant (60, 61, 62). Extenders were prepared to mimic the composition of seminal fluid of fish and contained numerous constituents. Fertility with cryopreserved sperm was poor and not easily repeated by others. Recently, workers suggested that simplified extenders improved storage of salmonid sperm (51, 14), however results are still variable. Dimethyl sulfoxide (DMSO) has gained popularity over other cryoprotectants such as glycerol, methanol, ethylene glycol and propylene glycol to protect salmonid sperm cells during the freeze-thaw process (37, 63, 14).

Traditionally, motility and fertility trials have been used to evaluate the efficacy of extenders and cryoprotectants. Motility ratings are subjective, require experience and are not precise enough to detect small differences between extenders. Fertility results have been variable in many cases. Differences in sperm concentration and variation in sperm and egg quality during the reproductive cycle may account for the difficulty of isolating superior extenders.

Measurement of enzyme leakage from cells following cryopreservation has been used to determine sperm viability in chickens (33) and has been evaluated for rainbow trout semen in Chapters 2 and 3. No enzyme detection following cryopreservation has been reported for Atlantic salmon sperm.

The fluorescence of genetic material of damaged cells (fluorometry) has been used to assess the viability of cryopreserved chicken, bull and human sperm (24, 25, 26). Results in Chapters 2 and 3 support the use of fluorometry to estimate the viability of stored rainbow trout sperm. Cells damaged during cryopreservation are detected by penetration of the fluorescent dye, ethidium bromide (EtBr) into the interior of the cell with resultant fluorescence of genetic material (24).

The method is reliable and repeatable. Fluorometry also allows the investigator to determine sperm concentration since the fluorescence of genetic material is directly proportional to the sperm concentration when cells are made fully permeable to EtBr by addition of digitonin (25). The simultaneous determination of sperm viability and concentration using fluorescent techniques will eliminate the need to perform optical density measurements to determine sperm concentration when in the laboratory.

Evidence suggests that sperm exhibit a wide variation in freezing ability between individual fish at the same stage in the reproductive cycle and between populations (64, 20). Selection of fish with superior freezing ability of sperm and selective breeding of such characteristics will advance cryopreservation in salmonids. The development of sperm cryopreservation through selection in chickens supports this concept (55).

The objectives of this study were to: (1) identify with the use of fluorometry, two successful diluents (composed of extender and cryoprotectant) for freezing Atlantic salmon sperm and, (2) determine the variability in freezing ability of spermatozoa amoung fish using fluorometry, motility and lactate dehydrogenase leakage when freezing sperm.

4.2 Materials and Methods

4.2.1 Evaluation of Extenders and Cryoprotectant Level - Experiment One

Fifteen diluents were evaluated to cryopreserve Atlantic salmon sperm; these consisted of five extenders, each with three levels of DMSO. Extender 1 was modified from Horton and Ott (56) and contained: NaCl, 0.137 M; KCl, 0.011 M; $Na_2HPO_4.7H_2O$, 0.004 M; and L-α-lecithin, 7.5 g/L; (237 mOsm/kg H2O). Extender 2 was modified from Stoss and Holtz (20) and contained: NaCl, 0.101 M; KCl, 0.023 M; $CaCl_2$, 0.007 M; $MgSO_4$, 0.001M; tris[hydroxymethyl]aminomethane, 0.200 M; bovine serum albumin, 4.000 g/L; (248 mOsm). Extender 3 was modified from Erdahl and Graham (63) and contained: NaCl, 0.100 M; KCl, 0.034 M; $MgCl_2.6H_2O$, 0.001 M; tris[hydroxymethyl]aminomethane, 0.165 M; citric acid, 0.0005 M; D-glucose, 0.056 M and KOH, 0.178 M; (408 mOsm). Extender 4, (65) contained: sucrose, 0.125 M; $KHCO_3$, 0.100 M and reduced-glutathione, 0.0 065 M; (275 mOsm). Extender 5, (10) contained: NaCl, 0.088 M; KCl,

0.022 M; $CaCl_2$, 0.002 M; $NaHCO_3$, 0.012 M; $NaH_2PO_4.H_2O$, 0.003 M; fructose, 0.0056 M; (177 mOsm). Extender 1 was previously reported to cryopreserve coho salmon sperm and was modified to include KCl. Extenders 2 was reported to cryopreserve rainbow trout sperm and was modified to contain bovine serum albumin instead of promine-D. Extender 3 was modified to contain tris instead of bicine. Extenders 4 and 5 were reported to cryopreserve salmon sperm. All extenders were adjusted to pH 7.5 with citric acid or sodium hydroxide and cooled to 4°C.

A volume of three millilitres of extender was dispensed into five sets of three tubes. DMSO was added to each set of tubes at 8, 10 and 12 percent of extender volume. Semen was collected from mature adult Atlantic salmon held in indoor tanks supplied with river water at 4°C. Each fish was anesthesized with MS-222 (100 mg/L), rinsed in fresh water, and wiped dry. Semen was collected by abdominal massage into glass jars and stored on crushed ice. A motility rating was performed on each sample as described in Chapter 2 and samples with ratings of 9 or 10 were pooled and held at 4°C. A volume of fresh semen was added to each set of tubes so that semen was diluted 1:3 (semen:diluent).

Four 0.5 ml clear straws (United Breeders Inc, Guelph, Ont.) were labelled and filled for each tube. Straws were heat sealed (Lorvic Corp. St. Louis, MO) and loaded into chamber furniture of a Kryo 10 Cell Freezer (Planer Biomed, Diamed Lab Supplies, Mississauga, Ont.). The freezer was programmed to cool straws from 4°C to -90°C at a rate of -30°C per minute. Frozen straws were placed in visutubes, which were attached to canes and stored in liquid nitrogen.

Fluorometry was used to estimate the viability of sperm stored under the fifteen treatments. Straws were thawed by immersion in a 4°C water bath for approximately 12 seconds. Straws were wiped dry and the ends clipped. Thawed semen was placed in capped vials on crushed ice during fluorometry. Fluorometric determination of percentage dead sperm was carried out as outlined in Chapter 2.

4.2.1.1 Statistical Analysis - Experiment One

Fluorometry values for the fifteen diluents (five extenders and three cryoprotectant levels per extender) were transformed by the arc sine transformation. One-way analysis of variance and

the Student-Newmans-Keuls test were used to isolate the two diluents with the lowest proportions of dead sperm.

4.2.2 Freezability of Sperm from Individual Fish - Experiment Two

Following isolation of the two best diluents to cryopreserve Atlantic salmon sperm, the variability of the success of cryopreservation of spermatozoa among fish was examined. Ten millilitres of the two extenders were added to two sets of six tubes. DMSO was added to each tube at the selected level. Extender and cryoprotectant were mixed and cooled to 4°C.

Semen was collected from 12 fish in the same tank as the fish used to evaluate extenders in Experiment One. Semen samples were rated by motility and six samples with a rating of 10 were selected for cryopreservation. Volumes of 3.73 ml of semen from Fish 1 - 6 were added to the six tubes containing the first diluent. Likewise, 3.73 ml of semen from Fish 1 - 6 were added to the six tubes containing the second diluent. Semen and diluent were mixed gently by inversion. Fifty 0.5 ml straws were filled from the semen and diluent mixture for each fish: 25 straws for each diluent selected in Experiment One. Straws were heat sealed, loaded into chamber furniture of the cell freezer and cryopreserved with the same program as Experiment One. Straws were transferred to liquid nitrogen following cryopreservation.

4.2.2.1 *In vitro* Tests of Sperm Viability

Motility, fluorometry and LDH leakage were used to evaluate the freezability of sperm among fish and to isolate the best diluent for freezing sperm from individual fish. Motility ratings, fluorometry measurements, and LDH activity of the seminal plasma were carried out by the methods described in Chapter 2. To determine LDH activity, seminal plasma was diluted 1:3 with 3.2 M $(NH_4)_2SO_4$ instead of 1:6 since semen was already diluted 1:3 with diluent. Each test was performed on three straws thawed in 4°C water.

In Experiment One, semen was pooled so that each straw contained approximately the same concentration of sperm. In Experiment Two, semen was cryopreserved from individual fish. Because sperm concentration varies from fish to fish, LDH activity in the seminal plasma following cryopreservation varies according to the concentration of cells preserved. Two methods were

evaluated to predict salmon sperm concentration to adjust LDH activity.

4.2.2.2 Salmon Sperm Concentration by Optical Density and Fluorometry

To determine the concentration of spermatozoa in each straw, regression equations relating optical density to sperm counts and fluorescense to sperm counts were evaluated. Because two extenders were used in Experiment Two, regression lines to predict sperm concentration by optical density and fluorometry were developed for each extender to determine if there was a significant influence of extender when predicting sperm concentration.

