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Successful fertilization of fresh steelhead trout, pink salmon, kokanee, chum salmon, fall chinook salmon, and coho salmon eggs with cryopreserved sperm is reported in this thesis. A mean percent fertilization greater than 50 percent was achieved for all species tested except chum salmon.

Differences in sperm viability from donor males was observed; sources of variability probably were degree of sexual maturity and inherent physical-chemical properties of semen. Urine contamination of sperm samples was not responsible for male variability.

In the cryopreservation of salmonid sperm, dilution ratios of 1:4 and 1:9 were acceptable; and when dimethyl sulfoxide was utilized as the solute moderator, equilibration was unnecessary. Extenders were developed for each species, with the most significant advance being development of a simplified diluent containing only

NaCl, $NaHCO_3$, and lecithin. The preferred life protector was dimethyl sulfoxide, and protection was accomplished at levels between 7.5 and 12.0% (v/v) depending on the species tested.

The preferred freezing procedure consisted of placing an ampoule (unsealed) of diluted semen in the vapor of liquid nitrogen (LN_2) for at least 1 min prior to immersion in LN_2 for storage. Storage times approaching one month did not affect sperm viability as determined by mean percent fertilization of fresh ova; longer holding times appear practicable.

The thawing procedure was determined to be critical in terms of sperm survival after freezing. The optimal technique consisted of filling the neck of an unsealed ampoule of frozen sperm with 4-10 C water, and then swirling the ampoule in a 50-60 C bath for 8 sec. When water was added to the ampoule of frozen sperm during thawing, cells equilibrated, thaw rate increased, agglutination decreased, and mean percent fertilization increased.

Recommendations for a cryogenic procedure suitable for salmonids were made; potential problems also were identified. Finally, one specific recommendation was proposed which involved production of normally and cryogenically produced embryos which would be monitored throughout their reproductive cycle in an attempt to determine the biological selectivity of the cryogenic procedure.

CRYOPRESERVATION OF PACIFIC SALMON AND STEELHEAD TROUT SPERM

by

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CRYOPRESERVATION OF PACIFIC SALMON AND STEELHEAD TROUT SPERM

GENERAL INTRODUCTION

Salmonids, especially the five species of Pacific salmon (Oncorhynchus sp.) and the steelhead trout (Salmo gairdneri), support extensive and valuable sport and commercial fisheries. Advances in fish cultural practices have maintained and enhanced specific stocks of these fish thus providing increases in both sport and commercial usage. In attempts to increase and enhance certain stocks, crossbreeding and selective breeding frequently has been utilized (Foerster 1935; Alm 1955; and Simon and Noble 1968). Because gamete availability is limited by maturation time and geographical location, these factors also limit the extent of selective and crossbreeding experiments. Long-term storage of fish sperm would partially eliminate such time and spatial problems. Thus, this research was conducted to determine the feasibility of developing and employing a cryogenic technique to store fish sperm for extended periods of time.

Numerous fishery researchers have attempted to preserve viable fish sperm (Ellis and Jones 1939; Blaxter 1953; Hodgins and Ridgway 1964; Sneed and Clemens 1956; Hoyle and Idler 1968; Mounib et al. 1968; Truscott et al. 1968; Graybill and Horton 1969;

Truscott and Idler 1969; and Ott and Horton (1971a, 1971b). Blaxter's (1953) work with herring (Clupea harrengus) was particularly significant as 80-85% fertilization of fresh ova with sperm frozen six months at -79 C was reported; Mounib, et al. (1968) achieved 36 ± 12% fertilization of fresh Atlantic cod (Gadus morhua) ova with sperm stored at -196 C. The most successful cryopreservation of salmonid sperm was achieved by Ott and Horton (1971a; 1971b), who reported a maximum mean fertilization of 77% with fresh coho salmon (Oncorhynchus kisutch) eggs and 82% with fresh steelhead trout eggs. Other investigations with cryopreserved salmonid sperm resulted in less than 20% fertilization of fresh ova.

Scope and Approach

This investigation was conducted on chum salmon (Oncorhynchus keta), coho salmon, fall chinook salmon (O. tshawytscha), kokanee (O. nerka), pink salmon (O. gorbuscha), and steelhead trout at state and federal facilities in Oregon, Washington, and Alaska during 1969, 1970, and 1971. Reference to the exact location where each species was collected appears in the individual Results sections.

The approach utilized was to develop an initial fish sperm extender and life protector combination for each species. Chemical components and their respective concentrations were based on the Cortland salt solution developed by Phillips et al. (1957, 1958) from analyses of the semen and/or blood serum of brown trout (Salmo trutta); the Cortland salt solution was compared with other physiological salines by Wolf (1963). Additional chemicals were tested, based on their reported effectiveness in preserving other types of cells and tissues. Concentrations of chemicals were varied to anticipated extremes and survival of sperm cells was determined by estimates of the sperm's motility or ability to fertilize fresh ova.

After development of the extender-protector combination, investigations were concentrated on potential sources of variability and the refinement of techniques. Methods of collecting sperm samples and the variability attributable to the male are examples of such factors. Because the optimal freeze rate of -30 C/min had been determined by Graybill and Horton (1969) and Ott (1970), this research was focused on thawing procedures as cellular damage occurs during both processes.

GENERAL METHODS AND PROCEDURES

Cryopreservation of viable fish sperm involved collecting sperm and ova, diluting sperm with an extender[1] containing a life protector[2], equilibrating[3] sperm to the extender, freezing sperm samples in liquid nitrogen (LN_2), storing sperm in a LN_2 refrigerator[4], and fertilizing ova with thawed spermatozoa. General methods and procedures follow, whereas specific techniques are presented by species in the individual Results sections.

Extenders and Protectors

Stock solutions of each chemical component of a particular extender (EXT) were prepared and held in a refrigerator at

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[1]Fish Sperm Extender (extender, diluent): A solution of salts, sometimes including organic compounds, which helps maintain the viability of cells during cryopreservation.

[2]Life Protector (protector, additive): An organic compound which protects the viability of cells during the freezing and thawing process.

[3]Equilibration Time: The time period between dilution (when sperm and extender are mixed) and the actual freezing of the sperm sample.

[4]Biostat, Model 1000-X, Cryogenic Engineering Co., 4955 Bannock Street, Denver, Colorado 80216.

0.5-3.0 C for a maximum of 14 days. After 14 days storage, remaining solutions were discarded and new stock solutions were mixed. Utilization of stock solutions facilitated the rapid and accurate preparation of extenders.[5]

The volume of protector added to the extender was determined by two factors: (1) the dilution ratio[6] and (2) the concentration of protector in the diluted sperm sample. If the final concentration of protector desired in the diluted sperm sample was 10%,[7] and the dilution ratio was 1:4, then an extender with 12.5% protector was prepared. Thus, when 1 ml of sperm was diluted with 4 ml of the extender, the concentration of the additive was 10% in the diluted sperm sample. Extender-protector combinations were held in a refrigerator at 0.5-3.0 C for a maximum of 24 hr.

Collecting, Diluting, and Equilibrating Sperm

Adult specimens were selected, examined for ripeness, and if mature were either killed or anesthetized with tricaine methanesulfonate (MS-222). Urine and some sperm were expressed from the

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[5] The chemical composition of all extenders tested during this study is presented in Appendix A.

[6] Dilution Ratio: The ratio of sperm to extender on a volume to volume basis.

[7] The percentage of all life protectors is expressed on a volume to volume basis.

fish by applying manual pressure to the posterior-dorsal area of the abdominal cavity. Fish were then rinsed with fresh water and wiped dry around the genital region before semen was collected in dry 2-oz glass receptacles or 10-ml test tubes by applying manual pressure to the gonadal area. As they were collected, sperm samples were placed in an ambient water bath. After all samples were collected, extenders and ice were added to the bath so that all solutions were homothermous when mixed.

After 30 min in the iced water bath, an extender and sperm were pipetted into a dry container and thoroughly mixed by swirling. The dilution process was performed completely (all at once) or progressively over a specified time period (i.e., 1 ml of diluent mixed with 1 ml of sperm followed by three 1-ml aliquots of diluent added every 10 min).

Generally, equilibration time was as short as possible. After dilution of the sperm sample, 1-ml aliquots were immediately pipetted into 1-ml glass ampoules. Immediately the unsealed ampoules were placed in LN_2 vapor for freezing.

Freezing and Thawing Sperm

Ampoules were placed on numbered canes (cane capacity was 4 ampoules coded from top to bottom) and canes were put in a specific canister (capacity 12 canes). Canisters were held in LN_2

vapor (approximately -179 C) where the sperm freezes at about -30 C/min (Graybill and Horton 1969). After 5 min in LN_2 vapor, the canister was lowered into LN_2 (-196 C) for storage.

Sperm samples were thawed at ambient air temperature or in a water bath of specific temperature. If thawed in a water bath, "dry" or "wet" procedures were used. In the dry method (DM), the base of the ampoule was immersed in a water bath until the semen thawed. In the wet method (WM), the neck of the ampoule (0.5 ml capacity) was filled with water of prescribed temperature and then the base of the ampoule was immersed in the water bath until the semen thawed.

Survival of Sperm

Percent motility[8] and sperm activity[9] were two measures utilized to estimate sperm survival. These estimates were utilized when time was not adequate to conduct a fertilization experiment. In estimating percent motility, a thin smear of thawed sperm was placed on a microscope slide with a glass rod which had the tip

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[8]Percent Motility: The percent of motile cells observed in a sperm sample.

[9]Sperm Activity: The time of maximal activity of spermatozoa following the addition of water to the sample.

drawn to a fine point. All samples were analyzed within 60 sec of the smear, because spermatozoa were motile when thawed and the cells survived for approximately 90 sec. When undiluted or diluted non-frozen samples were analyzed, the same procedure was used; however, water (1:1 ratio) was added to activate the immotile sperm. When estimating sperm activity, the procedure was the same except that the time (sec) of vigorous spermatozoa activity was recorded. These survival estimates were used primarily to evaluate experimental variables in the formulation of new extenders.

Collecting, Fertilizing, and Incubating Ova

Mature specimens were selected, killed, and bled by severing the caudal or the afferent branchial artery. The fish were rinsed with fresh water, wiped dry, cut open from anus to breast, and the ova collected in dry 1-gal containers (plastic). Eggs from a single specimen or the pooled ova from several fish were subdivided into aliquots of 50-100.

Aliquots of eggs were mixed with diluted frozen-thawed sperm, diluted non-frozen sperm, or non-diluted fresh sperm. If the dilution ratio was 1:9, 1 ml of diluted sperm or 0.1 ml of undiluted sperm was added to the ova; thus, the number of spermatozoa per aliquot was approximately equal. After mixing the gametes, water (10-50 ml) was immediately added if non-frozen sperm was

utilized. When ova were inseminated with cryopreserved sperm, water (10-50 ml) was added 5 min after the gametes were mixed (cryopreserved sperm are active when thawed). After the addition of water to an egg group, the ova were immediately transferred to numbered incubation boxes (wood frames 1 1/4-in high by 3-in square, covered top and bottom with 1/32-in mesh plastic screen). These incubation boxes were transferred to a hatching basket, secured in place, and submerged in a hatching trough.

Eggs were incubated 7-21 days or to the time when the embryonic shield was formed. Ova were removed from the incubation boxes and placed in numbered glass jars containing Carnoy's solution (1 part glacial acetic acid to 3 parts 100% ethanol). The number of eggs with visible embryonic development, and the total number of ova were recorded. Based on these observations, the percent fertilization was determined.

Data Analysis

In the statistical interpretation of percent fertilization data, all computations were performed with the percentages rounded to the nearest 0.01; values tabulated in the text were rounded to the nearest 0.1. In the experiments reported, variables were fixed and data were unpaired. Statistical computations and analyses were

performed in accordance with methods listed by Steel and Torrie (1960) and Snedecor and Cochran (1967).

RESULTS

Steelhead Trout

Introduction

This section summarizes research on the cryopreservation of steelhead trout sperm previously published by Ott and Horton (1971b) with permission from the OSU Graduate School (Letter, Dean H. P. Hansen, 13 Aug 1971). Adult steelhead were collected at the Eagle Creek National Fish Hatchery (BSFW) near Estacada, Oregon during the spring of 1969. Ott and Horton (1971a) determined that the successful cryopreservation of chinook and coho salmon sperm involved development of an extender which contained a specific concentration of DMSO as the life protector. In order to improve on methods of freezing steelhead trout sperm developed by Graybill and Horton (1969), the chemical composition of the extender and the concentration of DMSO in the diluent were tested. As a result, a mean percent fertilization (MPF) of 59.3 was obtained when fresh steelhead ova were inseminated with cryopreserved sperm.

Methods and Procedures

Sperm samples were collected, iced for 30 min, diluted 1:9 with the extender, and frozen in 1-ml glass ampoules (unsealed) in

the vapor above the surface of LN_2. After 5 min in LN_2 vapor, ampoules of semen were lowered into the LN_2 and stored for further analysis.

In preliminary experiments, extenders containing selected concentrations of chemical components were prepared and tested. Sperm was collected from two or three males, divided into 1-ml aliquots, diluted with each of the extenders, and frozen. Indices of viability of semen thawed in a 4 C water bath were estimated by microscopic examination for percent motile sperm. Selection of the preferred concentration of each chemical was based on mean percent motility (MPM) estimates. In this manner $NaHCO_3$, lecithin, mannitol, and DMSO were tested until an improved extender-protector combination was developed.

Final evaluation of the modified extender was based on results of a fertilization experiment. Sperm samples were collected from 10 fish, diluted with the extender containing DMSO, and frozen in LN_2 for 7 days. Three steelhead trout were killed, bled by cutting the caudal artery, and the eggs of each divided into 36 groups. After 6 days the frozen sperm samples were thawed in hatchery water, and 1-ml aliquots from each male were mixed with a group of eggs from each female (three replicates). For controls, three groups of eggs from each female were fertilized with fresh sperm from the same male and three additional groups were not fertilized.

Each egg group (n=108) was placed in an individual container in a hatchery trough and incubated 14 days. Eggs were then placed in Carnoy's solution and the percent of eggs with visible embryonic development was determined.

Results

An improved fish sperm extender (EXT 164) was developed in which $NaHCO_3$ was 750 mg/100 ml; lecithin was 750 mg/100 ml; and mannitol was 250 mg/100 ml (Table 1 and Appendix A). In these tests EXT 48, used with limited success with steelhead trout by Graybill and Horton (1969), was the solution modified as well as the control solution. This procedure was useful in the evaluation of treatments (chemicals) within an experiment, but I do not recommend comparing the percent of motile sperm between experiments.

When samples of steelhead trout sperm were diluted with EXT 164 containing 4, 8, and 12% DMSO, MPM (n=6) estimates were 09, 20, and 45, respectively. Consequently, 12% DMSO was used in subsequent preparation of extenders.

The MPF of fresh steelhead trout ova inseminated with frozen-thawed sperm diluted with EXT 164 was 59.3 (Table 2). Sperm samples were thawed in a 5 C water bath, mixed with a group of eggs, and after 5 min hatchery water was added to the ova and the egg groups placed in a hatchery water trough. The

Table 1. Mean percent motility of steelhead trout sperm frozen and stored 3 days in liquid nitrogen and thawed in a 4 C water bath (dry) method), when diluted 1:9 and protected (12% DMSO) with extenders containing various concentrations of $NaHCO_3$, lecithin, and mannitol.

Chemical Concentration (mg/100 ml)	Extender Number	Mean Percent Motility (n=8)
$NaHCO_3$		
0	150	10
100	48	11
250	155	13
500	129	33
750	156	43
1000	130	26
Lecithin		
0	157	11
250	158	11
500	156	21
750	160	28
1000	161	23
1500	162	23
Mannitol		
0	160	25
100	163	25
250	164	40
500	165	15

Table 2. Mean percent fertilization of steelhead trout ova inseminated with sperm frozen and stored 7 days in liquid nitrogen and thawed in a 4 C water bath (dry method), when diluted 1:9 and protected (12% DMSO) with EXT 164.

Male Number	Mean Percent Fertilization			
	Female One	Female Two	Female Three	Pooled Mean
351	53.6	64.8	64.8	61.1
352	39.4	66.9	47.9	51.4
353	48.8	70.6	57.3	58.9
354	85.4	82.6	80.4	82.8
355	59.4	75.3	60.2	65.0
356	63.4	75.4	66.8	68.5
357	36.4	60.1	43.8	46.8
358	46.2	50.1	43.4	46.5
359	36.5	58.9	58.4	51.2
360	48.7	74.1	58.8	60.5
Pooled Mean	51.8	67.9	58.2	59.3

Control (Fresh Sperm and Eggs)	Female One	95.2 (n=2)
Control (Fresh Sperm and Eggs)	Female Two	97.2 (n=2)
Control (Fresh Sperm and Eggs)	Female Three	97.3 (n=2)
Control (Eggs With No Sperm)		0.0 (n=6)
Number of Eggs per Experiment Unit + SE		60.2 $\pm$ 15.4

hypothesis that the MPF for individual males or females was equal was rejected ($P \leq .01$), whereas interaction between males and females was not significant ($P \geq 10$) (Appendix B, Table 1). The MPF was 82.8 when fresh steelhead trout ova were inseminated with cryopreserved sperm from male 354 (Table 2).

Summary

The general results of the cryopreservation of steelhead trout sperm were as follows:

(1) An improved fish sperm extender, EXT 164, and life protector (12% DMSO) combination was developed. EXT 164 contained NaCl (730 mg/100 ml), KCl (38 mg/100 ml), $CaCl_2 \cdot 2H_2O$ (23 mg/100 ml), $NaHCO_3$ (750 mg/100 ml), $Na_2HPO_4 \cdot H_2O$ (41 mg/100 ml), $MgSO_4 \cdot 7H_2O$ (23 mg/100 ml), fructose (100 mg/100 ml), lecithin (750 mg/100 ml), and mannitol (250 mg/100 ml).

(2) The MPF was 59.3 (n=90) when EXT 164 was used in the cryopreservation of steelhead trout sperm utilized to inseminate fresh ova. The null hypothesis that the MPF recorded for individual males and females were equal was rejected ($P \leq .01$).

Pink Salmon

Introduction

This chapter summarizes research on the cryopreservation of pink salmon sperm. Gametes were collected from pink salmon returning to Sashin Creek and Lovers Cove Creek near Little Port Walter on Baronoff Island, Alaska, during the fall of 1970. Experiments were conducted to determine if diluents (EXT 141) used successfully with coho salmon sperm (Ott and Horton 1971a) could be used to cryopreserve pink salmon sperm. When these attempts proved unsuccessful, experiments were conducted to develop an effective extender-protector combination for pink salmon. Equilibration time, dilution ratio, thawing procedure, and male variability also were investigated. After completion of basic tests, 20,000 pink salmon ova were collected at Lovers Cove, shipped on ice by air and truck from Alaska to Oregon, and successfully fertilized (30%) in Oregon with sperm collected and frozen in Alaska.

Methods and Procedures

Pink salmon were killed prior to gamete collection. Collected sperm and extenders containing DMSO were placed in a styrofoam cooler and partially immersed with water from the

cradle stream; ice was added to the water and the sperm samples were diluted when the temperature of the bath was 5-8 C. Diluted semen was frozen in LN_2 and stored for subsequent analysis.

Frozen sperm samples were removed from the LN_2 refrigerator, placed in a water bath (5-8 C), and viability of thawed spermatozoa estimated by MPM. Two alternate thawing methods were evaluated: (1) an ampoule of frozen sperm was placed in a 40-50 C water bath until the sperm began to thaw, then the cells were removed immediately and analyzed (DM); (2) 0.5 ml of 40-50 C water was pipetted into an ampoule of frozen sperm, the ampoule placed in the 40-50 C water bath until the sperm began to thaw, after which the ampoule was removed from the bath, shaken, and cells were removed immediately and analyzed (WM, 40-50 C).

Final evaluation of sperm survival after freezing and thawing was based on fertilizing capabilities of sperm cells. In preliminary tests, eggs mixed with thawed sperm were incubated 20-30 hr, preserved in Carnoy's solution, and fertility determined by the presence of normal cell cleavage.

