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THE POTENTIAL APPLICATION OF CRYOBIOLOGY TO AQUACULTURE.

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Introduction

The steadily increasing interest of science in long-term cryopreservation of fertile gametes grows not only from the need to attain a better understanding of basic life processes, but also from the pressing need to be able to manipulate natural resources for the benefit of people. In recent years the preservation of spermatozoa has become feasible in several mammalian species. It now appears that the preservation of fish spermatozoa and ova is also a coming reality.

During the past 30 years, the gradual development of techniques for cryopreservation of spermatozoa and ova from various mammalian species has led to dramatic reformation of the livestock industry. Frozen semen has already established itself as an integral tool of breeding programs throughout the world. The time has come when the field of aquaculture, long stagnant in use of artificial breeding techniques, has awakened to the enormous potential of cryopreserved spermatozoa and ova. As the world's demand for fish as a food source increases long practiced techniques of pisciculture must undergo drastic change if future needs are to be met. The utilization of cryopreserved fish gametes is one method of facilitating such necessary changes. The development of reliable techniques for preservation of fish gametes would offer both practical and economic advantages to aquaculture specialists. Benefits derived from the preservation of fish gametes would include:

1. Gametes from desired species could be shipped from hatchery to hatchery dependent upon the demands of each individual spawning season. Either ova or spermatozoa of the breeding population could be stored or shipped at very low cost. Furthermore, the risk of spreading infectious diseases would be greatly reduced using frozen gametes instead of live fish.

2. The number of males normally kept as brood stock fish from season to season could be reduced, or possibly eliminated entirely at some facilities.

3. If one sex ripened in advance of the other (a very common occurrence, particularly with males ripening before females), there would be assurance of having mature gametes of both sexes on hand at the proper time.

4. Gamete preservation could provide a powerful tool for genetic manipulation of fish populations. It would allow for the retention of desirable strains of fish. Genetically important fish lines could be retained indefinitely. Crossbreeding studies on species that normally mature at different seasons of the year would be possible.

5. Hatchery production could be established on a year-round basis rather than being limited to the short natural spawning seasons. Instead of a single all-or-none hatch, controlled hatches could be accomplished throughout the year in order to utilize production capabilities optimally.

6. The time element (short duration of viability of gametes), which has been the plague of artificial propagation of fishes since its beginning, would be reduced if not eliminated.

Objectives of Research

The objective of this study is to develop repeatable techniques that will allow for long-term cryopreservation of fish gametes. The study was initiated in order to enhance the reproductive potential of hatchery oriented spawning operations. It has involved a wide variety of species of fish found in Minnesota lakes and streams. The major thrust of the research is aimed at developing optimal chemical extender systems for both the short term (non-frozen) and long term (frozen) preservation of fish spermatozoa and ova.

Gamete Preservation: Past and Present

Even though the techniques used in the artificial propagation of fish have changed relatively little in modern times, research

concerning the breeding biology of important aquatic species has been an on-going process for more than a century (de Quatretages, 1853). However, it was not until the early 1930s that widespread interest relating to such research developed (Wolf, 1931; Bennington, 1936). Since then considerable work has been done on attempting to improve reproductive capacity and preserve viable gametes from fish semen. Involvement in this area of research initially peaked during the late 1960s, but because of renewed interest in the field, research efforts are greater today than ever before. Growing economic pressures have finally made increased reproductive performance imperative, and gamete preservation has assumed new importance. Furthermore, the need for such research appears to be well defined and its scope, worldwide.

Frozen Semen Preservation

Blaxter (1953) reported 80% fertility with Atlantic herring semen frozen in the testes. He was able to crossbreed herring which spawn in the spring with herring which spawn in the fall. Mounib et al. (1968) reported fertility with Atlantic cod semen stored at -79°C for 1 yr. Snead and Clemens (1956) reported post-thaw motility of carp semen but not fertility data.

