AN ABSTRACT OF THE THESIS OF

JAMES ROGER GRAYBILL (Name of student) for the Master of Science (Degree)

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Title: CRYO-PRESERVATION OF VIABLE FISH SPERM

Abstract approved: Redacted for Privacy
Howard F. Horton

Fishery researchers have attempted to preserve viable fish sperm for extended periods of time. Only limited success has been achieved with storage of spermatozoa from salmonids. The principal accomplishments of this investigation during the fiscal year 1966-1967 were the development of standard methods for the collection and evaluation of fish sperm, the development of an extender and life protector of spermatozoa, and the development of a freezing and thawing procedure for the live preservation of fish sperm at temperatures of liquid nitrogen (-196 C).

Collection of fish sperm was accomplished by means of a suction device with which semen was withdrawn from the genital pore of a fish into a test tube. A modification of the conventional hemocytometric technique was used to determine the concentration of sperm in fish semen. The use of vital stains to differentiate between live and dead sperm cells was investigated and abandoned due to inconsistent results. A subjective method of estimating the viability and relative motility of spermatozoa was established.

Experiments were conducted to prolong the viability of sperm cells in the unfrozen state. The deleterious effect of water on prolonging the viability of fish sperm was confirmed. I found that a high percentage of sperm cells remained viable for at least three days if they were refrigerated under aerobic conditions and kept from contact with water. Increased dilution of fish semen with extenders reduced the viability of sperm cells. The freezing point depression of solutions isotonic to fish spermatozoa was found to be -0.51 C. My evidence suggests that the key to maintaining viable fish sperm at 5 C is to provide substances in the extender which can be aerobically metabolized and stored as latent energy by the spermatozoa.

Experiments were conducted to prolong the viability of fish spermatozoa in the frozen state. Dimethyl sulfoxide (DMSO) gave the best results of the life protectors studied. Equilibration of the sperm cells in increasing concentrations of a life protector may have increased the viability of the spermatozoa. During several experiments, the optimum concentration of protector increased as the rate of freezing increased. The best survival of viable sperm was obtained in samples that were frozen in Solution 48 in combination with DMSO as a protector. Thawing the ampoules of semen in a 4 C water bath yielded the highest recovery of viable spermatozoa. Alevins were produced from eggs fertilized with cryogenically

preserved fish sperm. These alevins appeared to be as normal as young fish produced from unfrozen spermatozoa. The fertility rate was low (0-18%), but to my knowledge this is the first successful attempt to fertilize salmonid eggs with cryo-preserved spermatozoa. The findings in this study warrant further investigation.

Cryo-Preservation of Viable Fish Sperm

by

James Roger Graybill

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Associate Professor of Fisheries

in charge of major

Redacted for Privacy

Head of Fisheries and Wildlife Department

Redacted for Privacy

Dean of Graduate School

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I would like to express my appreciation to the following fish culturists who not only welcomed me to their stations, but gave me valuable assistance and suggestions. Mr. Charles Hiltz and his assistants at the Eagle Creek National Fish Hatchery (BSFW); Mr. Paul Vroman and his assistants at the Alsea Trout Hatchery (OSGC); Mr. William Wingfield and his assistants at the Roaring River Trout Hatchery (OSGC); Mr. Kenneth Bennett and his assistants at the Alsea River Salmon Hatchery (FCO); and Mr. Arthur Minney and his assistants at the Siletz River Salmon Hatchery (FCO).

Dr. Raymond C. Simon, Professor of Fisheries at Oregon State University, conducted the chromosome analysis and gave valuable suggestions throughout the investigation.

I would also like to thank Mr. Kurt E. Norlin, Laboratory Assistant, for his enthusiastic help with some of the unpleasant tasks around the laboratory.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

Figure Page

CRYO-PRESERVATION OF VIABLE FISH SPERM

INTRODUCTION

For the past century, fishery scientists have attempted to preserve the spermatozoa of fishes in a viable condition for extended periods of time (de Quatrefages, 1853; Ellis and Jones, 1939; Blaxter, 1953; Sneed and Clemens, 1956; Mitchum, 1963). Except for Blaxter's (1953) work with herring (Clupea), these attempts have either failed or yielded inconclusive results. On July 1, 1966, the U. S. Bureau of Commercial Fisheries contracted with Oregon State University for a study whose principal objective was to develop methodology for the cryo-preservation of viable salmonid sperm. Both contracting agencies recognized that the successful development of a sperm storage techniques would facilitate more efficient fish cultural practices and enhance research into fishery genetics and disease control.

The investigation was conducted during the fiscal year 1966-67, and had the following specific objectives: (1) Development of a best method of collecting semen; (2) selection of standard tests of viability of spermatozoa; (3) development of a life extender for the spermatozoa; (4) development of a life protector for the extended semen; (5) determination of a suitable period of equilibration of the sperm cells to the life protector; (6) determination of the best rate of freezing for storing sperm cells in liquid nitrogen; and (7) determination of the best

procedure for thawing the frozen spermatozoa. My study was culminated when techniques were developed which yielded normal steelhead trout (Salmo gairdneri) alevins from cryogenically preserved sperm cells.

One of the early attempts to preserve fish spermatozoa in a viable condition was conducted by Ellis and Jones (1939). They were able to hold viable sperm cells of Atlantic salmon (Salmo salar) parr for 7 days in the ovarian fluid of fish that was refrigerated to 0 C. Rucker (1949) held undiluted salmon sperm in a covered dish at refrigerator temperatures for 14 days with 93.2% of 133 eggs being fertilized with the preserved semen.

In one of the more successful experiments, Blaxter (1953) stored two sections of the testis of a March-spawning herring (Clupea harengus) in 80% sea water and 12.5% glycerol at -79 C. The stored sperm fertilized 80 and 85% of the eggs from autumn-spawning herring (C. harengus) collected six months later. Sneed and Clemens (1956) froze the spermatozoa of carp (presumably Cyprinus carpio) in frog Ringer's solution plus 6 to 12% glycerol for 60 days with 20% survival. Mitchum (1963) was unsuccessful in his attempts to store the sperm of rainbow trout (Salmo gairdneri) in Ringer's solution, glycerated yolk-citrate, skim milk, and homogenized milk extenders with either dry ice or liquid nitrogen as the refrigerant. Hodgins and Ridgway (1964) recovered motile and morphologically intact chinook salmon

(Oncorhynchus tshawytscha) and pink salmon (O. gorbuscha) sperm cells which had been frozen for 7 days with liquid nitrogen in a citrate-dextrose-sodium chloride solution with dimethyl sulfoxide as the protective agent. Hodgins reported, however, that the motility of the sperm cells decreased rapidly after thawing.[1] The fertilizing ability of the sperm cells which survived freezing was not tested.

Some terms used throughout this thesis which may be unfamiliar to the reader are:

Extender, life extender, diluent: A solution of salts, sometimes including organic compounds which maintain the viability of cells during refrigeration. Does not include additives. Used to dilute semen and preserve sperm cells.

Additive, life protector: An organic compound which protects the viability of cells during the freezing and thawing processes.

Percent motile: An estimate of the percent of cells observed in a sample that are motile (motile cells are assumed to be capable of fertilizing eggs).

Percent relative activity: The degree of activity of motile cells relative to the highest degree of motility ever observed (100%).

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[1] Personal communication. H. F. Horton, Associate Professor of Fisheries, Oregon State University, Corvallis. July 10, 1964.

The report that follows presents the results of my research under terms of the United States Department of the Interior Contract No. 14-17-0001-1546 between the Bureau of Commercial Fisheries and Oregon State University as represented by the Department of Fisheries and Wildlife under the guidance of Dr. Howard F. Horton.

METHODS

Approach to the Problem

The overall investigation was divided into a logical sequence of studies. Alternate hypotheses were established within each study. Using these hypotheses as guides, experiments were designed to test these suppositions so that the results would select only one hypothesis as true and identify those remaining as false. Critical path schedules were made for each study to more efficiently utilize time and further refine our methods of procedure and statistical design. All data were recorded on standardized forms from which IBM cards could be punched directly. Each study was planned on a statistical design so that if needed, statistical analysis could easily be conducted.

Sterilization Procedures

All glassware was washed several times with "7X," a commercial detergent developed specifically for cleaning apparatus to be used in the culture of living tissues. The glassware was then rinsed several times with distilled water and (glassware made entirely of glass) baked in an oven at 204 C (400 F) for two hours as suggested by Pelczar and Reid (1958). Glassware made with soft materials was autoclaved at 121 C (250 F) for 15 min. After being heated in the

oven, test tubes were sealed with aluminum foil until used. Sterile conditions were maintained as far as possible in all phases of the research.

Preparation of Extender Solutions

All extender solutions were prepared in the following manner. Each chemical was weighed on a Mettler balance accurate to ±0.2 mg. Stock solutions of each chemical were prepared in concentrations of 1, 5, or 10 g of chemical per 100 ml of distilled water, depending on the solubility and requirements of the extender. The appropriate weight of a chemical required by an extender was transferred to the extender bottle in the volume of stock solution calculated to contain the necessary weight of the chemical. This procedure was repeated for each chemical. Additional distilled water was added to the extender solution to produce the proper concentration of the chemicals. Sterile, disposable, cotton-plugged pipettes were used to transfer each solution. The constituents of each extender used in this investigation are listed in Appendix 1.

Source of Fish

The three species of fish from which sperm samples were taken in this investigation were collected at state and federal fish hatcheries in Oregon. Spring chinook salmon were obtained at the Eagle Creek

National Fish Hatchery (BSFW). Coho salmon (O. kisutch) were collected at the Alsea River Salmon Hatchery (FCO) and the Siletz River Salmon Hatchery (FCO). Fall-spawning rainbow trout were obtained from the Roaring River Trout Hatchery (OSGC). Steelhead trout were collected from the Alsea Trout Hatchery (OSGC) and the Eagle Creek National Fish Hatchery (BSFW).

Each male fish was checked for state of maturation. Mature male salmon were killed by a blow on the head. Mature male trout were anesthetized in either chlorotone or MS-222 (tricane methanesulfonate). Quieting the fish was necessary to expedite and facilitate the collection of semen.

Collection of Semen

A quieted fish was held in a vertical position with the head up by an assistant. A small sterile tube was then inserted into the genital pore. With the tube in place, vacuum was applied so as to draw the semen into a sterile collection tube.

Two apparati were used to collect the semen. The first apparatus tested was a "Vacutainer" blood collecting device (Fig. 1). The Vacutainer is composed of three parts: a hypodermic needle with two sharp ends and a plastic screw in between, a holder and a vacuum tube. A plastic sheath guards the sharp end of the needle. In use, the tip of the needle sheath was cut off, the needle screwed

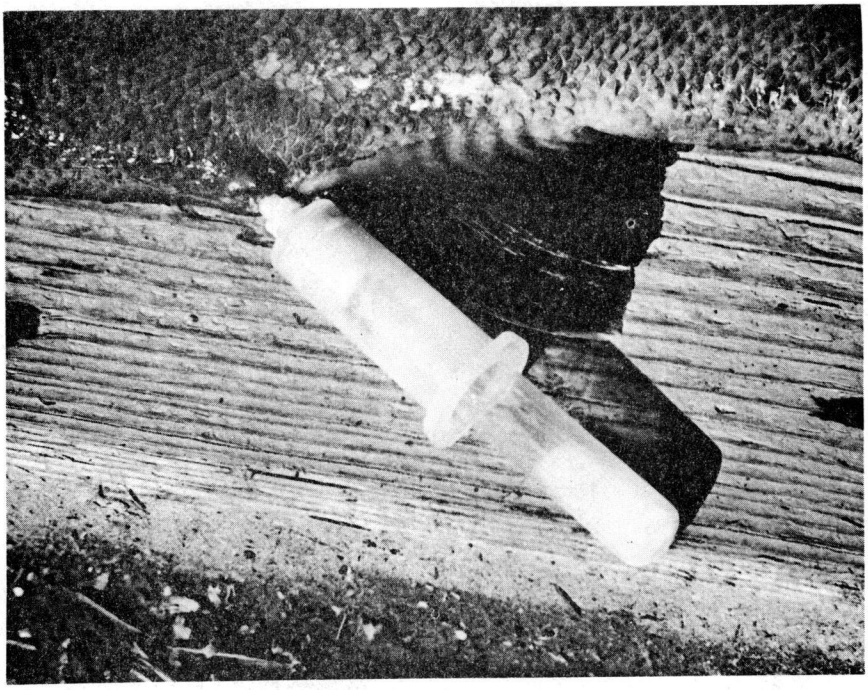

Figure 1. The "Vacutainer" used to collect semen from salmon and trout.

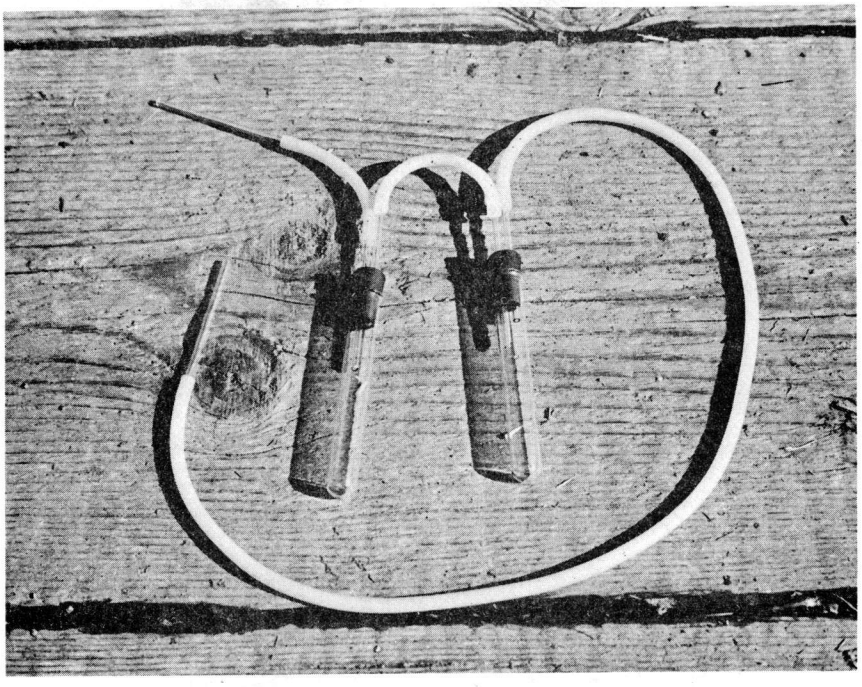

Figure 2. The dual tube suction device used to collect semen from salmon and trout.

into the holder and then the needle sheath with holder was placed into the genital pore. The vacuum tube was then pushed over the exposed end of the needle in the holder, thus relieving the vacuum and drawing the semen into the tube.

A second collection device tested was similar to the apparatus used by Miller (1965). This device is made of two sterilized test tubes connected in series between the two ends of a rubber tube. The ends of the rubber tube are connected to small diameter glass tubes (Fig. 2). The free end of the short rubber tube is inserted into the genital pore of the fish (Fig. 3 and 4). Suction is applied orally at the other end of the tubing by the collector. After the first test tube is full or collection is complete, the test tube is pulled free of the rubber stopper and sealed with a solid stopper. The second test tube acts as a sump to collect saliva or excess semen.

The Vacutainer method has the advantage that it is nearly free of contamination. Contamination can occur, however, when the needle sheath is first introduced through the genital pore. This technique has several disadvantages, none of which were found to apply to the dual test tube method. The Vacutainer holds a high vacuum which pulls the semen through the small diameter needle with a great deal of force causing tails of many of the spermatozoa to be broken. The thin walls of the seminal tract often collapsed around the end of the needle sheath and plugged the Vacutainer and/or which caused the

Figure 3. Collector using the dual tube suction device to collect semen from a steelhead trout.

Figure 4. Close-up view of the dual tube suction device being used to withdraw semen from a steelhead trout.

fish to bleed internally. With internal bleeding, blood was additionally collected with the semen. Occasionally, the fish flexed during the collection of the semen, which caused withdrawal of the apparatus and subsequently destroyed the vacuum. Semen left in the vacuum tube for extended periods of time lost viability more rapidly than did semen left in tubes without a vacuum. Because the dual test tube method had none of the above objections, it was the preferred method of collecting semen.

Either of the above methods is preferable to hand stripping the semen into a glass container. It is extremely difficult to keep urine out of the semen when hand stripping. Feces and fish slime are also apt to enter the semen sample. It was my experience that more semen can be collected with either suction device than can be collected by hand stripping.

Transportation of Semen

Once the semen was collected and the test tubes stoppered, the samples were placed into a test tube rack inside a styrofoam cooler filled with about four liters of stream water (Fig. 5). Stream water was used to avoid temperature shock to the spermatozoa. The semen was transported in this condition to the Fairplay Laboratory. Temperatures in the styrofoam cooler increased a maximum of 3 C on a warm day and a 3-hr trip.

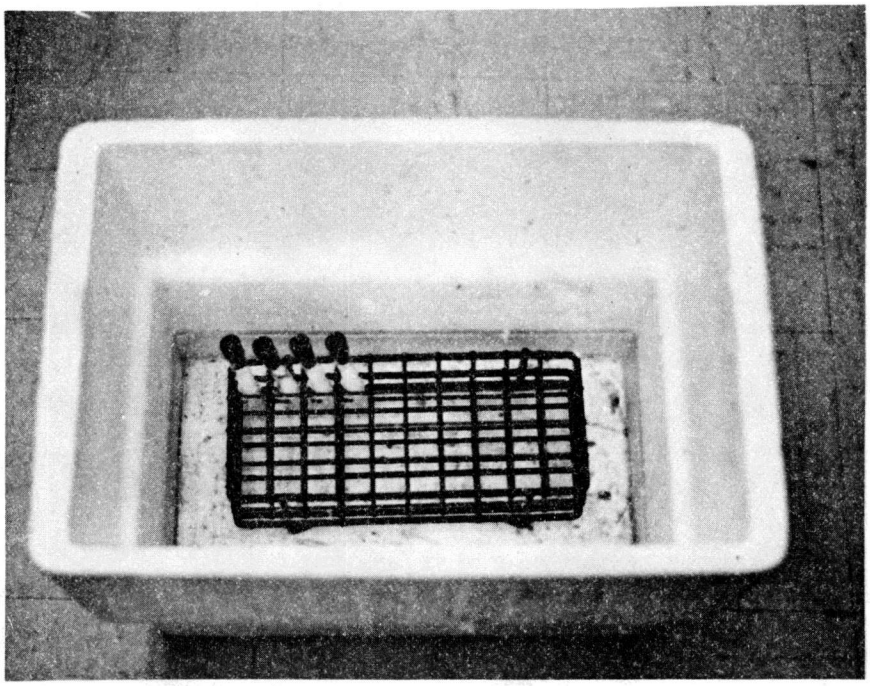

Figure 5. Interior view of the styrofoam cooler used for transporting semen. Shown are the water bath, test tube rack and test tubes containing collected semen. The same arrangement was used during the evaluation of semen in the laboratory.