In the final fertilization experiment, sperm samples were diluted 1:9 with EXT 189, protected with 10% DMSO, and frozen immediately in LN_2 vapor. Eggs from 10 females were placed in two glass jars (1-gal each) situated on ice in a styrofoam cooler, and shipped by air and truck from Little Port Walter to the Big

Creek Salmon Hatchery (FCO) near Knappa, Oregon. Eggs were inseminated at the Big Creek hatchery with sperm frozen in Alaska and thawed (WM, 40-50 C) in Oregon. Eggs were incubated to the eyed stage at the Big Creek hatchery, transported to the OSU salmon hatchery located on Netarts Bay (Tillamook County), and surviving fry were liberated into Whiskey Creek, a tributary to the bay.

Results

Initial attempts to cryopreserve sperm with EXT 141 (10% DMSO) were unsuccessful. When frozen-thawed sperm were mixed with fresh ova, no fertilization was recorded; in controls, however, 72% of fresh ova were fertilized.

Subsequent tests resulted in development of a fish sperm extender (EXT 189) and life protector combination in which $NaHCO_3$ was 500 mg/100 ml; NaCl was 730 mg/100 ml; mannitol was 500 mg/100 ml; lecithin was 750 mg/100 ml; fructose was 500 mg/100 ml; and DMSO was 10% (Table 3 and Appendix A). In each experiment sperm from two males was diluted 1:9, protected with 10% DMSO, frozen immediately in LN_2 vapor, thawed in a 9 C water bath (DM), and the best chemical concentration determined by comparing MPM. In experiments retesting DMSO, $NaHCO_3$, and mannitol, MPM was maximum at levels previously selected

Table 3. Mean percent motility of pink salmon sperm frozen and stored in liquid nitrogen and thawed in a 5-8 C water bath (dry method), when diluted 1:9 and protected (10% DMSO) with extenders containing various concentrations of $NaHCO_3$, NaCl, mannitol, lecithin, and fructose (% DMSO was varied in one test).

Chemical Concentration	Extender Number	Mean Percent Motility (n=8)
$NaHCO_3$ (mg/100 ml)		
500	141	40
1000	175	30
1500	176	20
2000	177	10
2500	178	00
NaCl (mg/100 ml)		
730	141	40
1500	179	30
2500	180	10
5000	181	00
Mannitol (mg/100 ml)		
0	134	20
100	141	30
250	166	30
500	142	42
1000	143	32
Lecithin (mg/100 ml)		
0	182	20
250	183	22
500	184	27
750	142	42
1000	185	35
1500	186	22
Fructose (mg/100 ml)		
0	187	10
100	142	20
250	188	25
500	189	35
1000	190	25
DMSO (% v/v)		
5	141	10
10	141	30
15	141	10

(Table 4).

A dilution ratio of 1:9 and an equilibration time as near to zero as possible gave best results. Sperm survival was maximized in samples diluted 1:9 as opposed to 1:4 or 1:19; and MPM decreased when sperm samples were equilibrated 5 min.

MPM was 25 and 45 when the temperature of the bath was 4 and 40-50 C (DM), respectively. MPF from sperm thawed in the warmer bath was 54.1 and 64.2, while 40.0 and 45.2% were recorded when eggs were fertilized with sperm thawed in a 4 C water bath (number of eggs per sample was 350).

A fertilization experiment was conducted to confirm results based on MPM estimates. Sperm samples from two males were diluted 1:9 with EXT 189, protected with 10% DMSO, frozen immediately in LN_2 vapor, and thawed in a 40-50 C water bath (WM). The MPF for individual males was 55.2 (52.0-61.0)[10] and 76.5 (71.1-83.2).[11] Ova inseminated with fresh sperm had 100% fertilization.

Two sources of variability in preceding experiments were "vigor" of the individual male and quality of the sperm sample.

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[10] N=4, SE=4.27, and number of eggs per sample = 85.

[11] N=4, SE=5.92, and number of eggs per sample = 95.

Table 4. Mean percent motility of pink salmon sperm frozen and stored in liquid nitrogen and thawed in a 5-8 C water bath (dry method), when diluted 1:9 and protected (10% DMSO) with extenders containing various concentrations of $NaHCO_3$ and mannitol (% DMSO was varied in one test).

Chemical Concentration	Extender Number	Mean Percent Motility (n=8)
$NaHCO_3$ (mg/100 ml)		
0	191	10
250	192	20
500	189	35
750	193	20
1000	194	15
1500	195	10
Mannitol (mg/100 ml)		
0	138	10
100	197	20
250	198	25
500	189	40
1000	199	20
1500	200	15
DMSO (%)		
5.0	189	10
7.5	189	20
10.0	189	40
12.5	189	25
15.0	189	10

While the quality of the sperm sample was determined, in part, by the donor, the possibility existed that each sample contained urine. Both the effect of urine and of factors associated with male variability on the survival and fertilizing ability of cryopreserved sperm were unknown.

In tests of the effects of urine, MPM after 24 hr storage was 30, 50, 60, 50, 50, and 0 for samples containing 0, 5, 10, 25, 50, and 100% urine, respectively (Table 5). Sperm and urine were collected by massage from the same male. Urine was added to sperm samples such that 1-ml aliquots of sperm contained 0, 5, 10, 25, 50, and 100% (v/v) urine. Samples were placed in a water bath, held at 5-7 C for 24 hr, and MPM estimated 0, 7, 12, and 24 hr after dilution. Results indicated that sperm viability was enhanced when samples contained low volumes of urine. As a result, a fertilizing experiment was conducted to determine the effect of urine on the fertilizing capability of stored sperm.

MPM and MPF were highest when sperm contained 5% urine (Table 6). As before, urine was added to sperm samples such that 1-ml aliquots contained 0, 5, 10, 25, and 50% (v/v) urine. Samples were placed in a water bath at 5-7 C for 48 hr; MPM was recorded, and ova were inseminated with the various sperm samples. Concentrations as high as 25% urine did not seem to reduce sperm motility or potency to any marked degree. Contamination of sperm

Table 5. Mean percent motility of pink salmon sperm containing various concentrations of urine from the donor fish when stored at 5-7 C for 0, 7, 12, and 24 hr.

Urine (% v/v)	Storage Time (hr)	Mean Percent Motility (n=4)
0	0	80
0	7	80
0	12	30
0	24	30
5	0	90
5	7	90
5	12	80
5	24	50
10	0	80
10	7	80
10	12	80
10	24	60
25	0	80
25	7	80
25	12	80
25	24	50
50	0	70
50	7	70
50	12	80
50	24	50
100	0	70
100	7	80
100	12	70
100	24	0

Table 6. Percent motility of pink salmon sperm containing various concentrations of urine from the donor fish when stored at 5-7 C for 48 hr; and the percent fertilization of fresh pink salmon ova inseminated with these stored urine-sperm samples.

Male Number	Urine (% v/v)	Percent Motility	Percent Fertilization
01	00	10	27.4
01	05	30	78.4
01	10	30	62.2
01	25	30	62.0
01	50	00	13.6
03	00	20	65.0
03	05	40	88.7
03	10	50	84.9
03	25	40	85.9
03	50	20	42.0

samples with urine did not appear to be an important variable in the cryoprotective procedure.

I next hypothesized that the variability in sperm samples was caused, in part, by the state of sexual maturity of the donor male. To test this hypothesis, five males fresh from the ocean were captured on September 1 and 1-ml sperm samples were collected from each. Each fish was marked, placed in a holding pond, and sperm samples from selected males were collected on September 3, 4, 6, 9, and 13. MPM of sperm stored at 5-7 C was monitored until estimates decreased to about 10%. The optimal date of sperm collection was based on the sample which maintained a motility

greater than 10% for the longest time.

Duration of sperm viability was maximal when samples from males 4, 5, 6 and 8 were collected on September 9; whereas maximum viability occurred when sperm was collected from male 7 on September 3 (Table 7).

A final experiment involved the shipment from Alaska of 20, 000 ova which were fertilized in Oregon with cryopreserved sperm. Sperm samples were collected at Lovers Cove, diluted with EXT 189 containing 10% DMSO, frozen immediately in LN_2 vapor, and transported to Oregon in a LN_2 refrigerator. Ova obtained at Lovers Cove were inseminated in Oregon 20 hr after collection with sperm frozen 7 days previously in Alaska. In this experiment, sperm samples were thawed in a 40-50 C water bath (WM, 40-50 C). A fertilization of 30% was achieved, and 6, 000 normal appearing fry were subsequently released from the Netarts Bay hatchery into Whisky Creek. No adult pink salmon returned to Whiskey Creek one and one-half years later.

Summary

The general results were:

(1) Pink salmon sperm were cryopreserved successfully with EXT 189 containing 10% DMSO.

Table 7. Percent motility of pink salmon sperm when collected at progressive dates from five males and stored at 5-7 C until percent motility estimates approached 10%.

Male Number (Date of Sperm Collection)	Storage Time (hr)							
	0	12	24	48	72	96	120	126
Male 4	Percent Motility							
9/1/70	100	90	70	40	10	-	-	-
9/6/70	100	60	40	40	10	-	-	-
9/9/70	90	80	70	70	70	30	10	-
9/12/70	90	80	70	40	10	-	-	-
Male 5								
9/1/70	100	60	50	30	10	-	-	-
9/4/70	100	40	50	40	20	10	-	-
9/6/70	100	40	30	10	-	-	-	-
9/9/70	90	60	70	70	70	50	10	-
9/13/70	90	90	80	50	40	10	-	-
Male 6								
9/1/70	100	100	50	40	00	-	-	-
9/6/70	100	50	40	30	10	-	-	-
9/9/70	95	60	40	30	20	10	-	-
9/13/70	90	80	80	30	10	-	-	-
Male 7								
9/1/70	100	95	90	80	50	20	10	-
9/3/70	95	90	90	70	70	60	60	10
9/6/70	100	70	70	60	10	-	-	-
9/9/70	90	80	40	30	20	10	-	-
9/13/70	90	70	70	30	10	-	-	-
Male 8								
9/1/70	100	100	70	40	10	-	-	-
9/6/70	100	60	40	40	10	-	-	-
9/9/70	90	60	50	30	20	10	-	-
9/13/70	90	50	40	10	-	-	-	-

(2) Survival was enhanced when sperm was diluted 1:9 as compared to 1:4 and 1:19 ratios. Freezing sperm immediately after dilution maximized viability of thawed cells. A comparison of the wet and dry method of thawing sperm indicated that the wet method was superior.

(3) The percent motility and fertility were increased in sperm samples containing 5, 10, and 25% (v/v) urine when concentrations of 0, 5, 10, 25, and 50% (v/v) urine were tested.

(4) Duration of sperm viability when stored at 5-7 C was maximum when samples were collected eight days (except for one male) after these fish entered fresh water. Four males probably were immature when captured; sperm viability increased after these fish were held in fresh water. Data also indicated that sperm viability decreased when males were held for an extended time period.

(5) The MPF was 30% when 20, 000 eggs were inseminated with cryopreserved sperm, and 6, 000 fry were released into Whiskey Creek. This was the first attempt to fertilize a large number of eggs with frozen-thawed sperm. In addition, the eggs and resulting yolk-sac fry were successfully reared and smolts released.

Chum Salmon

Introduction

This section summarizes research in the cryopreservation of chum salmon sperm which resulted in a maximum fertilization of 29.3% of fresh ova. Sperm and ova were collected from chum salmon returning to Whiskey Creek, OSU Hatchery at Netarts Bay, during the fall of 1970. Experiments were conducted to develop an EXT which contained the preferred concentration of chemical components. After basic tests were completed, chum salmon ova were collected and inseminated with cryopreserved sperm.

Methods and Procedures

Chum salmon returning to Whiskey Creek were anesthetized with MS-222, and sperm was obtained by applying manual pressure to the abdominal area. Sperm samples were held 30 min in a 4-8 C water bath, diluted 1:9 with an EXT, protected with DMSO, and frozen in LN_2 vapor. Samples were thawed either in a 5-8 C water bath (DM) or in a 40-50 C water bath (WM, 8-10C). In the fertilization experiment, ova were inseminated with 1 ml of cryopreserved sperm. In controls, 0.1 ml of fresh, undiluted sperm was mixed with an egg group to ensure similar volumes of spermatozoa in

both test and control units. Eggs were incubated 14 days, placed in Carnoy's solution, and percentage of eggs with visible embryonic development was recorded.

Results

MPM was highest when the EXT contained 10% DMSO. In this test, sperm was diluted with EXT 134 and protected with 2.5, 5.0, 7.5, 10.0, and 15.0% DMSO. Using EXT 134 and 10% DMSO, motility was estimated at 20-30% when frozen samples were thawed in a 5-8 C water bath (DM).

Subsequent tests resulted in selection of two comparable fish sperm extenders in which $NaHCO_3$ was 500 mg/100 ml, and lecithin was 0 mg/100 ml; however, MPM was maximal when the EXT contained either 100 mg/100 ml fructose or mannitol (Table 8 and Appendix A).

The MPF was 8.3, 9.2, and 8.7 when cryopreserved sperm was diluted with EXT 204, 210, and 196, respectively (Table 9). In control units the MPF was 95.1 and 93.0. In this experiment, sperm samples from two males were diluted with EXT 196, 204, and 210, protected with 10% DMSO, and frozen in LN_2 vapor. EXT 204 contained 100 mg/100 ml fructose, EXT 210 contained 100 mg/100 ml mannitol, whereas EXT 196 contained 100 mg/100 ml fructose and mannitol. Eggs were inseminated with cryopreserved

Table 8. Percent motility of chum salmon sperm frozen and stored in liquid nitrogen and thawed in a 5-8 C water bath (dry method), when diluted 1:9 and protected (10% DMSO) with extenders containing various concentrations of $NaHCO_3$, lecithin, fructose, and mannitol.

Extender Number	Chemical Tested	Concentration (mg/100 ml)	Preferred Concentration	Percent Motility
201, 202 134, 203	$NaHCO_3$	0, 250, 500, 1000	500	20
204, 205, 129 134, 135	Lecithin	0, 250 500 750, 1000	0	10
206, 204, 207 208, 209	Fructose	0, 100 250 500, 1000	100	30-40
206, 210 211 212, 159	Mannitol	0, 100, 250 500, 100	100	30-40

Table 9. Percent fertilization of chum salmon ova inseminated with sperm frozen and stored 7 days in liquid nitrogen and thawed in a 40-50 C water bath (wet method, 8-10 C) when diluted 1:9 and protected (10% DMSO) with extenders 196, 204, and 210.

Male Number	Extender Number	Ampoule Number	Percent Fertilization
48	204	01	14.1
		02	9.7
		03	0.0
48	210	01	20.5
		02	14.4
		03	0.0
48	196	01	2.3
		02	1.0
		03	0.0
49	204	01	7.6
		02	17.1
		03	1.2
49	210	01	12.2
		02	7.9
		03	0.0
49	196	01	29.3
		02	2.5
		03	17.1
Control (Fresh Sperm and Eggs)		Male 50	95.1 $\pm$ 3.3[a] (n=3)
Control (Fresh Sperm and Eggs)		Male 51	93.0 $\pm$ 7.0 (n=3)

[a]Standard error.

sperm, and thawed in a 40-50 C water bath (WM, 8-10 C).

There was no detectable difference between treatments; however, the location of the ampoule appeared to be significant. Ampoules were numbered according to their position on the cane with ampoule number three being nearest the LN_2 during the freezing process. The MPF for sperm in ampoules one, two and three was 14.3, 8.8, and 3.1, respectively (Table 10); however, the hypothesis that these means were equal was not rejected ($P \geq .05$) (Appendix B, Table 2).

Summary

(1) The most effective concentration of DMSO in experimental extenders was 10% (v/v).

(2) Motility of cryopreserved sperm was maximal when semen was diluted in EXT 204 and 210.

(3) Mean percent fertilization was 8.3, 9.2, and 8.7 when fresh ova were inseminated with sperm cryopreserved in EXT 196, 204, and 210, respectively.

(4) The relative position of the ampoule on the cane appeared to influence MPF, but differences were insignificant ($P \leq .05$).

Table 10. Effect of proximity of ampoule to liquid nitrogen on the mean percent fertilization of chum salmon eggs inseminated with cryopreserved sperm (see Table 9).

Ampoule Number	Proximity to Liquid Nitrogen	Mean Percent Fertilization (n=6) ± SE
01	Furthest	14.3 ± 9.5
02	Intermediate	8.8 ± 6.3
03	Nearest	3.1 ± 6.9

Kokanee

Introduction

This section summarizes research in the cryopreservation of kokanee sperm which resulted in an MPF of 63.2%. Gametes were collected at the Issaquah State Salmon Hatchery (WDF) in Issaquah, Washington, during the fall of 1971. Experiments were conducted to develop a suitable EXT-life protector combination. Also investigated were the effects of pooling sperm samples, freezing diluted samples in ampoules and whirl-pak bags,[12] various thawing procedures, and sperm storage time in LN_2.

[12] Nasco, Fort Atkinson, Wisc. 53538.

Methods and Procedures

Kokanee returning to Issaquah Creek were collected by seine, and males and females were held separately in covered hatchery troughs. Periodically, fish were collected from Issaquah Creek to replace those used in the investigation. In most tests sperm was collected from several males and pooled. Sperm samples were held in 10 ml test tubes in an ice bath for 30 min, diluted 1:9 with an EXT, pipetted in 1-ml aliquots into 6-oz whirl-pak bags or 1-ml glass ampoules, and samples immediately were frozen in LN_2 vapor.

Ampoules of frozen sperm were thawed in either a 5-8, 13-15, or 40-50 C water bath using both the DM and WM (13-15 C). Samples frozen in whirl-pak bags were thawed by placing frozen sperm (removed from the whirl-pak bag) in 1-ml of 13-15 C water (frozen sperm was crushed to promote melting).

In fertilization experiments, ova of 5-8 females were collected, pooled, and subdivided (average number of ova per experimental unit was 81 ± 32.8). Ova were inseminated either with 1-ml of diluted non-frozen sperm, or 0.1-ml of fresh semen.

Results

Utilizing EXT 48 as the initial control, EXT 129 containing 10% DMSO was developed in which $NaHCO_3$ was 500 mg/100 ml,

fructose was 100 mg/100 ml, lecithin was 500 mg/100 ml, mannitol was 0 mg/100 ml, and KCl was 38 mg/100 ml (Table 11 and Appendix A). Samples were thawed in a 5-8 C water bath (DM) and by comparison of MPM the preferred concentration of each chemical was selected.

The MPF was 18.4 $\pm$ 12.4 as compared to 12.2 $\pm$ 13.8 when sperm samples were diluted with EXT 129 and 48 (both contained 10% DMSO), respectively (Table 12). The null hypothesis that these means were equal was not rejected ($P \geq .15$) (Appendix B, Table 3). In control groups, ova were inseminated with fresh sperm and non-frozen sperm diluted with EXT 48 and 129 with 10% DMSO. The MPF recorded with these respective treatments was 86.1 $\pm$ 0.2, 62.6 $\pm$ 9.5, and 75.1 $\pm$ 8.3 (Table 12). The means 62.6 and 75.1 were not statistically different ($P \geq .10$) Appendix B, Table 3).

Experiments to determine the most effective thawing procedure were conducted utilizing sperm diluted with EXT 129 (10% DMSO). Variables tested were water bath temperature and the WM (13-15 C) and DM of thawing sperm. The MPF was maximal when samples were thawed in a 13-15 C (WM or DM) or in a 40-50 C (WM) water bath (Table 13). MPF recorded were 56.7, 55.3, and 55.6%, respectively. Fertilizations with sperm thawed in the 5-8 C (DM or WM or 40-50 C bath (DM) were 32.6, 38.8, and 36.4%, respectively.

Table 11. Mean percent motility of kokanee sperm frozen and stored in liquid nitrogen and thawed in a 5-8 C water bath (dry method), when diluted 1:9 and protected (10% DMSO) with extenders containing various concentrations of $NaHCO_3$, fructose, lecithin, mannitol, and KCl (% DMSO was varied in one test).