Semen of the salmonid family has been frozen with variable fertility. Hepworth (1962) and Mitchum (1963) were both unsuccessful in attempts to freeze rainbow trout semen. Hodgins and Ridgeway (1964) reported motility, but no fertility data on frozen spermatozoa from chinook and pink salmon. Hoyle and Idler (1968) reported 0-12% fertility on frozen Atlantic salmon semen. Truscott and Idler (1969) reported 5-19% fertility for the same species. Ott and Horton (1971) reported maximum fertilities of 38% for chinook salmon, 71% for coho salmon, and an average fertility of 59% for steelhead trout using frozen-thawed spermatozoa. Ott (1975) reported maximum fertilities with frozen semen of 83.2% (pink salmon), 29.3% (chum salmon), 63.2% (kokanee), 54.3% (fall chinook salmon, mean of four tests) and 77.7% (coho salmon, mean of three tests). Horton and Ott (1976) reported personal communication with Withler and Morley at Nanaimo, British Columbia. These researchers obtained the following maximum fertilities for frozen semen: pink salmon, 44.4%; sockeye salmon, 85.3%; chum salmon, 51%; and coho salmon, 56.4%. Erdahl and Graham (1978) reported success in freezing salmonid spermatozoa in the presence of dimethyl sulfoxide or ethylene glycol. They found glycerol to be ineffective as a cryoprotective agent. Stein and Bayrle (1978) attempted to cryopreserve the sperm of 7 freshwater fish using the pellet technique of Nagase (1964). The fertilization rate in the rainbow trout was about 80% when the sperm were equilibrated 15 minutes before deep freezing. Under the same conditions brown trout sperm yielded about 70% fertilization. Stoss and Holtz (1980) found that rainbow trout sperm yielded consistent fertilization rates of between 0-20% below controls after storage at -196°C. Erdahl and Graham (1980) reported that after storage at -79°C, post-freeze spermatozoa of both the brown trout (Salmo trutta) and rainbow trout (Salmo gairdneri) resulted in fertilization rates in excess of 80% when introduced to fresh ova. The semen was collected via aspiration, diluted 1:1 in an artificial seminal plasma extender containing 7% dimethyl sulfoxide, and frozen in either pellet or straw configuration to -79°C.

Ova Preservation

Considerable research has been done on successful preservation of mammalian ova, but preservation of sea animal ova has been attempted by only a few researchers. Hultin and Hoystrum (1954) and Okyama and Asahima (1972) reported viable sea urchin eggs after storage at -10°C for several hours. Staeger (1974) reported no viable oyster eggs after storage at -196°C. Zell (1978) cited evidence stating that salmonid eggs and embryos survived freezing to -55°C when frozen immediately after fertilization in a salt solution. Erdahl and Graham (1980) reported success in freezing rainbow trout and northern pike eggs to -20°C. The eggs were frozen in an extender system containing 14% dimethyl sulfoxide at a rate of -5°C/minute.

University of Minnesota Research

Research concerning the cryopreservation of fish gametes has been supported by the Minnesota Sea Grant Institute for the past three years. Initial work dealt mainly with salmonids, but has since been broadened to include other species commonly found in Minnesota lakes and streams. All research fish have been obtained with the cooperation of the Minnesota Department of Natural Resources. Gametes have been collected from brood stock fish reared at various hatcheries throughout the state, or from native populations of wildstock fish trapped during their spawning runs.

Gamete Collection

The key to the successful preservation of fish gametes, particularly spermatozoa, lies in the careful collection of gametes. Although the proper collection of gametes requires a number of necessary techniques, of primary importance in gamete preservation is the cautious prevention of allowing water to contact either sperm or eggs. The gametes must be kept dry. Research has shown that the spermatozoa

of most freshwater species remain viable (capable of fertilization) for only 30 to 60 seconds after the introduction of water. In fact, our own studies have shown that after 20 seconds of exposure to water, fertility of spermatozoa drops to 50% of initial levels, and is virtually nil after 30 seconds. Fish ova, although apparently somewhat more tolerant of the effects of exposure to water, also have an extremely short viable period after introduction to it. After 1 minute in water, ova fertility drops to less than 50% of original capability, and is almost non-existent after 2 minutes.

Aside from the extreme caution to prevent water from entering the system, the collection procedure in gamete preservation is easily adaptable to the normal techniques of pisciculture pertaining to gamete collection. Of course, extra care should be taken to prevent excess coelomic fluids, feces, or other foreign material from contacting the gametes.

