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Cryopreservation in Aquarium Fishes

Terrence R. Tiersch*

Aquaculture Research Station, Louisiana State University Agricultural Center, Louisiana Agricultural Experiment Station, 2410 Ben Hur Road, Baton Rouge, LA 70803, USA

Abstract: Few studies have addressed sperm cryopreservation in aquarium fishes (body sizes of 10 cm or less). There are several challenges inherent in developing cryopreservation procedures for these fishes. First, their small body size and sperm volume limit experimental replication and the numbers of treatments possible without pooling of samples. This hinders research, especially if many experimental variables are evaluated. The small sample volume necessitates identification of optimal sperm-to-egg ratios to maximize fertilization potential and places greater emphasis on increasing and maintaining sperm viability after thawing. Other technical problems include the use of 0.25-ml French straws, which increase difficulties in sample handling (automated straw fillers are more common for the 0.5-ml straw) and labeling. Sperm cryopreservation of live-bearing fishes (with internal fertilization) is essentially unexplored. The sperm of these fishes is sufficiently different in structure (e.g., head shape) and physiology (e.g., energy metabolism) from the sperm of other fishes that the need to develop specialized techniques is almost assured. The requirement for artificial insemination also introduces a new variable complicating the collection of data (e.g., assessing fertilization is not straightforward with internally held eggs). Cryopreservation in aquarium fishes will assist the development (e.g., through selective breeding), protection (e.g., through germplasm repositories), and distribution (e.g., through shipment of frozen sperm) of research lines and offers benefits for restoration of endangered species.

Key words: cryopreservation, aquarium fishes, sperm, germplasm repositories, genetic diversity.

INTRODUCTION

Sperm cryopreservation has been studied in fishes for more than 45 years since Blaxter (1953) used frozen sperm to hybridize spring and fall spawning herring and has been used in as many as 200 species (Leung and Jamieson, 1991; Rana, 1995a; Tiersch, 2000). Most work has focused on large-bodied culture and sport fishes, such as salmonids (e.g., Scott and Baynes, 1980), carps, and catfishes, and has yielded techniques that are being applied with varying levels of success around the world. However, only a handful of studies have addressed sperm cryopreservation in aquarium fishes (body sizes of 10 cm or less). The most notable of those studied is the zebrafish (*Danio rerio*) for which cryopreservation techniques using methanol were developed 20 years ago (Harvey et al., 1982) and the Japanese medaka (*Oryzias latipes*) (Aoki et al., 1997).

Cryopreservation research and application each require consideration of an interconnected series of activities and involve more than simply freezing samples. A successful program involves sample collection, refrigerated storage, freezing, thawing, rules for use and disposal, transfer agree-

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*Corresponding author: telephone 225-765-2848; fax 225-765-2877; e-mail ttiersch@agctr.lsu.edu

ments, and database development. Development of cryopreservation techniques for aquarium fishes is hindered by their small body sizes and sperm volumes, but given their utility in research and the existence of stock centers, many of the problems inherent in application of cryopreservation programs have existing solutions. For example, long-established breeding programs and detailed biological databases exist for lines of zebrafish, swordtails and platyfish (genus *Xiphophorus*), and Japanese medaka. In addition, these lines are routinely transferred among institutions for research, and agreements are in place concerning intellectual property. Advantages such as these do not exist for fishes of economic importance, such as channel catfish (*Ictalurus punctatus*) and common carp (*Cyprinus carpio*), for which research on cryopreservation is facilitated by their large bodies, but application is hindered by industry structure (e.g., lack of artificial spawning for channel catfish) and a lack of formal stock centers to maintain and distribute research lines.

At present, cryopreservation of eggs, embryos, and larvae of aquatic species has not been successful, except for the larvae of a marine polychaete (*Nereis virens*) (Olive and Wang, 1997, 2000) and the eastern oyster (*Crassostrea virginica*) (Paniagua-Chavez et al., 1998). Cryopreservation of fish eggs and embryos has received attention, and permeability barriers to water and cryoprotectants and other problems (e.g., chilling sensitivity) have been identified as sources of failure (Hagedorn et al., 1997; Hagedorn and Kleinhans, 2000). Accordingly this paper will focus on cryopreservation of sperm, although much of the material would also apply to eggs, embryos and larvae.