Chemical Concentration	Extender Number	Preferred Concentration	Mean Percent Motility
$NaHCO_3$ (mg/100 ml)			
0	154		
100	48		
250	155		
500	129	500	30
750	156		
1000	130		
Fructose (mg/100 ml)			
0	213		
100	129	100	30
250	214		
500	215		
750	216		
1000	217		
Lecithin (mg/100 ml)			
0	204		
100	132		
250	205		
500	129	500	25
750	134		
1000	135		
Mannitol (mg/100 ml)			
0	129		
100	218	0	35
250	219		
500	184		
750	220		
1000	221		
KCl (mg/100 ml)			
0	222		
38	129	38	30
250	223		
500	224		
DMSO (% v/v)			
5.0	48		
7.5	48		
10.0	48	10.0	15
15.0	48		

Table 12. Mean percent of fertilization of kokanee ova inseminated with frozen and non-frozen sperm diluted with either extender 48 or 129.

Extender Number		Mean Percent
Frozen	Non-Frozen	Fertilization ± SE
48	-	12.2 ± 13.8 (n=14)
129	-	18.4 ± 12.4 (n=14)
-	48	62.6 ± 9.5 (n=3)
-	129	75.1 ± 8.3 (n=3)
Control (fresh sperm and ova)		86.1 ± 0.2 (n=2)

Table 13. Mean percent fertilization of kokanee ova inseminated with sperm frozen and stored 1 day in liquid nitrogen and thawed in either a 5-8, 13-15, or 40-50 C water bath (dry method or wet method, 13-15 C), when diluted 1:9 and protected (10% DMSO) with extender 129.

Temperature Bath (C)	Thawing Method	Mean Percent Fertilization (n=5) ± SE
5-8	Dry	32.6 ± 12.6
5-8	Wet	38.8 ± 14.2
13-15	Dry	56.7 ± 16.0
13-15	Wet	55.3 ± 5.5
40-50	Dry	36.4 ± 16.7
40-50	Wet	55.6 ± 9.9
Control (Fresh Sperm and Eggs)		90.3 ± 5.1 (n=3)

The means of 56.7, 55.3, and 55.6% were pooled and compared with the means of 32.6, 38.8, and 36.4%. The pooled means were statistically different ($P \leq .01$) (Appendix B, Table 4).

The MPF was 33.4 and 44.3 when sperm samples were thawed in a 13-15 C water bath utilizing the DM and WM, respectively (Table 14). These means were significantly different ($P \leq .05$) (Appendix B, Table 5). A comparison also was made between samples thawed in a 13-15 C bath (WM) in which water was added to the sperm and ova mixture either immediately or after 5 min. When water was added immediately the MPF was 4.1; whereas when water was added after 5 min the MPF was 44.3. The hypothesis that 4.1 and 44.3 were equal was rejected ($P \leq .01$) (Appendix B, Table 5).

When sperm was thawed with the WM, survival was enhanced. Utilization of the WM seemingly would be more effective if sperm could be removed from the ampoule while still frozen. Because this was impractical using glass ampoules, sperm was frozen in whirl-pak bags from which frozen sperm could readily be removed prior to thawing.

A MPF of 63.2 was achieved when sperm samples (1-ml) frozen in whirl-pak bags were removed in a clump and thawed in 1-ml of 14 C water for 10 sec (Table 15). Ampoules and whirl-pak bags were removed in a clump and thawed in 1-ml of 14 C water for 10 sec (Table 15). Ampoules and whirl-pak bags containing

Table 14. Mean percent fertilization of kokanee ova inseminated with sperm frozen and stored 2 days in liquid nitrogen and thawed in a 13-15 C water bath (dry method or wet method, 13-15 C), when diluted 1:9 and protected (10% DMSO) with extender 129 and when water was added to the sperm-ova mixture either immediately or 5 min after mixing gametes.

Temperature Bath (C)	Thawing Method	Interval Before Water Added to Gametes (min)	Mean Percent Fertilization (n=5) ± SE
13-15	Dry	5	33.4 + 9.5
13-15	Wet	5	44.3 ± 4.1
13-15	Wet	0	4.1 ± 2.6
Control (Sperm diluted with EXT 129 with 10% DMSO, and Eggs)			48.4 ± 1.7 (n=2)
Control (Fresh Sperm and Eggs)			77.0 ± 0.0 (n=1)

Table 15. Mean percent fertilization of kokanee ova inseminated with sperm frozen and stored 2 days in liquid nitrogen and thawed in a 13-15 C water bath (dry method or wet method, 13-15 C), when diluted 1:9 and protected (10% DMSO) with extender 129 (sperm samples were frozen either in whirl-pak bags[a] or ampoules).

Freezing Method	Thawing Method	Mean Percent Fertilization (n=5) ± SE
Ampoule	Dry	48.6 ± 4.2
Whirl-pak Bag	Dry	53.3 ± 8.6
Whirl-pak Bag	Wet	63.2 ± 2.7
Control (Sperm diluted with EXT 129 with 10% DMSO, and Eggs)		69.8 ± 10.3 (n=2)
Control (Fresh Sperm and Eggs)		94.4 ± 0.0 (n=1)

[a] Nasco, Fort Atkinson, Wisc. 53538

frozen sperm also were thawed in a 14 C water bath (DM). The MPF observed was 48.6 and 53.3, respectively, and the hypothesis that these means were equal was not rejected ($P \geq .10$) Appendix B, Table 6). However, the hypothesis that 63.2 and 53.3 were equal was rejected ($P \leq .05$) (Appendix B, Table 6), indicating a beneficial effect of the WM with whirl-pak bags. In control units, the MPF was 69.8 when sperm was diluted 1:9 with EXT 129 (10% DMSO), whereas fresh sperm fertilized 94.4% of fresh ova.

In previous experiments with kokanee sperm, the concentration of DMSO in diluted sperm was 10%. Glycerol, a life protector, previously was judged unsatisfactory for cryopreservation of coho salmon sperm (Ott and Horton, 1971a). However, glycerol in combination with DMSO possibly could enhance sperm survival. Extenders containing DMSO and glycerol at concentrations of 5, 10, and 15% (factorial design) were tested for their effectiveness in a fertilization experiment. The MPF was 5.4 when DMSO was 10% and glycerol was 5% (Table 16). The only factor significantly effecting MPF was glycerol ($P \leq .05$) (Appendix B, Table 7). As the concentration of glycerol increased from 5-10-15%, the MPF decreased from 4.4-2.2-0.3, respectively. It was concluded that glycerol was an unsuitable component of the EXT.

Table 16. Mean percent fertilization of kokanee ova inseminated with sperm frozen and stored 3 days in liquid nitrogen and thawed in a 13-15 C water bath (wet method, 13-15 C), when diluted 1:9 and protected (5, 10, or 15% DMSO and glycerol, factorial design) with extender 129.

Glycerol (% v/v)	DMSO (% v/v)	Mean Percent Fertilization (n=3) ± SE
5	5	4.7 ± 2.4
10	5	3.5 ± 2.6
15	5	0.5 ± 0.9
5	10	5.4 ± 6.5
10	10	1.6 ± 1.8
15	10	0.4 ± 0.8
5	15	3.2 ± 1.1
10	15	1.4 + 2.9
15	15	0.0 ± 0.0
Control (Fresh Sperm and Eggs		95.6 ± 14.1 (n=3)

In theory, sperm viability remains constant when stored at -196 C. To test this assumption, sperm was diluted with EXT 129, protected with 10% DMSO and frozen. Fertilization experiments were conducted after 4, 9, and 16 days storage in LN_2. On each experimental date, viability of ova was determined by MPF achieved with fresh sperm. Adjustments in the MPF were made to compensate for any change in the viability of eggs between experiments. The initial day of the experiment (day 4) was the control for days 9 and 16. For example, if the fertilization percentage in the

control groups was 100 on day 4 and 80 on day 9, then 20% was added to each MPF recorded on day 9. Statistical analyses were performed on the adjusted values.

Sperm viability remained constant during 16 days storage in LN_2. After 4, 9, and 16 days, the adjusted MPF was 50.4, 66.0, and 45.1 with male 01 and 54.8, 52.5, and 50.8 with male 02 (Table 17). When a decrease in the MPF of 50.4 and 45.1; 54.8 and 52.5; and 54.8 and 50.8 were compared, the hypothesis that these means were equal was not rejected ($P \geq .10$) (Appendix B, Table 8).

In previous tests, sperm samples from several males were pooled. Pooling was employed because only 1-3 ml samples could be collected from each male. Because the effect of mixing sperm samples was unknown, an experiment comparing sperm viability from individual males and pooled males was conducted. The MPF was 32.9 (male 01), 26.3 (male 02), and 27.6 (male 01 + 02) when ova were inseminated with cryopreserved sperm (Table 18). Apparently, pooling sperm samples had no independent effect.

Individual male variability also was investigated; sperm was collected from six males and the MPF achieved with each male was compared with the volume of sperm obtained from each. The MPF with males which produced more than three ml or less than one ml of sperm was 21.6 and 24.2, respectively (Table 19).

Table 17. Mean percent fertilization of kokanee ova inseminated with sperm frozen and stored 4, 9, or 16 days in liquid nitrogen and thawed in a 40-50 C water bath (wet method, 13-15 C), when diluted 1:9 and protected (10% DMSO) with extended 129.

Male Number	Storage Time (Days)	Mean Percent Fertilization (n=5) ± SE	Adjusted Mean Percent Fertilization ± SE
01	4	50.4 ± 8.5	50.4 ± 8.5
02	4	54.8 ± 9.2	54.8 ± 9.2
01	9	50.5 ± 9.8	66.0 ± 9.8
02	9	37.0 ± 10.7	52.5 ± 10.7
01	16	33.0 ± 10.7	45.1 ± 10.7
02	16	38.7 ± 12.8	50.8 ± 12.8
Control (Fresh Sperm and Eggs) Day 4,			96.3 ± 0.6 (n=2)
Control (Fresh Sperm and Eggs) Day 9,			80.8 ± 2.8 (n=2)
Control (Fresh Sperm and Eggs) Day 16,			84.2 ± 0.9 (n=2)

Table 18. Mean percent fertilization of kokanee ova inseminated with pooled and individual sperm samples frozen and stored 2 days in liquid nitrogen and thawed in a 13-15 C water bath (wet method, 13-15 C), when diluted 1:9 and protected (10% DMSO) with extender 129.

Male Number	Mean Percent Fertilization (n=5) ± SE
01	32.9 ± 10.0
02	26.3 ± 14.9
01 + 02	27.6 ± 11.5
Control (Fresh Sperm and Eggs)	99.0 ± 1.4 (n=2)

Table 19. Mean percent fertilization of kokanee ova inseminated with sperm frozen and stored 4 days in liquid nitrogen and thawed in a 13-15 C water bath (wet method, 13-15 C), when diluted 1:9 and protected (10% DMSO) with extender 129.

Male Number	Sperm Sample (ml)	Mean Percent Fertilization (n=3) ± SE
05	3	13.9 ± 9.1
06	3	29.2 ± 6.3
09	3	21.6 ± 7.5
10	1	23.3 ± 17.3
07	1	19.5 ± 4.1
08	1	29.9 ± 15.3
Control (Fresh Sperm and Eggs)		84.2 ± 0.0 (n=2)

The hypothesis that these means were equal was not rejected ($P \geq .10$) (Appendix B, Table 9).

Summary

(1) Extender 129 with 10% DMSO was used to cryopreserve kokanee sperm and fertilized 63.2% of fresh ova.

(2) Mortality of a certain portion of spermatozoa occurred prior to the actual freeze-thaw process, since the MPF with diluted non-frozen sperm was less than with undiluted non-frozen semen.

(3) Fertilizing capability of sperm was maximized when thawed in 13-15 C bath (DM or WM) or a 40-50 C bath (WM, 13-15 C).

(4) The MPF was significantly greater when sperm was thawed in a 13-15 C bath using the WM as compared to the DM.

(5) As compared to adding water immediately, water added 5 min after ova and cryopreserved sperm were mixed resulted in a significantly greater MPF.

(6) Freezing sperm samples in whirl-pak bags gave better results than freezing in ampoules. A comparison of the WM and DM with this freezing technique resulted in a significant increase in MPF when the WM was utilized.

(7) Glycerol was determined to be an unsatisfactory protector of kokanee sperm.

(8) The fertilizing capability of sperm stored 16 days in LN_2 did not significantly decrease over that stored 4 and 9 days.

(9) The MPF resulting from pooled cryopreserved sperm samples was intermediate to that from individual males.

(10) The MPF using cryopreserved sperm could not be correlated with the volume of semen collected from individual males.

Fall Chinook Salmon

Introduction

This section summarizes initial research on the cryopreservation of fall chinook salmon sperm. The highest MPF obtained was 54.3 with sperm diluted 1:9 and protected (7.5% DMSO) with EXT 275. While research was centered on the development of a suitable EXT-protector combination, the effect of sperm storage time prior to dilution also was investigated. In addition, reliability of MPM estimates in selecting preferred concentration of chemical constituents of the diluent was tested in several fertilization experiments.

Methods and Procedures

Fall chinook salmon returning to Issaquah State Salmon Hatchery were killed prior to the collection of sperm and ova. Sperm samples were iced 15-30 min, diluted 1:4 or 1:9 with an EXT, protected with DMSO, frozen immediately in LN_2 vapor, and thawed in a 10-15 C water bath (DM). Sperm survival was evaluated by estimates of MPM, duration of sperm activity (DSA), and/or MPF.

Results

In tests evaluating the effects of five concentrations of DMSO (5.0, 7.5, 10.0, 12.5, and 15.0%) on sperm viability, MPM was maximal at 10%. For this experiment, sperm was diluted 1:9 with EXT 129.

Survival (MPM) was highest when sperm was diluted with EXT 202 containing the basic components of EXT 129 except that $NaHCO_3$ was decreased to 250 mg/100 ml and lecithin was increased to 750 mg/100 ml (Table 20). When the concentration of $NaHCO_3$ was re-tested at 0 (EXT 201), 100 (EXT 256), 200 (EXT 257), 300 (EXT 258), 400 (EXT 259), and 500 (EXT 134) mg/100 ml, MPM was highest at 400 mg/100 ml. In this test, sperm from four males was utilized and MPM with EXT 259 was 45. Replicating this experiment three times (each test involved two males) the MPM in all test groups was less than 10. The reason for decreased MPM was not apparent, but quality of sperm samples (male variability) appeared to be the causative factor. Experiments conducted between September 22 and 25 resulted in low MPM estimates whereas those conducted prior to the 22nd and after the 26th exhibited fairly high MPM. During the time interval between September 22 and 26 fall chinook salmon were not actively migrating, thus fresh males were not available.

Table 20. Mean percent motility of fall chinook salmon sperm frozen and stored in liquid nitrogen and thawed in a 3-5 C water bath (dry method), when diluted 1:9 and protected (10% DMSO) with extenders containing various concentrations of $NaHCO_3$ and lecithin.

Extender Number	Chemical Tested	Concentration (mg/100 ml)	Preferred Concentration	Mean Percent Motility
154, 155 129, 156 130	$NaHCO_3$	0, 250, 500, 750, 1000	250	30-50
253, 254, 155, 202 255	Lecithin	0, 250, 500, 750 1000	750	30-40

Table 21. Duration of activity of fall chinook salmon sperm stored 60 min in an ice water bath (0-4 C), when diluted 1:9 and protected (5.0, 7.5, 10.0, 12.5, or 15.0% DMSO) with extender 259.

DMSO (% v/v)	Duration of Sperm Activity (sec) (n=3) ± SE Storage Time (0-15 min)	Storage Time (45-60 min)
5.0	22.7 ± 1.5	23.7 ± 1.5
7.5	15.7 ± 3.2	21.7 ± 2.5
10.0	15.0 ± 1.0	17.3 ± 0.6
12.5	15.3 ± 1.2	17.3 ± 0.6
15.0	11.7 ± 2.5	10.0 ± 1.0
Control (Fresh Sperm)	26	21

In previous experiments survival of cryopreserved sperm was estimated by MPM. Because this technique was too subjective, a less subjective method, DSA, was employed to evaluate diluted non-frozen and fresh undiluted sperm. This technique has limited application with frozen-thawed sperm because addition of water causes an immediate cessation of sperm activity.

Fall chinook salmon sperm was diluted 1:9 with EXT 259, protected with 5.0, 7.5, 10.0, 12.5, and 15.0% DMSO, and placed in an ice water bath (0-4 C). The DSA (sec) was highest in samples containing 5.0% DMSO (Table 21). Similar results were recorded in past experiments; however, when samples were cryopreserved, MPM was maximal in diluents containing 10% DMSO. The DSA increased between the first and second sample period for all treatments except the one containing 15.0% DMSO (Table 21). Apparently sperm motility was inhibited by dilution, but the effect was reversible except at the highest concentration of DMSO.

In a similar experiment, five concentrations of $NaHCO_3$ were tested and DSA (sec) was estimated 0-30 and 60-120 min following dilution. Duplicate samples were frozen in LN_2 vapor and MPM estimated when samples were thawed in an ice water bath (0-4 C) (DM). The DSA was maximal after 0-30 min in samples diluted with EXT 260 (600 mg $NaHCO_3$/100 ml) whereas after 60-120 min EXT 261 (700 mg $NaHCO_3$/100 ml) proved best (Table 22). In

Table 22. Duration of activity of fall chinook salmon sperm stored 120 min in an ice water bath (0-4 C), when diluted 1:9 and protected (7.5% DMSO) with extenders containing various concentrations of $NaHCO_3$.

Extender Number	$NaHCO_3$ (mg/100 ml)	Duration of Sperm Activity (sec) (n=3) + SE	
		Storage Time (0-30 min)	Storage Time (60-120 min)
258	300	13.7 ± 1.7	15.0 ± 0.8
259	400	15.2 ± 1.9	15.0 ± 0.8
134	500	16.0 ± 0.8	14.2 ± 2.1
260	600	17.0 ± 1.0	17.2 ± 1.3
261	700	15.7 ± 1.3	17.7 ± 1.9
Control (Fresh Sperm)		24.2 ± 2.1	21.5 ± 0.6

the replicated samples frozen in LN_2, MPM was highest when sperm was diluted with EXT 260. In this trial, the initial estimate of DSA coincided with observations based on frozen-thawed spermatozoa; however, because final results were not consistent with preliminary findings (Table 20) the concentration of $NaHCO_3$ was subjectively set at 500 mg/100 ml (EXT 134).

When sperm samples were diluted with EXT 134 and 227, MPM of cryopreserved sperm was highest in EXT 227. Extender 134 contained NaCl, KCl, $CaCl_2 \cdot {}^{2H}{}_2{}^{0}$, $Na_2HPO_4 \cdot H_2O$, and $MgSO_4 \cdot 7H_2O$ in a combined concentration of 855 mg/100 ml.

This EXT also contained fructose, lecithin, and $NaHCO_3$. The concentrations of fructose, lecithin, and $NaHCO_3$ in EXT 227 were equivalent to those in EXT 134, but the only other salt was NaCl at 850 mg/100 ml. The MPM of sperm in EXT 227 and 134 was 35 and 25, respectively.

Working with EXT 227, the MPM was maximal when samples were diluted with EXT 225 containing 250 mg/100 ml $NaHCO_3$ (Table 23). The concentration of $NaHCO_3$ in the diluent was re-tested because conflicting results were obtained in previous tests (Tables 20 and 22). Utilizing EXT 225 as the control solution and varying the concentration of fructose, the MPM of thawed sperm was 40-50 with no difference observed between treatments (Table 23). Based on these results, EXT 264 containing 850 mg NaCl, 250 mg $NaHCO_3$, and 750 mg lecithin per 100 ml became the preferred diluent.

Utilizing EXT 263 as the control solution and varying the concentration of lecithin, MPM in cryopreserved samples was constant for all treatments; however, DSA was maximal when lecithin was 1000 mg/100 ml, EXT 270 (Table 24).

In experiments designed to retest the concentration of NaCl and $NaHCO_3$ in EXT 225, the MPM of frozen-thawed sperm was maximal when samples contained 850 mg/100 ml NaCl (EXT 225),

Table 23. Mean percent motility of fall chinook salmon sperm frozen and stored in liquid nitrogen and thawed in a 3-5 C water bath (dry method), when diluted 1:9 and protected (10% DMSO) with extenders containing various concentrations of $NaHCO_3$ and fructose.