Handling of Semen in the Laboratory

The styrofoam cooler containing the vials of semen was placed in a walk-in refrigerator at the Fairplay Laboratory. The temperature of the semen was allowed to decrease slowly to 4 C to avoid any temperature shock. Once the temperature of 4 C was reached, the rack of test tubes was removed from the water bath.

All samples of semen were extended and protected within the walk-in refrigerator. Extenders and protectors were always equilibrated to the temperature within the refrigerator before

they were mixed with the semen. A composite sample of semen from several fish was never tested during this investigation.

Evaluation of Semen

Four liters of water equilibrated to the temperature within the walk-in refrigerator was placed in the styrofoam cooler. Test tubes of semen to be evaluated were placed in this water bath and moved to a laboratory desk outside the refrigerator. Ampoules of frozen semen were thawed by placing them into an ampoule rack which was also submerged in four liters of water from the refrigerator. The ampoules were left in this water bath during the semen evaluation procedure. Cold well water (pH 7.1) was placed into two 250-ml jars. An eye dropper was placed into one jar and a 6-inch glass rod drawn to a fine point was placed into the other jar. A microscope slide was placed onto the stage of a binocular, zoom microscope. The fine tip only of the glass rod was dabbed into the selected sample of semen. The tip of the glass rod was then dabbed onto the microscope slide to produce a drop of semen about 2 to 4 mm in diameter.

To evaluate the semen, the microscope was focused on the outer edge of the drop of semen. Percent motility and percent relative activity of the sperm cells was estimated. A drop of water was then added to the semen and the percent motility and percent relative activity of the sperm cells was again estimated. Estimates were made

in intervals of 10%. With the microscope pre-focused, cells nearest the surface of the slide were clearly visible. I found this procedure to be the most rapid way of evaluating semen after the addition of water. Because sperm cells are short lived after exposure to water, expediency was necessary to obtain consistent evaluations. I agree with Sherman (1965) that this is the most valid method of laboratory evaluation of semen.

Methods of Freezing Semen

One milliliter of extended and protected semen was placed into a glass ampoule. The ampoule was sealed with a Kahlenberg-Globe sealer located inside the walk-in refrigerator (Fig. 6). Heat given off by the torch of the sealer or by the molten glass had no detectible effect on the semen. One ampoule was left unsealed to receive the thermocouple used to give continuous temperature read-out during the freezing process.

The ampoules of semen were frozen by either slow or rapid methods. If a slow rate of temperature decrease was desired (1 to 15 C/min), the ampoules were frozen in the Linde Biological Freezer unit (Fig. 7). Ampoules were removed from the walk-in refrigerator and placed in a wire basket inside the freezing chamber (Fig. 8). The long probe of the differential thermocouple was placed into the middle portion of a representative sample (Fig. 9). All samples

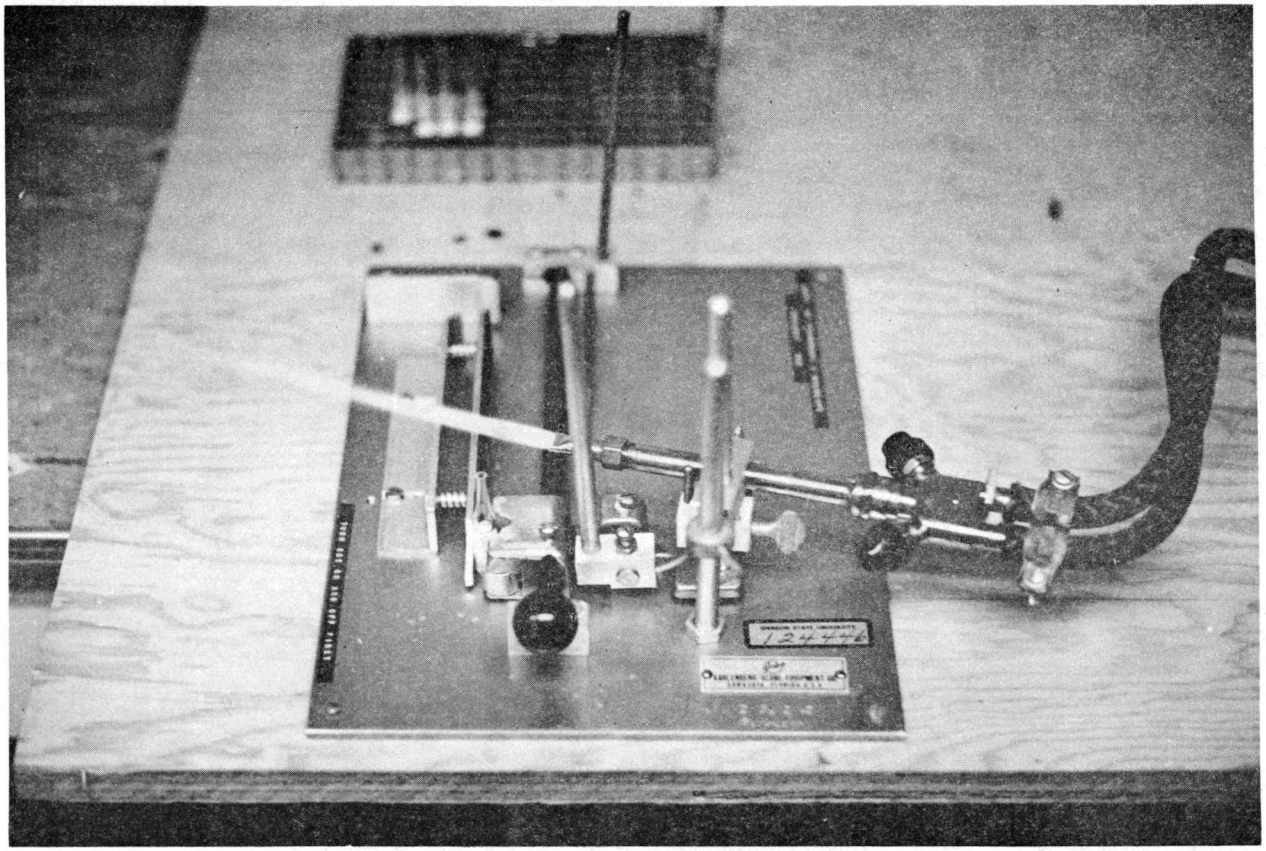

Figure 6. Kahlenberg-Globe automatic ampoule sealer with ampoule in position to be sealed.

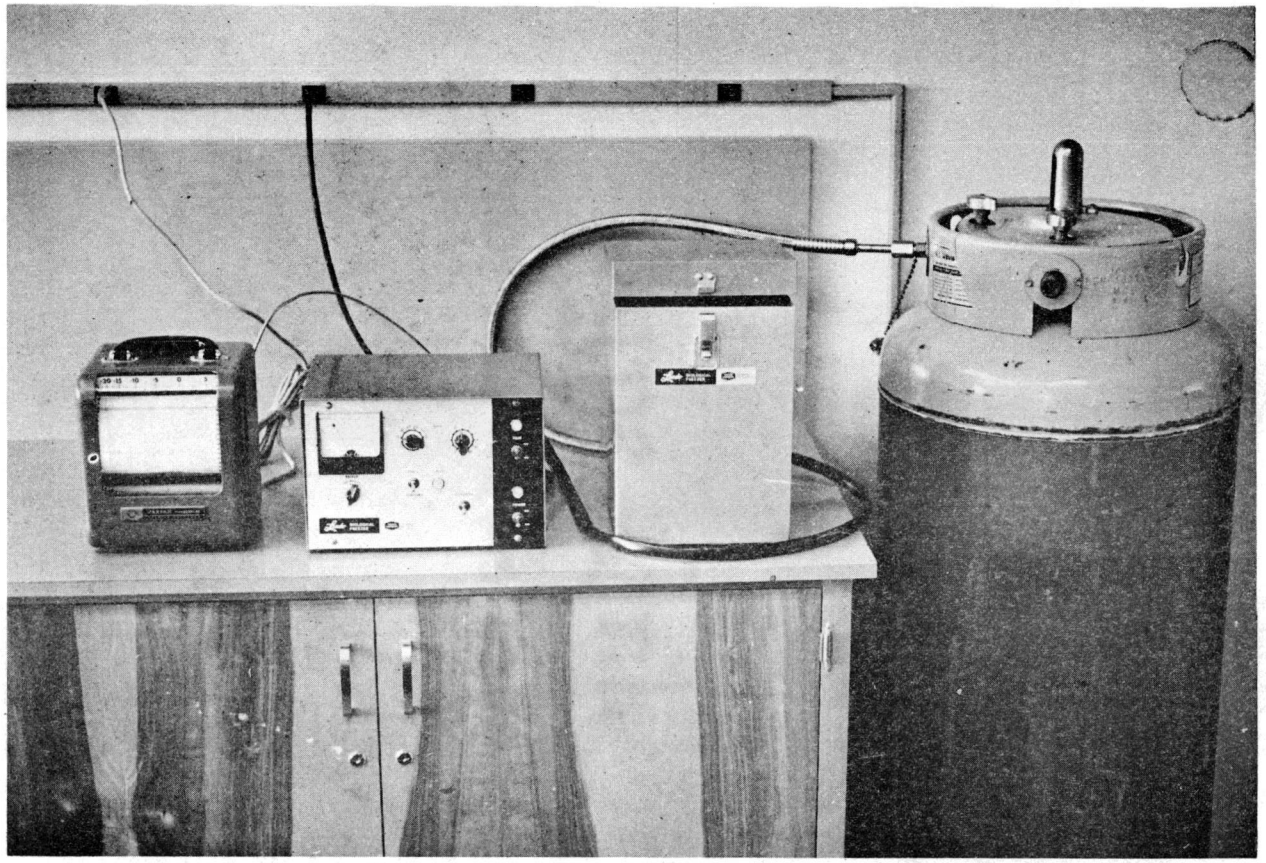

Figure 7. Equipment used to implement freezing at a constant rate of temperature decrease. From left to right: Varian strip-chart recording potentiometer, Linde Biological Freezer controller unit, Linde Biological Freezer chamber and Linde 160 liter liquid nitrogen container.

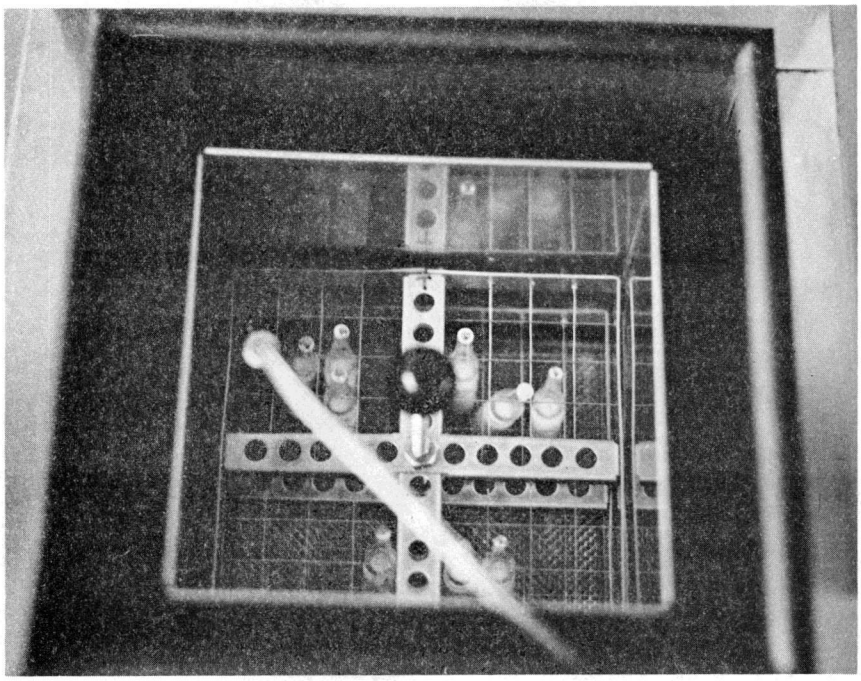

Figure 8. Interior view of the Linde Biological Freezer chamber with ampoules in the partioned basket and differential thermocouple in place.

Figure 9. Thermocouple positioned to provide continuous temperature read-out of the interior of an ampoule.

frozen at any one time were of the same thermal characteristics with regard to volume, amount of extender and amount of protector. Once all the ampoules were in place, the freezing cycle was initiated. Continuous temperature read-out was recorded on a Varian strip-chart recorder. The temperature of the freezing unit was lowered at a predetermined rate until the semen reached -120 C. At this point, the ampoules were removed from the freezing chamber, placed on canes and stored in a Cryenco liquid nitrogen (LN_2) refrigerator at -170 to -196 C (Fig. 10).

If a rapid temperature decrease of around 30 C/min was desired, the ampoules were frozen in the vapor above the surface of the liquid nitrogen. To accomplish this, the ampoules were placed in the top position of a cane. The cane was immediately placed in a canister which was hung inside the neck of the Cryenco liquid nitrogen refrigerator on a canister retrieving hook (Fig. 11). After 10 min, the canister was lowered into its position in the LN_2 refrigerator for storage.

Figure 10. Cryenco liquid nitrogen refrigerator. Cane with ampoules in place is leaning against the refrigerator. Canister containing canes is in the right foreground. Canister is placed through the opening in the rear of the refrigerator for storage.

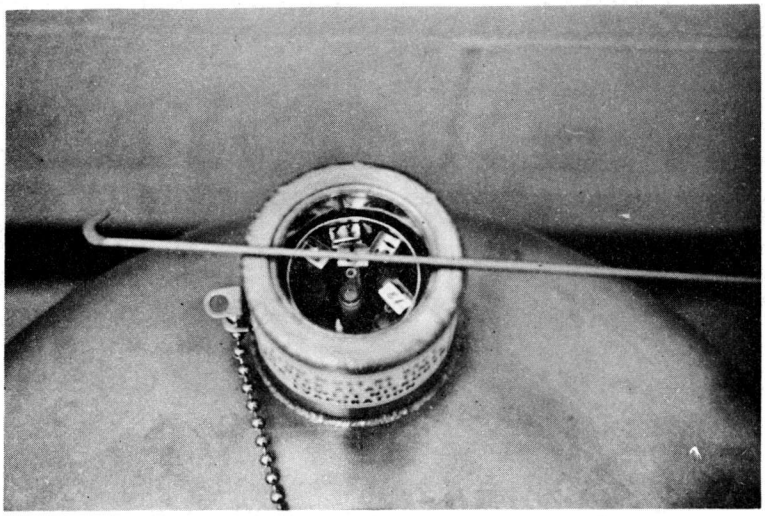

Figure 11. Canister with ampoules being frozen in the vapor above the surface of the liquid nitrogen. Canister is suspended from a retrieving hook.

RESULTS AND DISCUSSION

Morphology of Sperm Cells

I found no difference in the gross morphology of the spermatozoa between any of the three species of fish investigated. My observations of the spermatozoa of coho salmon agree with those of Lowman (1956) who gave the following morphological description of the coho spermatozoon:

> The salmon spermatozoon is entirely covered by a membrane 80A in thickness and, in addition, is composed of (1) the head, containing the nucleus, nuclear membrane, mitochondrial granules, and matrix; (2) the middle piece, composed of centrioles, mitochondrial granules, a tentatively identified Golgi remnant, and a matrix; and (3) the tail, consisting of the axial filament, mitochondrial granules, and a matrix.
>
> In the tail the cell membrane is formed into two oppositely placed longitudinal folds, spirally wound five to seven times around the axial filament throughout its length of 28 to 35 microns.
>
> The head is ovoidal with dimensions of about one and one-half by two microns. It is composed almost entirely of the nucleus covered by a 100A-thick nuclear membrane. In sections the nucleus is without visible structure and is of high density.
>
> The mitochondrial granules, 300A to 2000A in diameter, surround the nucleus, are concentrated in the middle piece near the origin of the axial filament, and are distributed throughout the length of the tail in the lateral folds of the cell membrane.

A schematic diagram of a sperm cell of a coho salmon is given by Lowman (1956).

A comparison of size and morphology of bovine sperm cells to

sperm cells of steelhead trout may be made by comparing Fig. 12 to Fig. 13. The spermatozoa of salmonids were difficult to work with because of their extremely small size (about the size of a cocci bacterium).

Morphology of the Genital Area of Salmonids

Urine instead of semen was occasionally collected due to improper placement of the sperm collection device. To reduce or eliminate the problem, an autopsy of several steelhead trout was performed. A diagrammatic view of the genital area of a steelhead trout 68 cm (FL) in length is given in Fig. 14. Note that the urethra opens into the genital papilla about 5 mm from the genital pore. The opening of the urethra can generally be avoided by inserting the tube from the collection device at an angle of about 30° from the long axis of the body. Also, the seminal ducts coming from the testes join about one inch from the genital pore. When the tube of the collection device was inserted deeply into the fish, sometimes only one seminal duct was drained of semen.

Viability of Undiluted Semen

The relationship between hours after collection and the average viability of undiluted, single samples of semen from each of three steelhead trout is given in Fig. 15. Obviously, undiluted sperm cells

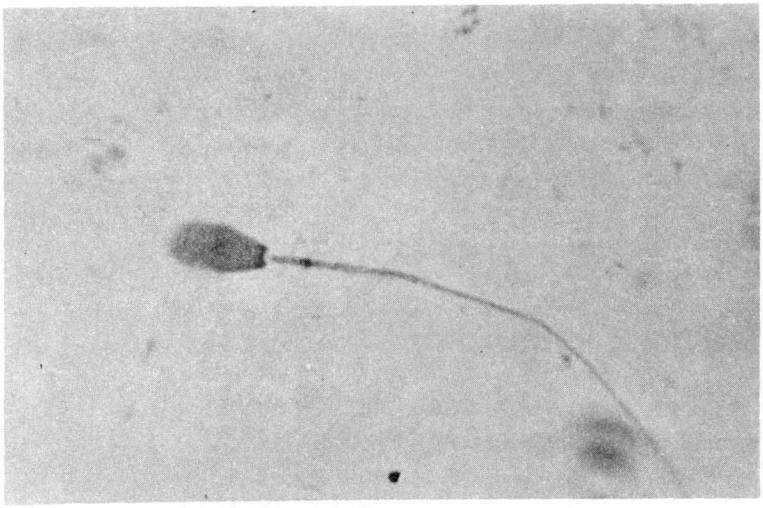

Figure 12. Photomicrograph (1,500 X) of a bovine spermatozoon.