The goal of this paper is to identify the essential practical steps for the development and application of effective cryopreservation procedures for aquarium fishes. The objectives are to: (1) review the basic steps in cryopreservation research relevant to aquarium fishes also considering those with internal fertilization; (2) review the basic requirements for development of cryopreservation programs, including those for endangered species; and (3) introduce some future opportunities in linking cryopreservation with related technologies.

Missing Connection Between Research and Application

Despite 50 years of research, application of cryopreservation is almost nonexistent for aquatic species (Rana, 1995a; Tiersch, 2000). Typically research is considered complete upon demonstration of the technical feasibility of fertilization by thawed sperm. Few studies have advanced beyond this to the level of application. Once techniques are in place for a particular species (or population), a process of transformation is necessary. The problems of research give way to the problems of application, including the need for standardization of protocols, scaling-up for commercial production, inventory procedures, quality control, economic analysis (Caffey and Tiersch, 2000a,b), and regulatory considerations (Jenkins, 2000a). These topics have recently been discussed with respect to the establishment of genetic conservation programs (Cloud and Thorgaard, 1993; Harvey et al., 1998).

As stated above, cryopreservation involves several basic activities (Figure 1), which will be addressed in relation to research and application in the following sections: (1) collection of sperm; (2) dilution of sperm; (3) refrigerated storage; (4) freezing; (5) frozen storage; (6) thawing; (7) fertilization, and (8) evaluation. Each activity is important, and just as links form a chain, failure at any single step can lead to failure for the entire project. These activities initially need to be developed through research and, depending upon the species, will have varying levels of difficulty. General practical information is available for topics such as cryopreservation and freeze-drying methods (Simione and Brown, 1991; Day and McLellan, 1995) and cryopreservation of algae (Morris, 1981) and the semen of boars (Johnson and Larsson, 1985; Johnson and Rath, 1991). Such sources can be valuable for developing cryopreservation protocols even though they are not intended for aquatic species. Basic methods for fishes have been summarized in several sources (e.g., Scott and Baynes, 1980; Leung and Jamieson, 1991; Billard et al., 1995; Rana, 1995b; Wayman and Tiersch, 2000), although optimal techniques are often developed on a species-by-species basis.

Collection of Sperm

It is important that the males are in good physical condition. Stressful conditions can reduce sperm quality and the survival of males after sperm collection. Consideration should also be given to detailed collection and archiving of biological data of donor males. It is not unlikely that sperm frozen for research will be thawed and used for breeding purposes not anticipated at the time of collection (Wachtel and Tiersch, 2000). Thus, it is essential to bear in mind that the value of frozen samples is directly related to the quality

Activity	Research	Application
Collection of sperm	Stripping of males Pooling of samples	Biological data on broodstock Permits and agreements
Dilution in extender	Extender composition Sperm quality evaluation	Disease screening Rules for use and disposal
Refrigerated storage	Dilution ratios Storage time	Inventory and quality control Sample transfer agreements
Freezing	Cooling rates and containers Cryoprotectants	Standardization of protocols Scaling up
Frozen storage	Plunge temperature Sample retrieval	Security considerations Repository system
Thawing	Thawing temperature Thawing duration	Standardization of protocols Scaling up
Fertilization	Sperm motility Sperm-to-egg ratio	Standardized protocols Internal insemination
Evaluation	Fertilization and hatching Growth and survival	Breeding database Select and develop broodstock

Figure 1. Activities involved in sperm cryopreservation, and examples of the corresponding needs for research and application in aquarium fishes.

and availability of the corresponding biological data (Kincaid, 2000) and sample labeling. It is also advisable to ensure that proper permits and agreements are in place to enable transfer and use of frozen samples (Jenkins, 2000a). These considerations should extend some years into the future given the potential for decades of frozen storage.

Generally, sperm are collected from males during the spawning season. The injection of natural or synthetic preparations of gonadotropins in combination with other drugs (e.g., dopamine antagonists) has been employed to increase the volume of sperm (Donaldson, 1996). Typically, care is taken to ensure that sperm are not contaminated with water, mucus, or feces during collection. Urine contamination is difficult to avoid without catheterization (Rana, 1995a) and, if fish are stripped by abdominal massage, it is useful to dilute the sperm with extender immediately to prevent activation of motility (e.g., Tiersch et al., 1998). Sperm collection from aquarium fishes is complicated by their small size, and procedures should be developed to maximize the volume of available sperm. Sperm can be collected from live male zebrafish by use of capillary tubes (Harvey et al., 1982). Males can also be killed to collect sperm by dissection and crushing of the testis, as was done for Japanese medaka (Aoki et al., 1997).