Extender Number	Chemical Tested	Concentration (mg/100 ml)	Preferred Concentration	Mean Percent Motility
226	$NaHCO_3$	0	250	40-50
225		250		
227		500		
228		750		
262		1000		
263	Fructose	0	0	40-50
225		100		
264		250		
265		500		
266		750		

Table 24. Mean percent motility of fall chinook salmon sperm frozen and stored in liquid nitrogen and thawed in a 3-5 C water bath (dry method), when diluted 1:9 and protected (10% DMSO) with extenders containing various concentrations of lecithin (duration of sperm activity estimated in subsamples stored 60 min in a 3-5 C water bath).

Extender Number	Lecithin (mg/100 ml)	Mean Percent Motility Frozen-Thawed Sperm	Duration of Sperm Activity (sec) Storage Time (Min) 0	15	30	45	60
267	0	30-40	15	16	15	10	08
268	250	30-40	18	14	20	12	13
269	500	30-40	14	15	18	14	13
263	750	30-40	17	16	18	14	12
270	1000	30-40	18	19	18	15	16

whereas the DSA in non-frozen subsamples was highest in EXT 272 containing 1050 mg/100 ml NaCl (Table 25).

In fertilization experiments designed to test previous results, the following six comparisons were made:

(1) EXT 134 versus EXT 227

(2) EXT 225 versus EXT 227

(3) EXT 225 versus EXT 272

(4) EXT 225 versus EXT 262

(5) EXT 263 versus EXT 275 versus EXT 276

(6) 5 versus 10 versus 15% DMSO

Each test involved the sperm of a different male(s), but the fertilization experiment was conducted with the same group of ova. Listed below are results of these tests, along with the predicted results based on MPM estimates and DSA observations.

(1) Based on MPM estimates, the MPF should be greatest when sperm was diluted with EXT 227 as compared with EXT 134. The recorded MPF was highest when cryopreserved sperm was diluted with EXT 227 (Table 26); however, treatment means for males 200 and 201 were not significantly different ($P \geq .10$) (Appendix B, Table 10).

(2) Based on MPM estimates, the MPF should be greatest when sperm was diluted with EXT 225 as compared with EXT 227. The recorded MPF was highest when frozen-thawed sperm was

Table 25. Mean percent motility of fall chinook salmon sperm frozen and stored in liquid nitrogen and thawed in a 3-5 C water bath (dry method), when diluted 1:4 and protected (10% DMSO) with extenders containing various concentrations of NaCl (duration of sperm activity estimated in subsamples stored 30 min in a 3-5 C water bath).

Extender Number	NaCl (mg/100 ml)	Mean Percent Motility Frozen-Thawed Sperm	Duration of Sperm Activity (sec) Storage Time (min) 0	10	20	30
271	650	20-30	00	00	08	04
225	850	30-40	13	16	15	18
272	1050	20-30	19	21	22	20
273	1250	20-30	18	16	00	00
274	1450	20-30	13	13	00	00

Table 26. Mean percent fertilization of fall chinook salmon ova inseminated with sperm frozen and stored 14 days in liquid nitrogen and thawed in a 10-15 C water bath (dry method), when diluted 1:9 and protected (10% DMSO) with extenders 134 or 227).

Extender Number	Male Number	Mean Percent Fertilization (n=6) $\pm$ SE
134	200	28.9 $\pm$ 16.5
227	200	35.1 $\pm$ 12.2
134	201	32.9 $\pm$ 11.0
227	201	35.0 $\pm$ 11.1
Number of Eggs per Experimental Unit		(49.0 $\pm$ 3.8)

diluted with EXT 225 (Table 27); however, the null hypothesis that means were equal was not rejected ($P \geq .10$) (Appendix B, Table 11).

(3) Based on MPM estimates, the MPF should be greatest when sperm was diluted with EXT 225 as compared with EXT 272; conversely based on DSA, the MPF should be highest with EXT 272. The MPF was 7.9 and 7.8 when cryopreserved sperm was diluted with EXT 225 and 272, respectively (Table 28). The hypothesis that the means were equal was not rejected ($P \geq .10$) (Appendix B, Table 12).

(4) When frozen-thawed sperm was diluted with EXT 225 and 263, the MPM estimates indicated similar survival of cells in both treatments. The MPF was 22.6 and 16.6 when cryopreserved sperm was diluted with the two diluents (Table 29). The null hypothesis that the treatment means were equal was not rejected ($P \geq .10$) (Appendix B, Table 13).

(5) The MPF was 23.1, 25.1, and 24.0 when ova were inseminated with frozen-thawed sperm diluted with EXT 263, 275, and 276, respectively (Table 30). It was anticipated that sperm survival, hence MPF, would be highest utilizing EXT 275, but the hypothesis that treatment means were equal was not rejected ($P \geq .10$) (Appendix B, Table 14).

(6) The MPF of ova inseminated with cryopreserved sperm was 22.8, 28.2, and 20.9 when sperm was protected with 5.0, 7.5,

Table 27. Mean percent fertilization of fall chinook salmon ova inseminated with sperm frozen and stored 12 days in liquid nitrogen and thawed in a 10-15 C water bath (dry method), when diluted 1:9 and protected (10% DMSO) with extenders 225 or 227.

Extender Number	$NaHCO_3$ (mg/100 ml)	Mean Percent Fertilization (n=6) ± SE
225	250	25.7 ± 7.6
227	500	20.6 ± 5.8
Number of Eggs per Experimental Unit		(51.2 ± 3.9)

Table 28. Mean percent fertilization of fall chinook salmon ova inseminated with sperm frozen and stored 7 days in liquid nitrogen and thawed in a 10-15 C water bath (dry method), when diluted 1:9 and protected (10% DMSO) with extenders 225 or 272.

Extender Number	NaCl (mg/100 ml)	Mean Percent Fertilization (n=6) ± SE
225	850	7.9 ± 5.9
272	1050	7.8 ± 7.7
Number of Eggs per Experimental Unit		(47.7 ± 4.8)

Table 29. Mean percent fertilization of fall chinook salmon ova inseminated with sperm frozen and stored 5 days in liquid nitrogen and thawed in a 10-15 C water bath (dry method), when diluted 1:9 and protected (10% DMSO) with extenders 225 or 263.

Extender Number	Fructose (mg/100 ml)	Mean Percent Fertilization (n=6) ± SE
225	100	22.2 ± 5.7
263	0	16.6 ± 8.2
Number of Eggs per Experimental Unit		(46.2 ± 5.9)

Table 30. Mean percent fertilization of fall chinook salmon ova inseminated with sperm frozen and stored 5 days in liquid nitrogen and thawed in a 10-15 C water bath (dry method), when diluted 1:9 and protected (10% DMSO) with extenders 263, 275, or 276.

Extender Number	$NaHCO_3$ (mg/100 ml)	Mean Percent Fertilization (n=6) ± SE
263	250	23.1 ± 4.6
275	375	25.1 ± 9.5
276	500	24.0 ± 6.7
Number of Eggs per Experimental Unit		(46.3 ± 3.7)

and 10.0% DMSO, respectively (Table 31). The predicted result was obtained as MPF was highest when samples contained 7.5% DMSO; however, the null hypothesis that treatment means were equal was not rejected ($P > .10$) (Appendix B, Table 15).

The controls for the previous six experiments involved the insemination of ova with fresh sperm and diluted (EXT 275, 7.5% DMSO) non-frozen sperm. The MPF was 87.5 and 91.6 when ova were mixed with diluted and fresh sperm, respectively (Table 32). The null hypothesis that treatment means were equal was not rejected ($P \geq .10$)(Appendix B, Table 16).

Three additional experiments were conducted on the sperm of fall chinook salmon. Two involved the chemical composition of the EXT, whereas one tested length of storage in an ice water bath. In previous experiments sperm samples were iced for 15-30 min prior to dilution and freezing; however, the effect of this or a longer interval of time was unknown.

The MPF was 42.1, 53.5, 54.3, 48.5, and 46.9 when cryopreserved sperm samples were iced 0, 15, 30, 60, or 120 min, respectively, prior to dilution and freezing (Table 33). The null hypothesis that treatment means were equal was not rejected ($P \geq .10$) (Appendix B, Table 17). In controls, the MPF was 96.4 and 99.6 when ova were inseminated with diluted (non-frozen) and

Table 31. Mean percent fertilization of fall chinook salmon ova inseminated with sperm frozen and stored 4 days in liquid nitrogen and thawed in a 10-15 C water bath (dry method), when diluted 1:9 and protected (5.0, 7.5, or 10.0% DMSO) with EXT 275.

Extender Number	DMSO (% v/v)	Mean Percent Fertilization (n=6) ± SE
275	5.0	22.8 ± 12.4
275	7.5	28.2 ± 9.6
275	10.0	20.9 ± 10.2
Number of Eggs per Experimental Unit		(49.7 ± 4.8)

Table 32. Mean percent fertilization of fall chinook salmon ova inseminated with fresh sperm or non-frozen sperm diluted 1:9 and protected (7.5% DMSO) with EXT 275.

Treatment	Mean Percent Fertilization (n=6) ± SE
Fresh Sperm	91.6 ± 4.6
Diluted Sperm	87.5 ± 5.1
Number of Eggs per Experimental Unit	(49.1 ± 4.3)

Table 33. Mean percent fertilization of fall chinook salmon ova inseminated with sperm frozen and stored 5 days in liquid nitrogen and thawed in a 10-15 C water bath (dry method), when diluted 1:9 and protected (7.5% DMSO) with EXT 275. Sperm was held 0, 15, 30, 60, or 120 min in a water bath at various temperatures prior to dilution and freezing.

Time in Water Bath (min)	Temperature (C)	Mean Percent Fertilization (n=4) ± SE
0	12.8	42.1 ± 21.3
15	7.8	53.5 ± 6.0
30	5.0	54.3 ± 9.1
60	2.8	48.5 ± 6.3
120	1.1	46.9 ± 6.1
Control (Fresh Sperm and Eggs)		99.6 ± 0.6
Control (Sperm diluted with EXT 275 with 7.5% DMSO and Eggs)		96.4 ± 5.3
Number of Eggs per Experimental Unit		(82.5 ± 6.6)

fresh sperm, respectively (Table 33). A comparison of these MPF was performed; the null hypothesis that the treatment means were equal was not rejected ($P \geq .10$) (Appendix B, Table 17).

Sperm samples were diluted with EXT 263, 265, and 266 containing 0, 500, and 750 mg/100 ml fructose, respectively, and the ordered MPF was 35.8, 27.2, and 32.4 (Table 34). An analysis of variance was performed; treatment means were not significantly different ($P \geq .10$) (Appendix B, Table 18). Results of this test were consistent with previous findings based on both MPM estimates (Table 23) and MPF observations (Table 29). In control groups, the MPF was 80.0 and 85.2 achieved with diluted (non-frozen) and undiluted fresh sperm, respectively (Table 34). The null hypothesis that control means were equal was not rejected ($P \geq .10$) (Appendix B, Table 18).

In the final experiment, fall chinook salmon sperm was diluted with EXT containing various concentrations of lecithin. After mixing frozen-thawed sperm and fresh eggs, the effect of adding water at various time intervals also was investigated.

Sperm diluted with extenders containing a 0 (EXT 267), 250 (EXT 268), 500 (EXT 269), 750 (EXT 263), and 1000 (EXT 270) mg/100 ml lecithin inseminated an average of 26.6, 31.6, 36.1, 35.7, and 32.5% of fresh ova, respectively (Table 35). Statistical comparison of these means was not conducted because of the

Table 34. Mean percent fertilization of fall chinook salmon ova inseminated with sperm frozen and stored 4 days in liquid nitrogen and thawed in a 55-65 C water bath (wet method, 10-15 C), when diluted 1:9 and protected (7.5% DMSO) with extenders containing various concentrations of fructose.

Extender Number	Fructose (mg/100 ml)	Mean Percent Fertilization (n=6) ± SE
263	0	35.8 ± 5.1
265	500	27.2 ± 6.8
266	750	32.4 ± 9.0
Control (Fresh Sperm and Eggs)		80.0 ± 17.2 (n=3)
Control (Sperm diluted with EXT 263 with 7.5% DMSO and Eggs)		85.2 ± 14.5 (n=3)
Number of Eggs per Experimental Unit		(64.2 ± 6.6)

magnitude of the standard error associated with the individual MPF. Addition of water immediately to sperm and ova resulted in MPF of 0.5, 3.0, 2.9, 2.1, and 0.5 as compared with 26.6, 31.6, 36.1, 35.7, and 32.5 when water was added after 5 min (Table 35). The null hypothesis that means of these groups were equal was rejected ($P \leq .10$) (Appendix B, Table 19). Variability in the MPF resulted from one low observation per treatment (Table 36). In the controls, sperm diluted with EXT 263 (non-frozen) inseminated an average of 98.8% of ova, whereas fresh sperm fertilized 97.9% (Table 35). The null hypothesis that

Table 35. Mean percent fertilization of fall chinook salmon ova inseminated with sperm frozen and stored 5 days in liquid nitrogen and thawed in a 10-15 C water bath (dry method), when diluted 1:9 and protected (7.5% DMSO) with extenders containing various concentrations of lecithin. Addition of water to sperm-ova mixture either occurred immediately or 5 min following insemination of ova.

Extender Number	Lecithin (mg/100 ml)	Time Water Added to Sperm-Ova Mixture (min)	Mean Percent Fertilization (n=3) ± SE
267	0	0	0.5 ± 0.9
268	250	0	3.0 ± 3.1
269	500	0	2.9 ± 1.3
263	750	0	2.1 + 3.6
270	1000	0	0.5 ± 0.9
267	0	5	26.6 ± 22.1
268	250	5	31.6 ± 25.9
269	500	5	36.1 ± 19.6
263	750	5	35.7 ± 8.8
270	1000	5	32.5 ± 26.8
Control (Fresh Sperm and Eggs)			97.9 ± 0.8
Control (Sperm diluted with EXT 263 with 7.5% DMSO and Eggs)			98.8 ± 1.0
Number of Eggs per Experimental Unit			(64.9 ± 4.6)

control means were equal was not rejected ($P \geq .10$) (Appendix B, Table 19.

Summary

(1) Duration of sperm activity was employed as a technique for estimating survival of spermatozoa. This technique, less subjective than MPM observations, appears useful in evaluating sperm samples. Observations recorded with DSA supported past findings based on MPM and MPF.

(2) A solution composed only of fructose, lecithin, $NaHCO_3$, NaCl, and DMSO proved suitable as an EXT. This diluent, EXT 227, compared favorably with EXT 134; the only difference being that the concentration of NaCl in EXT 227 was increased to 850 mg/100 ml in order to compensate for the omission of KCl, $CaCl_2 \cdot 2H_2O$, $Na_2HPO_4 \cdot H_2O$, and $MgSO_4 \cdot 7H_2O$.

(3) Extender 275 containing 850 mg NaCl/100 ml, 375 mg $NaHCO_3$/100 ml, and 750 mg lecithin/100 ml, and protected with 7.5% DMSO, was the best and most simple diluent developed.

(4) Mean percent fertilization was significantly increased when water was added to cryopreserved sperm and fresh eggs 5 min after the gametes were mixed as compared to the immediate addition of water.

Table 36. Percent fertilization of all chinook salmon ova inseminated with sperm frozen and stored in liquid nitrogen and thawed in a 10-15 C water bath (dry method when diluted 1:9 and protected (7.5% DMSO) with extenders containing various concentrations of lecithin. Addition of water to sperm-ova mixture occurred 5 min following insemination of ova.

Extender Number	Lecithin (mg/100 ml)	Percent Fertilization Observations (1)	(2)	(3)	(SE)
267	0	43.3	34.9	1.5	22.1
268	250	54.2	37.3	3.3	25.9
269	500	49.3	45.5	13.6	19.6
263	750	30.8	45.9	30.5	8.8
270	1000	55.1	39.7	2.9	26.8

Coho Salmon

Introduction

This chapter summarizes research on the cryopreservation of coho salmon sperm resulting in a maximum MPF of 74.1. Preliminary experiments conducted in 1968 by Ott and Horton (1971a) resulted in a MPF of 70.0. The present study involved modification of the diluent and a reevaluation of the protector (DMSO). Numerous experiments also were conducted to determine the cause(s) of variability in the fertilization of ova with cryopreserved sperm.

Methods and Procedures

Coho salmon returning to the Eagle Creek National Fish Hatchery and the Issaquah State Salmon Hatchery during 1969 and 1971 were either killed or anesthetized with MS-222, and sperm was collected in 2-oz glass receptacles. Sperm samples and diluents were held in an ice water bath for 5-30 min, diluted 1:4 or 1:9 and protected with either 7.5 or 8.0% DMSO (except when DMSO was tested). Equilibration time in all tests was zero except when this variable was evaluated. One-ml aliquots of diluted sperm were pipetted into unsealed glass ampoules (one test involved whirl-pak bags) and frozen in LN_2 vapor (except when the effect of liquid freezing was evaluated).

The evaluation of sperm cell survival was based on MPM and MPF. Sperm samples either were thawed by the WM or DM. Ova from females killed and bled were pooled and subdivided into experimental units.

Results

When coho salmon sperm was diluted 1:4, 1:9, 1:19, 1:39 or 1:79 the hypothesis that treatment means (MPF) were equal (Table 37) was rejected ($P \leq .01$) (Appendix B, Table 20). In this test, the effect of the male donor also was significant ($P \leq .05$)

Table 37. Mean percent fertilization of coho salmon ova inseminated with sperm frozen and stored 3 days in liquid nitrogen and thawed in a 5 C water bath (dry method), when diluted 1:4, 1:9, 1:19, 1:39, or 1:79 and protected (10% DMSO) with extender 141.

	Mean Percent Fertilization (n=3)				
	Dilution Ratio				
Male Number	1:4	1:9	1:19	1:39	1:79
400	21.5	24.3	25.6	4.9	2.9
401	46.7	40.7	39.9	7.7	7.1
402	41.9	47.1	27.2	8.7	4.6
Pooled Mean Percent Fertilization	36.7	37.4	29.2	7.1	4.9
Control (Fresh Sperm and Eggs)			70.6 (n=1)		
Number of Eggs per Experimental Unit			(38.0 ± 14.2[a])		

[a]Standard Error

(Appendix B, Table 20). Utilizing Duncans New Multiple Range Test (Steele and Torrie 1960), the MPF achieved with cryopreserved sperm subjected to various dilutions were compared. In the summarized data which follow, means underscored with the same line were not significantly different whereas means not connected by a line were significantly different ($P \leq .01$; Duncans Value, 16.8). There appears to be a critical maximum dilution ratio of about 1:19 with no detectable differences between 1:4, 1:9, and 1:19.

	Dilution Ratio				
	1:79	1:39	1:19	1:4	1:9
Mean Percent Fertilization	4:9	7:1	29.2	36.7	37.4

In experiments conducted to determine the preferred concentration of DMSO, MPF was 5.2, 27.8, 45.5, 42.7, 23.3, and 17.6 when sperm was protected with 2.5, 5.0, 7.5, 10.0, 12.5, and 15.0% DMSO, respectively (Table 38). When an analysis of variance was performed, variation attributed to males, DMSO, and males x DMSO was significant at $P \leq .01$ (Appendix B, Table 21). Utilizing Duncans New Multiple Range Test, the MPF achieved with sperm from each male subjected to various concentrations of DMSO were compared. In the summarized data which follow, means underscored with the same line were not significantly different;

Table 38. Mean percent fertilization of coho salmon ova inseminated with sperm frozen and stored 3 days in liquid nitrogen and thawed in a 5 C water bath (dry method), when diluted 1:9 and protected (2.5, 5.0, 7.5, 10.0, 12.5, and 15.0% DMSO) with extender 141.

	Mean Percent Fertilization (n=4)					
	DMSO (% v/v)					
Male Number	2.5	5.0	7.5	10.0	12.5	15.0
377	4.0	45.4	62.3	51.6	30.5	27.0
378	1.0	6.3	22.0	14.8	11.0	4.4
379	10.8	31.9	52.1	61.7	28.4	21.3
Pooled Mean Percent Fertilization	5.2	27.8	45.5	42.7	23.3	17.6
Control (Fresh Sperm and Eggs)				97.1 ± 2.3[a] (n=8)		
Number of Eggs per Experimental Unit				(46.1 ± 9.1)		

[a]Standard Error

conversely treatment means not connected with a line were significantly different ($P \leq .01$; Duncans Values: 20.4 male 377; 11.4 male 378; and 16.3 male 379). In general, DMSO at 7.5 or 10.0% offers suitable protection for cryopreserved coho salmon sperm. However, individual males differ with respect to the preferred concentration of the solute moderator.