Before the actual collection procedure, each fish is first anesthetized using methane tricane sulfonate (M.S.222). The use of an anaesthetic not only allows for greater ease in handling the fish and a decrease in trauma sustained by the fish, but also allows for the more complete removal of the gametes from the abdominal cavity.

Collection Apparatus

The apparatus that is used to collect the semen is similar in principle to the one described by Graybill and Horton in 1969. It consists of two sterilized vials connected in series by rubber tubing, so that one vial is used to hold the semen, and the other to collect saliva and/or excess semen. The vial that holds the collected semen is situated, immersed in a thermos flask that contains a mixture of hatchery water and crushed ice. The vial is held in position by a styrofoam ring around the collar of the flask. The vial for excess semen and saliva is simply taped to the side of the thermos flask. The section of tubing leading from the first vial is fitted with a "modified" transfer pipette to facilitate the selective collection of high quality semen. Oral suction on the tube leading to the second vial provides the mechanism for aspirating the semen.

Semen Collection

During the collection of spermatozoa, each fish is grasped by the tail (with gloved hand) and held belly-up, with the tail inclined downwards and the head positioned securely between the elbow and side. At this time the genital and surrounding area of each fish is carefully wiped dry. Gentle pressure is then applied by the holder of the fish with the free hand, and using a sort of "milking" action the semen is expressed. Initially, the pressure is concentrated on the anterior abdominal region along the midline of the fish, and is gradually relocated to the posterior abdominal area near the urino-genital opening. At this location pressure is applied along either side of the fish. At this time a second person, using the collection apparatus, collects what appears to be quality semen and allows inferior-appearing semen to be lost. Although not always the case, it is standard procedure to allow semen from different males to be mixed within the collection vial, since a single fish often produces an inadequate volume of semen.

Egg Collection

Eggs are collected without the aid of any special apparatus. After being anesthetized each fish is simply held belly down, and after the careful removal of excess water with a dry towel, the eggs are expressed using normal hatchery techniques. Best results are obtained when the pressure is maintained anteriorly for as long as possible, and relocated posteriorly only when egg flow begins to cease. The eggs are collected into clean, dry, plastic beakers. No attempt is made to remove coelomic fluids.

Chemical Analysis of Semen

Upon completion of the collection procedure, the semen is immediately brought into the lab for chemical analysis.

Each original sample of semen is divided into two portions. One portion is centrifuged using discontinuous density gradient centrifugation (Brown et al., 1971) to remove the spermatozoa from the seminal plasma with a minimum of damage to the cells. The seminal plasma is decanted and saved while the cells are discarded. These samples are labeled as control or minimum damage samples. The other portion of the original sample is placed in 15 ml polypropylene centrifuge tubes and plunged directly into liquid nitrogen to allow the sample to cool to -196°C. It is then thawed, and plunged and thawed twice more, in order to cause maximum membrane damage to the spermatozoa. The samples are then centrifuged at 22,000 x g for 10 minutes and the seminal plasma decanted and saved. These samples are labeled as freeze or maximum damage samples. The minimum damage seminal plasma is considered to contain only extracellular components of semen, while the maximum damage seminal plasma is understood to consist of both extracellular and intracellular components.

The seminal plasma samples are then placed in sealed, glass ampoules and stored at -20°C for subsequent analysis. At a later date analysis is then made for: (1) osmotic pressure;

(2) pH; (3) inorganic constituents, Na^{++}, K^{+}, P, Cl^{-}, Ca^{++}, Mg^{++}, Zn^{++}, Cu^{++}, and B; (4) organic constituents, lactic dehydrogenase, glutamic oxaloacetic transaminase, acid and alkaline phosphatase, reducing substances; (5) free amino acids and amino compounds; and (6) total amino acids--free amino acids and amino acids released from the degradation of protein (see Table 1).