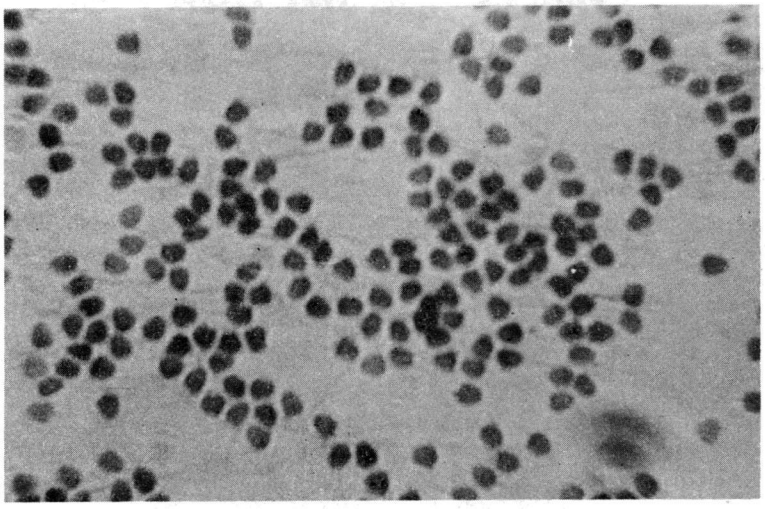

Figure 13. Photomicrograph (1,500 X) of spermatozoa of a steelhead trout.

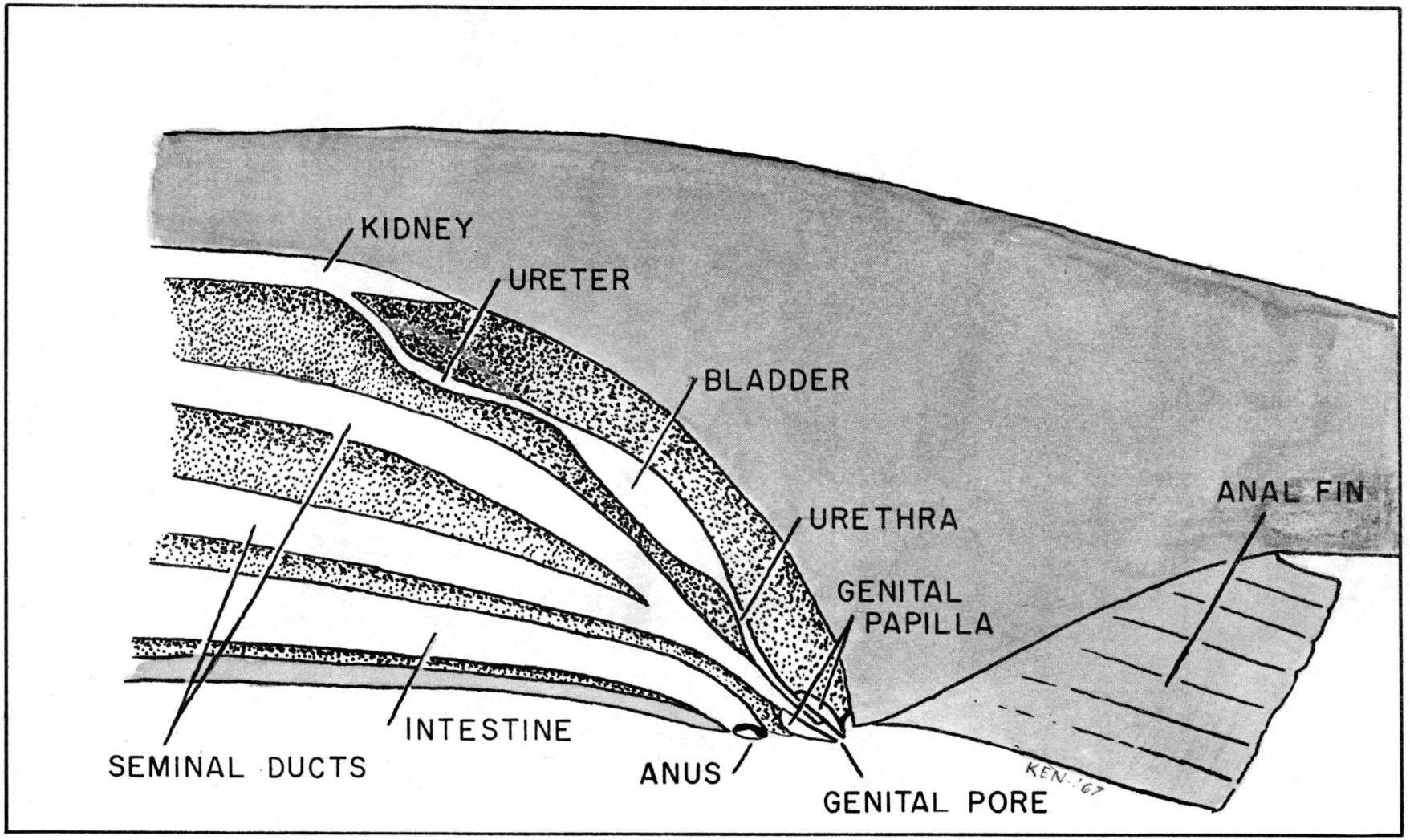

Figure 14. Sagittal section through the genital area of a steelhead trout (approximately life size).

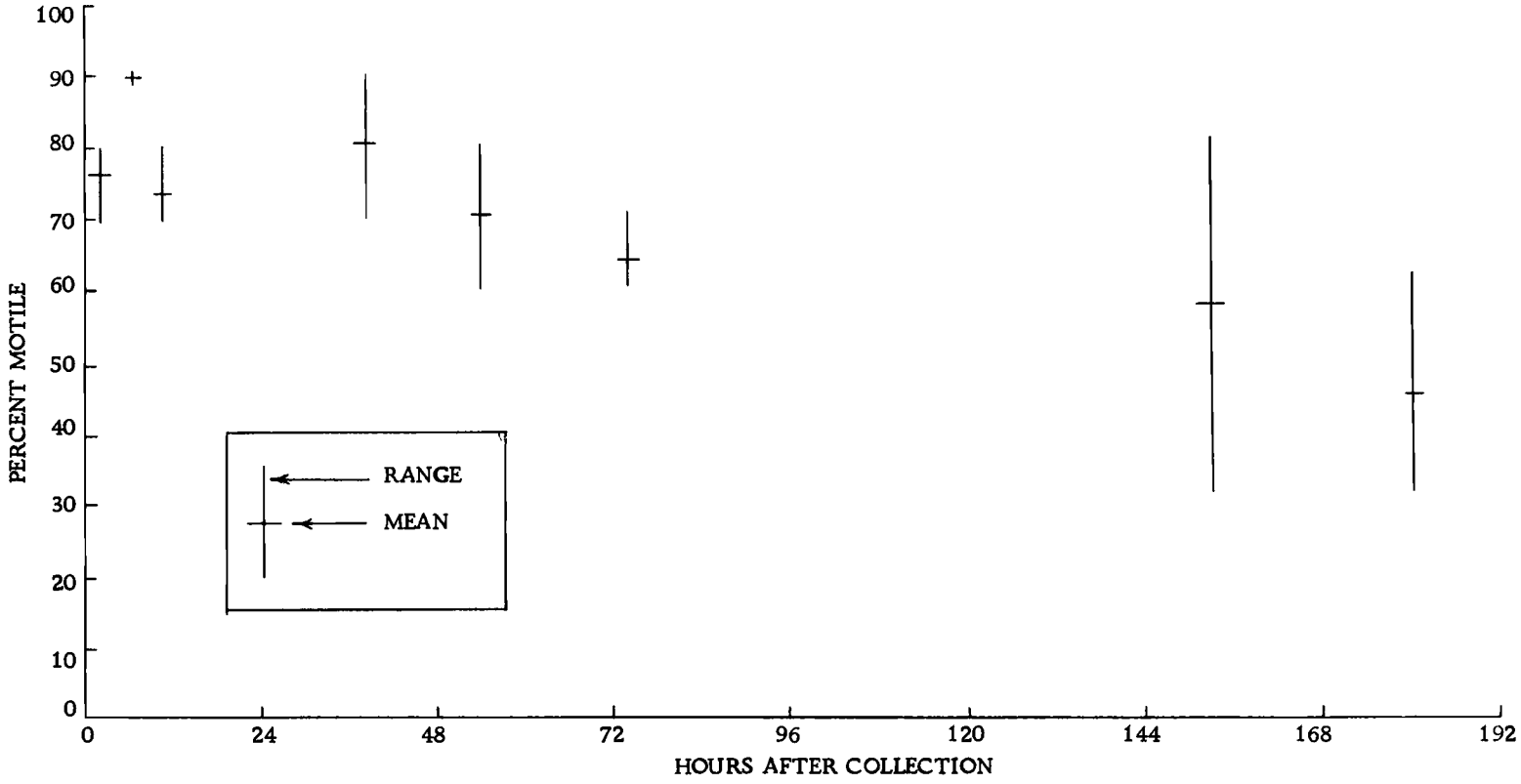

Figure 15. Relationship between the percent motile sperm cells, after the addition of water, and hours after the semen was collected from three steelhead trout, February, 1967.

remained highly viable for several days after collection. These results were substantiated in later tests and agreed with the findings of Rucker (1949). On the basis of these data, samples of semen were transported from field stations to the Fairplay Laboratory in an undiluted condition. Once at the laboratory, the semen was extended and tested.

The effect of a partial vacuum on the viability of undiluted semen was also investigated. Samples of semen were collected from each of five fish with the vacutainer method. Two samples of the undiluted semen were left in the vacuum tube, and the remaining three samples were exposed to atmospheric conditions. All samples were handled in the same manner except that the three samples exposed to air were evaluated for motility on each of six days. The two samples maintained in a partial vacuum were evaluated for motility on the sixth day. Spermatozoa exposed to air had high viability on the sixth day, while those in the samples maintained in the partial vacuum were without visible viability. These results suggest that a partial vacuum, lack of oxygen, or a combination of the two have a deleterious effect on the semen. I believe that the lack of oxygen to the sperm cells is the most harmful. This hypothesis is supported by Lowman (1956) who stated that "the metabolism of spermatozoa of animals practicing external fertilization in an aquatic environment is predominately aerobic."

Effect of Light on Undiluted Semen

Since the protection of fertilized fish eggs from sunlight is a widely used hatchery procedure, I tested the effect of the available light on the viability of undiluted semen. Semen collected from three steelhead trout was maintained in clear test tubes for four days. Semen collected from an additional three steelhead trout in the same manner was maintained in test tubes covered with aluminum foil for four days. The semen was evaluated for percent motile sperm and percent relative activity of spermatozoa, before and after the addition of water, on each of four days. There was no significant difference in percent motile sperm or percent relative activity of sperm cells between the samples that were either protected or unprotected from light.

Water Necessary to Activate Extended Semen

To standardize evaluation procedures, it was necessary to determine the volume of water in proportion to volume of semen that was necessary to activate the spermatozoa to full motility. Semen was collected from four coho salmon immediately prior to testing each sample for viability. The undiluted semen was extended two minutes after collection with an equal volume of Solution 1 (Appendix 1). For each sample of extended semen, 0.5 ml was placed into each of

six test tubes and maintained in a water bath. The semen in each test tube was evaluated for percent motile sperm and percent relative activity of spermatozoa immediately after the addition, in random order, of 0.05 (1 drop), 0.1, 0.2, 0.5, 1.0, or 5.0 ml of water.

The relationship between the amount of water added to 0.5 ml of extended semen and the percent of cells that were activated is presented in Fig. 16. I concluded that maximum motility of the extended semen was achieved when an equal or larger volume of water was added to the extended semen.

Assays of Sperm Cell Concentrations

The following standard procedure was established for determining the concentration of sperm cells in the semen of fish. One-half milliliter of undiluted semen was pipetted into 4.5 ml of extender or tap water and gently mixed. One-half milliliter of this mixture was pipetted into 49.5 ml of tap water in a test tube resulting in a final dilution of 1:1000. This solution was gently mixed. A hemocytometer and cover slip were examined microscopically and cleaned if necessary. A blood diluting pipette was rinsed several times in the diluted semen. The diluted semen was again gently mixed. Semen was then drawn into the blood diluting pipette while the pipette was moved slowly up and down in the solution. A small drop from the pipette was placed in the V-notch of the hemocytometer with the cover slip in place. The

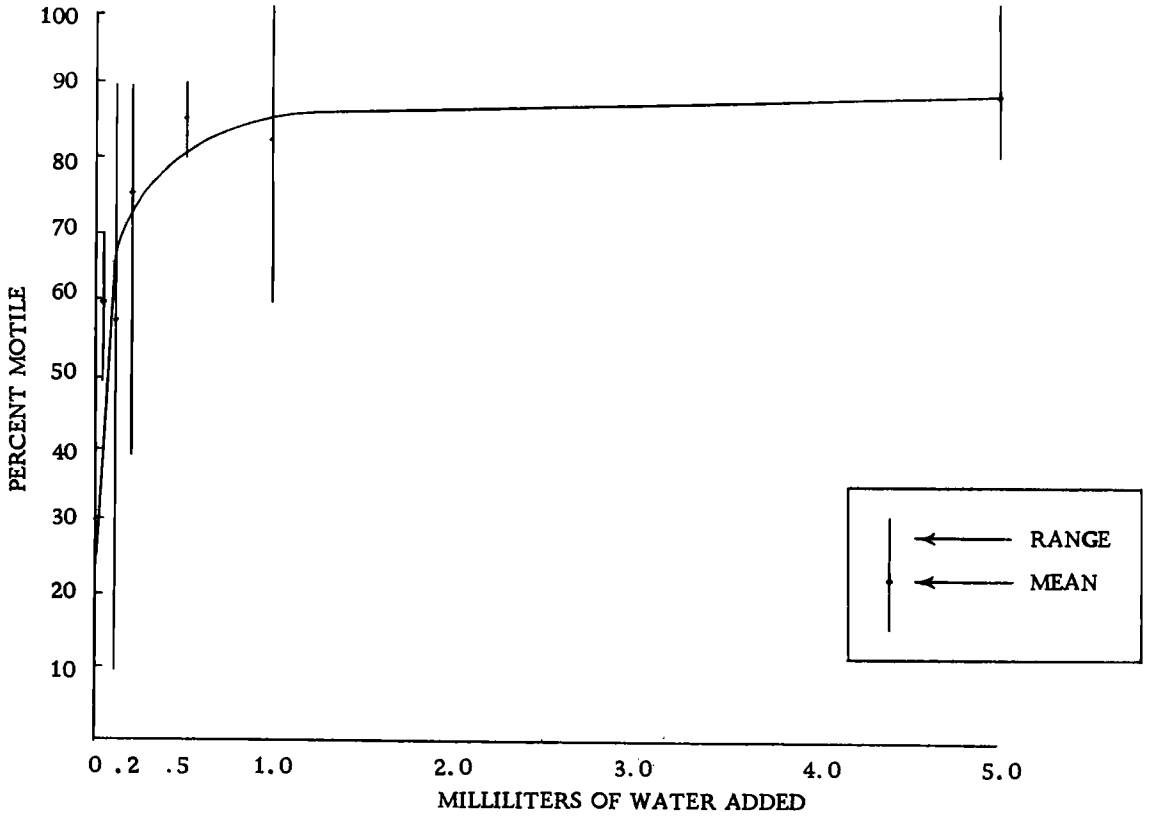

Figure 16. Relationship between the amount of water added to 0.5 ml of extended semen of coho salmon and the percent of sperm that were motile.

sperm cells were allowed to sink to the bottom of the counting chamber for several minutes. Examination of the counting chamber and adjacent areas showed that the sperm cells were evenly and randomly distributed over the entire surface. The sperm cells were then counted in five of the larger squares--the four corners and the middle of the largest square. Sperm cells resting on the top or right-hand borders were counted, while those resting on the bottom or left hand borders were not counted. The number of cells counted was multiplied by 0.05×10^9 to give the total number of sperm cells in one milliliter of the undiluted semen.

This technique varied in several respects from the technique used to estimate concentrations of spermatozoa in the semen of farm animals. Greater dilution was necessary because of the higher concentration of sperm cells in the semen of fish. Gram's iodine and carbyl fucsin were not added because they cause agglutination of the spermatozoa. The sperm cells were easily recognized which obviated the necessity of any staining.

When the sperm cells were added to water, osmotic pressures disrupted the nucleus from the cell membrane. The cell nucleus remained intact, however, and was the specific entity enumerated. Lowman (1956) also reported that the nuclear membrane did not separate from the nucleus and thus remained intact when placed into a hypotonic medium.

Estimates of the concentration of sperm cells in semen were made for 4 coho salmon and 12 fall-spawning rainbow trout. Concentrations of sperm cells from coho salmon ranged from 19.3×10^9 to 23.1×10^9 cells/ml and averaged 21.2×10^9 cells/ml. The concentration of sperm cells for rainbow trout ranged from 11.9×10^9 to 30.6×10^9 cells/ml and averaged 16.6×10^9 cells/ml. The concentrations of spermatozoa in fish semen were about 2.3 to 230 times greater than those reported for other animals (Table 1). Sperm cell concentrations in the semen of the sea urchin (Echinus esculentus) were reported to average about 20×10^9 cells/ml (Mann, 1964) which is comparable to my figures for coho salmon and fall-spawning rainbow trout.

Vital Stains

The use of vital stains for an objective test of viability is a standard practice in the evaluation of semen from farm animals. Vital stains theoretically stain only dead cells (Salisbury and Van Demark, 1961). The stain is mixed with the spermatozoa and is heat fixed very rapidly to prevent the stain from killing any additional cells.

Two vital stain mixtures were tested following the recommended procedures. One of the stains was composed of 2% fast-green, 0.2% eosin and 3% sodium citrate dissolved in distilled water

Table 1. Concentrations of sperm cells in the semen of various animals.

Species	Volume of Single Ejaculate		Sperm Density
	Normal Variation (ml)	Most Common (ml)	(average number/μliter)
Ass	10-80	50	4×10^5
Bat		0.05	6×10^6
Boar	150-500	250	10^5
Bull	2-10	4	3×10^5-2×10^6
Cock	0.2-1.5	0.8	3.5×10^6
Dog	2-15	6	3×10^6
Fox	0.2-4	1.5	7×10^4
Man	2-6	3.5	10^5
Rabbit	0.4-6	1	7×10^5
Ram	0.7-2	1	3×10^6
Stallion	30-300	70	1.2×10^5
Turkey	0.2-0.8	0.3	7×10^6
Coho salmon			21.2×10^6
Rainbow trout			16.6×10^6

(From Mann [1964] except for salmon and trout)

(Mayer, et al., 1951). The other stain was composed of 5% eosin and 10% nigrosin dissolved in distilled water (Williams and Pollak, 1950). A vital stain recommended by Fribourgh (1966) was also tested.

None of these stains gave satisfactory results during this investigation. In most cases it was difficult to differentiate the stained cells from those that were not stained. Estimates of viability determined with the use of vital stains did not correlate with estimates of viability determined from the observations of motility. Additional testing will be necessary to determine if vital stains will be useful in future investigations.

Development of Extenders

My approach to the development of an extender was to establish a list of prerequisites for a satisfactory diluent as a basis for experimentation. I then selected promising extenders from the literature and conducted tests in a way that the performance of the solutions could be compared. Finally, the extenders which performed well were successively modified and tested until a best extender was developed.