Frozen-thawed salmon sperm was used to prepare concentration curves. Straws were thawed in 4°C water, and the contents were centrifuged at 500g for 5 minutes at 4°C. The seminal plasma and diluent were discarded and the sperm pellets were pooled. Pooled sperm was dispensed into two sets of 15 tubes in volumes of 5 to 150 μl. The extenders used in Experiment Two were used to dilute sperm samples so the final volume of each tube was 150 μl. For each extender and dilution, three 25 μl samples were withdrawn and placed in 4.975 ml of a 1.1 M sodium citrate solution. The mixtures were inverted slowly 10 times and the optical density readings were determined by a spectrophotometer. In addition, three 10 μl samples were withdrawn from each tube and suspended in 3.990 ml of EtBr solution. A 25 μl sample of digitonin solution was added to the diluted sperm and EtBr solution, inverted slowly for one minute and a fluorescence reading recorded.

Triplicate sperm cell counts were made for each tube of sperm diluted with the extenders. A haemacytometer was filled by a non-heparinized capillary tube and sperm cell counts were made under a compound microscope. Sperm counts were corrected for the dilution factor and averaged. Linear regression analysis was used to determine a line equation for the two extenders by optical density and fluorometry. The slopes of the lines for optical density for the two extenders were compared by a Student-t test to see if there was a significant effect of extender when determining sperm concentration by optical density. The slopes of the lines for fluorometry for the two extenders were compared to see if there was a significant effect of extender when determining sperm concentration by fluorometry.

Correlation coefficients for optical density and fluorometry versus sperm counts were compared by Fisher Z transformation to isolate the better method to determine sperm cell concentration. This method was then used to adjust LDH activity of seminal plasma to account for the concentration of sperm in the freezing diluent. LDH activity was expressed as Units of LDH per litre of seminal plasma per billion of cryopreserved sperm cells per millilitre of semen.

A fertility trial of frozen-thawed sperm was attempted with eggs collected from adult salmon. There was no fertility in experimental lots or control lots suggesting eggs were either overripe before fertilization, were contaminated with blood or feces, or died prior to detection of the embryo as described in Chapter 2.

4.2.2.3 Statistical Analysis - Experiment Two

Data were analyzed by analysis of variance. Fluorometry values were transformed to arc sine as in Experiment One. Univariate analysis of variance was used to test the significance of the *in vitro* methods (motility, fluorometry and LDH activity) in detecting differences in the freezability of sperm from individual fish and differences between the extenders.

4.3 Results

Figure 9 is a histogram of the mean and standard deviation of fluorometry values for the five extenders and three cryoprotectant levels of Experiment One. One-way analysis of variance and the Student-Newman-Keuls test indicated that Extender 1 and Extender 4 at a 12 percent DMSO level resulted in significantly lower ($p<0.05$) percentages of dead sperm following cryopreservation than the other extenders and cryoprotectant levels. Therefore, the modified extender of Horton and Ott (56) and the extender of Mounib (65) were used in Experiment Two. Twelve percent DMSO resulted in a significantly lower percentage of dead cells than 8 and 10 percent DMSO for sperm cryopreserved with extenders 1, 4 and 5. There was no significant difference between percentage dead cells observed for 10 and 12 percent DMSO for extenders 2 and 3, although both levels were superior to 8 percent DMSO.

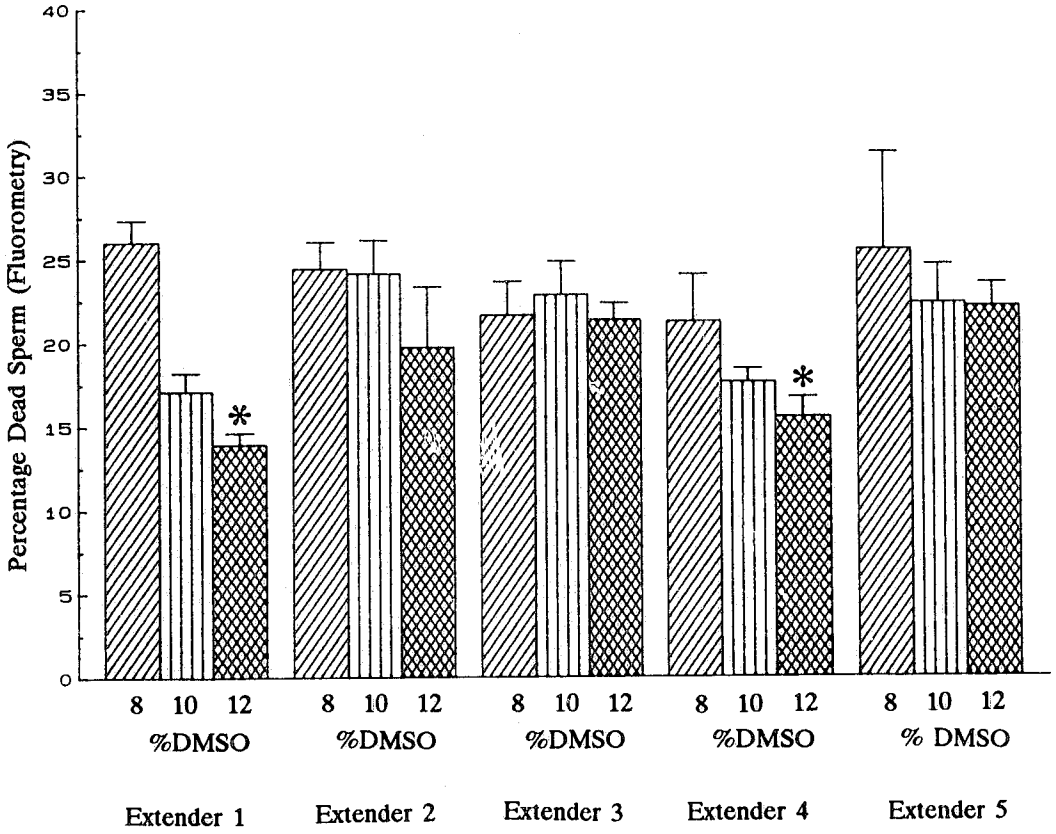

Figure 9. Mean and standard deviation of percentage dead sperm determined by fluorometry for Atlantic salmon sperm cryopreserved with five extenders and three levels of cryoprotectant (DMSO).

* significantly lower percentage of dead sperm by fluorometry

Table VI presents the mean values (-+SEM) for motility, fluorometry, and LDH activity of seminal plasma for the six fish and two extenders in Experiment Two. LDH activity was adjustedto account for sperm concentration (by fluorometry) of each straw following cryopreservation. The percentages of dead sperm and LDH activity of seminal plasma of semen cryopreserved with Extender 1 were consistently higher than cells cryopreserved with extender 4, suggesting extender 4 was superior. However, the motility ratings for sperm cryopreserved with extender 1 were higher than extender 4, suggesting extender 1 was superior, contradictory to fluorometry and LDH results. Figure 10 is a plot of the optical density and sperm concentration determined by a haemacytometer for the two extenders used in Experiment Two. Linear regression analysis of sperm concentration on optical density for Extender 1 revealed a highly significant trend ($p<0.001$) with an r^2 of 0.980. Linear regression analysis of sperm concentration on optical density for Extender 4 also revealed a highly significant trend ($p<0.001$) with an r^2 of 0.984. Comparison of the slopes for the two extenders indicated a significant ($p<0.05$) difference between the two regression lines.

Figure 11 is a plot of fluorometric intensity and sperm concentration determined by haemocytometer for the two extenders. Linear regression of sperm concentration on fluorometric intensity for Extender 1 revealed a highly significant relationship ($p<0.001$) with an r^2 of 0.976. Linear regression analysis of sperm concentration on fluorometric intensity for Extender 4 also revealed a highly significant relationship ($p<0.001$) with an r^2 of 0.986. Comparison of the slopes for the two extenders indicated there was significant difference ($p<0.05$) between the two regression lines. Comparison of the correlation coefficients of optical density versus sperm concentration and fluorometry versus sperm concentration for either extender indicated no significant difference between the two methods.

Analysis of variance of motility, fluorometry and LDH leakage on semen of different fish indicated non-significant relationships for motility and LDH leakage but a significant relationship

TABLE VI. **Estimation of sperm viability by motility, fluorometry and LDH activity in seminal plasma for Atlantic salmon sperm from six fish cryopreserved with two extenders (SEM for n=3).**

Fish	Extender	Motility Rating 0-10	Fluorometry % dead sperm	LDH activity units/L billion sperm[a]
1	1	4.1 (0.59)	31.0 (3.56)	18.1 (0.26)
1	4	3.0 (0.71)	19.2 (0.89)	8.5 (0.09)
2	1	3.4 (0.55)	19.4 (0.80)	16.6 (0.08)
2	4	1.9 (0.77)	16.2 (0.33)	12.4 (0.30)
3	1	4.0 (0.41)	30.7 (1.02)	27.2 (0.49)
3	4	2.3 (0.63)	20.0 (1.96)	13.3 (0.05)
4	1	4.0 (0.71)	33.6 (2.91)	28.1 (0.30)
4	4	0.9 (0.38)	20.4 (0.47)	14.7 (0.29)
5	1	2.3 (0.48)	24.8 (0.48)	15.1 (0.31)
5	4	0.8 (0.14)	17.1 (1.10)	12.8 (0.8)
6	1	3.5 (0.29)	23.9 (1.32)	14.4 (0.20)
6	4	2.1 (0.52)	14.3 (0.35)	12.2 (0.16)

[a] LDH activity in Units per liter of seminal plasma and diluent with a 1 to 3 dilution divided by sperm concentration of each fish expressed as billions of sperm per millimeter of straw content.