	DMSO (% v/v)					
	2.5	15.0	12.5	5.0	10.0	7.5
Mean Percent Fertilization (Male 377)	4.0	27.0	30.5	45.4	51.6	62.3

	DMSO (% v/v)					
	2.5	15.0	5.0	12.5	10.0	7.5
Mean Percent Fertilization (Male 378)	1.0	4.4	6.3	11.0	14.8	22.0

	DMSO (% v/v)					
	2.5	15.0	12.5	5.0	7.5	10.0
Mean Percent Fertilization (Male 379)	10.8	21.3	28.4	31.9	52.1	61.7

In experiments to retest DMSO, sperm was diluted with EXT 236, protected with 5.0, 7.5, 10.0, and 12.5% DMSO, and MPF was 31.1, 44.7, 24.6, and 28.9, respectively (Table 39). The predetermined control for DMSO was 7.5%, thus alternate treatments were compared with a MPF of 44.7%. In the analysis of variance, treatment effect (% DMSO) was significant ($P \leq .05$) (Appendix B, Table 22). Based on the calculated Least Significant Difference (12.7), the control MPF was significantly greater than fertilization achieved with the other three treatments.

Analysis of variance tests on the MPF achieved with cryopreserved sperm containing various concentrations of inositol, mannitol, or sorbitol (Tables 40, 41, and 42) revealed no significant effect attributable to the treatments compared ($P \geq .05$ (Appendix B, Tables 23, 24, and 25). However, in each experiment, there was a significant difference between males ($P \leq .01$) (Appendix B, Tables 23, 24, and 25).

As with fall chinook salmon, experiments were conducted to develop an EXT containing the minimal number of chemical components. The MPF achieved with cryopreserved sperm diluted with EXT 202 (a diluent developed based on previous experiments with fall chinook and coho salmon) was compared to that of EXT 225. EXT 225 contained 850 mg NaCl/100 ml whereas the combined salt concentration (NaCl, KCl, $CaCl_2 \cdot 2H_2O$, $Na_2HPO_4 \cdot H_2O$, and

Table 39. Mean percent fertilization of coho salmon ova inseminated with sperm frozen and stored 2 days in liquid nitrogen and thawed in a 5 C water bath (dry method), when diluted 1:4 and protected (5.0, 7.5, 10.0, or 12.5% DMSO) with EXT 236.

	Mean Percent Fertilization (n=8) + SE			
	DMSO (% v/v)			
Male Number	5.0	7.5	10.0	12.5
392	31.1 ± 17.4	44.7 ± 11.7	24.6 ± 8.2	28.9 ± 11.3
Control (Fresh Sperm and Eggs)	98.1 ± 0.9 (n=4)			
Number of Eggs per Experimental Unit	(99.6 ± 9.8)			

Table 40. Mean percent fertilization of coho salmon ova inseminated with sperm frozen and stored 9 days in liquid nitrogen and thawed in a 3-5 C water bath (dry method), when diluted 1:9 and protected (7.5% DMSO) with extenders containing various concentrations of inositol.

	Mean Percent Fertilization (n=3)					
Male Number	EXT 134 0[a]	EXT 171 100	EXT 172 250	EXT 173 500	EXT 174 1000	Pooled Mean
383	33.9	30.7	20.8	31.6	37.5	30.4
384	58.1	52.4	34.1	58.9	25.6	45.8
385	23.7	24.8	15.0	22.2	22.6	21.7
Pooled Mean	38.6	36.0	23.3	37.6	28.6	
Control (Fresh Sperm and Eggs)	88.7 ± 4.3[b] (n=3)					
Number of Eggs per Experimental Unit	(27.6 ± 5.8)					

[a] Concentration of inositol (mg/100 ml)

[b] Standard Error

Table 41. Mean percent fertilization of coho salmon ova inseminated with sperm frozen and stored 9 days in liquid nitrogen and thawed in a 3-5 C water bath (dry method), when diluted 1:9 and protected (7.5% DMSO) with extenders containing various concentrations of mannitol.

	Mean Percent Fertilization (n=3)					
Male Number	EXT 134 0[a]	EXT 141 100	EXT 166 250	EXT 142 500	EXT 143 1000	Pooled Means
386	31.8	42.8	54.9	54.6	51.9	47.2
387	41.8	40.9	40.8	46.2	35.6	41.0
388	38.1	35.5	39.1	31.7	28.5	34.6
Pooled Mean	37.2	39.7	44.9	44.2	38.6	
Control (Fresh Sperm and Eggs)				91.8 $\pm$ 6.2[b] (n=3)		
Number of Eggs per Experimental Unit				(53.2 $\pm$ 10.3)		

[a] Concentration of mannitol (mg/100 ml)

[b] Standard Error

$MgSO_4 \cdot 7H_2O$) in EXT 202 was 855 mg/100 ml. Additional chemical components ($NaHCO_3$, fructose, and lecithin) were equivalent in these diluents.

Cryopreserved coho salmon diluted with EXT 202 or 225 inseminated an average of 52.8 and 55.7% of fresh ova, respectively (Table 43.) The null hypothesis that treatment means were equal was not rejected ($P \geq .10$) (Appendix B, Table 26). Even though a significant difference was not recorded, EXT 225 was preferred because of a minimal number of chemical components.

Table 42. Mean percent fertilization of coho salmon ova inseminated with sperm frozen and stored 9 days in liquid nitrogen and thawed in a 3-5 C water bath (dry method), when diluted 1:9 and protected (7.5% DMSO) with extenders containing various concentrations of sorbitol.

	Mean Percent Fertilization (n=3)					
Male Number	EXT 134 0[a]	EXT 167 100	EXT 168 250	EXT 169 500	EXT 170 1000	Pooled Mean
389	69.8	69.5	65.4	58.5	66.6	66.0
390	63.7	61.0	58.4	61.3	61.5	61.1
391	17.5	21.5	14.9	24.5	30.4	22.8
Pooled Mean	50.3	50.7	46.2	49.7	52.8	
Control (Fresh Sperm and Eggs)				95.4 ± 3.0[b] (n=3)		
Number of Eggs per Experimental Unit				(53.7 ± 8.8)		

[a] Concentration of sorbitol (mg/100 ml)

[b] Standard Error

Table 43. Mean percent fertilization of coho salmon ova inseminated with sperm frozen and stored 4 days in liquid nitrogen and thawed in a 50-60 C water bath (wet method, 5-8 C), when diluted 1:9 and protected (7.5% DMSO) with EXT 202 or 225.

	Mean Percent Fertilization (n=3) ± SE	
Male Number	EXT 202	EXT 225
393	52.8 ± 15.5	55.7 ± 4.0
Control (Fresh Sperm and Eggs)		95.9 ± 0.8 (n=3)
Number of Eggs per Experimental Unit		(56.1 ± 7.9)

In experiments testing individual chemicals in EXT 225, the MPM was maximum when sperm was diluted with EXT 225 containing $NaHCO_3$ at 250 mg/100 ml as opposed to 0 (EXT 226), 500 (EXT 227), or 750 mg/100 ml (EXT 228). However, in diluted non-frozen samples the MPM after 2 hr storage in an ice water bath was highest in EXT 227. Based on these results EXT 227 was selected as the best diluent because of the high MPM in the diluted non-frozen sample, and because the preferred concentration of $NaHCO_3$ for other salmonids generally was 500 mg/100 ml.

The MPF was maximum when sperm from an adult coho salmon was diluted with EXT 225, whereas EXT 227 gave the best result when sperm from a jack coho salmon was utilized. Extenders tested were 226, 225, and 227 (0, 250, and 500 mg/100 ml $NaHCO_3$, respectively) and the MPF was 44.8, 65.5, and 42.7 for the adult, and 25.0, 43.7, and 76.0 for the jack, respectively (Table 44). The hypothesis that treatment means (MPF for the adult) were equal was not rejected ($P \geq .05$) (Appendix B Table 27). Absence of a significant treatment effect probably was due to the magnitude of the standard error associated with male 394. The hypothesis that treatment means (MPF) for the jack were equal was rejected ($P \leq .01$) (Appendix B, Table 27). A comparison of the means using Duncans New Multiple Range Test follows: Means underscored with the same line were not significantly different whereas means

Table 44. Mean percent fertilization of coho salmon ova inseminated with jack and adult sperm frozen and stored 7 days in liquid nitrogen and thawed in a 50-60 C water bath (wet method, 5-8 C), when diluted 1:9 and protected (7.5% DMSO) with EXT 226, 225, or 227.

Male Number	Mean Percent Fertilization (n=3) ± SE		
	EXT 226 0[a]	EXT 225 250	EXT 227 500
394 (Adult)	44.8 ± 3.4	65.5 ± 14.6	42.7 ± 27.8
395 (Jack)	25.0 ± 7.1	43.7 ± 12.6	76.0 ± 6.3
Control (Fresh Sperm and Eggs)		94.9 ± 0.9 (n=3)	
Control (Sperm diluted with EXT 227 with 7.5% DMSO, and Eggs)		92.4 ± 3.9 (n=3)	
Number of Eggs per Experimental Unit		(55.6 ± 5.8)	

[a]Concentration of $NaHCO_3$ (mg/100 ml)

not connected by a line were significantly different ($P \leq .05$; Duncans Value, 18.8). A significantly higher fertilization was achieved when cryopreserved sperm was diluted with the EXT containing 500 mg $NaHCO_3$/100 ml.

	Extender Number		
	226	225	227
Mean Percent Fertilization	25.0	43.7	76.0

In controls, the MPF was 95.9 and 92.4 when ova were inseminated with fresh and diluted non-frozen sperm, respectively (Table 44). The null hypothesis that these treatment means were equal was not rejected ($P > .10$) (Appendix B, Table 27).

Several thawing methods were evaluated; preliminary tests utilizing duplicate samples were conducted to determine the exact time interval required to thaw sperm (end point was determined as that instant when sperm was shaken from the ampoule). Times and thawing methods are presented in Table 45.

In a fertilization experiment conducted to evaluate the various thawing methods (Table 45), the MPF increased with temperature of the water bath when the WM was employed (Table 46). In the analysis of variance, the effect of water bath temperature and thawing method (WM or DM) were significant ($P \leq .05$) (Appendix B, Table 28). A comparison of the MPF relative to the various water bath temperatures was performed utilizing Duncans New Multiple Range Test. The summarized data follow ($P \leq .05$; Duncans Value = 14.6):

	Temperature Water Bath (C)			
	7-8	20-22	50-60	36-38
Mean Percent Fertilization	12.9	20.5	29.2	32.0

Table 45. Time required to thaw frozen sperm samples in water baths of various temperatures using either the dry (DM) or wet (WM, 7-8 C) method.

Temperature Water Bath (C)	Thawing Method	Time (sec)
7-8	DM	75
7-8	WM	60
20-22	DM	60
20-22	WM	25
36-38	DM	26
36-38	WM	14
50-60	DM	12
50-60	WM	08

Table 46. Mean percent fertilization of coho salmon ova inseminated with sperm frozen and stored 5 days in liquid nitrogen and thawed in various temperature water baths using either the wet (WM, 7-8 C) or dry (DM) method, when diluted 1:9 and protected (7.5% DMSO) with extender 227.

	Mean Percent Fertilization (n=4) ± SE	
Temperature Water Bath (C)	Wet Method (7-8 C)	Dry Method
7-8	18.7 ± 13.6	7.1 ± 8.0
20-22	26.5 ± 15.9	14.5 ± 11.8
36-38	38.0 ± 4.0	26.1 ± 21.6
50-60	39.4 ± 12.8	19.1 ± 9.2
Control (Fresh Sperm and Eggs)	79.9 ± 3.3 (n=2)	
Number of Eggs per Experimental Unit	(61.2 ± 6.5)	

Pooled MPF when the DM and WM (7-8 C) were used was 16.7 and 30.6, respectively. The thaw method selected for further use was the 50-60 C water bath (WM, 7-8 C) because fertilizing capacity was comparable with the other treatments and because a minimal time (8 sec) was required to thaw samples. Minimization of the time required to thaw a sample and inseminate a group of ova expedites completion of tests involving a large number of experimental units.

Further experimentation centered on optimizing the chemical components and their respective concentrations in the diluent. Results of these tests are summarized in Tables 47-50. In each case, the hypothesis that the treatment means were equal was tested at $P \leq .05$ (Appendix B, Tables 29-32). Whenever the treatment effect was significant, means were compared utilizing Duncans New Multiple Range Test. In the data which follow, means underscored with the same line were not significantly different ($P \geq .05$), whereas means not connected by a line were significantly different.

Based on data presented in Table 47, it was apparent that EXT 227 containing both NaCl and $NaHCO_3$ was the preferred diluent. The MPF achieved with EXT 227 was significantly higher than that achieved with either EXT 232 or 233. Data comparisons follow (Duncans Value, 15.5):

Table 47. Mean percent fertilization of coho salmon ova inseminated with sperm frozen and stored 3 days in liquid nitrogen and thawed in a 50-60 C water bath (wet method, 5-8 C), when diluted 1:9 and protected (7.5% DMSO) with extenders 227, 232, or 233.

	Mean Percent Fertilization (n=8) ± SE		
Male Number	EXT 227 850[a], 500[b]	EXT 232 1350, 0	EXT 223 0, 1350
396	67.5 ± 12.4		
397	69.3 ± 9.8	21.7 ± 6.3	15.2 ± 12.7
Control (Fresh Sperm and Eggs)		95.3 ± 2.8 (n=4)	
Control (Sperm diluted with EXT 227 with 7.5% DMSO, and Eggs)		92.9 ± 2.6 (n=4)	
Number of Eggs per Experimental Unit		(58.7 ± 5.1)	

[a] Concentration of NaCl (mg/100 ml)

[b] Concentration of $NaHCO_3$ (mg/100 ml)

Table 48. Mean percent fertilization of coho salmon ova inseminated with sperm frozen and stored 3 days in liquid nitrogen and thawed in a 50-60 C water bath (wet method, 5-8 C), when diluted 1:9 and protected (7.5% DMSO) with extenders containing various concentrations of fructose and/or lecithin.

Extender Number	Fructose (mg/100 ml)	Lecithin (mg/100 ml)	Mean Percent Fertilization (n=3) ± SE
234	0	0	42.5 ± 11.9
235	0	500	58.9 ± 16.6
236	0	1000	74.4 ± 2.1
237	500	0	38.5 ± 13.3
238	500	500	66.3 ± 3.3
239	500	1000	70.1 ± 10.8
240	1000	0	56.3 ± 4.6
241	1000	500	64.3 ± 17.9
242	1000	1000	77.7 ± 9.7
Control (Fresh Sperm and Eggs)			94.3 ± 2.1 (n=3)
Number of Eggs per Experimental Unit			(67.0 ± 6.6)

Table 49. Mean percent fertilization of coho salmon ova inseminated with sperm frozen and stored 3 days in liquid nitrogen and thawed in a 50-60 C water bath (wet method, 5-8 C), when diluted 1:9 and protected (7.5% DMSO) with extenders containing various concentrations of lecithin.

Extender Number	Lecithin (mg/100 ml)	Mean Percent Fertilization (n=3) ± SE
235	500	39.6 ± 11.7
236	1000	46.4 ± 13.6
251	1500	57.2 ± 6.4
252	2500	47.9 ± 9.9
Control (Fresh Sperm and Eggs)		91.2 ± 3.0 (n=4)
Number of Eggs per Experimental Unit		(73.1 ± 6.1)

Table 50. Mean percent fertilization of coho salmon ova inseminated with sperm frozen and stored 5 days in liquid nitrogen and thawed in a 50-60 C water bath (wet method, 5-8 C), when diluted 1:9 and protected (7.5% DMSO) with extenders containing various concentrations of NaCl and $NaHCO_3$.

Extender Number	NaCl (mg/100 ml)	$NaHCO_3$ (mg/100 ml)	Mean Percent Fertilization ± SE
243	650	300	2.3 ± 3.1
244	650	500	35.5 ± 23.4
245	650	700	57.0 ± 8.6
246	850	300	39.3 ± 12.9
235	850	500	63.3 ± 10.7
247	850	700	58.8 ± 16.6
248	1050	300	64.7 ± 6.4
249	1050	500	47.0 ± 25.1
250	1050	700	66.2 ± 15.1
Control (Fresh Sperm and Eggs)			96.1 ± 2.1 (n=3)
Number of Eggs per Experimental Unit			(75.4 ± 7.6)

	Extender Number		
	233	232	227
Mean Percent Fertilization	15.2	21.7	69.3

When fructose and lecithin were tested at 0, 500, and 1,000 mg/100 ml in a factorial arrangement of treatments the effect of fructose and the interaction of fructose-lecithin was not significant, but the effect of lecithin was significant ($P \leq .05$) (Appendix B, Table 30). The pooled MPF when lecithin was 0, 500, and 1000 mg/100 ml was 45.8, 63.1, and 74.1, respectively (Table 48). In the comparison of these means, pooled MPF was significantly greater when lecithin was either 500 or 1000 mg/100 ml. The summarized data follow (Duncans Value, 11.8):

	Lecithin (mg/100 ml)		
	0	500	1000
Pooled Mean Percent Fertilization	45.8	63.1	74.1

Based on the results of the fructose-lecithin experiment (Table 48), lecithin was retested. Again the treatment effect was significant (Appendix B Table 31). A comparison of treatment means (Duncans Value, 14.1) follows:

	Lecithin (mg/100 ml)			
	500	1000	2500	1500
Mean Percent Fertilization	39.6	46.4	47.9	57.2

In the experiment testing NaCl and $NaHCO_3$ (Table 50), treatments (NaCl, $NaHCO_3$, and NaCl x $NaHCO_3$) were significant at $P \leq .05$ (Appendix B, Table 32). Statements concerning the preferred level of a chemical were limited to one concentration of the other chemical because the interaction term was significant. Thus, if the concentration of $NaHCO_3$ was 300 mg/100 ml, the preferred concentration of NaCl was 1050 mg/100 ml. When the $NaHCO_3$ concentration was 500 mg/100 ml, the preferred level of NaCl was 850 mg/100 ml. Finally, when the concentration of $NaHCO_3$ was 700 mg/100 ml, the effect of NaCl was not significant at the three levels tested. The summarized data follow:

	$NaHCO_3$ (300 mg/100 ml) Level of NaCl (mg/100 ml)		
	650	850	1050
Mean Percent Fertilization	2.3	39.3	64.7

Duncans Value ($P \leq .05$) = 21.9

	$NaHCO_3$ (500 mg/100 ml		
	Level of NaCl (mg/100 ml)		
	650	850	1050
Mean Percent Fertilization	35.5	63.3	47.0

Duncans Value ($P \leq .05$) = 21.9

	$NaHCO_3$ (700 mg/100 ml)		
	Level of NaCl (mg/100 ml)		
	650	850	1050
Mean Percent Fertilization	57.0	58.8	66.2

Results, of the past four experiments, indicate that the EXT should contain NaCl, $NaHCO_3$, and lecithin. The preferred level of lecithin was between 500 and 1500 mg/100 ml whereas the best concentration of NaCl was determined by the level of $NaHCO_3$.

Contamination of coho salmon semen with up to 25% urine had no detrimental effect on the MPF of ova inseminated with cryopreserved or refrigerated sperm (Table 51). The effect of 0-25% urine on the MPF resulting from semen stored 27 hr at 0.5-2.0 C was not significant ($P \geq .05$) (Appendix B, Table 33). However, when cryopreserved sperm was similarly tested, the urine effect

Table 51. Mean percent fertilization of coho salmon ova inseminated either with unfrozen sperm or sperm frozen and stored 27 hr in liquid nitrogen and thawed in a 50-60 C water bath (wet method, 5-8 C), when diluted 1:4 and protected (7.5% DMSO) with extender 235. The frozen and unfrozen sperm samples contained various concentrations of urine.