Desired Characteristics of an Extender

The requirements for a life extender of semen of most farm animals are given by Salisbury and Van Demark (1961) as: (1) osmotic pressure isotonic with blood and capable of maintaining that approximate pressure during storage; (2) provide a proper balance of mineral elements essential to the life of sperm cells; (3) provide sperm cell nutrients for both aerobic and anaerobic metabolic processes; (4) provide lipoproteins and/or lecithin to protect sperm cells against cold shock; (5) provide chemical means for buffering toxic end-products of sperm cell metabolism; (6) provide a source of reducing substances to protect the sulfhydryl-containing cellular enzymes; and (7) be free of substances, bacterial products, or infectious organisms harmful to spermatozoa, the fertilization process, and the development of the fertilized ovum. These requirements were used as guides in my search for a life extender of the spermatozoa of fish.

Isotonicity of Salmonid Sperm

I believed it was important that the extenders developed be nearly isotonic to the spermatozoa of salmonids. Solutions isotonic to the sperm cells of most freshwater teleosts were reported to have a freezing point depression of about -0.58 C (Wolf, 1963). To

determine the isotonicity of the sperm cells of a salmonid, a series of sucrose solutions was prepared. To 100 ml of distilled water were added 0, 5, 8, 9, 10, 11, 12, 15 and 20 g of sucrose, which resulted in solutions calculated to have freezing point depressions of 0.0, -0.28, -0.45, -0.51, -0.56, -0.62, -0.68, -0.85 and -1.15 C respectively. To each solution, 0.1 ml of semen from one of two steelhead trout obtained at the Alsea Trout Hatchery was added. The semen was left in the solutions for seven days. Visual observations of the morphology of sperm cells placed into these concentrations of sucrose were then made. Examination of the spermatozoa was made under oil immersion (1,940 X) while the sperm cells were suspended in a hanging drop. The results of this experiment are given in Table 2. Based on the absence of atypical sperm cells, the 100 ml solution containing 9 g of sucrose appears to be isotonic to the sperm cells. At this concentration of sucrose, an equivalent osmolarity of sodium chloride would be 0.148g mole/liter. The freezing point depression of this solution was calculated to be -0.51 C. I concluded that future extenders should have this freezing point depression and an osmolarity equivalent to that given here for sodium chloride.

Use of pH Indicators

Since tissue culture workers customarily include a pH indicator in their culture media, I conducted experiments to determine if

Table 2. Observations of the morphology of spermatozoa of steelhead trout held for seven days in various concentrations of sucrose.

	Gms Sucrose per 100 ml Water	Freezing Point Depression	Equivalent Osmolarity of NaCl g mole/liter	Tails Absent	Tails Coiled Around Head	Tails Wider Than Normal	Tail Configuration Distorted	Cell Membrane Shrunk	Mid-piece Enlarged	Row Totals
	0.0	0.0	0.0	X	X		X			3
	5.0	-0.28	0.081	X		X	X			3
	8.0	-0.45	0.130			X	X			2
Fish	9.0	-0.51	0.148							0
Number	10.0	-0.56	0.164		X					1
74	11.0	-0.62	0.178		X			X		2
	12.0	-0.68	0.197						X	1
	15.0	-0.85	0.248					X	X	2
	20.0	-1.15	0.336					X	X	2
	0.0	0.0	0.0	X	X		X			3
	5.0	-0.28	0.081	X		X	X			3
	8.0	-0.45	0.130			X	X			2
Fish	9.0	-0.51	0.148							0
Number	10.0	-0.56	0.164		X					1
75	11.0	-0.62	0.178		X			X	X	3
	12.0	-0.68	0.197	X					X	2
	15.0	-0.85	0.248	X				X	X	3
	20.0	-1.15	0.336					X		1

small amounts of phenol red or bromothymol blue were detrimental to the viability of fish spermatozoa. The semen from each of two coho salmon was divided into three subsamples by putting 1 ml of semen into 1 ml each of the following extenders: Solution 1, Solution 10 (Solution 1 plus 4 drops of phenol red) and Solution 11 (Solution 1 plus 4 drops of bromothymol blue) (Appendix 1). The semen of another coho salmon was divided into three 1 ml subsamples. One subsample was placed into 1 ml each of Solution 7, Solution 8 (Solution 7 and 4 drops of phenol red) and Solution 9 (Solution 7 and 4 drops of bromothymol blue) (Appendix 1). Another sample of semen was collected from a fourth coho salmon and was divided into three 0.5 ml subsamples. One subsample was placed into 4.5 ml each of Solutions 7, 8 and 9. Extension of the semen took place at the Alsea River Salmon Hatchery. The semen was then transported in a cooler to the Fairplay Laboratory for evaluation.

The results of this experiment indicated that neither phenol red nor bromothymol blue was detrimental to the viability of the spermatozoa and either pH indicator could safely be included in an extender.

Tests of Extender Solutions

From the available literature, I selected several promising extenders and started a series of comparative tests. Solution 1

(Table 3, Appendix 1) was a fish Ringer solution developed by T. Yamamoto (Rothschild, 1958). Solution 2 (Appendix 1) was a 33% solution of sucrose and was reported to extend the life of unfrozen spermatozoa of Atlantic salmon (Ellis and Jones, 1939).

Table 3. Constituents of Solution 1.

Component	Molarity
NaCl	0.128
KCl	0.00256
$CaCl_2 \cdot 2H_2O$	0.00183
$NaHCO_3$	0.000238

For the test, semen was collected from several spring chinook salmon and extended with Solutions 1 and 2 at dilutions of 1:1 and 3:5 (semen to extender). The dilutions were made within two minutes after the semen was collected. Spermatozoa in the semen extended with Solution 2 lost almost all viability within one hour, while cells extended in Solution 1 maintained some viability for over 24 hr. Solution 1 was compared to many extenders during the year and performed better than most.

Because Ellis and Jones (1939) were able to keep the sperm of Atlantic salmon alive for several days in the ovarian fluid of the same species, I elected to try the ovarian fluid of spring chinook salmon as an extender (Solution 3, Appendix 1). Three milliliters of a sample

of semen collected from a spring chinook salmon were placed into 5 ml each of Solution 1 and Solution 3 (ovarian fluid). The semen remained viable less than 48 hr in either solution. Sperm cells extended in Solution 3 were highly active in this fluid prior to the addition of water for semen evaluation. Under a state of high activity, energy sources would seemingly be exhausted rapidly. Since motility lasted for more than 24 hr, it is possible that the spermatozoa were able to metabolize some of the substrates available in the ovarian fluid.

The successful culture of testis cells of fish in Cortland Salt Solution (Solution 12, Appendix 1) was reported by Wolf (1963). I was also encouraged to try a commercially prepared tissue culture medium, Eagle's Minimum Essential Medium with Spinner salts and glutamine added (Solution 14, Appendix 1), which was used to grow leucocytes of salmonids.[2] One-half milliliter of semen from three fall-spawning rainbow trout was added at the hatchery to 4.5 ml each of Solutions 1, 12 and 14. The extended semen was then transported back to the Fairplay Laboratory for evaluation. Because viability of all sperm cells extended in Solutions 12 and 14 ceased within seven hours, further testing of these two solutions was discontinued. It is surprising that neither of these solutions maintained viability of fish

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[2] Personal communication. R. C. Simon, Professor of Fisheries, Oregon State University, Corvallis. December 15, 1966.

spermatozoa although they have supported life in most other types of animal cells.

Because some solutions activated the spermatozoa upon their addition to the semen, I felt the energy of the cells could easily become exhausted. Since the spermatozoa of rainbow trout oxidize only small amounts of glucose (Terner and Korsh, 1963), I suspected that the spermatozoa of salmonids did not have the enzymes needed to incorporate into the cell the substrates available in most extenders. If this were true, solutions which activated the cells would not be suitable for the preservation of fish semen.

To observe what activity was produced when a solution was added to semen, the following procedure was used. Semen was collected from fall-spawning rainbow trout. The undiluted semen was then transported back to the Fairplay Laboratory. A drop of semen was placed on a microscope slide. The microscope was focused on the edge of the drop at a magnification of 200. A drop of the selected extender (Solutions 1, 12, 15-29; Appendix 1) was then placed on the semen. Estimates of percent motile sperm and percent relative activity of spermatozoa were then made.

The results of these tests indicated that most solutions activated the spermatozoa, with the exception of Solutions 22, 23, 24, 25 and 26 (Table 4). These were the only solutions tested that contained glycine. I noted also that the sperm cells in Solutions 23 and 24

Table 4. Observations of percent motile sperm and percent relative activity of spermatozoa of rainbow trout immediately after the addition of selected extenders.

Solution Number	Percent Motile	Percent Relative Activity	Comment
Water	100	100	
1	100	100	
12	100	100	Cells very active for 15 min.
15	100	100	Cells active for 15-30 sec.
16	80	100	Cells active for 10 sec.
17	80	100	Cells active for 15 sec.
18	80	100	Cells active for 15 sec.
19	80	100	Cells active for 10 sec.
20	80	100	Cells active for 10 sec.
21	80	100	Cells active for 30 sec.
22	20	70	Cells active for 15 sec.
23	10	30	Cells active for 5 sec. Very active after the addition of water, but quiet after Soln. 23 added again.
24	10	30	Same as above except Soln. 24 was added after water.
25	0	0	Soln. 25 didn't break up initial group of sperm. Few active cells after water was added.
26	10	30	Soln. 26 didn't break up sperm group. Activity of cells barely increased after water was added.
27	80	100	Cells active for 15 sec.
28	80	100	Cells active for 15 sec.
28	10	10	Cells active for 2 sec, but not activated by water.
29	80	100	Cells active for 5 sec, but not activated by water.

were activated by water and then put into a state of quiescence by addition of more extender.

I tested these glycine-containing extenders against Solution 1 by extending the semen of three fall-spawning rainbow trout. One-half milliliter of semen was added to 4.5 ml each of Solutions 1, 22, 23 and 24 for each of three fish. The results of this experiment are presented in Table 5. Motile cells were still present after 52 hr. Solutions 22, 23 and 24 all appeared to be better extenders than Solution 1, with Solution 24 being the best tested to this point. The constituents of Solution 24 are listed in Table 6.

Table 5. Average percent motile sperm and average percent relative activity of spermatozoa before and after the addition of water to the sperm of three fall-spawning rainbow trout.

Solution Number	Before Addition of Water		After Addition of Water	
	Average % Motile	Average % Relative Activity	Average % Motile	Average % Relative Activity
1	7.5	31.7	18.3	15.8
22	0	0	22.5	12.5
23	0	0	20.8	15.8
24	0	0	31.7	16.7

Rothschild (1958) presented a review of literature pertinent to substances that may inhibit the motility of spermatozoa of salmonids. He inferred that an abundance of potassium ions may bring about a lack of motility. Sneed and Clemens (1956) also noted that the sperm

from mature carp (Cyprinus carpio) were immobilized when placed in a solution of 200 to 500 mg of potassium chloride added to 100 ml of frog Ringer's solution. These authors felt that their results were due in part to the increased molarity of the solution caused by the addition of potassium chloride.

Table 6. Constituents of Solution 24.

Component	Molarity
NaCl	0.128
KCl	0.00255
$CaCl_2 \cdot 2H_2O$	0.00137
$NaHCO_3$	0.0893
Citric Acid	0.00469
Glycine	0.0675
Glucose	0.0277

The effect on spermatozoan motility when potassium chloride ions were exchanged for sodium chloride ions was tested. Solution 1 was the basic extender and from it several other extenders (Solutions 31, 32, 33, 34 and 35; Appendix 1) were made by exchanging potassium chloride for sodium chloride on a weight to weight basis.

Samples of semen were collected from each of two steelhead trout. One-half milliliter of semen was added to 4.5 ml each of the above solutions. This procedure was repeated for each fish. The results of the semen evaluations are given in Table 7. The greatest spermatozoan activity was caused by the addition of water to semen

Table 7. Average percent motile sperm and average percent relative activity of spermatozoa before and after the addition of water to the semen from two steelhead trout extended in solutions with different concentrations of sodium and potassium ions.

Solution Number	Amount of Ion		Before Addition of Water		After Addition of Water	
	NaCl (g/100 ml)	KCl (g/100 ml)	Average % Motile	Average % Relative Activity	Average % Motile	Average % Relative Activity
Control	0.000	0.000	7.5	37.5	52.5	80.0
1	0.748	0.019	7.5	5.0	10.0	7.5
31	0.727	0.040	5.0	2.5	0.0	0.0
32	0.667	0.100	15.0	27.5	27.5	17.5
33	0.467	0.300	10.0	7.5	15.0	10.0
34	0.167	0.600	0.0	0.0	27.5	20.0
35	0.767	0.000	2.5	27.5	10.0	20.0

extended in Solutions 32 and 34. No motility was noted prior to the addition of water in the spermatozoa extended in Solution 34.

For an additional test, a drop of extender was added to a drop of undiluted semen placed on a microscope slide, and the motility induced by the extender was noted. This was repeated for all extenders. The results of this test are given in Table 8. Solution 34 induced the least amount of activity among the cells. Solution 34 had the highest concentration of potassium ions and correspondingly the lowest concentration of sodium ions. These results may partially confirm the supposition that potassium inhibits the motility of spermatozoa of fish. These particular solutions did not warrant further testing as extenders because they maintained viability of the sperm less than 20 hr.

Table 8. Percent motile sperm and percent relative activity of spermatozoa of two steelhead trout when added to solutions containing different concentrations of sodium and potassium ions.

Solution Number	Percent Motile	Percent Relative Activity
Water	50	80
01	50	100
31	30	100
32	20	20
33	10	40
34	10	20
35	50	100

Fructose was substituted for glucose in Solution 12 to test whether fructose would increase the viability of sperm in the extended semen. This substitution resulted in Solution 36 (Appendix 1). The results indicated that this substitution did not change the viability of sperm in the extended semen.

I noted earlier that glycine might have beneficial effects on the viability of extended semen. My observations of the sperm cells from fall-spawning rainbow trout treated with glycine appeared to be similar to those of Tyler and Rothschild (1951) who reported that the sperm of sea urchins (Lytechinus pictus) did not produce acid anaerobically when treated with glycine. After six hours in anaerobic conditions, the sea urchin sperm treated with glycine still maintained high motility (50 to 75%) and capability of fertilization. The sea urchin spermatozoa acting as controls (without glycine) lost their motility and capacity to fertilize. The beneficial action of glycine was believed to depend primarily on its metal-binding capacity (Tyler and Rothschild, 1951). Lecithin was also reported to give added protection to semen upon freezing (Salisbury and Van Demark, 1961).

The effects of including glycine and lecithin in an extender was tested simultaneously. Varying amounts of glycine and lecithin were added to Solution 36 in a factorial design. For each level of lecithin, four levels of glycine were investigated. Since I used four levels of

lecithin, a total of 16 solutions were tested. The four levels of either chemical were 0.0, 0.05, 0.20 and 0.50 g/100 ml. The number and the constituents of each solution are listed in Appendix 1. Semen was collected from three steelhead trout. At the Fairplay Laboratory, 0.5 ml of undiluted semen from a single fish was added to 0.5 ml of each extender. This was repeated for each of the three fish. Results indicated that as the level of lecithin was increased so was the viability of the sperm cells, whereas as the level of glycine was increased, the viability of the sperm cells decreased.

Effect of Dilution on Viability of Semen

The effect on the viability of sperm cells produced by an increased dilution of the semen was tested. Semen was collected from two steelhead trout at the Alsea Trout Hatchery. The semen from each fish was diluted at the Fairplay Laboratory according to the following schedule:

Ratio of Semen to Extender	Amount of Semen Added to Extender	Amount of Solution Z5
1:0.5	1 ml	0.5 ml
1:1	1 ml	1.0 ml
1:1.5	1 ml	1.5 ml
1:3	1 ml	3.0 ml
1:9	0.5 ml	4.5 ml
1:49	0.5 ml	24.5 ml
1:99	0.5 ml	49.5 ml

After these dilutions were made, one milliliter was removed from each solution of the extended semen and placed in separate test tubes.

Table 9. Percent motile spermatozoa, averaged for two steelhead trout, held at various volumes and dilutions of Solution 25.

Final Volume (ml)	Dilution (Parts Soln. 25 to 1 part semen)	Before Addition of Water		After Addition of Water	
		Average Percent Motile	Average Percent Relative Activity	Average Percent Motile	Average Percent Relative Activity
0.0	0.0	30.0	62.5	65.0	77.5
0.5	0.5	37.5	72.5	65.0	85.0
1.0	1.0	7.5	37.5	45.0	55.0
1.5	1.5	0	0	37.5	32.5
3.0	3.0	0	0	30.0	10.0
4.0	9.0	0	0	27.5	12.5
24.0	49.0	0	0	15.0	7.5
49.0	99.0	0	0	5.0	5.0
1.0	0.5	12.5	45.0	50.0	67.5
1.0	1.0	5.0	20.0	55.0	77.5
1.0	1.5	0	0	40.0	55.0
1.0	3.0	5.0	22.5	52.5	60.0
1.0	9.0	0	0	32.5	25.0
1.0	49.0	0	0	20.0	10.0
1.0	99.0	0	0	25.0	10.0

The results of dilution of the semen held at both volumes are given in Table 9. In every case, the average of percent motile sperm for both fish was highest for the sperm cells maintained in the smallest volume. This higher percent motile sperm could be due to better gas saturation between the extended semen and the atmosphere in the smaller volumes than in the larger volumes. Fig. 17 shows the relationship between the average percent motile sperm after the addition of water, and the ratio of dilution. A suitable extender should not decrease the viability of spermatozoa as dilution is increased.

At this point in my study, I selected an extender for use in my sperm freezing experiments. Solution 48 was chosen as the best extender because it maintained a high percent motile sperm and a high percent relative activity of spermatozoa after water was added to the extended cells. The constituents of Solution 48 are listed in Table 10.

Table 10. Constituents of Solution 48.