[b] Raw data for unadjusted LDH activity, fluorometric intensity and predicted sperm concentrations for the six Atlantic salmon is contained in Appendix C.

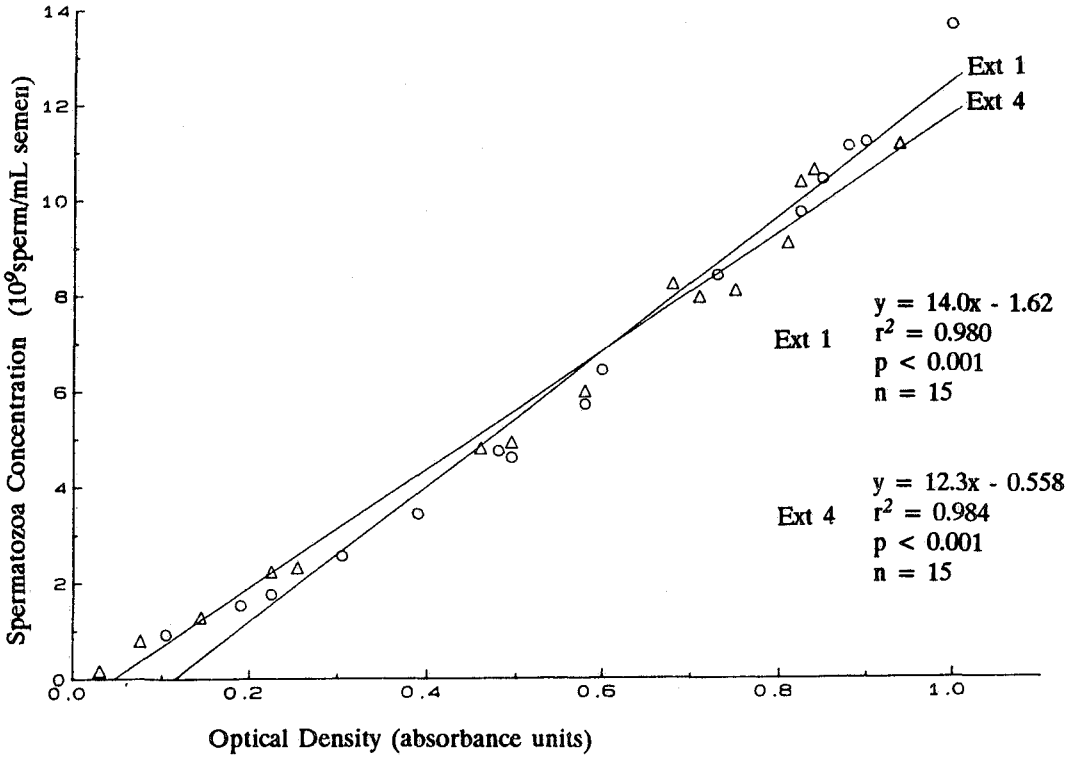

Figure 10. Linear regression lines to predict spermatozoa concentration by optical density (550 nm) for two extenders (Ext 1 and Ext 4) used to cryopreserve Atlantic salmon sperm. (○ Extender 1; △ Extender 4)

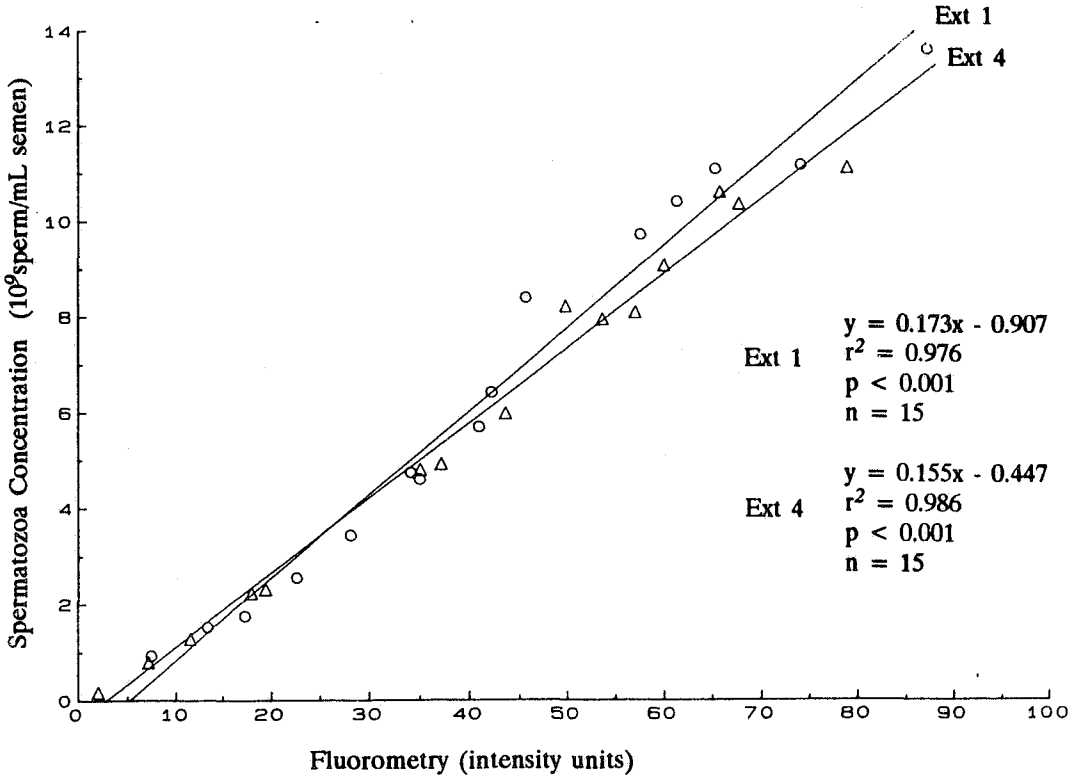

Figure 11. Linear regression lines to predict spermatozoa concentration by fluorometry (360 nm and 575 nm) for two extenders (Ext 1 and Ext 4) used to cryopreserve Atlantic salmon sperm. (o Extender 1; Δ Extender 4)

for fluorometry. Using fluorometry, there were significant differences ($p<0.05$) observed among the frozen-thawed semen samples from the six fish in Experiment Two.

Analysis of variance of motility, fluorometry and LDH leakage on the diluents indicated significant differences ($p<0.05$) between Diluent 1 and Diluent 4 by all three methods.

4.4 Discussion

In Experiment One, using fluorometry we identified Extender 1 and Extender 4 with 12 percent DMSO as the most successful diluents for freezing salmon sperm. Horton and Ott (1976) had poor success using Extender 1, however the addition of KCl to Extender 1 in our study prevented the activation of sperm cells prior to freezing and the addition of 12 percent DMSO for cryopreservation resulted in low numbers of dead cells after freezing as supported by fluorometry. Extender 4 contained constituents as outlined by Mounib (65) and in combination with 12 percent DMSO also contained low numbers of killed sperm following cryopreservation. Horton and Ott (56) recommended the concentration of each constituent in extenders be determined experimentally. Ott and Horton (64) found significantly better motility of rainbow trout sperm cryopreserved with 12 percent DMSO. Stoss and Holtz (20) found fertility of cryopreserved rainbow trout sperm higher with 10 percent DMSO than 5.0, 13.8, 15.0 and 20.0 percent; they did not report the use of 12 percent DMSO.

In Experiment Two, one-way analysis of variance of fluorometry results indicated significant differences in the post-thaw viability of the semen amoung the six fish. Fluorometry is highly sensitive in detecting the percentage of dead cells in a sample as determined in Chapter 2 and supported by Bilgili and Renden (24) for killed proportions of avian sperm. Horton and Ott (56) observed a variable fertilizing capacity of frozen semen from individual male rainbow trout and accounted for variation by suggesting that the fish were at different stages of maturity. However, these authors failed to account for the concentration of sperm in the fertilization process which may have varied among fish, thus affecting the fertility results. They had insufficient evidence to conclude that the sperm of some fish will withstand the freezing or thawing process better than

sperm of other fish.

Salmon used in Experiment Two were selected from a tank of broodstock that were graded and scheduled to spawn at about the same time. Semen selected for the experiment contained sperm with excellent motility ratings. The accuracy of fluorometry to detect cell damage following cryopreservation indicates that there was a true difference in freezability among the sperm of the six fish.