Urine (% v/v)	Mean Percent Fertilization (n=4) ± SE	
	Frozen-Thawed Sperm	Unfrozen Sperm Stored 27 hr at 0.5-2.0 C
0	68.0 ± 8.6	100.0 ± 0.0
2.5	47.1 ± 8.9	99.7 ± 0.5
5.0	65.7 ± 10.6	99.2 ± 0.5
10.0	53.1 ± 13.9	99.5 ± 1.1
25.0	64.9 ± 8.8	99.1 ± 1.4
Control (Fresh Sperm and Eggs)		100.0 ± 0.0 (n=2)
Number of Eggs per Experimental Unit		(99.6 ± 9.8)

was significant ($P \leq .05$) (Appendix B, Table 33). Because the MPF resulting from samples containing 0, 5, or 25% urine were highest, significance of urine contamination may be beneficial rather than detrimental.

Equilibration times of up to 60 min had no significant effect on the fertilizing success of cryopreserved coho salmon sperm (Table 52). When treatment means were compared the hypothesis that the means were equal was not rejected at $P \geq .05$ (Appendix B, Table 34). A similar conclusion was reached when MPF in control groups were compared ($P \geq .10$) (Appendix B, Table 34).

Table 52. Mean percent fertilization of coho salmon ova inseminated with sperm frozen and stored 3 days in liquid nitrogen and thawed in a 50-60 C water bath (wet method, 5-8 C), when diluted 1:4 and protected (7.5% DMSO) with extender 235 at various equilibration times.

Equilibration Time (min)	Mean Percent Fertilization (n=5) ± SE
0	53.3 ± 20.3
3	66.1 ± 9.6
6	63.3 ± 5.9
15	48.0 ± 9.3
30	52.5 ± 14.4
60	71.7 ± 10.6
Control (Fresh Sperm and Eggs)	89.5 ± 2.6 (n=3)
Control (Sperm diluted with EXT 235 with 8.0% DMSO, and Eggs)	86.1 ± 2.9 (n=3)
Number of Eggs per Experimental Unit	(85.0 ± 9.5)

When ampoules of sperm were placed directly in the LN_2 for freezing, the resulting MPF was zero as compared to 43-66 when ampoules were frozen in the vapor above the LN_2 (Table 53). Sperm samples should be allowed to freeze a minimum of 1 min in LN_2 vapor prior to submersion in LN_2 for storage (Table 54). The treatment effect (time in LN_2 vapor) was significant at $P \leq .05$ (Appendix B, Table 35) and a comparison of MPF utilizing Duncans New Multiple Range Test was performed. The MPF resulting from

Table 53. Mean percent fertilization of coho salmon ova inseminated with sperm frozen in liquid nitrogen (LN_2) or LN_2 vapor (-179 C) and stored 2 days in LN_2 and thawed in a 50-60 C water bath (wet method, 5-8 C), when diluted 1:4 and protected (7.5% DMSO) with extender 235.

Replication	Mean Percent Fertilization ± SE	
	Sperm Frozen in LN_2	Sperm Frozen in LN_2 Vapor
1	0.0 (n=2)	51.8 ± 10.5 (n=2
2	0.0 (n=2)	50.8 ± 19.0 (n=2)
3	0.0 (n=1)	43.0 ± 22.6 (n=3)
4	0.0 (n=1)	54.2 ± 4.7 (n=3)
5		66.0 ± 9.2 (n=4)
Control (Fresh Sperm and Eggs)	88.5 ± 0.7 (n=2)	
Number of Eggs per Experimental Unit	(76.4 ± 6.9)	

Table 54. Mean percent fertilization of coho salmon ova inseminated with sperm frozen in liquid nitrogen (LN_2) vapor (-179 C) for specified intervals and then submerged and stored 3 days in LN_2 and thawed in a 50-60 C water bath (wet method, 5-8 C), when diluted 1:4 and protected (7.5% DMSO) with extender 235.

Time in LN_2 Vapor (min)	Mean Percent Fertilization (n=4) ± SE
0.5	9.8 ± 10.2
1.0	58.7 ± 11.6
2.0	59.4 ± 7.1
5.0	65.9 ± 5.7
15.0	68.8 ± 13.3
60.0	61.7 ± 10.5
Control (Fresh Sperm and Eggs)	95.8 ± 2.2 (n=3)
Number of Eggs per Experimental Unit	(60.6 ± 7.1)

sperm held in vapor <1 min before submersion in LN_2 was significantly lower than that resulting from samples held in vapor for 1-60 min (Duncans Value, $P \leq .05 = 16.7$). Summarized data follow:

	Time in LN_2 Vapor (min)					
	0.5	1.0	2.0	60.0	5.0	15.0
Mean Percent Fertilization	9.8	58.7	59.4	61.7	65.9	68.8

Within the range of 1.00-6.25 inches, the distances of the ampoule above the LN_2 (in vapor) during the freezing process was not related to subsequent MPF of fresh ova (Table 55). Position of the ampoule as determined by MPF was not significant at $P \leq .05$ (Appendix B, Table 36).

Contrary to results obtained with kokanee, whirl-pak bags were not satisfactory containers for the cryopreservation of coho salmon sperm. The MPF was 10.2 and 49.4 when whirl-pak bags and ampoules were used, respectively (Table 56). The null hypothesis that the means were equal was rejected at $P \leq .01$ (Appendix B, Table 37).

The most advisable time to add water to fresh ova and cryopreserved sperm was about 2 min after gametes were mixed (Table 57). The null hypothesis that treatment means were equal was rejected at $P \leq .05$ (Appendix B, Table 38). Treatment means were compared utilizing Duncans New Multiple Range Test.

Table 55. Mean percent fertilization of coho salmon ova inseminated with sperm frozen various distances above liquid nitrogen (LN_2), stored 8 days in LN_2 and thawed in a 50-60 C water bath (wet method, 5-8 C), when diluted 1:4 and protected (7.5% DMSO) with extender 235.

Distance of Ampoule Above LN_2 (in)	Mean Percent Fertilization (n=2)			
	Replication 1 (Four Males)			
6.25	22.4	48.1	47.3	70.4
4.50	35.7	39.7	50.5	71.8
2.75	37.1	61.4	56.0	67.8
1.00	45.2	60.0	60.8	76.2
Control (Fresh Sperm and Eggs)		84.8 ± 8.2[a] (n=4)		
Number of Eggs per Experimental Unit		(80.3 ± 9.4)		
	Replication 2 (Four Males)			
6.25	27.0	22.1	25.6	15.3
4.50	26.0	24.8	23.6	59.1
2.75	23.1	21.9	42.6	49.9
1.00	30.4	38.7	16.6	64.4
Control (Fresh Sperm and Eggs)		100.0 ± 0.0 (n=4)		
Number of Eggs per Experimental Unit		(53.9 ± 5.8)		

[a]Standard Error

Table 56. Mean percent fertilization of coho salmon ova inseminated with sperm frozen in ampoules or whirl-pak bags, stored 2 days in liquid nitrogen and thawed in a 50-60 C water bath (wet method, 5-8 C), when diluted 1:4 and protected (7.5% DMSO) and extender 236.

Freezing Container	Mean Percent Fertilization (n=7) ± SE
Ampoules	49.4 + 11.8
Whirl-pak bags	10.2 ± 5.0
Control (Fresh Sperm and Eggs)	88.5 ± 0.7 (n=2)
Number of Eggs per Experiment Unit	(75.6 ± 7.8)

A valid conclusion, either statistically or biologically cannot be made on a basis of these data; however, apparently the addition of water to gametes should be delayed 1-2 min, but gametes should be diluted prior to 5 min. The summarized data follow (Duncan Value, $P \leq .05 = 20.5$):

	Time Water Added to Gametes (min)					
	0.0	0.5	1.0	5.0	10.0	2.0
Mean Percent Fertilization	18.2	26.1	30.6	30.9	39.7	48.4

Storage times of 15 and 28 days in LN_2 and the interaction of time and males did not significantly affect MPF using cryopreserved sperm (Table 58) ($P \geq .01$; Appendix B, Table 39). The effect of

Table 57. The effect of various time intervals between mixing gametes and the addition of water on the mean percent fertilization of coho salmon ova inseminated with sperm frozen and stored 4 days in liquid nitrogen and thawed in a 50-60 C water bath (wet method, 5-8 C), when diluted 1:4 and protected (7.5% DMSO) with extender 251.

Time Water Added to Sperm and Eggs (min)	Mean Percent Fertilization (n=6) ± SE
0.0	18.2 ± 22.1
0.5	26.1 ± 16.5
1.0	30.6 ± 7.9
2.0	48.4 ± 23.3
5.0	30.9 + 6.4
10.0	39.7 ± 4.8
Control (Fresh Sperm and Eggs)	97.5 ± 1.4 (n=2)
Number of Eggs per Experimental Unit	(65.2 + 19.0)

males, however, was significant at $P \leq .01$ (Appendix B, Table 39).

In tests of fish randomly collected thoughout the spawning season, neither condition factor of the male ($R = .01$), date the sperm samples were obtained ($R = .04$), nor water temperature ($R = .05$) were strongly correlated with MPF of fresh ova inseminated with cryopreserved sperm (Tables 59-60). In these tests, identical procedures were used to collect, dilute, protect, and freeze sperm. After all samples had been cryopreserved, they were individually thawed by the same process and immediately

Table 58. Mean percent fertilization of coho salmon ova inseminated with sperm frozen and stored 15 and 28 days in liquid nitrogen and thawed in a 50-60 C water bath (wet method 5-8 C), when diluted 1:4 and protected (7.5% DMSO) with extender 235.

Days of Storage in LN_2	Male Number	Mean Percent Fertilization (n=8) ± SE	Adjusted Mean Percent Fertilization
15	400	26.6 ± 7.7	48.6[a]
28	400	35.1 + 23.7	35.1
15	401	26.9 ± 9.1	48.9
28	401	52.4 ± 15.7	52.4
15	402	27.1 ± 14.7	49.1
28	402	53.7 ± 8.4	53.7
15	403	57.2 ± 10.8	79.2
28	403	71.6 ± 5.4	71.6
Control (Fresh Sperm and Eggs) for Day 15			73.1 ± 8.2 (n=4)
Control (Fresh Sperm and Eggs) for Day 28			95.1 ± 5.1 (n=4)
Number of Eggs for Experimental Unit for Day 15			(80.3 ± 9.4)
Number of Eggs per Experimental Unit for Day 28			(53.9 ± 5.8)

[a]Mean percent fertilizations for day 15 were adjusted by adding 22%.

Table 59. Date of sperm collection, condition factor of the donor male, and percent motility of sperm in relation to the mean percent fertilization of coho salmon ova inseminated with sperm frozen and stored from 2-32 days in liquid nitrogen and thawed in a 50-60 C water bath (wet method, 5-8 C), when diluted 1:9 and protected (7.5% DMSO) with extender 141.

Date of Sperm Collection (1971)	Percent Motility Fresh Sperm	Condition Factor of Donor Male (K)[a]	Mean Percent Fertilization (n=4) ± SE	Pooled Mean Percent Fertilization
11/1	50-60	1.058	40.9 ± 13.4	
	40-50	1.100	25.5 ± 3.3	40.9
	60-70	1.214	44.7 ± 8.3	
	80-90	1.086	52.3 ± 12.7	
11/7	70-80	1.069	56.0 ± 21.8	
	70-80	1.245	50.6 ± 3.3	50.5
	60-70	1.000	47.9 ± 12.0	
	70-80	1.064	47.6 ± 6.6	
11/10	70-80	1.095	32.5 ± 6.8	
	70-80	1.122	61.5 ± 6.4	53.3
	70-80	1.197	53.3 ± 6.6	
	70-80	0.911	65.7 ± 12.7	
11/13	80-90	1.212	63.5 ± 11.1	
	80-90	1.128	57.2 ± 3.7	50.0
	80-90	1.024	49.2 ± 13.1	
	60-70	1.480	30.1 ± 9.5	
11/16	90-100	1.137	74.1 ± 5.6	
	90-100	1.138	54.2 ± 15.7	55.9
	90-100	1.107	56.4 ± 6.3	
	90-100	1.061	39.0 ± 4.0	

Continued

Table 59 (Continued)

Date of Sperm Collection (1971)	Percent Motility Fresh Sperm	Condition Factor of Donor Male (K)[a]	Mean Percent Fertilization (n=4) ± SE	Pooled Mean Percent Fertilization
11/19	90-100	0.996	35.0 ± 22.6	
	40-50	1.066	29.3 ± 11.3	35.1
	80-90	1.181	42.5 ± 20.8	
	70-80	1.199	33.6 ± 13.4	
11/25	90-100	1.072	54.5 ± 15.2	
	90-100	1.199	67.2 ± 3.0	56.9
	80-90	1.197	43.5 ± 9.4	
	90-100	1.182	62.3 ± 7.0	
11/28	70-80	0.992	47.4 ± 8.5	
	90-100	0.915	49.8 ± 10.7	53.9
	80-90	0.883	60.9 ± 5.1	
	70-80	0.983	57.3 ± 18.6	
12/1	70-80	0.919	48.0 ± 5.6	
	70-80	0.985	35.2 ± 6.5	35.5
	40-50	0.957	10.5 ± 5.8	
	80-90	0.866	48.1 ± 7.3	
Control (Fresh Sperm and Eggs)			91.6 ± 3.8 (n=4)	
Number of Eggs per Experimental Unit			(78.3 ± 5.4)	

[a] K (Condition Factor) = Weight Grams $\times 10^5$ / Length $(mm)^3$

Table 60. The temperature of Issaquah Creek from October 23 to December 1, 1967 related to the mean percent fertilization of fresh coho salmon ova inseminated with cryopreserved sperm collected on the respective dates.

Date of Recording (1971)	Mean Daily Water Temperature (C)	Mean Percent Fertilization (n=16)
10/23	7.78	-
10/24	7/78	-
10/25	7.78	-
10/26	7.22	-
10/27	6.11	-
10/28	5.55	-
10/29	5.00	-
10/30	5.00	-
10/31	5.00	-
11/1	5.00	40.9
11/2	5.55	-
11/3	6.67	-
11/4	6.67	-
11/5	5.55	-
11/6	5.00	-
11/7	5.55	50.5
11/8	6.67	-
11/9	7.22	-
11/10	7.22	53.2
11/11	8.33	-
11/12	8.33	-
11/13	8.33	50.0
11/14	7.78	-
11/15	7.22	-
11/16	6.11	55.9
11/17	6.11	-
11/18	6.11	-
11/19	7.78	35.1
11/20	7.22	-
11/21	7.22	-
11/22	7.22	-
11/23	6.67	-
11/24	7.22	-
11/25	6.67	56.9
11/26	7.22	-
11/27	6.11	-
11/28	5.55	53.9
11/29	5.55	-
11/30	5.55	-
12/1	5.55	35.5

used to inseminate a sample of fresh eggs taken from the pooled ova of four females (Table 59). While MPM was significantly correlated with MPF (R = .63), none of the variables measured offer an acceptable explanation for the significance of the donor male in previous experiments.

Summary

(1) Sperm cell survival was maximal when samples were diluted 1:4, 1:9, or 1:19 and protected with DMSO at 7.5%.

(2) A solution composed only of lecithin, NaCl, $NaHCO_3$, and DMSO proved suitable as an EXT. The preferred concentration of lecithin was between 500 and 1500 mg/100 ml, whereas the level of NaCl was determined by the amount of $NaHCO_3$ utilized in the diluent.

(3) In thawing frozen sperm samples, it was evident that the wet method was preferable. The technique selected was a 50-60 C water bath (WM, 7-8 C) since sperm cell survival was maximal and time requirements to complete this procedure were minimal.

(4) Contamination of sperm with urine (up to 25%) and equilibration time (up to 60 min) had no effect on survival of cryopreserved sperm.

(5) Freezing sperm samples directly in LN_2 caused 100% mortality of spermatozoa. When samples were allowed to freeze in LN_2 vapor for at least 1 min, regardless of position relative to the surface of the LN_2, sperm cell survival was improved.

(6) Mean percent fertilization was significantly higher when sperm samples were frozen in ampoules as compared to whirl-pak bags.

(7) Sperm viability as determined by MPF was not affected by storage in LN_2 for a period of 28 days.

(8) Condition factor (K), motility of sperm, date of sperm collection, and water temperature were factors monitored throughout the spawning season of the coho salmon. The resulting MPF with cryopreserved sperm only was correlated with the percent motility of the sample when collected.

DISCUSSION

Pre-Freeze Process

Male Selection

In general, males were selected for experimentation on the basis of similarity of phenotypic characteristics; however, the MPF achieved with cryopreserved sperm from individual males often were significantly different. This problem is not unique to salmonid sperm as Mounib et al. (1968) observed similar variability with frozen-thawed Atlantic cod sperm and Salamon (1968) reported large differences in the freezability of ejaculates from individual rams (Bovidae). Apparently, adult fish sampled during the peak of the spawning season consistently had superior sperm (for example, initial experiments with fall chinook salmon sperm were unsuccessful).

Experiments were conducted with coho and pink salmon in an attempt to determine the source(s) of male variability. However, when a detailed experiment was conducted on coho salmon males sampled throughout the spawning season, results were inconclusive (Tables 59 and 60). This test involved an attempt to correlate variability in MPF with sperm collection date, condition factor of donor male, and/or MPM of spermatozoa. Utilizing a similar approach, survival of stored pink salmon sperm was correlated

with collection date (Table 7). While MPM was responsible for a portion of the viability in MPF (R = .63), neither condition factor (R = .01), date of sperm collection (R = .04), nor water temperature (R = .05) were strongly correlated with MPF in the coho salmon test; whereas, duration of sperm viability in the pink salmon experiment was related to collection date. Remaining variability, I believe, can be explained by inherent physical-chemical differences between donor males.

Truscott et al. (1968) reported a low correlation between sperm motility and fertilizing capability,but these authors did not recommend extensive use of MPM in determining sperm cell survival. According to Graham and Pace (1967), survival of sperm cells does not indicate that fertilization will ensue. In tests conducted with kokanee gametes, motile sperm were incapable of fertilizing fresh ova; however, in the majority of tests a correlation existed between MPF and MPM. Thus, I recommend utilization of MPM as a general guide to estimating sperm cell survival, but caution that critical factors such as the concentration of DMSO must be tested with fertilization experiments.

Sperm Collection

An important aspect of the cryogenic procedure is obtaining uncontaminated sperm samples. Because of the close proximity

of the genital pore to the urinary tract in salmonids (Graybill 1968), it was impossible to determine the amount of urine possibly collected with the semen even though an attempt was made to express urine prior to sperm collection. When tested, specific urine levels exhibited a beneficial effect on unfrozen pink salmon sperm (Table 5), whereas no effect was recorded with unfrozen coho salmon sperm (Table 49). The MPF achieved with cryopreserved coho salmon sperm containing specific concentrations of urine were significantly different at $P \leq .05$ (Table 51) (Appendix B, Table 33), but no beneficial or detrimental effect was determined. Apparently contamination of sperm with small amounts of urine is not an important variable affecting the MPM or MPF.

The mechanism for the beneficial effect of urine with pink salmon sperm probably involves a reduction in spermatozoa agglutination. However, urine exhibited no effect with coho salmon semen. Species differences may be related to osmotic and/or ionic properties of the urine. Because pink salmon were collected in an estuarine environment, the ionic concentration of their urine probably was greater than that of the coho salmon which were collected in freshwater. Characteristically, urine from species in freshwater is copius and dilute, whereas urine from saltwater species is low in volume and concentrated (Krogh 1939). The urine of pink salmon probably was ionically and osmotically similar to sperm plasma,

whereas the urine from coho salmon was not. Poon and Johnson (1970) and Plosila et al. (1972) determined that the addition of water to stored sperm immediately prior to mixing with ova increased the MPF. The clumping of spermatozoa during storage may inhibit fertilization of ova, thus adding water just prior to gamete mixing reduces agglutination and leads to increased fertilization. I surmised that urine may function in a similar manner.

Dilution Ratio

Dilution ratos of 1:4, 1:9, and 1:19 were suitable for frozen-thawed coho salmon sperm (Ott and Horton 1971a), however, when these ratios were utilized with pink salmon, a 9-fold dilution was optimal. Further tests revealed that 4-, 9-, and 19-fold dilutions were satisfactory for all species, but higher ratios (1:39, 1:79) with coho salmon sperm resulted in significantly lower MPF (Table 37, Appendix B; Table 20).