Components	Molarity
NaCl	0.125
KCl	0.00509
$CaCl_2 \cdot 2H_2O$	0.00157
$NaHCO_3$	0.00119
$Na_2H_2PO_4$	0.00297
$MgSO_4 \cdot 7H_2O$	0.000933
Fructose	0.00555
Lecithin (Soy Bean)	0.00716

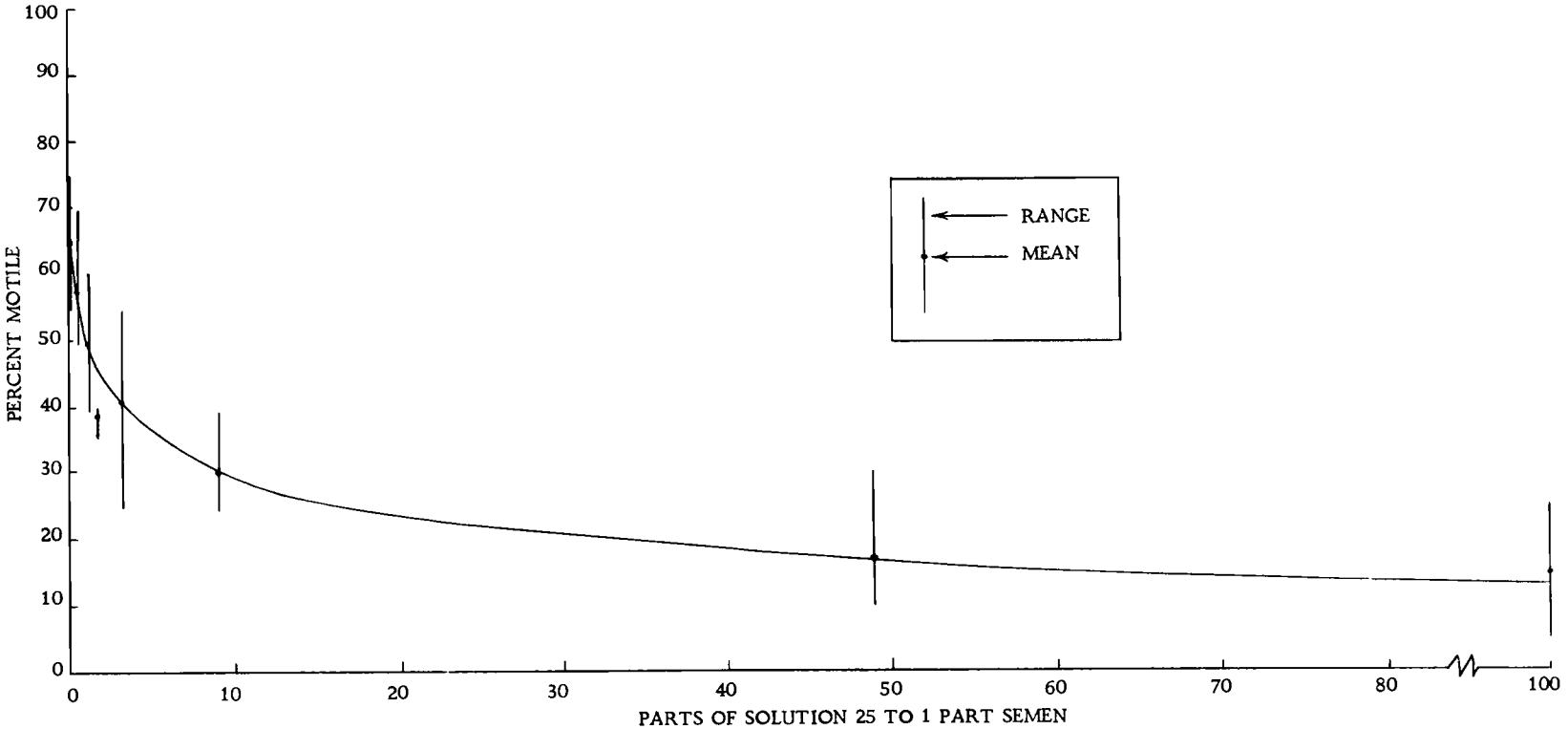

Figure 17. Relationship of percent motile spermatozoa, after the addition of water, to the amount of dilution of the semen of steelhead trout.

An optimum extender was not developed during this investigation, although Solutions 24, 25 and 48 appear to be useful diluents. The ideal extender should maintain viability of sperm cells when the semen is greatly diluted. Based on my investigation, I cannot say precisely what formulation would increase the viability of greatly diluted semen. I recommend that the search for an ideal extender be pursued further, with special attention given to testing the effects of the presence or absence of oxygen in the extender on viability of spermatozoa.

Techniques of Freezing and Thawing Sperm Cells

In general, the cells of animals are able to survive freezing if cooling is effected so as to by-pass the zone of crystallization and assume the vitreous state. Rapid warming of the frozen cells also avoids the zone of crystallization. By-passing the zone of crystallization prevents colloidal changes such as denaturation and coagulation of proteins, protoplasmic precipitation, release of enzymes and structural disarrangement (Mann, 1964).

The principal protective action of life protectors reportedly results from their ability to prevent excessive concentration of electrolytes and other substances that occur on freezing. This protective property is limited to substances that are non-toxic, have a low molecular weight, have a high solubility in aqueous electrolyte

solutions and have an ability to permeate living cells (Lovelock and Bishop, 1959). The chemicals I tested during this investigation were DMSO (dimethyl sulfoxide), glycerol, and PVP (polyvinyl pyrrolidone).

A standard procedure was used to test the effect of various additives on the viability of spermatozoa. Undiluted semen was brought from the field to the Fairplay Laboratory where it was extended at a ratio of 1 part semen to 2 parts extender. Three parts of this mixture were then mixed with 2 parts of the same extender which included a proportion of a specified additive. At each step in the mixing process, a small sample of each mixture was set aside so that long term evaluation of the semen could be accomplished. Evaluation of the spermatozoa took place just prior to and immediately after the semen was mixed with each new solution.

Determination of Rate of Thaw

Rapid thawing of semen from farm animals is generally accomplished by placing the ampoule of semen into a water bath of from 0 to 5 C (Sherman, 1965; Salisbury and Van Demark, 1961).

I tested the effect of thawing semen from fish at a fast rate (water bath) and at a slow rate (air). Semen was collected from three steelhead trout at the Alsea Trout Hatchery and frozen in a mixture of Solution 25, of which 5% was DMSO. One ampoule from each fish

was thawed in the air (22 C), while the other ampoule was thawed in a water bath (4 C). (Ampoules thawed more quickly in the water bath than in the air due to the latent heat of the water.) Motile cells were found only in those ampoules thawed in the water bath, which agrees with the generalized practices reported for domestic mammals. Based on these findings, all ampoules of frozen semen were thawed in a water bath maintained at from 4 to 6 C. The rates of temperature increase for ampoules of frozen semen thawed in a water bath and in air are compared in Fig. 18. The semen was completely thawed at -3 C.

Selection of Best Protectors

The experimental procedure used to test additives was employed to evaluate the effects of the addition of DMSO, glycerol or PVP each at a concentration of 5% in the final extender. Semen was collected from three steelhead trout at the Alsea Trout Hatchery. In the laboratory, 1.5 ml of semen was added to 3.0 ml of Solution 25. This was repeated for each fish. From each of these mixtures, 3.0 ml was added to 2.0 ml of Solution 25 of which 10% was either DMSO, glycerol or PVP. This gave a concentration of 5% of an additive in the final extender. All samples containing the same additive were frozen at an initial temperature decrease of 1 C/min until a temperature of -30 C was reached. From -30 C to -120 C, a temperature decrease

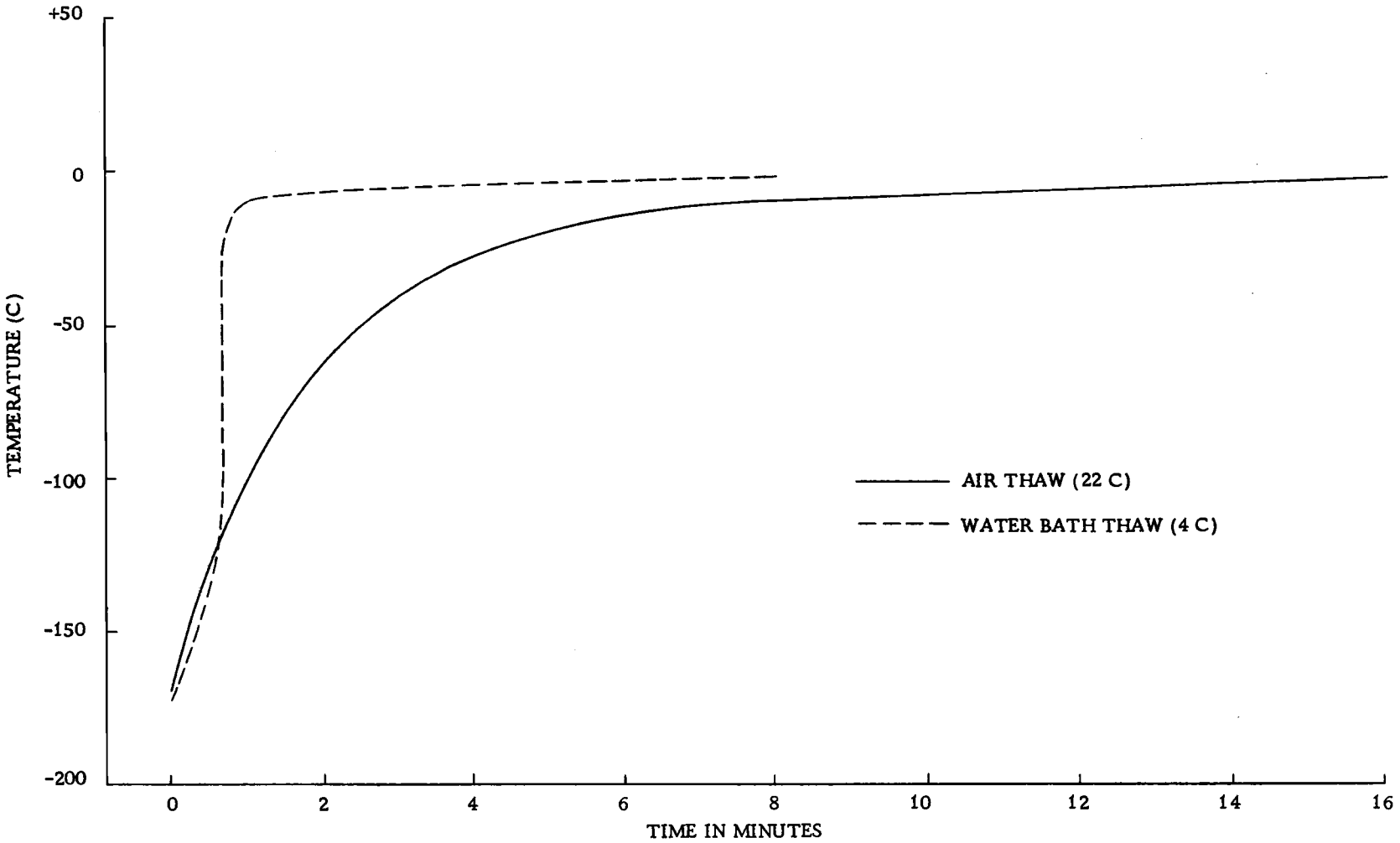

Figure 18. Relationship between rate of temperature increase and time for ampoules of steelhead semen thawed in air or in water.

of 5 C/min was implemented. Once a temperature of -120 C was reached, the ampoules were placed in the liquid nitrogen refrigerator (-196 C). This temperature regime was used for samples protected by each additive.

The effect of adding the extender to the undiluted semen and of adding the extender with additive to the extended semen is presented in Fig. 19. Shortly after the addition of the extender, the activity of the sperm cells was reduced. Cellular activity increased about an hour later, but was never again as high as that of the control cells. The effect produced by the addition of all three additives was generally the same. PVP may have the least detrimental effect of the three additives on the semen, but this was not definitely established from these tests. The reduction and subsequent increase of activity noted could be due to inaccurate observations by the evaluator, or it could be the result of a period of equilibration by the sperm cells to a new environment. This fluctuation of activity was noted several times later in the investigation and appeared to be caused by the effect of equilibration to a new environment by the sperm cells.

The semen frozen in the above experiment was evaluated in the usual way, and the results are given in Fig. 20. During the freezing and thawing process, the solution containing DMSO maintained viability of the spermatozoa, while the solutions with glycerol or PVP did not maintain any visible viability of the sperm cells. The inclusion of

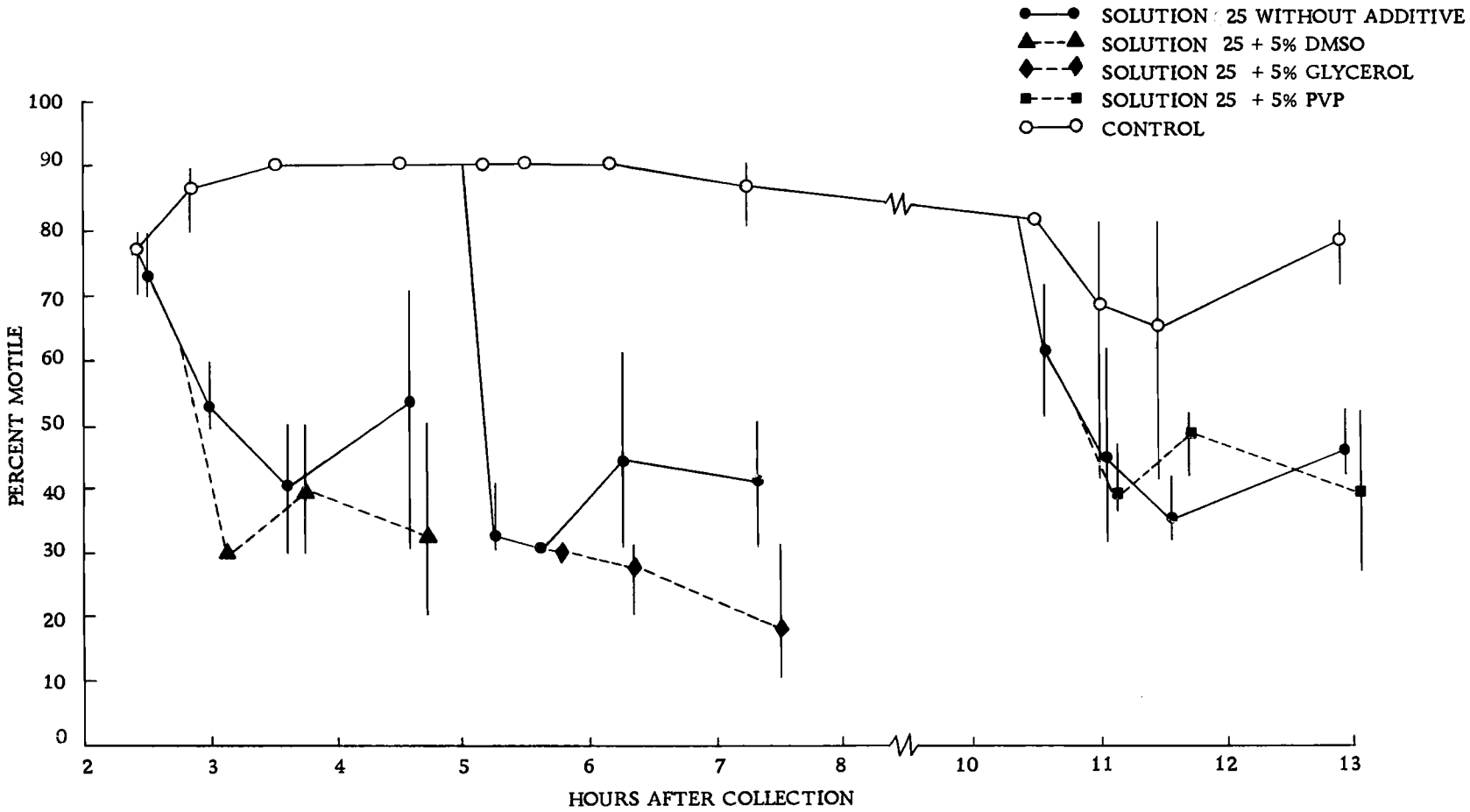

Figure 19. Relationship between percent motile sperm, after the addition of water, and hours after collection for three samples of semen of steelhead trout extended in Solution 25 and protected with either DMSO, glycerol or PVP. Ranges are indicated by vertical lines.

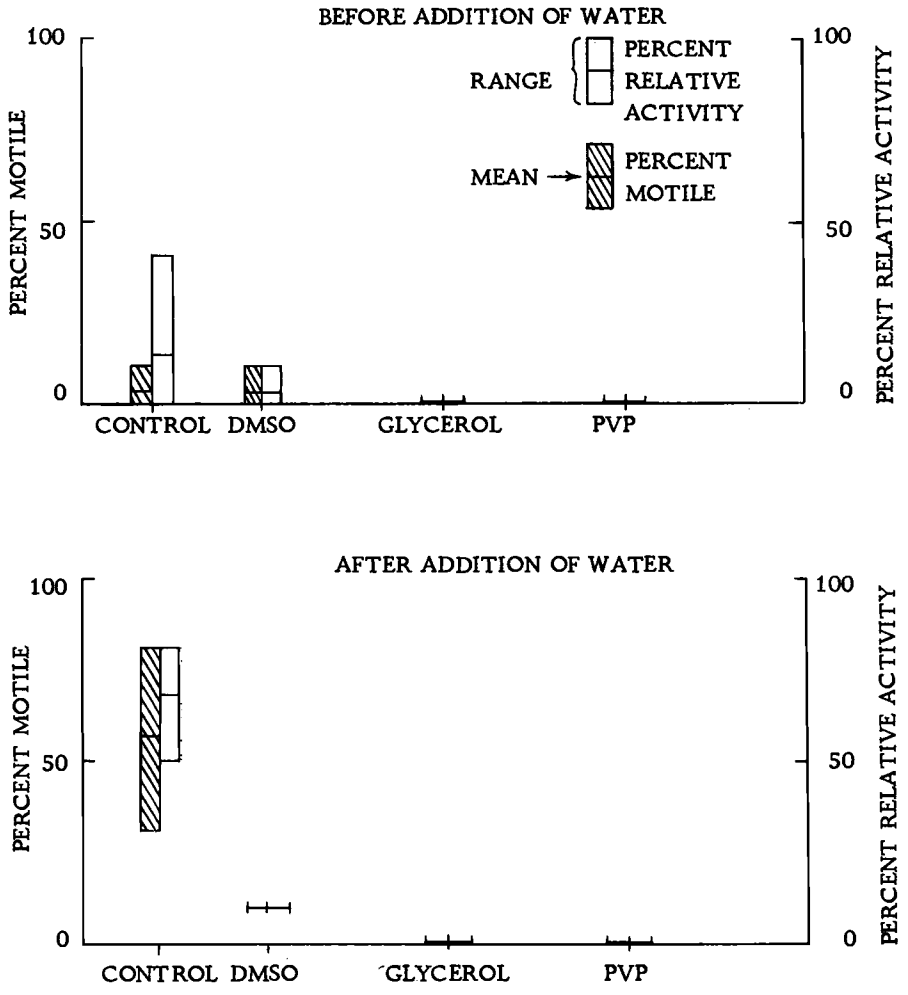

Figure 20. Percent motile sperm and percent relative activity of spermatozoa before and after the addition of water to samples of semen of steelhead trout protected with several additives.

DMSO in the extender did not cause any clumping of the sperm cells, as occurred when glycerol or PVP was added. Examination of the thawed spermatozoa under oil immersion at a magnification of 1,940 X revealed the following: (1) Cells in the extender containing DMSO appeared normal; (2) cells in the extender containing glycerol usually had lost their tails; and (3) some cells in the extender containing PVP were without tails and the heads of other spermatozoa were abnormally shaped. DMSO had none of the objections that glycerol or PVP had and was the best protector tested.