Traditionally, motility has been used to evaluate freezing success in salmonids and to detect differences in extenders and cryoprotectants. However motility ratings may lead to variation in results. Ott and Horton (64) concluded that motility estimates decrease the time required to evaluate extenders within an experiment, but advised against using motility estimates to compare experiments. The unexpected positive correlation observed between motility and fluorometry and LDH leakage indicates motility may be a misleading indicator of sperm viability. The lower motility ratings for Diluent 4 may be explained if Diluent 4 allowed less damage during cryopreservation but the chemical composition of the extender was not favorable to sperm activation following thawing. Likewise, diluent 1 may not have protected sperm as well during cryopreservation as indicated by fluorometry and LDH activity but the chemical composition of the extender allowed surviving cells to be fully activated. A fertility trial with sperm from each fish and diluent would test this hypothesis. The obscure motility results support the conclusions of Truscott *et al.* (61) who concluded motility should be supplemented by a more reliable indicator of sperm viability.

LDH activity did not detect significant differences in the post-thaw viability of the semen of the fish even when activity was adjusted to account for sperm concentration.

Both optical density and fluorometry are good methods to predict sperm concentration of cryopreserved sperm as indicated by the significance. Fluorometry is a valuable tool because in addition to determining percentage dead cells, fluorometry is a convenient technique to simultaneously determine sperm concentration. However, preparation of solutions for fluorometry is time consuming and expensive. Optical density is equally effective to determine sperm

concentration and is recommended when only sperm concentration is required. Spectrophotometers are commonly purchased by hatcheries to monitor water chemistry and algal growth so that the determination of sperm concentration by optical density in the field is quite practical. The significant difference between the slope of the regression lines for Extender 1 and Extender 4 when predicting sperm concentration by optical density may be explained by the optical density of the extenders. Extender 1 was a 'cloudy' solution, thus absorbing some light during the optical density test. Extender 4 was a 'clear' solution probably absorbing very little light. Although the volume of semen and extender was very small (25 μl) compared to the volume of sodium citrate (4.975 ml) to determine optical density, there was an effect of the extender on the concentration prediction. This concept was not tested during the experiment. The significant difference between the slope of the regression lines for Extender 1 and Extender 4 when predicting sperm concentration by fluorometry may also be explained by the optical density of the extenders. Extender 1 may have absorbed more light than Extender 4 during the excitation beam of fluorometry and prevented more fluorescense from being read during the emmision beam. These concepts were not tested in the experiment. It is recommended that a separate regression line be developed for each extender used to cryopreserve Atlantic salmon sperm when predicting sperm concentration by optical density or fluorescense. In Experiment Two, one-way analysis of variance indicated that fluorometry was highly significant ($p<0.001$) in detecting a difference between the extenders while motility and LDH leakage were less significant ($p<0.05$). This supports the concept that fluorometry is a more sensitive tool to detect small differences between extenders.

Experiment Two contained greater percentages of dead cells as determined by fluorometry for Diluent 1 and 4 as compared to Experiment One. A greater number of straws were filled in Experiment Two thus increasing the equilibration time prior to freezing. Stoss and Holtz (51) found that when using DMSO increased equilbration time resulted in decreased fertility following storage. The higher percentage of dead cells in Experiment Two may reflect the damaging effects of DMSO before cryopreservation. Cells may be rendered more susceptable to damage during storage if the duration between mixing with diluent and freezing is prolonged.

5. CRYOPRESERVATION OF RAINBOW TROUT SPERMATOZOA

5.1 Introduction

The advance of cryopreservation of trout sperm has been hampered by the unavailability of gametes during most of the year. Semen is often available for several months after spawning; however, eggs become overripe shortly after the fish matures. Fertility trials have been the most prominent way to test sperm viability following cryopreservation but egg availibility may limit the amount of research during one reproductive season. Early fertility results using stored sperm were poor and inconsistent. Hoyle and Idler (62) cryopreserved Atlantic salmon sperm with an extender and ethylene glycol and reported fertilities of zero to 12%. Graybill and Horton (8) cryopreserved rainbow trout sperm with a salt solution and dimethyl sulfoxide (DMSO) in liquid nitrogen and achieved fertilities of zero to 18%. Expansion in the aquaculture industry has led to a more concentrated effort to refine techniques and improve fertility results.

Many extenders have been developed to cryopreserve trout sperm, however inconsistencies in sperm concentrations used to fertilize eggs have made evaluation of extenders difficult. Stein and Bayrle (18) reported 80% fertility with sperm cryopreserved in pellets and fertilization with a concentration of 4-6 x 10^6 cryopreserved sperm per egg. Rainbow trout with a normal sperm concentration of 9 x 10^9 sperm/ml would represent greater than 1 x 10^9 sperm per egg. Legendre and Billard (66) achieved a maximum fertility of 71.4% with Mounibs diluent (65) but failed to report the number of pellets or the concentration of sperm used to fertilize eggs.

Other workers have reported sperm concentrations in fertility trials but much variation exists. Stoss and Holtz (20) obtained fertility levels of approximately 80% in lots of 123 - 140 eggs fertilized with 625 x 10^6 cryopreserved sperm representing 4.5 - 5.0 x 10^6 sperm per egg. Baynes and Scott (34) fertilized lots of 100 eggs with semen diluted 1:3 with a modified extender of Mounib (65). Maximum fertility achieved was 67% with two 0.5 ml straws representing

approximately 1.125 x 10^9 sperm per egg. Ideally, extenders should be evaluated prior to fertilization. Variability in egg quality and difficulty in consistent dispersal of sperm over eggs add unwanted variation to fertility results. Motility has been used to evaluate extenders but with limited success (61). Legendre and Billard (66) found that motility gave an estimate of fertility results but recommended research be oriented to analyzing the ionic or organic constituents of seminal fluid following cryopreservation to predict fertility. Analysis of enzymes leaking out of damaged cells has been discussed in Chapters 2, 3 and 4. Truscott et al., (61) reported that the most motile sperm were the most fertile but could not quantify their findings. Fluorometry, a technique of fluorescing the genetic material of cells damaged during cryopreservation and discussed in Chapters 2, 3 and 4, may be an alternative to fertility and motility to evaluate extenders and cryoprotectants. Development of such a technique would allow for rapid evaluation of extenders at a much lower cost than fertility trials.

Many compounds have been evaluated to cryopreserve rainbow trout sperm; however, until recently DMSO has been recognized as the superior cryoprotectant (7, 37). DMSO penetrates cells very quickly to offer protective action during cryopreservation. The use of DMSO to cryopreserve Atlantic salmon sperm was investigated in Chapter 4. DMSO may exhibit detrimental effects to sperm cells if equilibration time is prolonged when freezing many straws or when filling straws in the field and transporting them prior to cryopreservation. Other cryoprotectants may be more successful under these conditions. Lake and Ravie (67) used dimethylacetamide (DMA) to cryopreserve fowl sperm and concluded its performance was superior to DMSO or glycerol. DMA is a relatively new cryoprotectant and has not been investigated for fish sperm.

The objectives of this study were to: 1) evaluate dimethyl acetamide as a cryoprotectant for rainbow trout sperm, 2) determine by the *in vitro* methods, fertility determination and proportion of eyed eggs, which diluent(s) perform best for sperm cryopreservation, 3) evaluate motility, fluorometry and LDH leakage as predictors of the proportion of eggs fertilized and eyed and 3) determine the superior *in vitro* method(s) to predict fertility.

5.2 Materials and Methods

Combinations of four extenders and two cryoprotectants at two levels (16 diluents) were evaluated to cryopreserve rainbow trout sperm. Extenders 1, 2, 3 and 4 were prepared as outlined in Chapter 4 with osmolarities of 290, 290, 390, and 225 mOsm/kg H_2O respectively (Wescor Osmometer, Logan, Utah). All extenders were adjusted to a pH of 7.5 with citric acid or sodium hydroxide. Nine millilitres of each extender were dispensed into four sets of four tubes. DMSO was added to two tubes of each set in volumes of 0.9 ml and 1.08 ml (10% and 12% vol./vol. extender). DMA was added to the remaining two tubes of each set at the same volumes. Extender and cryoprotectant were mixed and cooled to 4°C.

Mature rainbow trout held in outdoor circular cement ponds fed river water at 4°C were anesthesized in MS-222 at a concentration of 100 mg/L, rinsed in fresh water following anaesthesia, wiped dry and semen was collected by abdominal massage. Semen samples were collected into glass jars and placed on crushed ice. Samples were rated in the laboratory as outlined in Chapter 2 and samples with motility ratings of 9 or 10 were pooled for cryopreservation. An optical density reading was taken on a sample of the pooled semen after a 1:200 dilution of semen with sodium citrate as outlined in Chapter 2. The regression equation in Chapter 2 ($y = 12.3x - 0.372$) was used to calculate sperm concentration. Fifteen, 0.5 ml clear plastic French straws were filled for each treatment and cryopreserved as outlined in Chapter 4. Straws were placed in aluminum canes and stored in liquid nitrogen prior to evaluation.

5.2.1 *In vitro* Evaluation of Cryopreserved Sperm

Motility, fluorometry and LDH leakage were used to estimate the viability of cryopreserved sperm. Motility ratings were performed as outlined in Chapter 2 by activating sperm from three straws thawed in a 4°C water bath. Fluorometric determinations of the percentage of dead cells were performed on three straws using the method described in Chapter 2. LDH activity of the seminal plasma was measured for three straws using the procedure described in Chapter 2. Seminal

plasma was diluted 1:3 instead of 1:6 as in Chapter 2 since semen to was diluted with extender prior to freezing.