TABLE 1

Analysis of Seminal Plasma

_____ Osmotic Pressure
_____ pH

_____ Inorganic Constituents
- Na^{++} - Cl^{-} - Zn^{++}
- K^{+} - Ca^{++} - Cu^{++}
- P - Mg^{++} - B

_____ Organic Constituents
- Lactic Dehydrogenase (LDH)
- Glutamic Oxaloacetic Transaminase (GOT)
- Acid and Alkaline Phosphatase
- Reducing Substances

_____ Free Amino Acids and Amino CMPS.

_____ Total Amino Acids
- Free AA
- AA Released from Protein

TABLE 2

Optimal Chemical Extender System for Trout Semen (System #6)

In 2 Liters of Deionized Water		
$CaCl_2 \cdot 2H_2O$	0.205	g
$MgCl_2 \cdot 6H_2O$	0.440	g
Na_2HPO_4	0.530	g
KCl	5.115	g
NaCl	11.682	g
Citric Acid	0.200	g
Glucose	20.000	g
KOH (1.27 g/100 ml)	20	ml
Bicine (5.3 g/100 ml)	40	ml
Osmotic Pressure--345 mOsm		
pH 7.8		

Artificial Extender Systems

The chemical analysis of the semen is used to formulate artificial chemical extender systems for the spermatozoa (see Table 2). The extender system attempts to mimic the composition of seminal plasma, the natural environment of the spermatozoa, as closely as possible. However, it is hoped that the artificial system is even more beneficial to the longevity of the cells than raw seminal plasma.

Of major concern in the formulation of the artificial extender is the proper balance of electrolyte composition. Electrolytes of primary concern are Na^{++}, K^{+}, P, Cl^{-} and Mg^{++}. Potassium hydroxide is added to the extender in order to increase the concentration of K^{+} ions, as it has been shown that increased levels of K^{+} help to maintain the spermatozoa in an inactive state. Glucose is added to provide an energy substrate for the spermatozoa. Bicine functions to buffer the extender system against pH changes resulting from metabolic processes.

The extender system has been designed to maintain spermatozoa in an inactive state and prolong their viability. It has been possible to store extended semen at 5°C in an oxygen atmosphere for up to 19 days with no apparent loss of fertilizing capacity. This extender system is also used as the dilution medium in the freezing process.

Cryoprotective Agents

In order to provide protection against intracellular ice crystal formation and increasing solute concentration that occur during the freeze procedure, a cryoprotective agent must be added to the extender when sperm cells are to be frozen. It has been found that either dimethyl sulfoxide (Me_2SO_4) or ethylene glycol provides optimal protection during the freeze process. Conversely, glycerol has been found to be less effective. Tests have shown a level of 7% (vol/vol) cryoagent in the extender to be optimal. Increased levels of cryoprotective material appear to cause cell toxicity, while decreased amounts offer inadequate freeze protection. Low temperature, non-frozen preservation is best without the addition of cryo-agent.

Semen Preservation

Semen that is to be frozen is mixed with extender plus cryoprotectant (1:2) immediately following the collection process. The extender

and semen are maintained at ambient hatchery water temperature before and after the extension process. The extender is added to the semen at the controlled rate. Initially, 1 ml of extender is added to 1 ml of semen. After several minutes equilibration time a second ml of extender is added. Total equilibration time for the extended semen should be approximately 20 minutes. The samples are then frozen on dry ice in either 0.1 ml pellets or 0.25 ml straws. Pellet frozen semen is deposited a drop at a time onto a specially prepared block of dry ice. In either case, the samples are cooled at a rate of approximately -85°C/minute during transition through the critical range of temperatures from -5°C to -30°C. After 30 minutes storage at -79°C the samples are placed in liquid nitrogen. The pellets are thawed in an additional 1 ml of extender at 5°C. The straws are thawed by immersion in water at 5°C with no further dilution of the semen.

Following thawing, the samples are stored at 5°C while eggs are stripped from ripe females. Immediately following egg collection, fertilization of individual samples is conducted employing dry techniques (Truscott, et al., 1968). The eggs are then placed into incubators and left undisturbed for 4 to 5 weeks. At this time fertility data are obtained based upon either the number of eggs in the eyed state, or upon the number of hatched fry.

After storage at -79 or -196°C post-freeze spermatozoa of both the brown trout (Salmo trutta) and rainbow trout (Salmo gairdneri) have resulted in fertilization rates in excess of 80% when introduced to fresh ova (see Table 3). Spermatozoa of the brown trout have consistently yielded slightly better fertility data than the rainbow trout. These comparisons hold true for control samples, extended semen samples, and frozen semen samples.