Selection of Best Extenders for Freezing

During the conduct of this study, two extenders, Solutions 25 and 48, maintained viable spermatozoa at 4 C for reasonably long periods of time (3-4 days). The performance of these extenders during freezing and thawing of sperm cells was investigated. Semen was collected from three steelhead trout at the Eagle Creek National Fish Hatchery and extended at the Fairplay Laboratory. Undiluted semen (1.5 ml) from each fish was added to 3.0 ml each of Solutions 25 and 48. Three milliliters of extended semen from each mixture was added to 2 ml of the same extender, of which 10% was DMSO, resulting in a final concentration of 5% DMSO in the extender. One-half of the samples were frozen at an initial temperature decrease of 1 C/min until the temperature of the sample reached -30 C. From

-30 to -120 C, a temperature decrease of 5 C/min was implemented. At -120 C, the ampoules were transferred to the liquid nitrogen refrigerator. The remaining one-half of the samples were frozen in the vapor above the liquid nitrogen interface.

The initial and long term effect on viability of the addition of each solution to the undiluted semen and of the addition of the extender with protector to the extended semen is presented in Fig. 21. Solution 25 maintained higher viability of the spermatozoa than did Solution 48. The addition of the protector increased viability of cells in both extenders. The percent viability of the spermatozoa extended in Solution 25 with 5% DMSO was higher after 68 hr than the percent viability of the controls.

The observed viability of semen frozen at two rates in Solutions 25 and 48 (with 5% DMSO) and refrigerated for 116 days before being thawed is presented in Fig. 22. Solution 48 maintained higher viability after freezing at either freezing rate than did Solution 25, and was the best extender developed. I could not conclude whether one freezing rate was better than the other. In any case, freezing in the vapor above the surface of the liquid nitrogen did not appear to be detrimental to viability of the spermatozoa and appears to be a suitable method of freezing semen in the field.

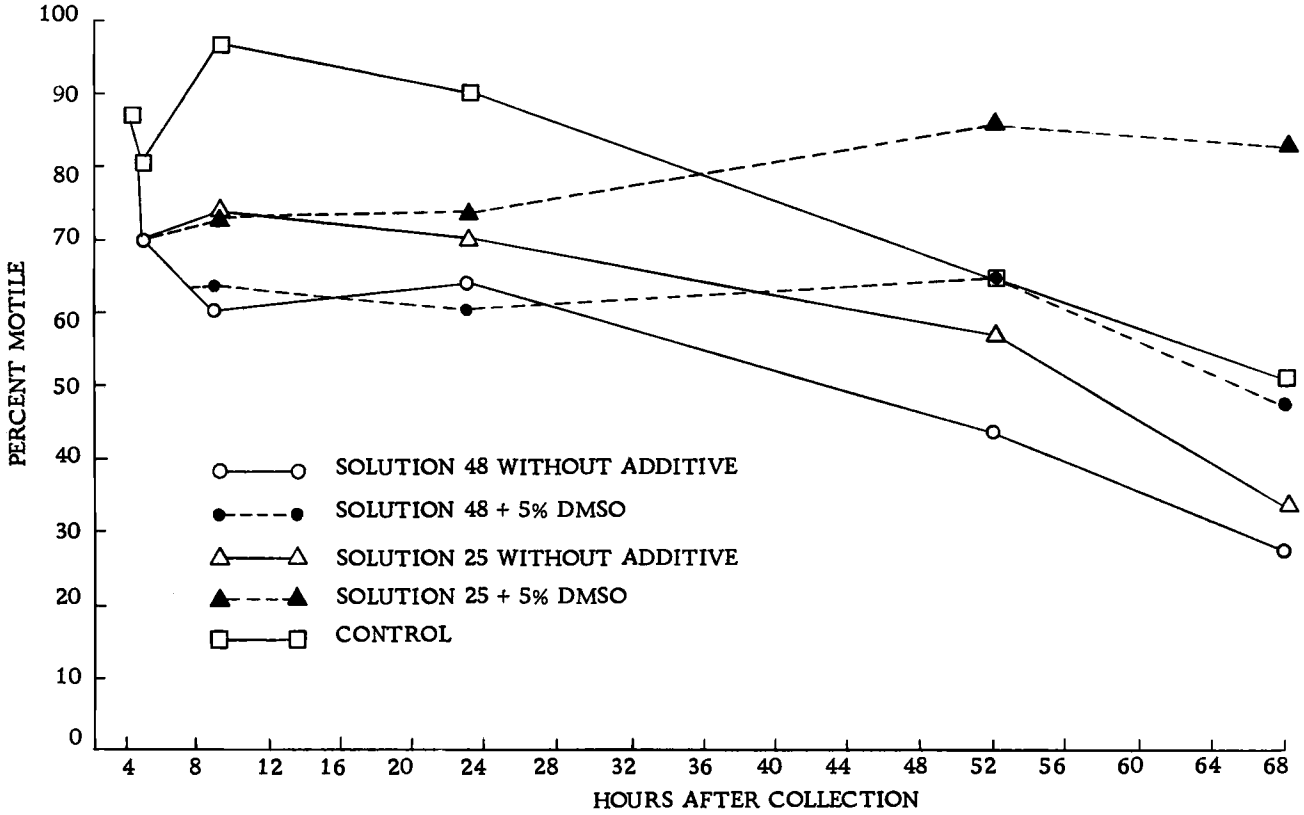

Figure 21. Relationship between percent motile sperm, after the addition of water, and the number of hours after collection for samples of steelhead trout semen maintained in various extenders and protectors.

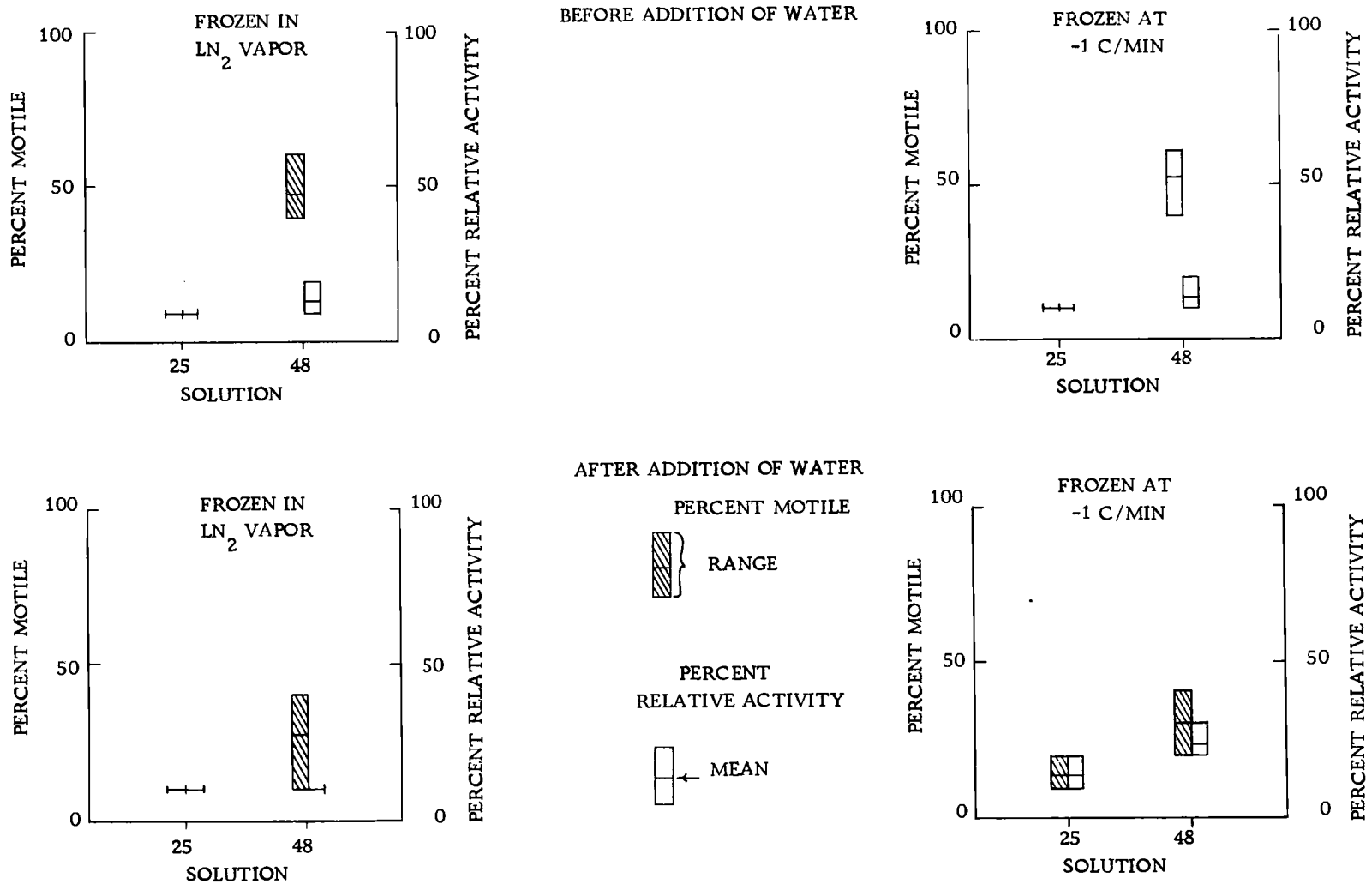

Figure 22. Comparison of the effect of two solutions containing 5% DMSO on the percent motile sperm and the percent relative activity of spermatozoa of steelhead trout frozen at two rates and refrigerated for 116 days.

Selection of Best Rate of Freezing

The effect of various rates of freezing on the viability of semen extended in a standard solution was investigated. Semen was collected from three steelhead trout at the Alsea Trout Hatchery. One-half milliliter of undiluted semen was added to each of two ampoules. Into each of these ampoules was added 0.5 ml of Solution 25 of which 10% was DMSO. The ampoules were then gently shaken to mix the contents. This process was repeated just prior to the freezing procedure for each selected rate of temperature decrease. The freezing rates implemented were: (1) Ampoules were submerged directly in the liquid nitrogen; (2) ampoules were suspended in the vapor above the surface of the liquid nitrogen; and (3) ampoules were frozen in the Linde controlled rate liquid nitrogen freezer at temperature decreases of 30, 15 and 5 C/min, and at an initial temperature decrease of 1 C/min. The test involving a 1 C/min decrease was conducted in two steps. During the first step, the ampoules were frozen at a temperature decrease of 1 C/min until the temperature within the ampoule reached -30 C. At that time, a temperature decrease of 5 C/min was implemented until the end of the cycle.

The entire experiment was duplicated with the following modifications: (1) The semen was extended and frozen the day after the semen was collected; and (2) a concentration of 20% DMSO was used in the extender.

The freezing rates actually obtained during the experiment, with the exception of the rate obtained when an ampoule was submerged in liquid nitrogen, are given in Fig. 23. The observations on viability of the thawed sperm cells after being frozen 3 to 5 days are presented in Fig. 24 for the first experiment and in Fig. 25 for the second. Of those ampoules submerged directly in the liquid nitrogen, all but one shattered and could not be evaluated. A temperature decrease of 5 C/min was optimal for those spermatozoa protected with 5% DMSO. A temperature decrease of 15 C/min was optimal for those spermatozoa protected with 10% DMSO. I cannot say from these experiments that one level of DMSO maintained higher viability than the other, since the second experiment was run a day after the semen was collected.

Equilibration Time

The effect of time for equilibration and on the protection provided to the sperm cells by two additives (DMSO and glycerol, each at four concentrations) was investigated. Semen was collected from four steelhead trout at the Eagle Creek National Fish Hatchery. The undiluted semen was transported to the Fairplay Laboratory for extension. From each of the four fish, 2 ml of semen was placed into each of 2 test tubes. Four milliliters of Extender 48 was mixed with each 2 ml sample of semen. One-half (3 ml) of the extended semen

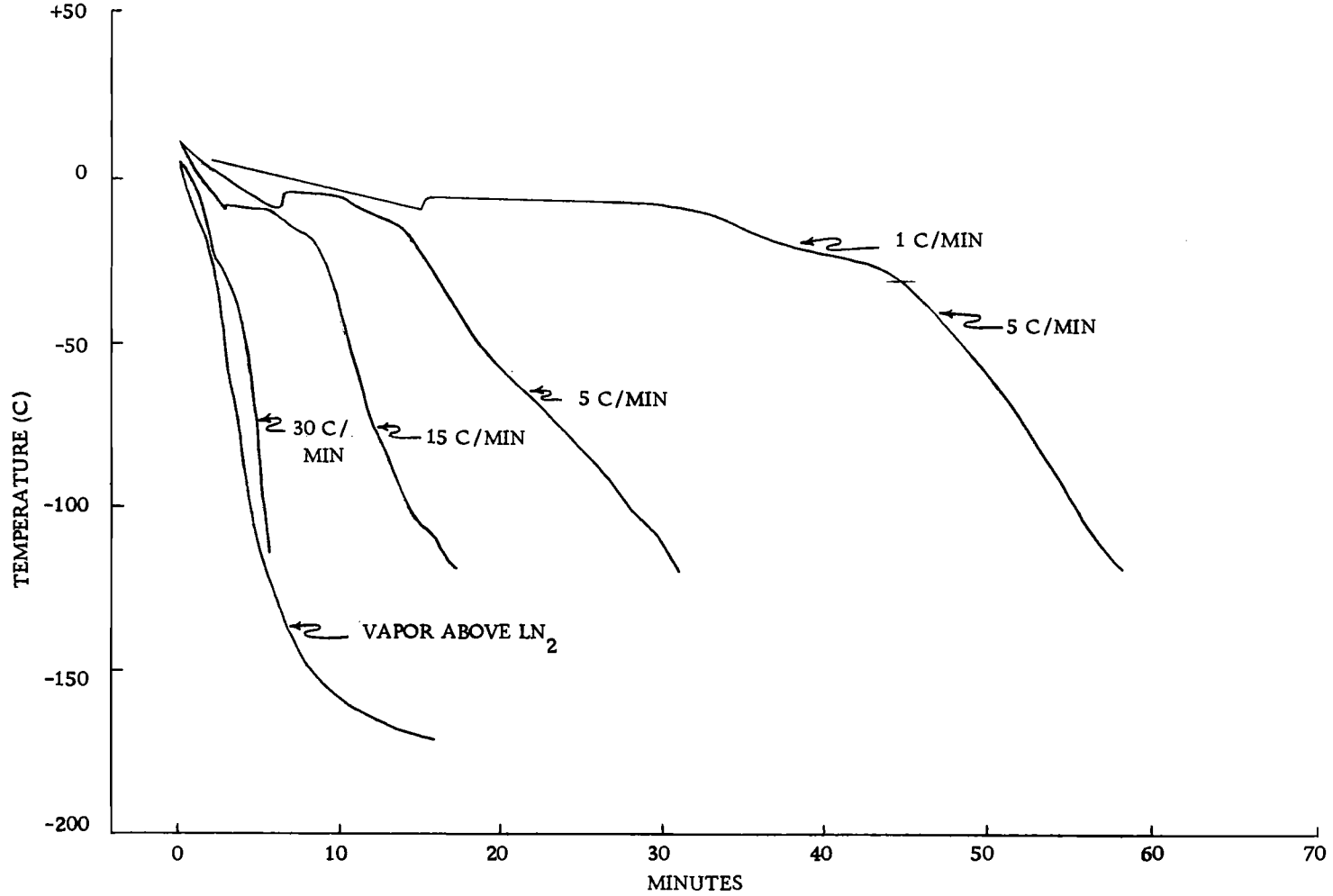

Figure 23. Relationship between temperature decrease and time for five rates of freezing of ampoules of steelhead trout semen.

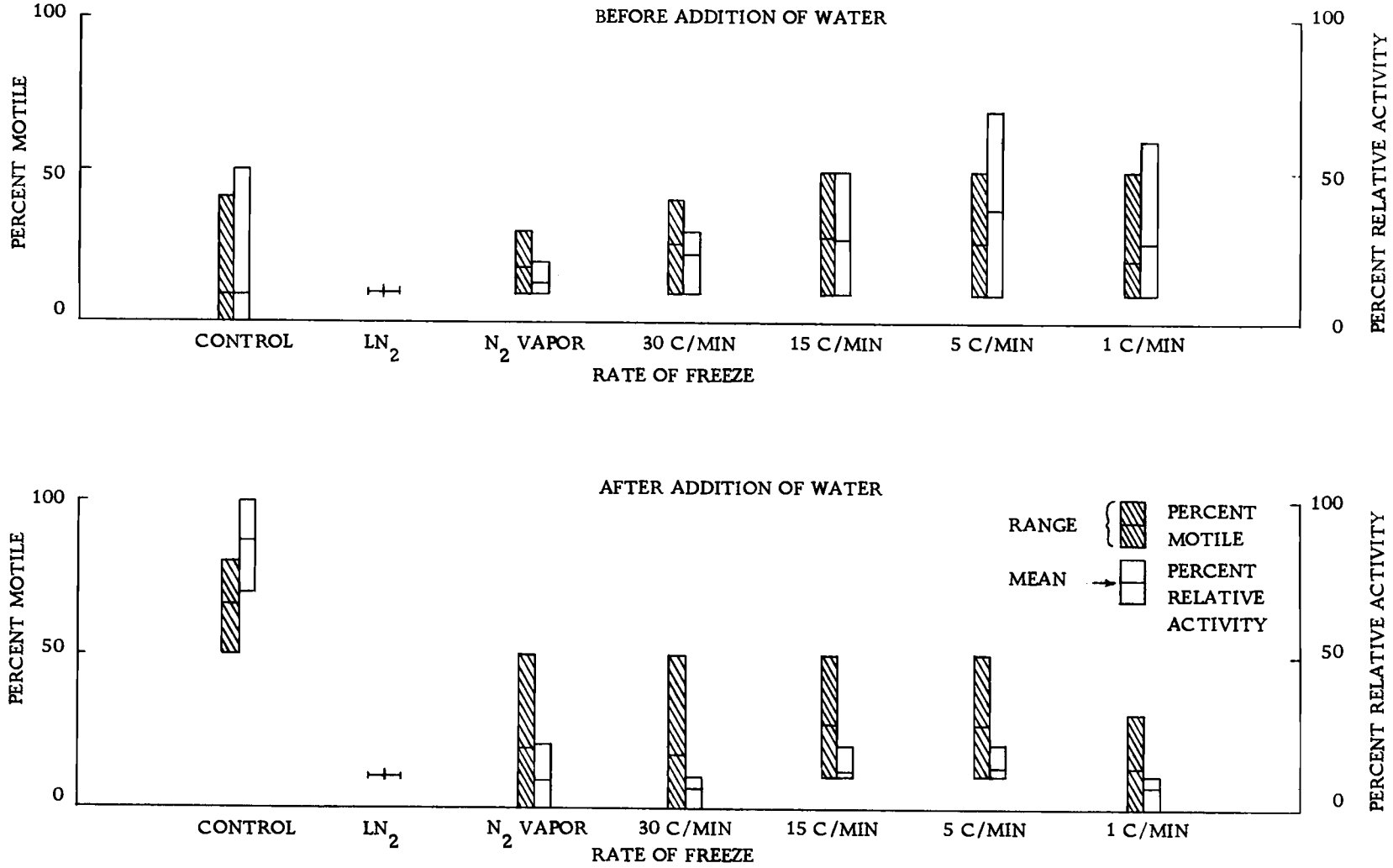

Figure 24. Relationship between percent motile sperm and percent relative activity of thawed spermatozoa before and after the addition of water to samples of semen of steelhead trout extended in Solution 25, of which 5% was DMSO, and frozen at several rates of temperature decrease.