5.2.2 Fertility of Cryopreserved Sperm

Fresh rainbow trout eggs were fertilized with cryopreserved sperm from thawed straws to determine fertility rates. Six mature female rainbow trout held in outdoor ponds fed river water at 2°C were anesthesized with MS-222, rinsed in fresh water and eggs were collected by abdominal massage into clean dishes. Eggs were pooled and mixed thoroughly. Air temperature of the hatchery was 5°C and well water used for incubation was 7°C. The eggs were allowed to to acclimate to the hatchery air temperature over a 30 minute period. The eggs were divided into batches of approximately 300 eggs and three batches were fertilized for each of the 16 treatments. To fertilize each batch of eggs, two straws were thawed by immersion in a 5°C water bath for approximately 12 seconds. The contents were placed on a batch of eggs, mixed gently and let stand for three minutes. A small quantity of hatchery water was then added and after three minutes, the eggs were rinsed in hatchery water and placed randomly in trays of Heath Incubators divided into small compartments by wood laths.

Control batches of eggs were fertilized with 0.250 ml of pooled semen (the approximate volume of semen contained in two 0.5 ml straws excluding diluent) collected from mature rainbow trout by abdominal massage.

Dead eggs that turned opaque were picked regularly over the incubation period and cleared in a solution of methanol, acetic acid and water described in Chapter 2. Dead eggs with a whitened embryo after clearing were recorded as fertilized eggs. Dead eggs with an eyed embryo were recorded as fertilized and eyed. After live eggs had reached the eyed stage, just prior to hatching, the number of live eyed eggs in each batch was recorded. From these values the percentage of eggs fertilized and the percentage of eyed eggs were calculated for each batch. Percentage fertilized and percentage eyed eggs were expressed as percentages of control values.

5.2.3 Statistical Analysis

Fluorometry, fertility and eyed egg percentages were transformed to arc sine for all analyses. Two-way analysis of variance was performed to determine the effect of extender, cryoprotectant and the interaction between the two. Data was also analysed by one-way analysis of variance and Student-Newman-Kuels tests were used to isolate a superior diluent based on the *in vitro* methods, fertility and eyed eggs. Linear regression analysis was used to examine the relationships between the *in vitro* methods and the percentages of fertilized and eyed eggs. Correlation coefficients were compared by the Fisher Z statistic to determine the superior *in vitro* method to predict fertility.

5.3 Results

The optical density of pooled semen was 0.70 absorbance units indicating a sperm concentration of 8.24 x 10^9 sperm/ml of semen. Based on a dilution of 1:3 for cryopreserved semen, each straw contained approximately 1.03 x 10^9 sperm and eggs were fertilized with approximately 6.9 x 10^6 sperm/egg. The percentages of eggs fertilized and reaching the eyed stage in control batches were 94.3 and 94.1 with standard deviations of 3.5 and 3.74, respectively.

Table VII presents the mean and standard deviation for motility, fluorometry, LDH activity, fertility and eyed egg values for the four extenders and two cryoprotectants evaluated in the experiment. Motility ratings ranged from 0.5 to 4.3 on the scale of 0 to 10. Extender 1 with 12% DMA exhibited the greatest motility rating. Fluorometry values ranged from 35.4% dead sperm for Extender 1 with 12% DMA to 67.2% dead for Extender 4 with 12% DMSO. LDH activity of the seminal plasma ranged from 25.3 Units/litre for Extender 4 with 12% DMA to 70 Units/litre for Extender 1 with 10% DMSO. Fertility results ranged from 0% for Extender 2 with 10% DMSO to 66.2% for Extender 1 with 10% DMA. Eyed egg values had similar ranges.

Analysis of variance for the motility and fluorometry estimations of sperm viability, fertility and eyed eggs by the variables extender, cryoprotectant and level of cryoprotectant indicated highly significant differences between extenders and cryoprotectants ($p<0.001$) and a significant interaction

Table VII. Estimation of sperm viability by *in vitro* methods, fertility and eyed eggs for rainbow trout sperm cryopreserved with four extenders and two cryoprotectants at two levels. Mean values ± (st.dev)

Extender	Cryoprotectants levels	Motility rating	Fluorometry (% dead sperm)	LDH U/Lit	Fertility[a] %	Eyed Eggs[b] %
1	10% DMSO	0.7 (0.3)	65.0 (4.1)	70.0 (1.7)	1.0 (1.3)	0.9 (1.4)
1	12% DMSO	2.0 (1.3)	66.9 (1.3)	62.7 (2.9)	0.1 (0.2)	0.1 (0.2)
1	10% DMA	3.7 (0.6)	35.5 (5.6)	42.3 (5.9)	66.2 (2.[illegible]	62.5 (3.8)
1	12% DMA	4.3 (0.6)	35.4 (4.6)	27.7 (4.0)	53.9 (8.2)	50.6 (5.5)
2	10% DMSO	3.0 (0.0)	48.6 (2.6)	51.0 (0.0)	0.0 (0.0)	0.0 (0.0)
2	12% DMSO	3.3 (0.6)	49.2 (6.4)	59.0 (4.3)	0.3 (0.6)	0.3 (0.6)
2	10% DMA	4.0 (1.0)	39.6 (2.0)	45.0 (2.7)	19.9 (2.6)	18.5 (2.7)
2	12% DMA	4.0 (1.0)	41.5 (1.0)	43.3 (0.6)	8.0 (7.8)	8.0 (7.8)
3	10% DMSO	0.5 (0.0)	55.2 (1.8)	47.7 (2.5)	0.1 (0.2)	0.1 (0.2)
3	12% DMSO	0.5 (0.0)	57.6 (2.2)	51.3 (3.2)	0.1 (0.2)	0.1 (0.2)
3	10% DMA	0.7 (0.3)	51.8 (0.4)	53.7 (6.4)	0.0 (0.0)	0.0 (0.0)
3	12% DMA	0.7 (0.3)	54.8 (0.4)	43.3 (8.1)	1.5 (2.3)	1.4 (2.4)
4	10% DMSO	0.5 (0.0)	55.8 (5.1)	54.0 (7.6)	0.2 (0.3)	0.0 (0.0)
4	12% DMSO	0.5 (0.5)	67.2 (2.9)	58.0 (4.4)	1.2 (2.0)	1.2 (2.0)
4	10% DMA	0.8 (0.3)	40.7 (0.6)	26.7 (2.1)	1.2 (0.8)	1.1 (0.8)
4	12% DMA	0.7 (0.3)	43.7 (4.1)	25.3 (2.1)	4.4 (2.2)	4.4 (2.2)

[a] Fertility expressed as a percentage of control lots of eggs.

[b] Eyed eggs expressed as a percentage of control lots of eggs.

between extender and cryoprotectant by motility, fluorometry and LDH leakage. DMA performed significantly better than DMSO under all treatments by all *in vitro* methods and fertility.

Figure 12 presents the mean motility ratings for sperm cryopreserved with the sixteen diluents. A comparison of diluents by SNK tests for motility revealed sperm in Extender 1 with 10% and 12% DMA, Extender 2 with 10% and 12% DMA and Extender 2 with 10% and 12% DMSO had significantly higher motility ratings than sperm in other diluents. Figure 13 presents the mean percent dead sperm determined by fluorometry for the sixteen diluents. SNK tests for fluorometry indicated sperm in Extender 1 with 10% and 12% DMA, Extender 2 with 10% and 12% DMA and Extender 4 with 10% and 12% DMA had significantly lower percentages of dead sperm following cryopreservation than sperm in other diluents. Figure 14 presents the mean LDH activity measured for sperm cryopreserved in the 16 diluents. SNK tests indicated that sperm cryopreserved in Extender 1 with 10% and 12% DMA and Extender 4 with 10% and 12% DMA had significantly lower LDH activity in the seminal plasma than other diluents used to cryopreserve sperm. Figure 15 presents the mean fertility results for sperm cryopreserved in the 16 diluents and indicated sperm in Extender 1 with 10% DMA exhibited significantly better fertility results than sperm in all other diluents.

Motility, fluorometry and LDH results indicated Extender 1 and Extender 2 gave greater numbers of viable sperm than all other diluents. Extender 1 with 10% DMA gave promising results by all parameters. Eyed egg values were not significantly different than fertility values for the 16 diluents.