Definite advantages are attained at higher dilution ratios. Percent motility estimates are easier to determine because sperm cell concentration is decreased. Agglutination probably is reduced and treatment effects are more pronounced since each cell is exposed to a greater degree to the EXT. Replications are enhanced in some tests particularly when semen volume is small.

Galtsoff (1964) reported high sperm cell concentrations may

result in polyspermy thus leading to aberrant embryos (American oyster, Crassostrea virginica), and Truscott et al. (1968) suggested that inactive sperm, through weight and numbers, could be attracted to the micropyle thus preventing normal fertilization. In cryogenic work with the Pacific oyster (Crassostrea gigas), Staeger (1973) observed abnormal larvae when spermatozoa concentration was high. During my investigation, aberrant salmonid embryos seldom were observed; however, dilution ratios of 1:9 generally were employed and minimal amounts of fresh sperm were mixed with control groups.

Equilibration Time

Ott and Horton (1971a) determined an equilibration time of 1 hr had no beneficial effect on survival of frozen-thawed coho salmon sperm as determined by MPF. Further, viability of pink salmon and kokanee sperm appeared to decrease when samples were equilibrated. Additional tests confirmed preliminary findings as equilibration times between 0 and 1 hr did not significantly alter MPF of cryopreserved coho salmon sperm (Table 52; Appendix B, Table 34. Apparently, when DMSO serves as the solute moderator, equilibration is unnecessary because the rapid penetration of this chemical through the cellular wall affords protection during freezing and thawing. Decrease in viability of pink salmon and kokanee

sperm probably was due to an inadequate diluent. Diluted and unfrozen kokanee sperm equilibrated less than 5 min resulted in a reduced MPF as compared with fresh semen (Tables 14 and 15), whereas with either fall chinook or coho salmon significant differences in MPF between diluted unfrozen and fresh sperm were not observed. In essence, when the EXT tested is not compatible with sperm storage, the effect of equilibration becomes apparent.

Extender-Life Protector

Chemical constituents of diluents developed for each species were presented in the individual Results section. The obvious question is did these various EXT result from differences in species, variability in cryogenic techniques, or the gradual development of preferred and simplified diluent? Because experiments with chinook and coho salmon were the last tests conducted, the simplified EXT developed for these species were not tested with the other salmonids.

Observations throughout this research indicated differences in "cryopreservativeness" exist between species. Based on the highest MPF achieved and on general observations, cryopreservation procedures were more successful with coho salmon and steelhead trout than with the other salmonids. In particular, EXT developed for other salmon and trout completely were ineffective when utilized

to dilute kokanee sperm.

Graybill and Horton (1969) and Ott and Horton (1971a, 1971b) utilized the basic Cortland saline solution (Wolf 1963) in development of initial EXT. Following their lead and the findings of others, I tested various chemicals and intensively analyzed those exhibiting a differential response in sperm cell survival. This approach differs from that of Gregory (1968, 1970), Hwang and Idler (1969), and Truscott and Idler (1969) who studied the chemical composition of spermatozoa and plasma as an aid to development of diluents.

In contrast, Smeed and Clemens (1956) reported that carp (Cyprinus carpio) sperm diluted with seminal plasma did not survive cryopreservation, and Ott (1970) determined that coho salmon sperm diluted with seminal plasma and protected with DMSO did not survive freezing and thawing. These results indicate plasma is not a desirable diluent; nevertheless, I believe the approach employed by Gregory (1968, 1970), Hwang and Idler (1969), and Truscott and Idler (1969) is essential in the ultimate perfection of a diluent and freezing procedure. However, some effective and essential components of EXT probably will be discovered by alternate means.

Basic chemical components of an EXT for salmonids include NaCl, KCl, $CaCl_2 \cdot 2H_2O$, $NaHCO_3$, $NaH_2PO_4 \cdot H_2O$, $MgSO_4 \cdot 7H_2O$, and fructose. Components added to the diluting medium

because beneficial effects were observed include lecithin and mannitol. In general, fructose, mannitol, lecithin, and $NaHCO_3$ were tested for preferred concentration because these constituents exhibited the greatest effect on viability of spermatozoa.

Barner (1965) reported mannitol functions as an osmotic antagonist to DMSO in mammalian systems. Staeger (1973), however, found no decrease in DMSO toxicity when mannitol was added to Pacific oyster sperm. Mannitol enhanced MPM of cryopreserved steelhead trout (Table 1), pink salmon (Tables 3 and 4), and chum salmon sperm (Table 8), but in a fertilization experiment with coho salmon the level of mannitol did not affect recorded MPF (Table 41). Fertilization tests with steelhead trout, pink salmon, and chum salmon sperm containing mannitol were not conducted. Based on the coho salmon test and supported by results reported by Staeger (1973), I concluded that mannitol was not essential in the diluent. Nevertheless, the assessment of mannitol as an osmotic antagonist to DMSO as determined by a factorial arrangement of treatments may yet reveal a beneficial aspect of this chemical.

Gregory (1968) reported the occurrence of fructose in trout seminal plasma and Graybill and Horton (1969) and Ott (1970) determined the suitability of this sugar as a component of the EXT. When a simplified diluent eventually was developed, lecithin and

fructose were evaluated in a factorial arrangement by treatments. Observations (MPF) revealed that fructose was not aiding survival of cryopreserved coho sperm (Table 48). In previous experiments, the effect of fructose probably was masked by the beneficial effects of lecithin. Nagase et al. (1964), as cited in Salamon (1968), suggested that high molecular weight sugars offer superior protection to frozen-thawed cells as compared to low molecular weight sugars. Salamon (1968) working with deep freezing of ram semen found lactose and raffinose superior to either glucose or fructose. Future experiments with salmonid sperm preservation should test the possibility of utilizing lactose or raffinose to enhance survival of spermatozoa.

Giese (1968) stated that lecithin, a phospholipid, is especially important as a structural component of cellular membranes. Hydrophilic groups maintain continuity between the aqueous extra- and intra-cellular components (Giese 1968). In addition, lecithin, because of hydrophobic groups, will dissolve fat-soluble materials thus allowing these materials to permeate the cell wall (Giese 1968). Suitability of lecithin as a component of EXT probably is due to its importance as a cellular component and its inherent chemical properties. Graybill and Horton (1969), Ott (1970), and Ott and Horton (1971a, 1971b) utilized lecithin in fish sperm diluents in order to enhance survival of cryopreserved

spermatozoa. Specifically, the fertilization test with cryopreserved coho salmon sperm (Tables 48 and 49) revealed the beneficial effects of lecithin as exhibited by increased MPF.

Finally, specific levels of $NaHCO_3$ were tested with each species; preferred concentrations ranged from 375-750 mg/100 ml with 500 mg/100 ml selected for coho salmon, pink salmon, chum salmon, and kokanee. However, the combined effects of NaCl and $NaHCO_3$ were not determined until a simplified EXT was developed and tests were made with coho salmon sperm. Initially, I concluded that utilization of either NaCl or $NaHCO_3$ alone was not effective, as MPF was significantly lower when only one salt was used (Table 47, Appendix B, Table 29). When NaCl was held at 300 and 500 mg/100 ml, survival of frozen-thawed sperm (determined by MPF) was maximal at 850 and 1050 mg/100 ml $NaHCO_3$, respectively (Table 50). These results indicated tonicity was the critical factor, however, when NaCl was 700 mg/100 ml the MPF was not affected by the three levels of $NaHCO_3$ tested (650, 850, and 1050 mg/100 ml).

Sneed and Clemens (1956) suggested tonicity and composition of the EXT are as important as the protector. In preparing a balanced medium such as blood, Giese (1968) stated that the total salt concentration and the composition of each constituent in relation to the other are important. Results with coho salmon

support these hypothesis as tonicity and concentration were significant.

According to Doebbler and Cowley (1964), one method of protecting cells is through the addition of chemicals which inhibit ice formation until cells reach preservation temperatures. It appears that when a higher proportion of vitreous ice is achieved, cellular protection is enhanced. Nash (1967) reported that a high hydrogen-bonding (H-B) capacity was extremely important in cryopreservation. Chemicals exhibiting H-B ability reduce the quantity of non-crystalline (amorphous) ice (Doebbler 1966). DMSO, a cryophylactic agent, functions intracellularly (Doebbler 1966; Rowe 1966) and provides extracellular protection during fast freezing due to formation of vitreous ice (Litvan 1972). Glycerol also possesses H-B capacity (Giese 1968), thus this chemical functions in promoting the proportion of vitreous ice.

Glycerol has been employed successfully as a protector with mammalian sperm, and several researchers (Blaxter 1953; Mounib et al. 1968) have utilized this additive with saltwater fish species (marine spawning) and achieved successful cryopreservation. However, research with freshwater fish, particularly salmonids, revealed that glycerol is unsuitable for these species (Graybill and Horton 1969; Mitchum 1963; Ott 1970; Sneed and Clemens 1956; Truscott et al. 1968). DMSO, however, has been

used successfully by various researchers in the cryopreservation of salmonid sperm. Since both glycerol and DMSO function similarly, it appears that glycerol is either toxic to fish sperm or interferes with the actual fertilization of fresh ova. Giese (1968) reported that glycerol is toxic to colon bacillus (Eschericola cola) and pneumonia coccus (Diplococcus pneumonia), and that it must be removed by dialysis upon thawing. Spermatozoa (salmonid) are active when thawed and have an expected motility of less than 60 sec, rending this technique impractical. DMSO is toxic at higher levels (Table 38), but protection at certain levels results in viable frozen-thawed spermatozoa. The level used (7.5-12.0%) depends on the species and may be variable within a species as was determined with coho salmon (Table 38).

Freeze Process

As discussed previously, spermatozoa survival also is affected by the freezing process. In initial experiments Graybill and Horton (1969) and Ott and Horton (1971a) determined that freezing sperm samples at -30C/min resulted in the highest survival. During the freezing process, water freezes as pure ice, dehydration begins as solutes concentrate, and cellular damage occurs (Doebbler and Cowley 1964). Additives, such as DMSO, modify these phases by increasing vitreous ice; however, controlled freezing rates

also are utilized to reduce ice crystal formation. Implementation of fast-freezing rates will decrease exposure of solutes to the cell (time) and if adequate cell permeability is retained intracellular ice formation is unlikely (Doebbler and Cowley 1964). Even faster freezing rates will reduce formation of ice crystals but thermal shock and intracellular ice probably will occur (Doebbler and Cowley 1964). It also was suggested by these authors that faster thawing rates are preferable when utilizing a fast-freezing procedure.

In several experiments (Tables 9 and 35), the magnitude of the standard error associated with MPF resulted from the extremely low fertilization in one of the samples within a treatment. I hypothesized that one of the ampoules in each group was exposed directly to LN_2 during the freezing process. As a result of tests with coho salmon (Table 53), I concluded that exposure of sperm samples to LN_2 during freezing resulted in 100% mortality of spermatozoa. I also determined that sample position (Table 55) relative to the LN_2 did not alter survival, and when samples were allowed to freeze for at least 1 min (Table 54) in LN_2 vapor prior to submersion in LN_2 survival was not affected.

Survival of spermatozoa during storage in LN_2 was tested with kokanee and coho salmon. Based on results of these tests, the MPF did not decrease (significantly) during storage for 16 and 28 days with kokanee (Table 17; Appendix B, Table 8) and coho

salmon (Table 58; Appendix B, Table 39), respectively. Of particular interest was the assumption with control groups that if MPF in the controls (fresh sperm and ova) differed, the MPF with cryopreserved sperm could be adjusted accordingly. In the coho salmon experiment, differences in MPF achieved with frozen-thawed sperm were equivalent to the changes observed between control groups. This leads to the assumption that if an egg is viable, the probability of successful fertilization with cryopreserved sperm is not affected by the proportion of viable versus non-viable eggs. Utilizing this assumption, individual MPF could be recomputed on a basis of control MPF (diluted and fresh sperm). This would facilitate comparisons between experiments and the actual effect of the cryogenic procedure would be more accurately evaluated.

Thawing and Fertilization Processes

As discussed in the Methods and Procedures chapter of this thesis, the WM and DM were utilized to thaw sperm samples. In terms of the freezing rate, I determined that a fast thaw rate resulted in significantly higher MPF.

In preliminary tests, Ott and Horton (1971a) determined that the DM of thawing in an ice water bath was preferred to a slower procedure of thawing at ambient air temperature. In tests with coho salmon, increases in water bath temperature resulted in a

higher survival of spermatozoa (Table 45). In addition, when sperm samples were thawed in either a 36-38 or 50-60 C bath (WM, 7-8 C), the MPF was higher than when the DM was employed (Table 46).

Ott and Horton (1971a, 1971b) found that the addition of water to thawed spermatozoa resulted in a complete cessation of cellular activity. These results were reconfirmed with fall chinook salmon (Table 35). Mechanism of sperm activation appears to be osmotic because when semen is placed in a hypotonic solution such as water, spermatozoa become active. During freezing and/or thawing processes, osmotic changes apparently activate sperm by changes in cell permeability. Water addition, which normally activates fresh sperm, has the reverse effect on thawed cells. During freezing, membrane permeability may be altered as cells dehydrate; thus when water is added to thawed cells, they swell quickly (almost instantaneously) until the membrane ruptures. However, if water is added during thawing, cells equilibrate, thaw rate is increased, agglutination decreased, thus MPF increases.

Recommendations

In the cryopreservation of salmonid sperm the following procedures are recommended. Sperm should be collected dry in sterilized glass containers (when the volume of air to semen is

10:1, motility generally remains constant for 24 hr) from males selected on a basis of phenotypic characteristics during the peak of the spawning season. Sealed sperm samples and the sealed extender-protector solution should be placed in a water bath isothermic with the fish holding water. After sperm sample collection, the bath should be iced and sperm diluted and frozen within 2 hr. Sperm should be diluted 1:9 (1:4-1:19), not equilibrated, pipetted into 1-ml unsealed glass ampoules, and frozen immediately in LN_2 vapor (-30 C/min). I recommend that for most salmonids the extender-protector combination contain lecithin (500-1500 mg/100 ml), NaCl (850 mg/100 ml), $NaHCO_3$ (500 mg/100 ml), and DMSO (7.5-10.0% v/v). The concentration of each constituent should be determined experimentally if time permits. After the sperm samples are held in LN_2 vapor for at least 1 min, they should be immersed in LN_2 for storage. Sperm can be stored for at least 28 days without changes in viability; furthermore, I can see no reason why longer storage times should adversely affect sperm survival. When thawing sperm, the ampoule should be removed from the LN_2, filled with 4-10 C water, and swirled in a 50-60 C water bath for 8 sec. The sperm can then be shaken from the ampoule and immediately added to a group of fresh ova (50-100 eggs/unit). The gamete mixture should be swirled gently and allowed to stand approximately 2 min. Eggs should then be

washed with fresh water and placed in an incubation facility.

When problems are encountered the following steps are recommended:

(1) A thorough check should be made to confirm that all laboratory methodology, such as sterilization of sample containers, were performed correctly.

(2) A check should be made on the quality of all chemical components of the diluent including the solute moderator (DMSO).

(3) Replicate sampling from various males should be conducted if viability of frozen-thawed sperm is low because the male may be the source of the low viability.

When these factors can be accounted for, I recommend testing the chemical composition of the diluent. Initial tests should involve the life protector, thereafter other chemical components can be evaluated.

In the design of experiments in which ova are inseminated with cryopreserved sperm, treatment replications should equal the control replicates for both fresh sperm and diluted-unfrozen sperm. Diluted-unfrozen sperm should be held at least 15 min prior to the insemination of ova. Utilizing equal replications, the MPF in treatment groups can be adjusted based on control groups (MPF), thus the actual freeze-thaw process is accurately assessed.

If I had but one recommendation to propose, it would be to design and conduct an experiment to test the possible biological selectivity of the freeze-thaw process. The test would involve

production of two groups of fish both with identical parentage, only one group would be cryogenically produced whereas the other would be produced by normal insemination procedures. Both groups would be monitored throughout the incubating, rearing, and smolting periods and sperm-ova from returning adults would be evaluated.

The use of cryopreserved sperm in fish culture, genetics, and disease control was reviewed by Sneed and Clemens (1956), Truscott et al. (1968), Graybill and Horton (1969), and Ott and Horton (1971a). In salmon culture, it would be possible to store sperm of fish selected for breeding purposes and eliminate the need for holding male brood stock. At hatcheries where spring chinook salmon are held for up to three months, mortality associated with crowded conditions would thus be reduced. Research into genetic improvement of fishes would be facilitated by utilizing cryopreserved sperm from males with desired characteristics without regard to the time or place of their availability. For example, crosses could be made between odd and even year runs of pink salmon, spring and fall spawning trouts, and father and successive generations of daughters. In disease control, males from disease resistant stocks could be crossed with those from disease-susceptible populations. In bioassay work, production of homogenous fry in terms of the male donor would be possible with cryopreserved sperm. Finally, centralization of resident male

brood stocks would increase available rearing space at hatcheries while simultaneously decreasing costs of production.

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APPENDICES

Appendix A. The chemical composition of fish sperm extenders tested

Extender Number	Chemical Concentration (mg/100 ml)[a]											
	NaCl	KCl	$CaCl_2 \cdot 2H_2O$	$NaHCO_3$	$Na_2HPO_4 \cdot H_2O$	$MgSO_4 \cdot 7H_2O$	Fructose	Lecithin	Mannitol	Glycine	Sorbitol	Inositol
48	730	38	23	100	41	23	100	500	-			
128	730	38	23	200	41	23	100	500	-			
129	730	38	23	500	41	23	100	500	-			
130	730	38	23	1000	41	23	100	500	-			
131	730	38	23	2500	41	23	100	500	-			
132	730	38	23	500	41	23	100	100	-			
133	730	38	23	500	41	23	100	300	-			
134	730	38	23	500	41	23	100	750	-			
135	730	38	23	500	51	23	100	1000	-			
136	730	38	23	500	41	23	100	1500	-			
137	730	38	23	500	41	23	100	2500	-			
138	730	38	23	500	41	23	500	750	-			
139	730	38	23	500	41	23	1000	750	-			
140	730	38	23	500	41	23	2500	750	-			
141	730	38	23	500	41	23	100	750	100			

[a] Milligrams of solute per 100 milliliters total solution.