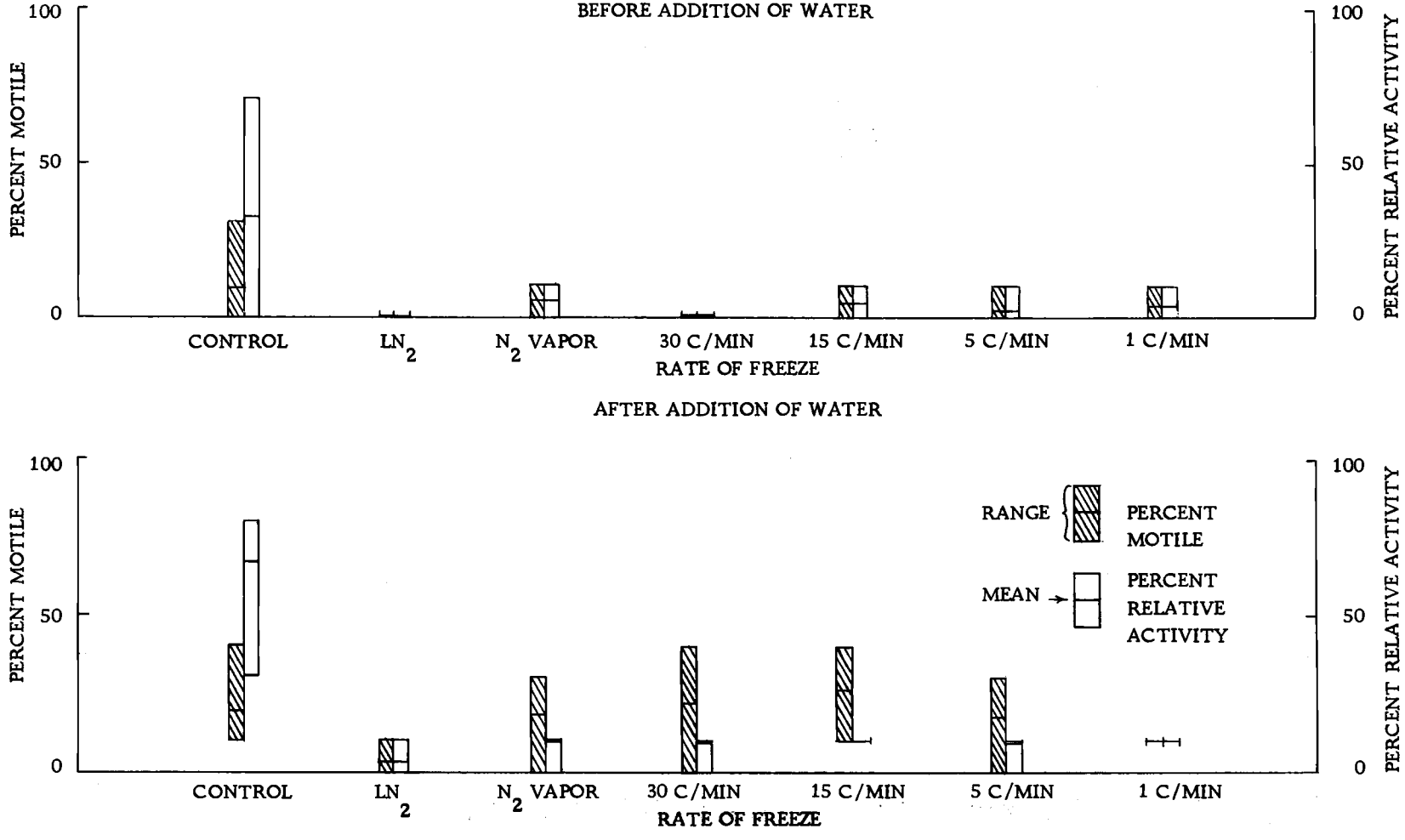

Figure 25. Relationship between percent motile sperm and percent relative activity of thawed spermatozoa before and after addition of water to samples of semen of steelhead trout extended in Solution 25, of which 10% was DMSO, and frozen at several rates of temperature decrease.

from each tube was placed into another test tube. Into two of these test tubes respectively for each fish was placed 2 ml of the extender containing 10% additive and 2 ml of the extender containing 15% additive. Into the remaining two test tubes for each fish was placed 1 ml of the extender containing 20% additive and 1 ml of the extender containing 25% additive. About 45 min later, an additional 1 ml of like extender and additive was placed into each of these last two test tubes. This last step was used to avoid shock to the sperm cells when too much additive was introduced at one time. Glycerol and DMSO each protected the semen of two fish. The final concentration of each additive in the extender was 5.0, 7.5, 10.0 and 12.5%. At this point, there were 5 ml of extended and protected semen in each of four test tubes. From each test tube, 2 ml was removed of which 1 ml was placed into each of two 1 ml ampoules. The two ampoules from each test tube were frozen in the vapor seven inches above the surface of the liquid nitrogen. The remaining 3 ml of extended and protected semen was refrigerated and used for a duplicate test the following day. All mixing of solutions of semen took place in the walk-in refrigerator. A diagram of the above procedure for one fish is presented in Table 11.

The observations of viability of spermatozoa frozen in four concentrations of each additive, with and without a period for equilibration, are presented in Fig. 26 and 27. Equilibration did not increase visible viability of sperm cells in those samples protected with DMSO.

Table 11. Diagram of experimental procedure used for testing time of equilibration and four levels each of two protectors of semen of steelhead trout frozen in the vapor above the surface of liquid nitrogen.

Fish No.	Undiluted Semen	Extender 48	Extended Semen	Extender With Protector	Level of Protector	Extender With Protector	Level of Protector	Total Extended Semen	Level of Protection in Final Extender	Frozen Same Day	Frozen Next Day
1	2 ml	+ 4 ml	3 ml	+ 2 ml	10%			5 ml	5.0%	1 ml + 1 ml	1 ml + 1 ml
			3 ml	+ 2 ml	15%			5 ml	7.5%	1 ml + 1 ml	1 ml + 1 ml
	2 ml	+ 4 ml	3 ml	+ 1 ml	20%	+ 1 ml	20%	5 ml	10.0%	1 ml + 1 ml	1 ml + 1 ml
			3 ml	+ 1 ml	25%	+ 1 ml	25%	5 ml	12.5%	1 ml + 1 ml	1 ml + 1 ml

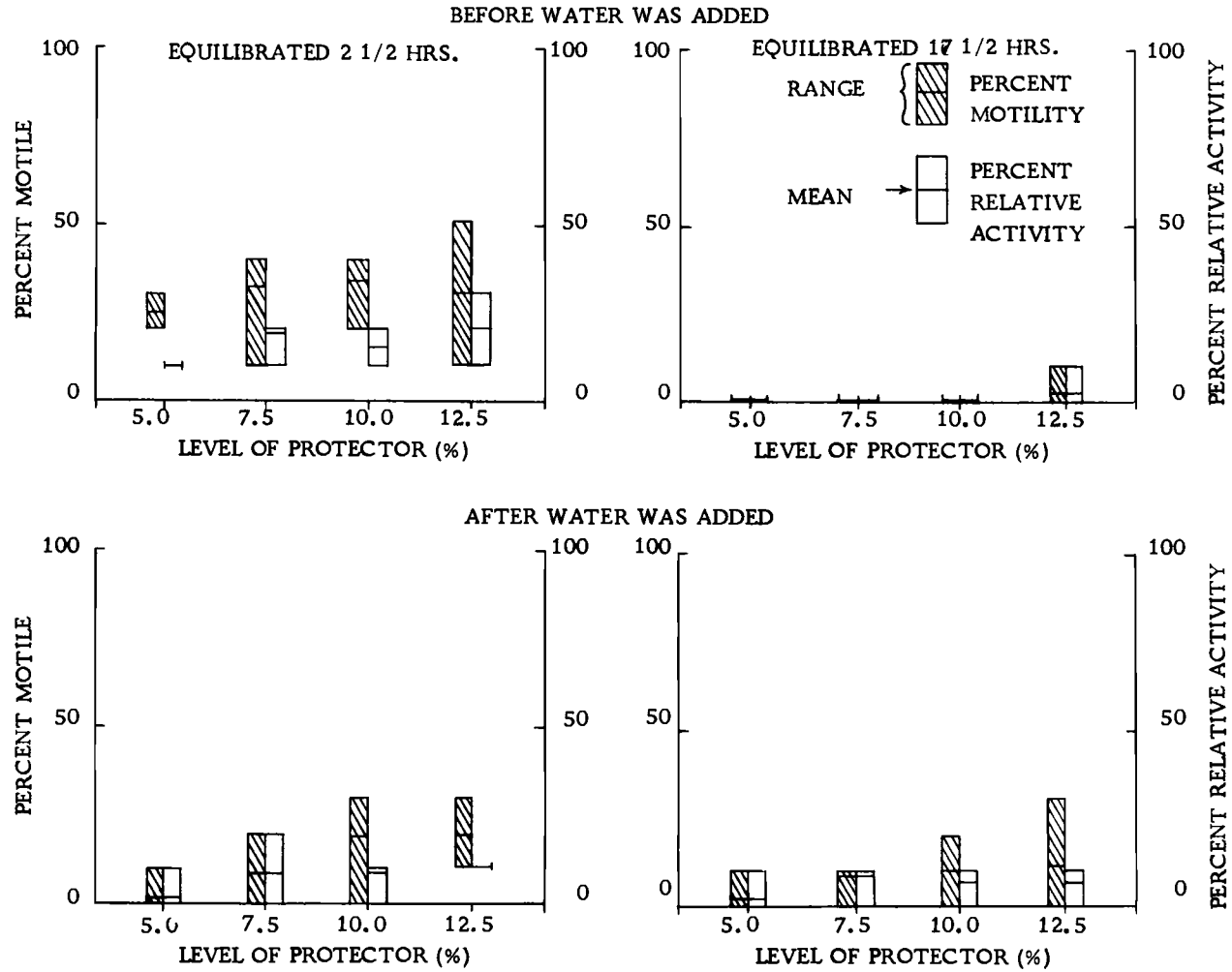

Figure 26. Percent motile sperm and percent relative activity of spermatozoa before and after the addition of water to samples of semen of steelhead trout equilibrated for two time periods with several levels of DMSO and frozen in the vapor above the surface of liquid nitrogen.

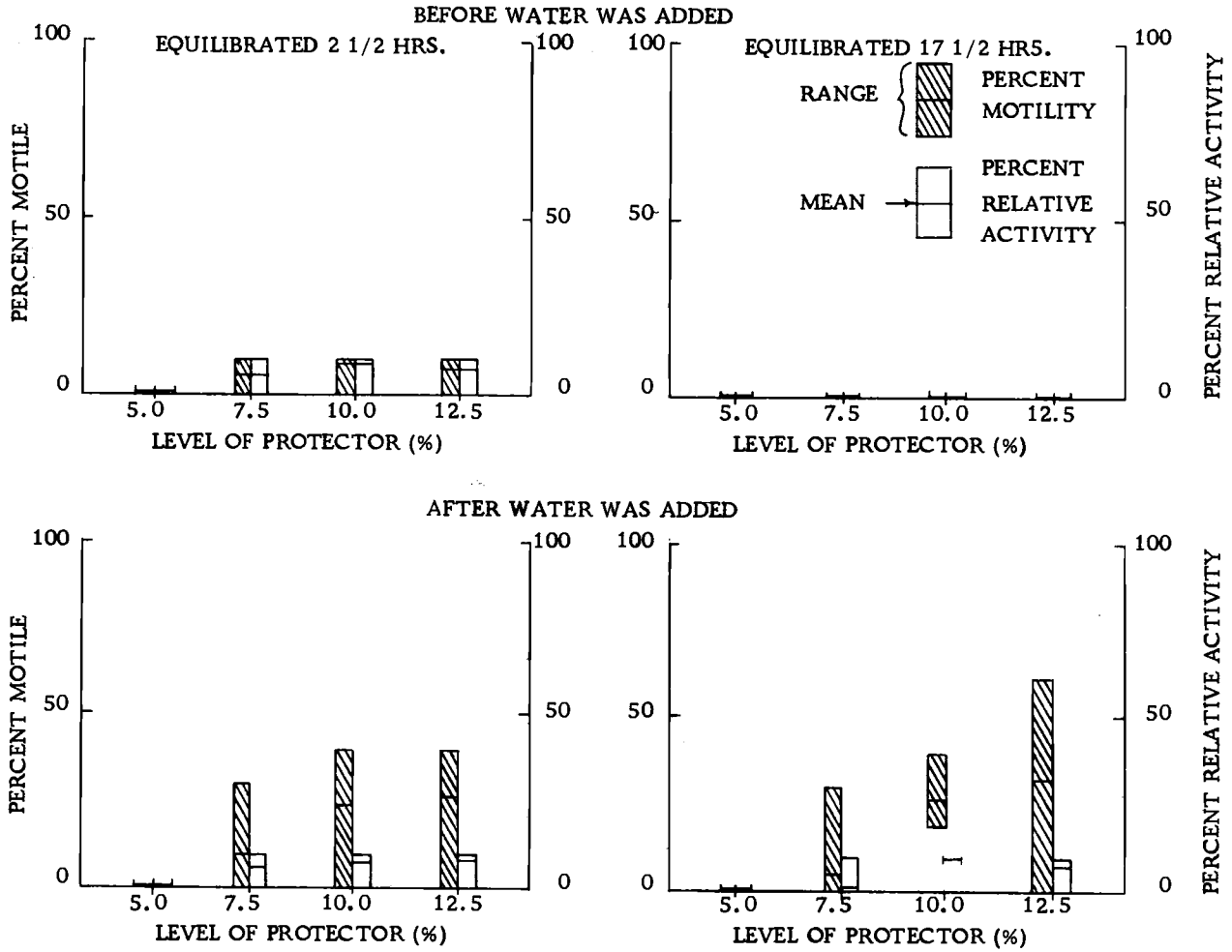

Figure 27. Percent motile sperm and percent relative activity of spermatozoa before and after the addition of water to samples of semen of steelhead trout equilibrated for two time periods with several levels of glycerol and frozen in the vapor above the surface of liquid nitrogen.

Equilibration may have increased viability of spermatozoa protected with glycerol even though motility prior to the addition of water was low. The highest concentrations of either protector were beneficial to survival whether or not the spermatozoa were equilibrated. The samples protected with glycerol were agglutinated and did not appear to be suitable for fertilization of eggs; this supposition should be tested.

Based on my tests, DMSO appears to be a satisfactory protector. The percentage of cells that were motile was high (30-50%) in numerous cases, but percent relative activity of the thawed cells was generally low (10%). Unfrozen cells from the same samples normally expressed a high percent relative activity (40-90%). This suggests that some damage to the cell occurs on freezing or thawing. The cause of the low percent relative activity after thawing remains open to further investigation.

Throughout the testing of DMSO at various concentrations and at various rates of temperature decrease, a general hypothesis was developed: As the concentration of additive is increased, the rate of temperature decrease needed for maximum survival is also increased. At a final concentration of 5% DMSO, the optimum rate of temperature decrease was 5 C/min. At a final concentration of 10% DMSO, the optimum rate of temperature decrease was 15 C/min. The optimum concentration of DMSO was 12.5% in the final extender when the

ampoules were frozen at a rate of about 30 C/min. This is an important conclusion from the standpoint of practical application. There is no evidence from this study that freezing sperm cells in the vapor above the surface of the liquid nitrogen cannot be used as a field technique. Portable freezing equipment could be used in places where electricity is not available.

Fertilizing Capacity of Frozen Spermatozoa

The true test of viability of "frozen" spermatozoa is not whether the cells are motile, but whether they will fertilize eggs. The capacity of cryo-preserved spermatozoa to fertilize eggs was tested and compared with the fertilizing capacity of fresh spermatozoa. Semen was collected from a steelhead trout at the Eagle Creek National Fish Hatchery. The sample of fresh semen was placed into a water bath (5 C) and then evaluated for motility. Two ampoules of semen frozen two weeks previously from each of two steelhead trout were selected. One ampoule from each fish had been frozen in Solution 48, of which 5% was DMSO, at a temperature decrease of 1 C/min to -30 C, and then at a rate of 5 C/min until the end of the freezing process. The other ampoule from each fish had been frozen in the vapor above the surface of the liquid nitrogen. All four of the selected ampoules were thawed in the water bath and evaluated for motility before being mixed with fresh eggs. About 200 eggs from a steelhead trout were placed

into each of three, 4-ounce jars. Two-tenths milliliter of fresh semen was placed into the first jar of eggs and gently mixed. One milliliter of thawed semen (frozen in nitrogen vapor) from each fish was placed into each of the two remaining jars. The jars were gently swirled to mix the eggs and sperm. Excess sperm was then washed from the eggs.

About 200 eggs from another steelhead trout were placed into each of three more jars. Again, 0.2 ml of fresh semen from the same male as above was placed into the first jar of eggs and gently mixed. One milliliter of thawed semen (frozen initially at 1 C/min) was added to each of the two remaining jars and gently mixed. Excess semen was washed away after all eggs from one female had been mixed with the semen. Once washing was complete, the eggs were placed in incubators having separate water supplies.

Two weeks later, a duplicate experiment was performed. Semen was again collected from a steelhead trout and tested in the same manner. Duplicate samples of frozen semen were thawed and evaluated for motility. About 200 eggs were stripped into each of four jars. This time, 1 ml of thawed semen (frozen in nitrogen vapor) from each fish was introduced into separate jars of eggs. Semen was not added to the third jar which served as a control against possible contamination by other sperm cells. Two-tenths milliliter of fresh semen was added to the fourth jar. Washing was accomplished by

running water from a hose over the eggs contained in a jar.

Eggs from another steelhead trout were stripped into each of four jars. Again, 1 ml of thawed semen (frozen initially at 1 C/min) from each fish was introduced into separate jars of eggs. Fresh semen (0.2 ml) was added to the third jar of eggs. No semen was added to the fourth jar of eggs which again served as a control for contamination by other sperm cells.

About 200 eggs from a third steelhead trout were stripped into each of three jars. Two samples of semen from the same fish, each frozen in the vapor above the liquid nitrogen but protected at different levels of DMSO (5 and 12.5%), were each placed in a jar of eggs. Fresh semen (0.2 ml) was added to the third jar of eggs. All eggs were then moved to incubators having separate water supplies.