Linear regression analysis of fertility on motility, fluorometry and LDH activity values revealed significant ($p<0.05$) relationships with r^2 values of 0.364, 0.469, and 0.202 respectively. Analysis of correlation coefficients by the Z statistic for fluorometry and LDH leakage correlated to fertility indicated fluorometry was significantly better than LDH leakage for predicting fertility ($p<0.05$). There was no significant difference between correlation coefficients for motility and

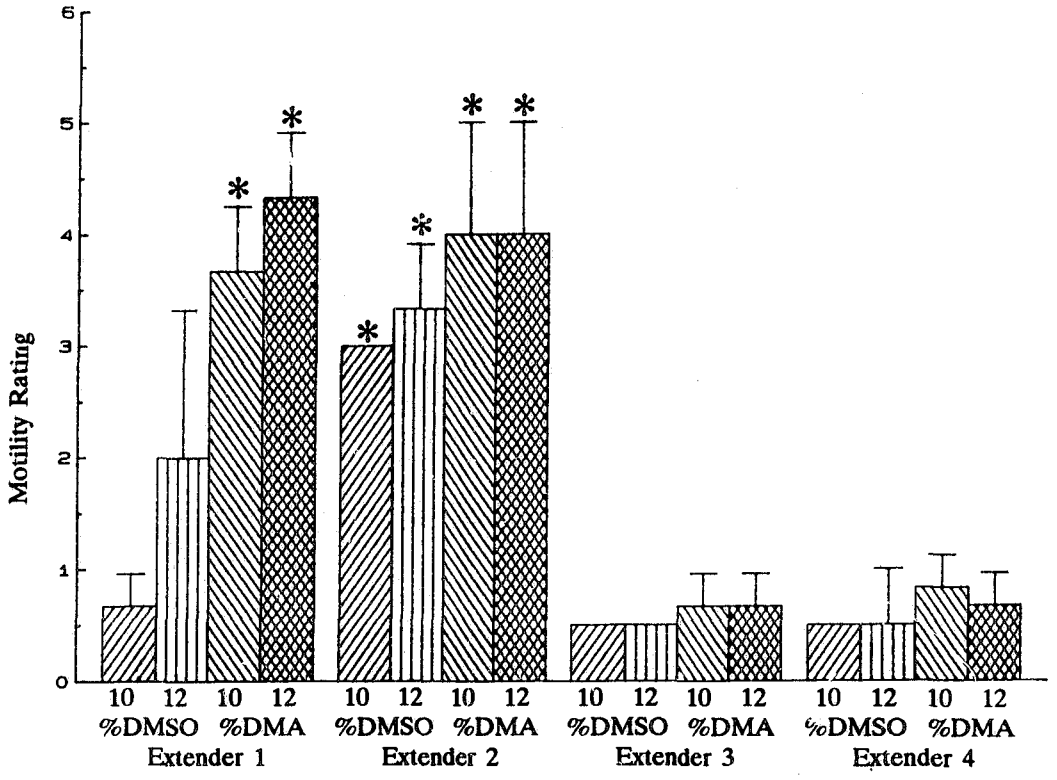

Figure 12. Mean and standard deviation of motility ratings for rainbow trout sperm cryopreserved with four extenders and two cryoprotectants at two levels. (n = 3)

* significantly higher motility rating

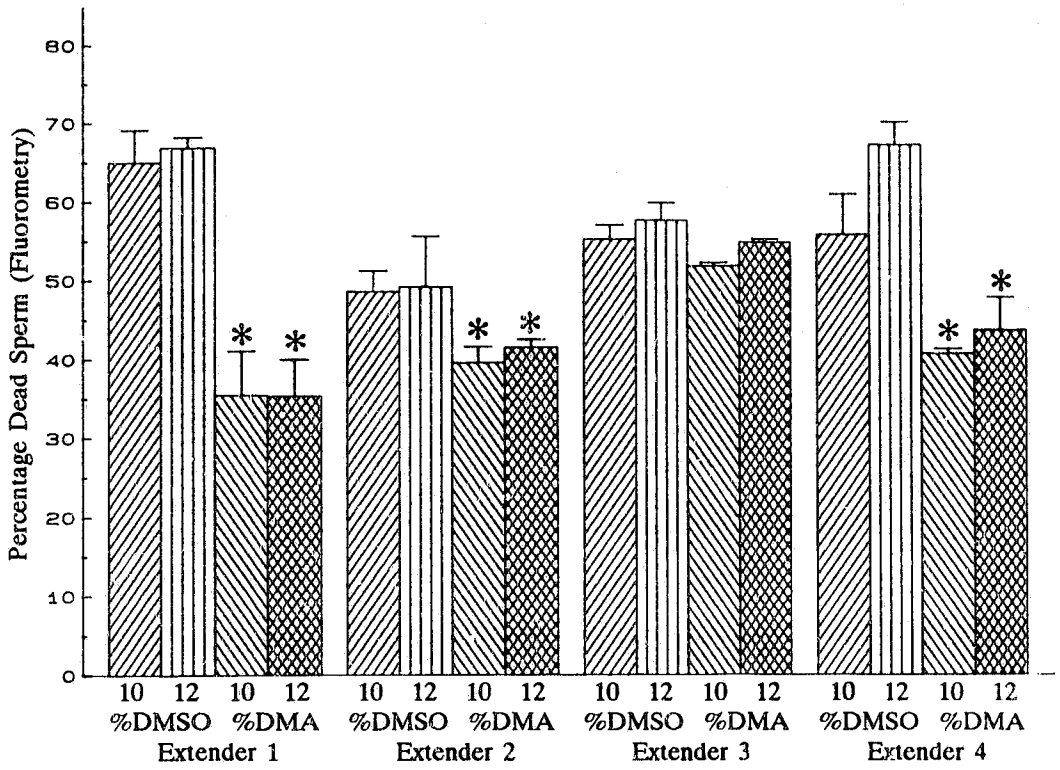

Figure 13. Mean and standard deviation of percentage dead sperm determined by fluorometry for rainbow trout sperm cryopreserved with four extenders and two cryoprotectants at two levels. (n = 3)

* significantly lower percentage of dead sperm by fluorometry

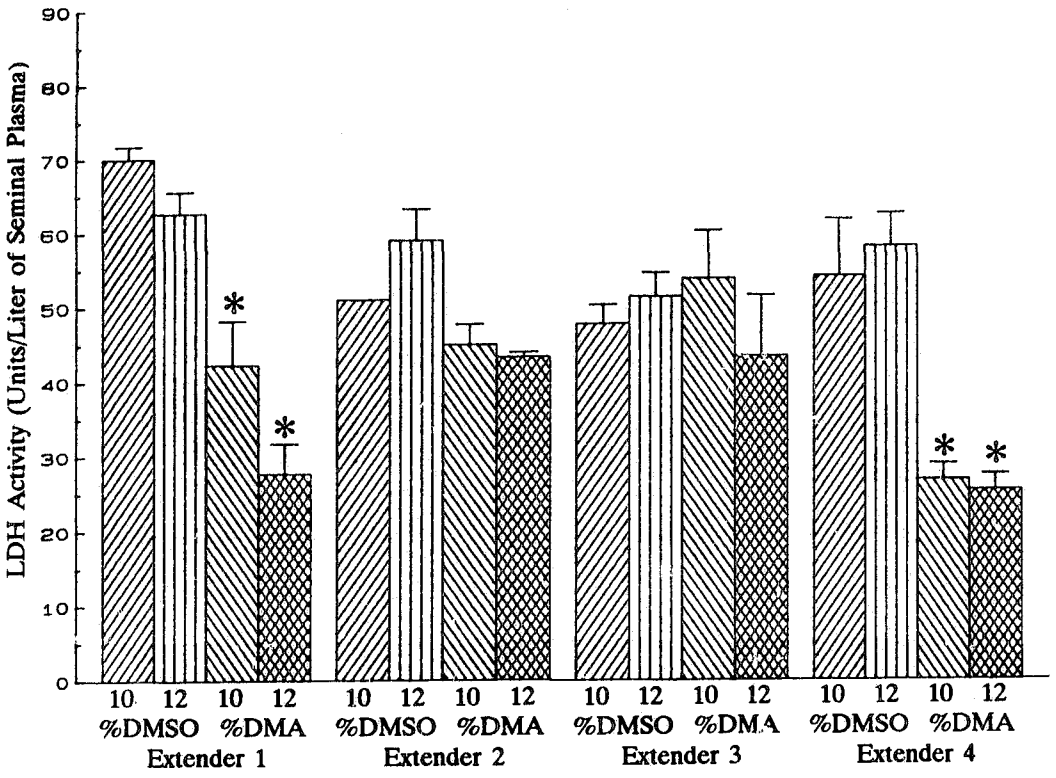

Figure 14. Mean and standard deviation of LDH activity of seminal plasma for rainbow trout sperm cryopreserved with four extenders and two cryoprotectants at two levels. (n = 3)