Continued

Appendix A (Continued)

Extender Number	NaCl	KCl	$CaCl_2 \cdot 2H_2O$	$NaHCO_3$	$Na_2HPO_4 \cdot H_2O$	$MgSO_4 \cdot 7H_2O$	Fructose	Lecithin	Mannitol	Glycine	Sorbitol	Inositol
142	730	38	23	500	41	23	100	750	500	-		
143	730	38	23	500	41	23	100	750	1000	-		
144	730	38	23	500	41	23	100	750	2500	-		
145	730	38	23	500	41	23	100	750	100	100		
146	730	38	23	500	41	23	100	750	100	250		
147	730	38	23	500	41	23	100	750	100	500		
148	730	38	23	500	41	23	100	750	100	1000		
149	730	-	23	500	41	23	100	750	100	-		
150	730	100	23	500	41	23	100	750	100	-		
151	730	250	23	500	41	23	100	750	100	-		
152	730	500	23	500	41	23	100	750	100	-		
153	730	1000	23	500	41	23	100	750	100	-		
154	730	38	23	-	41	23	100	500	-	-		
155	730	38	23	250	41	23	100	500	-	-		
156	730	38	23	750	41	23	100	500	-	-		
157	730	38	23	750	41	23	100	-	-		-	-
158	730	38	23	750	41	23	100	250	-		-	-
159	730	38	23	500	41	23	-	-	1000		-	-
160	730	38	23	750	41	23	100	750	-		-	-
161	730	38	23	750	41	23	100	1000	-		-	-
162	730	38	23	750	41	23	100	1500	-		-	-
163	730	38	23	750	41	23	100	750	100		-	-
164	730	38	23	750	41	23	100	750	250		-	-
165	730	38	23	750	41	23	100	750	500		-	-
166	730	38	23	500	41	23	100	750	250		-	-
167	730	38	23	500	41	23	100	750	-		100	-
168	730	38	23	500	41	23	100	750	-		250	-
169	730	38	23	500	41	23	100	750	-		500	-
170	730	38	23	500	41	23	100	750	-		1000	-
171	730	38	23	500	41	23	100	750	-		-	100

Continued

Appendix A (Continued)

Extender Number	NaCl	KCl	$CaCl_2 \cdot 2H_2O$	$NaHCO_3$	$Na_2HPO_4 \cdot H_2O$	$MgSO_4 \cdot 7H_2O$	Fructose	Lecithin	Mannitol	Glycine	Sorbitol	Inositol
172	730	38	23	500	41	23	100	750	-			250
173	730	38	23	500	41	23	100	750	-			500
174	730	38	23	500	41	23	100	750	-			1000
175	730	38	23	1000	41	23	100	750	100			-
176	730	38	23	1500	41	23	100	750	100			-
177	730	38	23	2000	41	23	100	750	100			-
178	730	38	23	2500	41	23	100	750	100			-
179	1500	38	23	500	41	23	100	750	100			-
180	2500	38	23	500	41	23	100	750	100			-
181	5000	38	23	500	41	23	100	750	100			-
182	730	38	23	500	41	23	100	-	500			-
183	730	38	23	500	41	23	100	250	500			-
184	730	38	23	500	41	23	100	500	500			-
185	730	38	23	500	41	23	100	1000	500			-
186	730	38	23	500	41	23	100	1500	500			-
187	730	38	23	500	41	23	-	750	500			
188	730	38	23	500	41	23	250	750	500			
189	730	38	23	500	41	23	500	750	500			
190	730	38	23	500	41	23	1000	750	500			
191	730	38	23	-	41	23	500	750	500			
192	730	38	23	250	41	23	500	750	500			
193	730	38	23	750	41	23	500	750	500			
194	730	38	23	1000	41	23	500	750	500			
195	730	38	23	1500	41	23	500	750	500			
196	730	38	23	500	41	23	100	-	100			
197	730	38	23	500	41	23	500	750	100			
198	730	38	23	500	41	23	500	750	250			
199	730	38	23	500	41	23	500	750	1000			
200	730	38	23	500	41	23	500	750	1500			
201	730	38	23	-	41	23	100	750	-			

Continued

Appendix A (Continued)

Extender Number	NaCl	KCl	$CaCl_2 \cdot 2H_2O$	$NaHCO_3$	$Na_2HPO_4 \cdot H_2O$	$MgSO_4 \cdot 7H_2O$	Fructose	Lecithin	Mannitol	Glycine	Sorbitol	Inositol
202	730	38	23	250	41	23	100	750	-			
203	730	38	23	1000	41	23	100	750	-			
204	730	38	23	500	41	23	100	-	-			
205	730	38	23	500	41	23	100	250	-			
206	730	38	23	500	41	23	-	-	-			
207	730	38	23	500	41	23	250	-	-			
208	730	38	23	500	41	23	500	-	-			
209	730	38	23	500	41	23	1000	-	-			
210	730	38	23	500	41	23	-	-	100			
211	730	38	23	500	41	23	-	-	250			
212	730	38	23	500	41	23	-	-	500			
213	730	38	23	500	41	23	-	500	-			
214	730	38	23	500	41	23	250	500	-			
215	730	38	23	500	41	23	500	500	-			
216	730	38	23	500	41	23	750	500	-			
217	730	38	23	500	41	23	1000	500	-			
218	730	38	23	500	41	23	100	500	100			
219	730	38	23	500	41	23	100	500	250			
220	730	38	23	500	41	23	100	500	750			
221	730	38	23	500	41	23	100	500	1000			
222	730	-	23	500	41	23	100	500	-			
223	730	250	23	500	41	23	100	500	-			
224	730	500	23	500	41	23	1000	500	-			
225	850	-	-	250	-	-	100	750	-			
226	850	-	-	-	-	-	100	750	-			
227	850	-	-	500	-	-	100	750	-			
228	850	-	-	750	-	-	100	750	-			
229	-	-	-	500	-	-	100	750	-			
230	450	-	-	500	-	-	100	750	-			
231	1200	-	-	500	-	-	100	750	-			

Continued

Appendix A (Continued)

Extender Number	NaCl	KCl	$CaCl_2 \cdot 2H_2O$	$NaHCO_3$	$Na_2HCO_4 \cdot H_2O$	$MgSO_4 \cdot 7H_2O$	Fructose	Lecithin	Mannitol	Glycine	Sorbitol	Inositol
232	1350	-	-	-	-	-	100	750	-			
233	-	-	-	1350	-	-	100	750	-			
234	850	-	-	500	-	-	0	0	-			
235	850	-	-	500	-	-	0	500	-			
236	850	-	-	500	-	-	0	1000	-			
237	850	-	-	500	-	-	500	0	-			
238	850	-	-	500	-	-	500	500	-			
239	850	-	-	500	-	-	500	1000	-			
240	850	-	-	500	-	-	1000	0	-			
241	850	-	-	500	-	-	1000	500	-			
242	850	-	-	500	-	-	1000	1000	-			
243	650	-	-	300	-	-	-	500	-			
244	650	-	-	500	-	-	-	500	-			
245	650	-	-	700	-	-	-	500	-			
246	850	-	-	300	-	-	-	500	-			
247	850	-	-	700	-	-	-	500	-			
248	1050	-	-	300	-	-	-	500	-			
249	1050	-	-	500	-	-	-	500	-			
250	1050	-	-	700	-	-	-	500	-			
251	850	-	-	500	-	-	-	1500	-			
252	850	-	-	500	-	-	-	2500	-			
253	730	38	23	250	41	23	100	-	-			
254	730	38	23	250	41	23	100	250	-			
255	730	38	23	250	41	23	100	1000	-			
256	730	38	23	100	41	23	100	750	-			
257	730	38	23	200	41	23	100	750	-			
258	730	38	23	300	41	23	100	750	-			
259	730	38	23	400	41	23	100	750	-			
260	730	38	23	600	41	23	100	750	-			
261	730	38	23	700	41	23	100	750	-			

Continued

Appendix A (Continued)

Extender Number	NaCl	KCl	$CaCl_2 \cdot 2H_2O$	$NaHCO_3$	$Na_2HPO_4 \cdot H_2O$	$MgSO_4 \cdot 7H_2O$	Fructose	Lecithin	Mannitol	Glycine	Sorbitol	Inositol
262	850	-	-	1000	-	-	100	750				
263	850	-	-	250	-	-	-	750				
264	850	-	-	250	-	-	250	750				
265	850	-	-	250	-	-	500	750				
266	850	-	-	250	-	-	750	750				
267	850	-	-	250	-	-	-	-				
268	850	-	-	250	-	-	-	250				
269	850	-	-	250	-	-	-	500				
270	850	-	-	250	-	-	-	1000				
271	650	-	-	250	-	-	100	750				
272	1050	-	-	250	-	-	100	750				
273	1250	-	-	250	-	-	100	750				
274	1450	-	-	250	-	-	100	750				
275	850	-	-	375	-	-	-	750				
276	850	-	-	500	-	-	-	750				

APPENDIX B

The analysis of variance of data contained in the individual Results sections of this thesis.

Table 1. The analysis of variance of data contained in Table 2 of the Results section for steelhead trout.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-Value
Males	09	10089.879	1121.098	6.26*
Females	02	3948.222	1974.111	11.03*
Males x Females	18	1892.498	105.139	0.59
Error	60	10742.668	179.044	
Total	89	26673.267		

*Significant at $\alpha = .01$

Table 2. The analysis of variance of data contained in Table 10 of the Results section for chum salmon.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-Value
Treatments (Ampoule Position)	02	381.743	190.871	3.19
Error	15	898.477	59.898	
Total	17	1280.220		

Table 3. The analysis of variance of data contained in Table 12 of the Results section for pink salmon.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-Value
Treatments (Extender 48 versus 129)	1	273.000	273.000	1.59
Error	26	4461.207	171.585	
Total	27	4734.207		
Treatments (Diluted versus fresh sperm)	1	234.500	234.500	2.96
Error	4	317.269	79.317	
Total	5	551.769		

Table 4. The analysis of variance of data contained in Table 13 of the Results section for kokanee.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-Value
Treatments (Thawing Method)	1	2626.978	2626.978	17.85*
Error	28	4121.635	147.201	
Total	29	6748.613		

*Significant at $\alpha = .01$

Table 5. The analyses of variance of data contained in Table 14 of the Results section for kokanee.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-Value
Treatments (Thawing Method)	1	300.743	300.743	5.59*
Error	8	430.289	53.786	
Total	9	731.032		
Treatments (Water Addn.)	1	4042.914	4042.914	338.43**
Error	8	95.569	11.946	
Total	9	4138.483		

* Significant at $\alpha = .05$
**Significant at $\alpha = .01$

Table 6. The analyses of variance of data contained in Table 15 of the Results section for kokanee.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-Value
Treatments (Ampoules versus Whirl-Paks)	1	54.709	54.709	1.19
Error	8	367.577	45.947	
Total	9	422.286		
Treatments (Thawing Method)	1	248.602	248.602	6.12*
Error	8	324.981	40.623	
Total	9	573.583		

*Significant at $\alpha = .05$

Table 7. The analysis of variance of data contained in Table 16 of the Results section for kokanee.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-Value
DMSO	2	8.770	4.385	0.59
Glycerol	2	76.434	38.217	5.15*
DMSO x Glycerol	4	6.825	1.706	0.23
Error	18	133.514	7.417	
Total	26	225.543		

*Significant at $\alpha = .05$

Table 8. The analyses of variance of data contained in Table 17 of the Results section for kokanee.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-Value
Treatments (50.4 versus 45.1)	1	71.182	71.182	0.76
Error	8	748.242	93.530	
Total	9	819.424		
Treatments (54.8 versus 50.8)	1	42.684	42.684	0.34
Error	8	1002.346	125.293	
Total	9	1045.030		
Treatments (54.8 versus 52.5)	1	13.502	13.502	0.13
Error	8	808.262	101.033	
Total	9	821.764		

Table 9. The analysis of variance of data contained in Table 19 of the Results section for kokanee.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-Value
Treatments (Sperm Volume)	1	32.080	32.080	0.26
Error	16	197.364	123.710	
Total	17	2011.444		

Table 10. The analyses of variance of data contained in Table 26 of the Results section for fall chinook salmon.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-Value
Treatments (Extender 134 versus 227 for Male 200)	1	113.837	113.837	0.54
Error	10	2103.344	210.334	
Total	11	2217.181		
Treatments (Extender 134 versus 227 for Male 201	1	12.526	12.526	0.10
Error	10	1214.466	121.447	
Total	11	1226.992		

Table 11. The analysis of variance of data contained in Table 27 of the Results section for fall chinook salmon.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-Value
Treatments (Extender 225 versus 227)	1	75.501	75.501	1.65
Error	10	456.854	45.685	
Total	11	532.355		

Table 12. The analysis of variance of data contained in Table 28 of the Results section for fall chinook salmon.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-Value
Treatments (Extender 225 versus 272	1	0.024	0.024	0.00
Error	10	473.614	47.361	
Total	11	473.638		

Table 13. The analysis of variance of data contained in Table 29 of the Results section for fall chinook salmon.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-Value
Treatments (Extender 225 versus 263)	1	94.248	94.248	1.90
Error	10	495.144	49.514	
Total	11	589.392		

Table 14. The analysis of variance of data contained in Table 30 of the Results section for fall chinook salmon.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-Value
Treatments (Extender 263 versus 275 versus 276)	2	12.176	6.088	0.12
Error	15	790.024	52.668	
Total	17	802.200		

Table 15. The analysis of variance of data contained in Table 31 of the Results section for fall chinook salmon.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-Value
Treatments (DMSO 5.0 versus 7.5 versus 10.0)	2	169.827	84.913	0.73
Error	15	1749.469	116.631	
Total	17	1919.296		

Table 16. The analysis of variance of data contained in Table 32 of the Results section for fall chinook salmon.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-Value
Treatments (Diluted versus Fresh Sperm)	1	49.573	49.573	2.07
Error	10	239.282	23.928	
Total	11	288.855		

Table 17. The analyses of variance of data contained in Table 33 of the Results section for fall chinook salmon.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-Value
Treatments (Time in Ice Water Bath)	4	402.904	100.726	0.77
Error	15	1951.461	130.097	
Total	19	2354.365		
Treatments (Diluted versus Fresh Sperm)	1	15.585	15.585	1.09
Error	4	56.998	14.249	
Total	5	72.583		

Table 18. The analyses of variance of data contained in Table 34 of the Results section for fall chinook salmon.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-Value
Treatments (Extender 263 versus 265 versus 266)	2	227.313	113.657	2.24
Error	15	761.895	50.793	
Total	17	989.208		
Treatments (Diluted versus Fresh Sperm	1	40.560	40.560	0.19
Error	4	851.145	212.786	
Total	5	891.705		

Table 19. The analyses of variance of data contained in Table 35 of the Results section for fall chinook salmon.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-Value
Treatments (Water Addn.)	1	7081.268	7081.268	40.22*
Error	28	4929.948	176.070	
Total	29	12011.216		
Treatments (Diluted versus Fresh Sperm)	1	1.197	1.197	1.41
Error	4	3.385	0.846	
Total	5	4.582		

* Significant at $\alpha = .01$

Table 20. The analysis of variance of data contained in Table 37 of the Results section for coho salmon.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-Value
Dilution	4	9133.706	2283.426	15.93*
Males	2	1226.205	633.103	4.42**
Dilution x Males	8	1217.242	152.155	1.06
Error	30	4299.825	143.328	
Total	44	15916.978		

* Significant at $\alpha = .01$
**Significant at $\alpha = .05$

Table 21. The analysis of variance of data contained in Table 38 of the Results section for coho salmon.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-Value
DMSO	5	13985.934	2797.187	28.52**
Males	2	10623.722	5311.861	54.16**
DMSO x Males	10	3161.350	316.135	3.22**
Error	54	5295.858	98.071	
Total	71	33066.864		

**Significant at $\alpha = .01$

Table 22. The analysis of variance of data contained in Table 39 of the Results section for coho salmon.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-Value
Treatments (DMSO)	3	1816.236	605.412	3.82*
Error	28	4438.831	158.530	
Total	31	6255.067		

*Significant at $\alpha = .05$.

Table 23. The analysis of variance of data contained in Table 40 of the Results section for coho salmon.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-Value
Inositol	4	1573.551	393.388	2.46
Males	2	4462.167	2231.084	13.96*
Inosital x Males	8	1805.574	225.697	1.41
Error	30	4794.012	159.800	
Total	44	12635.304		

*Significant $\alpha = .01$

Table 24. The analysis of variance of data contained in Table 41 of the Results section for coho salmon.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-Value
Mannitol	4	420.885	105.221	1.00
Males	2	1200.028	600.014	5.69*
Mannitol x Males	8	1159.289	144.911	1.37
Error	30	3164.526	105.484	
Total	44	5944.728		

*Significant at $\alpha = .01$

Table 25. The analysis of variance of data contained in Table 42 of the Results section for coho salmon.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-Value
Sorbitol	4	206.495	51.624	0.59
Males	2	16825.508	8412.754	96.52*
Sorbitol x Males	8	672.074	84.009	0.96
Error	30	2614.956	87.165	
Total	44	20319.033		

*Significant at $\alpha = .01$

Table 26. The analysis of variance of data contained in Table 43 of the Results section for coho salmon.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-Value
Treatments (Extender 202 versus 225)	1	12.298	12.298	0.01
Error	4	513.674	128.419	
Total	5	525.972		

Table 27. The analyses of variance of data contained in Table 44 of the Results section for coho salmon.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-Value
Treatments (Extender 266 versus 225 versus 227 for Male 395)	2	3997.518	1998.759	24.10*
Error	6	497.537	82.923	
Total	8	4495.055		
Treatments (Extender 226 versus 225 versus 227 for Male 394)	2	956.019	478.010	1.44
Error	6	1994.194	332.366	
Total	8	2950.213		
Treatments (Diluted versus Fresh Sperm)	1	19.082	19.082	2.33
Error	4	32.783	8.196	
Total	5	51.865		

*Significant at $\alpha = .01$

Table 28. The analysis of variance of data contained in Table 46 of the Results section for coho salmon.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-Value
Temperature	3	1815.157	605.052	3.53*
With or Without Water	1	1556.262	1556.262	9.09*
Temperature x With or Without Water	3	106.433	35.478	0.21
Error	24	4108.178	171.174	
Total	31	7586.030		

*Significant at $\alpha = .05$

Table 29. The analysis of variance of data contained in Table 47 of the Results section for coho salmon.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-Value
Treatments (Extender 227 versus 232 versus 233 for Male 397	2	6990.368	3495.184	40.42*
Error	9	778.182	86.465	
Total	11	7768.550		

*Significant at $\alpha = .05$

Table 30. The analysis of variance of data contained in Table 48 of the Results section for coho salmon.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-Value
Fructose	2	350.082	175.041	1.36
Lecithin	2	3658.296	1829.148	14.17*
Fructose x Lecithin	4	346.449	86.612	0.67
Error	18	2324.216	129.123	
Total	26	6679.043		

*Significant at $\alpha = .05$

Table 31. The analysis of variance of data contained in Table 49 of the Results section for coho salmon.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-Value
Treatments (Lecithin)	3	1491.854	497.285	3.05*
Error	28	4558.467	162.802	
Total	31	6050.321		

*Significant at $\alpha = .05$

Table 32. The analysis of variance of data contained in Table 50 of the Results section for coho salmon.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-Value
NaCl	2	5420.975	2710.487	13.13*
$NaHCO_3$	2	3828.481	1914.240	9.27*
NaCl x $NaHCO_3$	4	4244.185	1061.046	5.14*
Error	27	5575.171	206.488	
Total	35	19068.812		

*Significant at $\alpha = .05$

Table 33. The analyses of variance of data contained in Table 51 of the Results section for coho salmon.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-Value
Treatments (Refrigerated Sperm with Urine)	5	2.968	0.594	1.00
Error	18	10.696	0.594	
Total	23	13.664		
Treatments (Frozen Sperm with Urine)	4	1341.937	335.484	3.13*
Error	15	1607.815	107.188	
Total	19	2949.752		

*Significant at $\alpha = .05$

Table 34. The analyses of variance of data contained in Table 52 of the Results section for coho salmon.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-Value
Treatments (Equilibration Time)	5	1934.350	386.870	2.45
Error	24	3788.091	157.837	
Total	29	5722.441		
Treatments (Diluted versus Fresh Sperm)	1	16.534	16.534	2.21
Error	4	29.916	7.479	
Total	5	46.450		

Table 35. The analysis of variance of data contained in Table 54 of the Results section for coho salmon.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-Value
Treatments (Freezing Method)	5	9693.423	1938.685	19.09*
Error	18	1828.268	101.570	
Total	23	11521.691		

*Significant at $\alpha = .05$

Table 36. The analyses of variance of data contained in Table 55 of the Results section for coho salmon.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-Value
Males (Replication 1)	3	5319.259	1773.086	5.78*
Ampoules (Position)	3	888.887	296.296	0.97
Males x Ampoules	9	566.719	62.969	0.21
Error	16	4905.531	306.596	
Total	31	11680.396		
Males (Replication 2)	3	5511.641	1837.214	14.73*
Ampoules (Position)	3	116.426	38.809	0.31
Males x Ampoules	9	1281.312	142.368	1.14
Error	16	1996.201	124.763	
Total	31	8905.580		

*Significant at $\alpha = .05$

Table 37. The analysis of variance of data contained in Table 56 of the Results section for coho salmon.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-Value
Treatments (Ampoules versus Whirl-Paks)	1	5382.945	5382.945	65.31**
Error	12	988.998	82.417	
Total	13	6371.943		

**Significant at $\alpha = .01$

Table 38. The analysis of variance of data contained in Table 57 of the Results section for coho salmon.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-Value
Treatments (Water Addn.)	5	3346.626	669.325	2.81*
Error	30	7153.318	238.444	
Total	35	10499.944		

*Significant at $\alpha = .05$

Table 39. The analysis of variance of data contained in Table 58 of the Results section for coho salmon.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-Value
Males	3	9902.125	3300.708	18.91*
Days storage	1	169.000	169.000	0.97
Males x Days Storage	3	926.111	308.704	1.77
Error	56	9776.824	174.586	
Total	63	20774.059		

*Significant at $\alpha = .01$