Embryo Preservation

Research on fish ova (or embryo) preservation is still in the early stages of the Minnesota Sea Grant Institute project. The majority of research to this point has concentrated on semen preservation. However, based upon apparent interest in this aspect of cryopreservation research (e.g., United States Trout Association, Minnesota Department of Natural Resources, National Sea Grant Program Project Review Committee), future efforts will concentrate on the preservation of fertilized ova. At this time eggs of a number of different species, including the brown trout, rainbow trout, pink salmon, chinook salmon, and northern pike have been examined. A freeze procedure has been developed that allows for ova survival after cooling to -20°C.

TABLE 3

Typical Frozen Spermatozoa Fertilization Trial (Brown Trout).
Spermatozoa Frozen in Straws to - 79°C.

Treatment		Number of Eggs	Number Fertile	Number Infertile	Percent Fertile
Control (neat semen)		388	350	38	90
Extended w/o Cryo Agent		1666	1442	224	87
Extended + 7% Me_2SO_4		294	250	44	85
Extended + 7% Ethylene Glycol		300	270	30	90
Extended + 7% Glycerol		350	160	190	45
Extended + 7% Me_2SO_4	Frozen to -79°C	533	513	20	96
Extended + 7% Ethylene Glycol	Frozen to -79°C	549	484	65	88
Extended + 7% Glycerol	Frozen to -79°C	720	248	472	34
	Totals	4800	3717	1083	

Embryo Preservation Technique

Ova are collected using the normal hand stripping procedure on anesthetized females, taking special care to keep water from contacting the eggs. The eggs are then mixed with neat semen. The egg-sperm mixture is then placed in a bath of extender (plus cryoprotectant) at 5°C and allowed to equilibrate for 30 minutes (note: water is still entirely absent from the system). The extender is the same as that used for semen preservation. Dimethyl sulfoxide or ethylene glycol is added to the extender at a level of 14% (vol/vol). The eggs are then aspirated into specially prepared straws and placed into the Linde Biological Freezer. Eggs are then cooled at a rate of -5°C/minute down to -25°C. Thawing of the eggs is accomplished by placing straws in a water bath at 5°C. The eggs are then placed into incubators and left undisturbed until eye-up. At this point fertility is determined.

To date, it has been possible to freeze fish eggs to -20°C without adversely affecting fertility (see Table 4). However, attempts to cool the eggs further have resulted in a traumatic loss of viability. In most cases, eggs cooled to -25°C or lower have yielded extremely low fertility. Apparently, during this stage of the freeze process crystallization is causing severe membrane damage, since yolk proteins will precipitate and the eggs will turn white immediately after being placed in the incubator. In most species examined, the eggs can tolerate approximately 20% cryoprotectant without loss of viability. However, there are species differences that should be examined. In particular, the pink and chinook salmon eggs appear to be quite sensitive (negatively) to even low levels of cryoprotective agents.

Problems Associated with the Study of Fish Gamete Preservation

Even though recent research has made marked progress in understanding the principles of gamete preservation in fish, substantial work remains to be done. Furthermore, certain problems exist which make such research extremely difficult. Not only do most researchers struggle against basic physiological problems commonly associated with mammalian gamete preservation, but also attempt to overcome the constraints imposed by an aquatic environment. At present some of the most difficult problems facing researchers in this field are: (1) the short duration of individual

TABLE 4

Typical Egg Freeze Fertilization Trial (Northern Pike)

TREATMENT	FINAL TEMPERATURE	ALIVE/DEAD	PERCENT FERTILE
1. Control (Neat Semen, Neat Egg, H_2O)		46/7	87
2. Extender + 14% Ethylene Glycol	non-frozen	38/10	79
3. Extender + 14% Ethylene Glycol	-5°C	32/10	76
4. Extender + 14% Ethylene Glycol	-10°C	24/12	67
5. Extender + 14% Ethylene Glycol	-15°C	18/16	53
6. Extender + 14% Ethylene Glycol	-20°C	7/34	17
7. Extender + 14% Ethylene Glycol	-25°C	3/48	6

Commonly Encountered Problems

1. Physical trauma to the eggs via handling before water hardening.
2. Species variability with respect to cryoprotective material toxicity.
3. Non-uniform egg freezing within individual straws.
4. Unsatisfactory freeze rates.

spawning seasons; (2) the limited viability of fish gametes; (3) the lack of adequate facilities for research; and (4) the lack of cooperation and correspondence among workers.