*significantly lower LDH activity of the seminal plasma

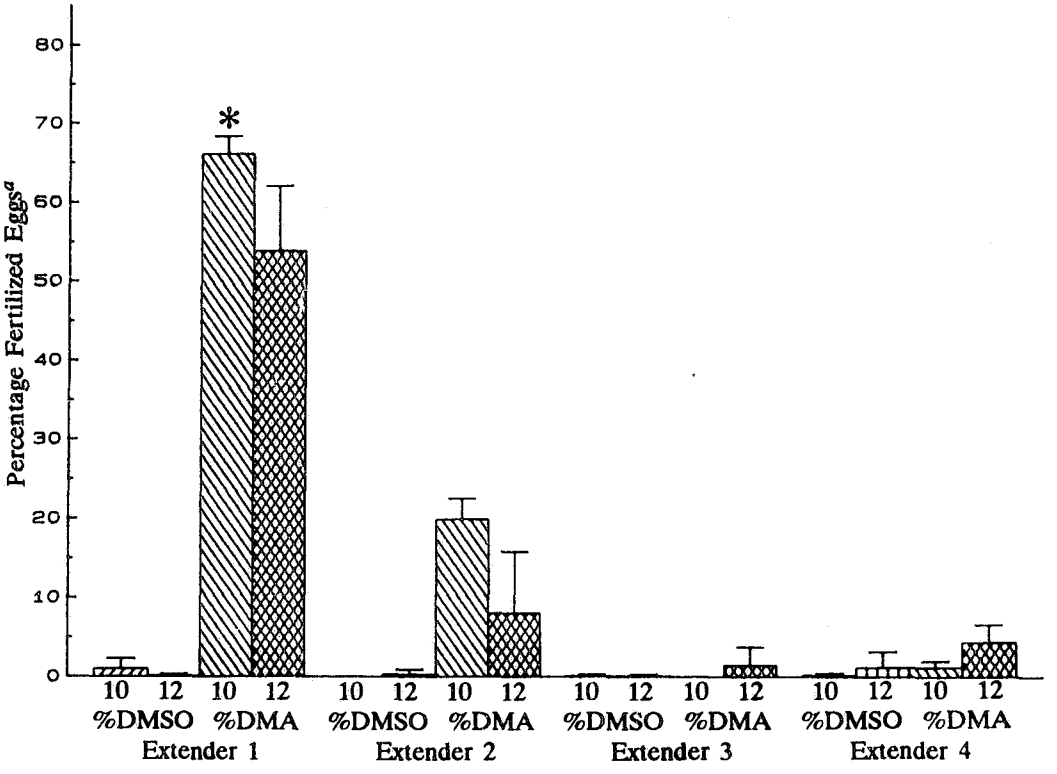

Figure 15. Mean and standard deviation of the percentage of eggs fertilized with rainbow trout sperm cryopreserved with four extenders and two cryoprotectants at two levels. (n = 3)

[a] expressed as a percentage of control batches of eggs.

* significantly greater percentage of fertilized eggs

fluorometry when predicting fertility or between motility and LDH leakage when predicting fertility.

5.4 Discussion

DMA performed better than DMSO as a cryoprotective agent for rainbow trout sperm as indicated by the *in vitro* methods and fertility results. DMSO has been demonstrated as a successful cryoprotectant for small batches of sperm cryopreserved in the laboratory with minimal equilibration time. Stoss and Holtz (20) found that DMSO concentrations between 10% and 20% provided high post-thaw fertility (77%) when sperm equilibration time was kept to a minimum. At higher concentrations of DMSO, prolonged equilibration time had detrimental effects on sperm survival. Commercial freezing procedures would require large numbers of straws of semen to be loaded and frozen at one time, thus increasing equilibration times. DMA is a promising alternative cryoprotectant for commercial freezing procedures. Further investigation into optimal concentration and equilibration time is warranted.

Motility ratings should not be the only method used to evaluate extenders. Motility ratings were high for sperm cryopreserved with Extender 1 and 10% DMA and fertility results for the diluent were superior to others. However, motility ratings for sperm cryopreserved in Extender 2 with DMSO were also high but fluorometry and LDH results indicated damage to the cells and the sperm did not fertilize any eggs. These findings support the conclusions of Legendre and Billard (66) who found motility ratings subjective and misleading.

The maximum fertility achieved in this experiment was 66.2 percent with approximately 6.9 x 10^6 sperm/egg. Stoss and Holtz (20) reported 80 percent fertility with 5-10 x 10^6 sperm/egg; however, they cryopreserved sperm in pellets, used smaller egg lots, and a thawing solution which diluted sperm cells after cryopreservation, thus allowing easy dispersal of sperm over all eggs. It is not practical to use a thawing solution when sperm is frozen in straws because the solution can not be added to the frozen semen. However, additional diluent could be added to the thawed semen to aid in dispersal of sperm to all eggs especially when small volumes of semen such as in Chapter 2 are used to fertilize eggs. The time period between thawing and insemination should be

minimized. Stoss and Holtz (20) found that a delay of 30 seconds between thawing pellets and addition of sperm to eggs reduced the fertilization rate. Control eggs were fertilized with 250 µl of semen and fertilities were high. When fertilizing eggs with small quantities of semen it is essential that eggs and sperm be mixed thoroughly to ensure sperm comes in contact with all eggs.

The application of cryopreservation for commercial breeding programs in fish is justified by the fertility results using Extender 1 with 10% DMA. The use of three straws to fertilize 300 eggs would probably have improved fertility results since the concentration of live sperm to egg would be increased. On a practical basis, eggs from a five pound rainbow trout brood fish (approximately 3000 eggs) would require cryopreserved sperm from 30-0.5 ml straws to achieve good fertility. The volume of semen required to fill these 30 straws using a 1:3 dilution with extender would be approximately 3.75 ml, which is still considerably less than most hatcheries use during pair matings. The use larger straws for cryopreservation would allow fertilization in the field to proceed at a rapid pace.

Sperm cryopreserved with Extender 4 and 10% and 12% DMA showed low percentages of dead sperm by fluorometry and low activities of LDH in the seminal plasma, suggesting the diluents were superior to other diluents. However, the sperm from these treatments showed poor motility and poor fertility. In Chapter 4, sperm in Extender 4 exhibited the same trend. It seems that Extender 4 caused little damage to sperm during cryopreservation as estimated by fluorometry and LDH leakage, however the chemical composition of the Extender was not favourable to sperm activation after thawing. The low fertility with Extender 4 is supported by poor motility. Measurement of motility in combination with fluorometry may be beneficial to predicting fertility when extenders perform as Extender 4 did in this experiment and in Chapter 4.

Sperm cryopreserved with Extender 1 and 10% DMA exhibited low percentages of dead sperm by fluorometry, low LDH activity in the seminal plasma and higher motility ratings than sperm in other diluents. Fertility results with sperm from Extender 1 with DMA were better than other diluents. The chemical composition of Extender 1 appeared favourable for sperm activation

and fertilization of eggs. The addition of KCl to the Extender 1 of Horton and Ott (56) inhibited activation of sperm before cryopreservation but once diluted by ovarian fluid allowed sperm to become active. Ott (17) reported greater than 50% fertilization with sperm cryopreserved with the unmodified Extender 1 and a DMSO concentration between 7.5 and 12 percent. The modified extender with DMSO did not perform as well as the modified extender with DMA in our experiment. Further work to identify optimal levels of DMA is warranted.

Fluorometry, motility and LDH leakage were good predictors of fertility for cryopreserved rainbow trout sperm. The value and limitations of these estimators of sperm viability has been discussed in Chapter 2. LDH leakage lacks baseline values to determine the degree of success of freezing sperm. Motility gives an indication of what fertility results to expect but lacks precision and repeatability. Fluorometry is precise and repeatable.

Fluorometry values in this experiment seemed to be lower what one would expect; that is, diluents with low percentages of dead sperm fertilized only small percentages of eggs. Bakst *et al.*,(68) have identified a similar phenomenon with the fluorometry method and have suggested that when sperm are stored in isotonic buffers (as we did in this experiment) up to 25% of the dead cells contain labile membranes capable of excluding EtBr. When these sperm are stressed in a hypotonic buffer (of 60 mOsmol/kg H_20) EtBr penetrates the non-viable sperm and presents a more accurate assessment of sperm viability. In Chapter 2, fluorometry values were very close to the proportions of live/killed sperm indicating that sonication allowed good penetration of EtBr into non-viable sperm. The evaluation of extenders and cryoprotectants in the present chapter was not affected by the consistently low fluorometry values, however further research is warranted to verify that fish sperm cells might exclude EtBr under certain conditions. Motility estimations should be used with fluorometry to present a complete picture of sperm viability, especially for frozen-thawed sperm.

6. SUMMARY OF RESULTS AND CONCLUSIONS

Development of reliable techniques for fresh and frozen storage of salmonid spermatozoa is essential to improve breeding programs for salmonid fish. Fresh storage of salmonid semen will allow genetic crossing of semen and eggs isolated geographically, allow semen of desirable males that mature before the females to be utilized and allow semen to be held and evaluated during storage to indicate the viability before fertilization. Frozen storage or cryopreservation of salmonid spermatozoa will allow gene pools to be preserved for future use, ensure availability of gametes over a broader time period and guard against lack of broodstock resulting from disease or natural disaster. Although many attempts have been made to develop repeatable techniques for fresh and frozen storage of salmonid spermatozoa, few have been successful.