About 36 to 39 temperature units after the semen was mixed with the eggs, samples of six eggs were removed from each lot and preserved in Carnoy's fixative (1 part glacial acetic acid to 3 parts 95% ethanol). These eggs were examined by Dr. Raymond C. Simon for fertilization and for numbers and morphology of chromosomes.

Chromosome analysis for those eggs fertilized on May 12, 1967, was conducted in the following manner: Two eggs from each group were examined for diploid development, rate of development, chromatin bridges and lagging chromosomes. Blank cells were found in all eggs except controls and one egg from female No. 3 fertilized with thawed

semen from male No. 95. The egg from this group was the only ovum found that had been fertilized with the cryo-preserved sperm. Based on cell numbers and size, the rate of development for this egg was the same as for the controls. No chromatin bridges nor lagging chromosomes were found in cells in anaphase, and no haploid cells were found. From all indications, the egg fertilized with cryo-preserved semen was as normal as the controls.

The percent of eggs fertilized by the various samples of semen, as determined by count of alevins for the first experiment and number of eyed eggs in the second experiment, is given in Table 12. There was no possible way for sperm cells to contaminate the eggs of the second experiment, and only a remote chance in the first. Those offspring occurring from eggs fertilized with cryogenically preserved semen had to come from cells that had been frozen. Only those eggs exposed to sperm cells which had been frozen in nitrogen vapor were fertilized. With the particular test used, I cannot say whether percent fertilization was related to the way the sample of semen was frozen, or whether percent fertilization was correlated with the time elapsed between thawing and addition of the sperm to the eggs. There was no correlation between motility observed and the percentage of eggs fertilized, which supports the hypothesis that percent fertilization was affected by the time elapsed between thawing the cells and fertilizing the eggs.

Table 12. Percent fertilization of eggs and evaluations of selected samples of semen of steelhead trout frozen at two rates of temperature decrease in Solution 48 of which 5% or 12.5% was DMSO.

Female Number	Percent of DMSO	Male Number	Freeze Rate	Date Fertilized	Percent Fertilized at Eyed Egg Stage	Evaluation			
						Before Addition of Water		After Addition of Water	
						Percent Motile	Percent Relative Activity	Percent Motile	Percent Relative Activity
1		110-Control	Fresh, not frozen	April 28, 1967	87.2	0	0	100	100
	5	95	LN_2 vapor[1]	April 28, 1967	18.0	60	10	10	10
	5	101	LN_2 vapor[1]	April 28, 1967	3.3	40	10	40	10
2		110-Control	Fresh, not frozen	April 28, 1967	95.0	0	0	100	100
	5	95	1 C/min	April 28, 1967	0.0	60	10	30	10
	5	101	1 C/min	April 28, 1967	0.0	60	10	40	10
3		115-Control	Fresh, not frozen	May 12, 1967	98.3	0	0	100	100
	5	95	LN_2 vapor[1]	May 12, 1967	6.1	0	0	30	10
	5	101	LN_2 vapor[1]	May 12, 1967	0.0	10	10	30	10
4		115-Control	Fresh, not frozen	May 12, 1967	97.6	0	0	100	100
	5	95	1 C/min	May 12, 1967	0.0	10	10	70	20
	5	101	1 C/min	May 12, 1967	0.0	10	10	70	20
5		115-Control	Fresh, not frozen	May 12, 1967	99.3	0	0	100	100
	5	106	LN_2 vapor[1]	May 12, 1967	0.5	10	10	10	10
	12.5	106	LN_2 vapor[1]	May 12, 1967	0.8	30	10	50	20

[1] Vapor above surface of liquid nitrogen.

To my knowledge, this is the first successful attempt to fertilize the eggs of salmonids with cryo-preserved spermatozoa. Alevins were produced from eggs fertilized with frozen sperm and appeared to be as normal as young fish produced from unfrozen spermatozoa. I believe that the percent fertilization can be increased by placing the sperm with the eggs immediately after the semen is thawed. This hypothesis will be tested under an extension of this study.

CONCLUSIONS

1. The dual tube device was preferred for collecting fish semen.

2. Undiluted semen held in test tubes can be transported in a water bath cooler for three hours without any detectable loss of viability.

3. Precautions should be taken to prevent rapid temperature changes in the semen during laboratory procedures.

4. Observations of percent motile sperm and percent relative activity of spermatozoa, before and after the addition of water, will give reasonable estimates of viability of sperm cells.

5. No differences were detected in the gross morphology of spermatozoa of coho salmon, chinook salmon, rainbow trout and steelhead trout.

6. Spermatozoa in undiluted semen will remain viable for at least several days when refrigerated at 4 C.

7. The concentration of sperm in the semen of coho salmon and rainbow trout is 2.3 to 230 times greater than for most farm animals. Sperm concentrations of the two fish investigated are comparable to that reported for the sea urchin (Echinus esculentus).

8. An extender should be developed which will maintain viable sperm in greatly diluted semen.

9. There is a direct relationship between the concentration of

additive in the extender and the optimum rate of freezing samples of semen.

10. Freezing semen in the vapor above the surface of the liquid nitrogen could prove useful as a field method.

11. Solution 48 with DMSO as an additive was the best extender-protector combination tested for the preservation of spermatozoa at very cold temperatures.

12. The eggs of steelhead trout were successfully fertilized with spermatozoa that had been frozen and refrigerated in liquid nitrogen for 14 and 28 days. These eggs developed into alevins which appeared to be normal.

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APPENDICES

APPENDICES

Appendix 1. Constituents of each extender used in this investigation. Strength of components is in molarity except where noted. Inorganic compounds are listed by chemical formula and organic compounds are listed by name.

Sol. No.	pH	Component	Molarity
1	7.3	NaCl	0.128
		KCl	0.00256
		$CaCl_2 \cdot 2H_2O$	0.00183
		$NaHCO_3$	0.000238
2		Sucrose	0.9737
3		Spring chinook ovarian fluid	100%
4		NaCl	0.0620
		KCl	0.0124
		$CaCl_2 \cdot 2H_2O$	0.00886
		$NaHCO_3$	0.00115
5		Distilled water	100%
6		Sucrose	0.4869
7	8.1	NaCl	0.120
		KCl	0.00300
		$CaCl_2 \cdot 2H_2O$	0.00205
		$NaHCO_3$	0.00540
8		NaCl	0.109
		KCl	0.00273
		$CaCl_2 \cdot 2H_2O$	0.00186
		$NaHCO_3$	0.00492
9		NaCl	0.109
		KCl	0.00273

Appendix 1 (continued)

Sol. No.	pH	Component	Molarity
		$CaCl_2 \cdot 2H_2O$	0.00186
		$NaHCO_3$	0.00492
		Bromothymol blue	10%
10		NaCl	0.114
		KCl	0.00228
		$CaCl_2 \cdot 2H_2O$	0.00163
		$NaHCO_3$	0.00205
		Phenol red	10%
11		NaCl	0.114
		KCl	0.00228
		$CaCl_2 \cdot 2H_2O$	0.00163
		$NaHCO_3$	0.00205
		Bromothymol blue	10%
12		NaCl	0.124
		$CaCl_2 \cdot 2H_2O$	0.00156
		KCl	0.00509
		$NaH_2PO_4 \cdot H_2O$	0.00297
		$NaHCO_3$	0.0119
		$MgSO_4 \cdot 7H_2O$	0.000933
		D-glucose	0.005549
14	7.3	NaCl	0.6800
		KCl	0.0400
		$NaH_2PO_4 \cdot 2H_2O$	0.125
		$MgSO_4 \cdot 7H_2O$	0.0200
		Glucose	0.1000
		L-arginine HCl	0.0126

Appendix 1 (continued)

Sol. No.	pH	Component	Molarity
		L-tyrosine	0.0036
		L-cystine	0.0024
		L-histidine HCl· H_2O	0.0042
		L-glutamine	0.0292
		L-isoleucine	0.00525
		L-leucine	0.00524
		L-lysine HCl	0.0073
		L-methionine	0.0015
		L-phenylalanine	0.0033
		L-threonine	0.0048
		L-tryptophane	0.0010
		L-valine	0.0047
		Choline Cl	0.0001
		Folic acid	0.0001
		i-inositol	0.0002
		Nicotinamide	0.0001
		Ce-D-Panthothenate	0.0001
		Pyridoxal HCl	0.0001
		Riboflavine	0.00001
		Thiamine HCl	0.0001
		Phenol red	0.0010
15	7.3	NaCl	0.127
		KCl	0.00268
		$CaCl_2 \cdot 2H_2O$	0.00136
		$NaHCO_3$	0.00179
		$MgSO_4 \cdot 7H_2O$	0.000811
16	7.3	NaCl	0.127

Appendix 1 (Continued)

Sol. No.	pH	Component	Molarity
		KCl	0.00509
		$CaCl_2 \cdot 2H_2O$	0.00136
		$NaHCO_3$	0.00143
17	7.3	NaCl	0.127
		KCl	0.00255
		$CaCl_2 \cdot 2H_2O$	0.00273
		$NaHCO_3$	0.00119
18	7.3	NaCl	0.127
		KCl	0.00255
		$CaCl_2 \cdot 2H_2O$	0.00137
		$NaHCO_3$	0.00119
19	7.3	NaCl	0.137
		KCl	0.00255
		$CaCl_2 \cdot 2H_2O$	0.00137
		$NaHCO_3$	0.00119
20	7.3	NaCl	0.127
		KCl	0.00255
		$CaCl_2 \cdot 2H_2O$	0.00137
		$NaHCO_3$	0.0595
		Citric acid	0.0104
21	7.3	NaCl	0.120
		KCl	0.00255
		$CaCl_2 \cdot 2H_2O$	0.00137
		$NaHCO_3$	0.0714
		Citric acid	0.0104

Appendix 1 (Continued)

Sol. No.	pH	Component	Molarity
22	7.3	NaCl	0.120
		KCl	0.00268
		$CaCl_2 \cdot 2H_2O$	0.00137
		$NaHCO_3$	0.0893
		Citric acid	0.00469
		Glycine	0.0675
23	7.1	NaCl	0.128
		KCl	0.00255
		$CaCl_2 \cdot 2H_2O$	0.00137
		$NaHCO_3$	0.0893
		Citric acid	0.00469
		Glycine	0.135
24	7.2	NaCl	0.128
		KCl	0.00255
		$CaCl_2 \cdot 2H_2O$	0.00137
		$NaHCO_3$	0.0893
		Citric acid	0.00469
		Glycine	0.0675
		Glucose	0.0277
25	7.1	NaCl	0.127
		KCl	0.00255
		$CaCl_2 \cdot 2H_2O$	0.00137
		$NaHCO_3$	0.0893
		Glycine	0.0675
		Ascorbic acid	0.00284
		Citric acid	0.00463
		Glucose	0.0277

Appendix 1 (Continued)

Sol. No.	pH	Component	Molarity
		Lecithin (soy bean)	0.000716
26	7.2	NaCl	0.103
		KCl	0.00255
		$CaCl_2 \cdot 2H_2O$	0.00137
		$NaHCO_3$	0.0893
		Glycine	0.0675
		Ascorbic acid	0.00284
		Citric acid	0.00463
		Glucose	0.0277
		Lecithin (soy bean)	0.000716
		Isoleucine	0.00381
27	7.3	NaCl	0.128
		KCl	0.00255
		$CaCl_2 \cdot 2H_2O$	0.00137
		$KHCO_3$	0.00120
28	7.5	NaCl	0.137
		$Na_3C_6H_5O_7 \cdot 2H_2O$	0.0136
		Glucose	0.114
		KCl	0.00268
29	7.6	NaCl	0.137
		$Na_3C_6H_5O_7 \cdot 2H_2O$	0.0136
		Glucose	0.114
30	7.3	NaCl	0.124
		$CaCl_2 \cdot 2H_2O$	0.00157
		KCl	0.00509
		$NaH_2PO_4 \cdot H_2O$	0.00297

Appendix 1 (Continued)

Sol. No.	pH	Component	Molarity
		$NaHCO_3$	0.0119
		$MgSO_4 \cdot 7H_2O$	0.000933
		Glucose	0.00555
		Whole egg ultrafiltrate	5%
		Fetal calf serum	5%
31	7.2	NaCl	0.124
		KCl	0.00536
		$CaCl_2 \cdot 2H_2O$	0.00137
		$NaHCO_3$	0.000238
32		NaCl	0.114
		KCl	0.0134
		$CaCl_2 \cdot 2H_2O$	0.00137
		$NaHCO_3$	0.000238
33		NaCl	0.0800
		KCl	0.0402
		$CaCl_2 \cdot 2H_2O$	0.00137
		$NaHCO_3$	0.000238
34		NaCl	0.0286
		KCl	0.0804
		$CaCl_2 \cdot 2H_2O$	0.00137
		$NaHCO_3$	0.000238
35		NaCl	0.137
		KCl	0.000
		$CaCl_2 \cdot 2H_2O$	0.00137
		$NaHCO_3$	0.000238
36		NaCl	0.125

Appendix 1 (Continued)

Sol. No.	pH	Component	Molarity
		KCl	0.00509
		$CaCl_2 \cdot 2H_2O$	0.00157
		$NaHCO_3$	0.00119
		$NaH_2PO_4 \cdot H_2O$	0.00297
		$MgSO_4 \cdot 7H_2O$	0.000933
		Fructose	0.00555
37		NaCl	0.125
		KCl	0.00509
		$CaCl_2 \cdot 2H_2O$	0.00157
		$NaHCO_3$	0.00119
		$NaH_2PO_4 \cdot H_2O$	0.00297
		$MgSO_4 \cdot 7H_2O$	0.000933
		Fructose	0.00555
		Glycine	0.00675
38		NaCl	0.125
		KCl	0.00509
		$CaCl_2 \cdot 2H_2O$	0.00157
		$NaHCO_3$	0.00119
		$NaH_2PO_4 \cdot H_2O$	0.00297
		$MgSO_4 \cdot 7H_2O$	0.000933
		Fructose	0.00555
		Glycine	0.0270
39		NaCl	0.125
		KCl	0.00509
		$CaCl_2 \cdot 2H_2O$	0.00157
		$NaHCO_3$	0.00119
		$NaH_2PO_4 \cdot H_2O$	0.00297

Appendix 1 (Continued)

Sol. No.	pH	Component	Molarity
		$MgSO_4 \cdot 7H_2O$	0.000933
		Fructose	0.00555
		Glycine	0.0675
40		NaCl	0.125
		KCl	0.00509
		$CaCl_2 \cdot 2H_2O$	0.00157
		$NaHCO_3$	0.00119
		$NaH_2PO_4 \cdot H_2O$	0.00297
		$MgSO_4 \cdot 7H_2O$	0.000933
		Fructose	0.00555
		Lecithin	0.000716
41		NaCl	0.125
		KCl	0.00509
		$CaCl_2 \cdot 2H_2O$	0.00157
		$NaHCO_3$	0.00119
		$NaH_2PO_4 \cdot H_2O$	0.00297
		$MgSO_4 \cdot 7H_2O$	0.000933
		Fructose	0.00555
		Glycine	0.00675
		Lecithin	0.000716
42		NaCl	0.125
		KCl	0.00509
		$CaCl_2 \cdot 2H_2O$	0.00157
		$NaHCO_3$	0.00119
		$NaH_2PO_4 \cdot H_2O$	0.00297
		$MgSO_4 \cdot 7H_2O$	0.000933
		Fructose	0.00555

Appendix 1 (Continued)

Sol. No.	pH	Component	Molarity
		Glycine	0.0270
		Lecithin	0.000716
43		NaCl	0.125
		KCl	0.00509
		$CaCl_2 \cdot 2H_2O$	0.00157
		$NaHCO_3$	0.00119
		$NaH_2PO_4 \cdot H_2O$	0.00297
		$MgSO_4 \cdot 7H_2O$	0.000933
		Fructose	0.00555
		Glycine	0.0675
		Lecithin	0.000716
44		NaCl	0.125
		KCl	0.00509
		$CaCl_2 \cdot 2H_2O$	0.00157
		$NaHCO_3$	0.00119
		$NaH_2PO_4 \cdot H_2O$	0.00297
		$MgSO_4 \cdot 7H_2O$	0.000933
		Fructose	0.00555
		Lecithin	0.00287
45		NaCl	0.125
		KCl	0.00509
		$CaCl_2 \cdot 2H_2O$	0.00157
		$NaHCO_3$	0.00119
		$NaH_2PO_4 \cdot H_2O$	0.00297
		$MgSO_4 \cdot 7H_2O$	0.000933
		Fructose	0.00555

Appendix 1 (Continued)

Sol. No.	pH	Component	Molarity
		Glycine	0.00675
		Lecithin	0.00287
46		NaCl	0.125
		KCl	0.00509
		$CaCl_2 \cdot 2H_2O$	0.00157
		$NaHCO_3$	0.00119
		$NaH_2PO_4 \cdot H_2O$	0.00297
		$MgSO_4 \cdot 7H_2O$	0.000933
		Fructose	0.00555
		Glycine	0.0270
		Lecithin	0.00287
47		NaCl	0.125
		KCl	0.00509
		$CaCl_2 \cdot 2H_2O$	0.00157
		$NaHCO_3$	0.00119
		$NaH_2PO_4 \cdot H_2O$	0.00297
		$MgSO_4 \cdot 7H_2O$	0.000933
		Fructose	0.00555
		Glycine	0.0675
		Lecithin	0.00287
48		NaCl	0.125
		KCl	0.00509
		$CaCl_2 \cdot 2H_2O$	0.00157
		$NaHCO_3$	0.00119
		$NaH_2PO_4 \cdot H_2O$	0.00297
		$MgSO_4 \cdot 7H_2O$	0.000933
		Fructose	0.00555

Appendix 1 (Continued)

Sol. No.	pH	Component	Molarity
		Lecithin	0.00716
49		NaCl	0.125
		KCl	0.00509
		$CaCl_2 \cdot 2H_2O$	0.00157
		$NaHCO_3$	0.00119
		$NaH_2PO_4 \cdot H_2O$	0.00297
		$MgSO_4 \cdot 7H_2O$	0.000933
		Fructose	0.00555
		Glycine	0.00675
		Lecithin	0.00716
50		NaCl	0.125
		KCl	0.00509
		$CaCl_2 \cdot 2H_2O$	0.00157
		$NaHCO_3$	0.00119
		$NaH_2PO_4 \cdot H_2O$	0.00297
		$MgSO_4 \cdot 7H_2O$	0.000933
		Fructose	0.00555
		Glycine	0.0270
		Lecithin	0.00716
51		NaCl	0.125
		KCl	0.00509
		$CaCl_2 \cdot 2H_2O$	0.00157
		$NaHCO_3$	0.00119
		$NaH_2PO_4 \cdot H_2O$	0.00297
		$MgSO_4 \cdot 7H_2O$	0.000933
		Fructose	0.00555
		Glycine	0.0675
		Lecithin	0.00716