Limited Duration of Spawning Seasons

The relatively short duration of individual spawning runs is the major problem facing researchers attempting to study fish gametes. In Minnesota, in a given location, the brown trout completes spawning in a three-week period, the northern pike in a one-week period, the walleye in 4 or 5 days, and the muskellunge in only 2 or 3 days. Other species spawn with equal rapidity. To complicate matters, the spawning seasons of many species overlap, which only serves to shorten the already brief period of gamete collection. As a result, every season, certain species are likely to be overlooked completely. Furthermore, within each species there is a deterioration of gametes within the spawning season. Optimal quality gametes may, in fact, only be present for a 1 or 2 day period. There is quite simply too little time to complete even a fraction of planned research. Without question, the time available for active fish gamete research is extremely short and constitutes a major obstical to research endeavors.

Limited Viability of Gametes

The limited duration of viability of fish gametes also poses an obstacle to researchers. Although fish gametes are in an inactive state *in vivo* and immediately following collection, upon dilution with water they pass from an inactive state to an active state, and then into an exhausted condition within a very brief period of time. As alluded to earlier, it has been shown that spermatozoa, upon dilution with water, attain a peak motility within 5 seconds, and are dead by the time 45 seconds have elapsed. Ova survive somewhat longer in water, yet after only 1 minute they begin to lose fertility rapidly. Thus, it becomes very difficult to develop any sort of laboratory test assay for fish gametes (particularly sperm). The gametes are inactive before dilution with water, and become inactive again so quickly after dilution with water, that both qualitative and quantitative assessment is extremely difficult.

Limited Facilities

Adequate facilities, for both laboratory research and fish population maintenance, are rare. While either type of facility may be fairly commonplace, to find the two in combination or even in proximity, frequently poses a problem for fish gamete research. Too often the major effort is put forth in time and travel to acquire fish and not in quality laboratory research. Furthermore, there is nothing more disheartening than to see the fish die, for one reason or another, before experimental use. And, even if the fish do survive transportation and handling, the quality of the gametes may be greatly reduced. To maintain fish gametes in a viable state is a difficult task in itself. To battle inadequate facilities while attempting to hold ripe, spawning fish only complicates the research effort.

Limited Communication

At this time, communication between researchers studying fish gametes is unquestionably poor. Basically, the sole means of communication is by publication of journal articles. By no means does this constitute adequate correspondence between researchers. In order for maximum progress to occur in the field of fish gamete preservation there must be more interaction and cooperation between individuals. Often many important facts and/or possible insights cannot be transmitted via normal journal publication. Personal communication is necessary if the proper exchange of findings and ideas is to be accomplished. Possibly, the organization of some type of annual meeting for persons interested in fish gamete research would facilitate such an exchange of ideas.

Summary

Gamete preservation in aquatic species appears to be assuming new importance in fisheries research. Researchers have been interested in the study of fish gametes for a number of years, but widespread attention to their efforts has not come until recently. Particular interest in gamete preservation can be found in laboratories in France and Germany.

Up to this point the majority of the work has concentrated on the preservation of spermatozoa. The sperm of many salmonid species has been examined, and fertility values in excess of 50% using frozen-thawed semen are quite common. Preservation of ova (or embryos) has only been attempted by a few researchers, and work is still in the initial stages. Success in freezing ova to -20°C has been reported.

As in any research area, a number of basic problems exists relating to fish gamete preservation. They must be solved if desired objectives are to be achieved. However, if interest and participation in this area of research continue to grow as they have in recent times, many of these problems will likely be resolved. Hopefully then, the use of cryopreserved fish gametes will in turn eliminate

many problems often experienced during normal spawning seasons, and will prove to be a useful tool in the artificial propagation of fish populations the world over. The challenge to cryobiologists to meet the needs of the fisheries industry is only exceeded by the value of the potential products.

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