Perhaps the greatest hindrance to the development of accurate techniques for fresh and frozen storage of salmonid spermatozoa has been the lack of methodology to estimate the viability of stored gametes. Traditionally, only motility and fertility have been used to evaluate storage techniques. Motility estimates vary with the experience of the evaluator and fertility trials are expensive to carry out, time consuming and vary with the concentration of sperm used to fertilize eggs. In Chapter 2 we compared four methods to estimate the viability of stored spermatozoa. Various proportions of live and killed spermatozoa were mixed and motility, fluorometry, LDH leakage and fertility were used to estimate the viability of the spermatozoa. Motility, fluorometry and LDH leakage significantly predicted fertility, however, fertility results were variable and warrant further research. Fluorometry was the most simple of the three methods and gave very reliable, repeatable results demonstrating its use as a potential method to evaluate many techniques for fresh and frozen storage of spermatozoa at one time.

Fresh storage of salmonid spermatozoa is a valuable hatchery tool to allow time for evaluation of semen prior to insemination to improve the efficiency of the spawning process. Many attempts have been made to develop reliable techniques for fresh storage of salmonid semen

but few have been successful. Most have identified a vital role of oxygen and low temperature in sperm survival but have used complicated apparatus to maintain sperm viability. In Chapter 3 we evaluated several of these techniques for fresh storage of salmonid semen and developed new techniques. Fluorocarbons are inert, non-toxic compounds that possess a great capacity to hold oxygen and carbon dioxide. A simple technique of storing semen above an oxygenated fluorocarbon layer maintained sperm viability for up to 37 days at 4°C at which time spermatozoa expressed a fertility of 81.2%. The *in vitro* methods of fluorometry and motility and evaluation of fertility isolated the semen stored on top of the fluorocarbon layer in the moisture-saturated air atmosphere as the superior treatment. The use of fluorocarbon for transport of semen in the field and short-term storage shows much promise for future breeding programs. Also, fluorocarbons may greatly assist in maintaining quality semen for brief periods of time before cryopreservation of salmonid spermatozoa, techniques that have been hampered by the deterioration of semen quality between collection and freezing.

Many extenders and cryoprotectants (diluents) have been evaluated to cryopreserve salmonid spermatozoa but results have been variable and unpredictable. Variation has existed between different batches of semen and between eggs used to evaluate cryopreservation success. In Chapter 4 we evaluated 15 diluents to cryopreserve Atlantic salmon semen (5 extenders x 3 levels of DMSO) and used fluorometry to isolate two superior diluents. Semen was cryopreserved with the two superior diluents and it was demonstrated that there was a significant difference in post-thaw viability of spermatozoa from different fish. This may account for the variability in results that is presently reported in the literature.

In Chapter 5, rainbow trout spermatozoa was cryopreserved with 16 diluents (four extenders x two cryoprotectants x two levels of cryoprotectant). Dimethyl acetamide outperformed dimethyl sulfoxide as a cryoprotectant as indicated by motility, fluorometry, LDH leakage and fertility. An extender reported by Horton and Ott (56) modified to include KCl and mixed with 10% DMA performed better than other diluents in the experiment. Frozen-thawed spermatozoa expressed a

fertility of 66.2% with 6.9 x 10^6 sperm/egg. DMSO has been traditionally used as the accepted cryoprotective agent for rainbow trout spermatozoa but this experiment has demonstrated DMA as a potential cryoprotective agent. Fluorometry, motility and enzyme leakage significantly predicted fertility of frozen-thawed rainbow trout spermatozoa.

In conclusion, this thesis has contributed to many areas of semen storage for salmonid fish. New techniques to evaluate the storage success of sperm, namely fluorometry and enzyme leakage, will help standardize results of storage trials in the future and add to motility ratings which have been traditionally used as the only assessment of stored sperm. Techniques of fluorometry will allow many extenders to be evaluated at one time and greatly increase the volume of work that can be carried out in one breeding season. Establishing concentration curves for each extender used to cryopreserve sperm cells will help document the concentration of frozen thawed sperm used to fertilize eggs so future work can be compared to develop more efficient extenders and cryoprotectants. Fluorocarbon compounds show much promise to improve short-term storage of salmonid sperm which will greatly aid hatcheries during the breeding season. New cryoprotectants such as dimethyl acetamide will improve the fertilization rate of frozen-thawed spermatozoa.

7. APPENDIX A - SEMEN VOLUME CALCULATION

The volume of semen required from each treatment (proportion of killed spermatozoa) to fertilize a lot of 300 eggs was calculated in the following manner. The optical density of semen in each treatment was used to calculate the concentration of spermatozoa in billions of sperm/ml semen from the equation:

Sperm Concentration = 12.3 x Optical Density - 0.372

Using this concentration the volume of semen to fertilize 300 eggs with 200,000 sperm/egg was calculated. For example, the first set of live/killed sperm proportions contained the following percentages of killed semen and average optical densities:

% Killed	Optical Density (n=3)
0	0.66
25	0.63
50	0.64
75	0.62
100	0.63

Semen with 0 percent killed sperm (no semen sonicated) had an optical density of 0.66 units indicating a sperm concentration of 7.38×10^9 sperm/ml of semen. The volume of semen required to fertilize 300 eggs was then:

$$\frac{200{,}000 \text{ sperm/egg} \times 300 \text{ eggs}}{7.38 \times 10^9 \text{ sperm/ml semen}}$$

$= 8.13 \times 10^3$ ml of semen

$= 8.13$ μl of semen

8. APPENDIX B - REGRESSION ANALYSIS STATISTICS FOR FRESH STORAGE OF RAINBOW TROUT SEMEN

Estimations of sperm viability by motility, fluorometry and LDH over the duration of the experiment were analyzed by regression analysis to predict the number of days for semen stored under five treatments and two atmospheres to reach a motility of 0, a proportion of dead sperm of 95% and LDH activity of 700 U/L. Following are the regression equations for each treatment and atmosphere and the associated p value. The regression equations are in the general form y= b + mx.

Semen treatment	Atmosphere	Regression Equation	p value
Non-diluted	MSA	Motility = 10.7-1.17 day	p=0.018
		Fluor. = 15.7-3.01 day	p=<0.001
		LDH = 55 + 39.5 day	p = 0.050
Non-diluted	NSA	Motility = 10.7-1.36 day	p=0.033
		Fluor. = -0.98 - 5.11 day	p<0.001
		LDH = 54.7 + 34.0 day	p=0.005
Diluted 1:1 with FC	MSA	Motility = 9.80 - 0.117 day	p=0.001
		Fluor. = 3.83 + 1.34 day	p=<0.001
		LDH = 119 +9.30 day	p<0.001
Diluted 1:1 with FC	NSA	Motility = 8.48 - 0.110 day	p<0.001
		Fluor. = 3.08 + 1.54 day	p<0.001
		LDH = 28.4 + 11.8 day	p<0.001
Diluted 1:1 with FC - extender emulsion	MSA	Motility = 8.71 - 0.200 day	p<0.001
		Fluor. = 5.05 + 2.63 day	p<0.001
		LDH = 0.2 + 13.5 day	p<0.001
Diluted 1:1 with FC - extender emulsion	NSA	Motility = 4.83 - 0.124 day	p=0.015
		Fluor. = -0.28 + 2.26 day	p<0.001
		LDH = 20.7 + 8.43 day	p<0.001
Diluted 1:1 with 2:1 FC - extender emulsion	MSA	Motility = 8.70 - 0.188 day	p<0.001
		Fluor. = -3.11 + 2.83 day	p<0.001
		LDH = 12.4 + 12.9 day	p<0.001
Diluted 1:1 with 2:1 FC - extender emulsion	NSA	Motility = 4.57 - 0.128 day	p=0.014
		Fluor. = 5.81 + 2.40 day	p<0.001
		LDH = 27.4 +12.6 day	p<0.001
Diluted 1:1 with extender	MSA	Motility = 4.95 - 0.147 day	p=0.004
		Fluor. = 1.72 + 3.19 day	p<0.001
		LDH = 72.0 + 17.7 day	p<0.001
Diluted 1:1 with extender	NSA	Motility = 4.95 - 0.266 day	p=0.010
		Fluor. = -3.12 + 3.06 day	p<0.001
		LDH = 86.1 + 11.7 day	p=0.001

9. APPENDIX C. - ADJUSTMENT OF LDH ACTIVITY OF THE SEMINAL PLASMA FOR DIFFERENT FISH

To adjust for varying LDA activity from different fish due to varying sperm concentrations, raw LDH activities were divided by sperm concentrations predicted by fluormetry from the regression equations in Figure 12 for extender 1 and extender 4.

Fish	Extender	Unadjusted LDH activity U/L	Fluorometric Intensity	Sperm Concentration x 10^9 sperm/ml Semen	Adjusted LDH Activity
1	1	46.3	20.00	2.55	18.1
1	4	23.0	20.31	2.70	8.5
2	1	55.2	24.48	3.33	16.6
2	4	41.4	24.40	3.34	12.4
3	1	94.7	25.37	3.48	27.2
3	4	47.7	26.09	3.60	13.3
4	1	96.2	25.05	3.43	28.1
4	4	50.2	24.90	3.41	14.7
5	1	44.6	24.78	3.38	15.1
5	4	40.3	23.20	3.15	12.8
6	1	53.1	26.64	3.70	14.4
6	4	46.2	27.37	3.80	12.2

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