Experimental designs that utilize fully replicated combinations of treatments (e.g., cryoprotectants and concentrations) will not be possible with the small volumes of sperm obtainable from aquarium fishes (10–100 μl). An alternative is to pool samples from several males. This approach could be especially useful for species with highly inbred lines. For example, research populations of *Xiphophorus maculatus* maintained since the 1930s are now in their 98th generation of sibling matings (R. Walter, Southwest Texas State University, *Xiphophorus* Genetic Stock Center, personal communication). The effects of male-to-male variation would be minimized in these lines, allowing pooling or even the interchangeable use of individual males as replicates within and among treatments.

Conversely, in outbred lines of fish, pooling would obscure male-to-male variation and could have significant effects on experimental results and the genetic composition of progeny. For example, when equal volumes of sperm were pooled from groups of three chinook salmon (*Oncorhynchus tshawytscha*), the results were variable, and it was found that individual males fertilized between 1% and 76% of the eggs (Withler, 1988). However, a more recent study found that equal contributions to fertilization success were made by sperm from pooled males (Babiak et al., 1998). Many variables can affect the response of sperm to cryopreservation, which can introduce hidden sources of variation within pooled samples. Researchers will need to carefully weigh the purpose of experiments against the costs of pooling, this being of primary importance in programs that employ cryopreserved sperm to conserve genetic variation in applications such as endangered species. Indeed, after research has identified suitable procedures, it would seem

essential for conservation of genetic diversity to cryopreserve and use sperm from individual males to produce specific crosses that could be pooled after the offspring have hatched.

Dilution in Extender

Considerable attention has been given to the development of extender solutions for fish sperm (e.g., Ott and Horton, 1971; Scott and Baynes, 1980; Kerby, 1983). These solutions are used to dilute and enhance the storage of sperm by maintaining a nonactivated, nonmotile state. Formulations range from complex to simple, and standardization has not yet been accepted even for well-studied species such as rainbow trout (*Oncorhynchus mykiss*). Formulations are sometimes based on the chemical composition of seminal plasma, but osmotic pressure, ionic composition and pH seem to be the most important considerations with respect to control of motility (e.g., Morisawa et al., 1983; Stoss, 1983; Billard et al., 1995). Sperm of internally fertilizing species possess atypical features, such as well-developed mitochondrial sheaths in the midpiece of spermatozoa (Stoss, 1983) and glycolytic activity comparable to that of mammalian sperm (Gardiner, 1978). These may be adaptations for movement or survival in the female reproductive tract and suggest physiological differences from the sperm of externally fertilizing fishes sufficient to influence the composition of extender solutions.

Evaluation of sperm quality is not standardized for aquatic species, although subjective estimation of motility (Billard et al., 1995) and fertilization are commonly employed (Tiersch, 2000). Other methods include the use of computer-assisted motility analysis (e.g., Toth et al., 1997), the identification of physiological and biochemical correlates of postthaw fertility (e.g., Lahnsteiner et al., 1996), and use of fluorescent dyes to evaluate damage to various cellular components (e.g., Segovia et al., 2000). Potential transfer of techniques from other vertebrates, such as boars (Johnson and Larsson, 1985) and poultry (Bakst and Wishart, 1996) also is possible.

Numerous commensal and pathogenic microorganisms survive in cryopreserved sperm of domesticated animals, and many such organisms can occur in the sperm of aquatic species (Jenkins, 2000b). Cryopreservation programs will need to establish protocols for disease screening and to establish rules for use and disposal of contaminated samples. Antibiotics can be added to extend the storage time of sperm samples (Stoss, 1983), but the potential for disease transmission with cryopreserved sperm remains unstudied. Clearly, the most effective approach would be to ensure that collections were made from disease-free certified stocks.

Refrigerated Storage

Short-term, nonfrozen storage of sperm is desirable for several reasons. Sperm can be collected at a stock center or hatchery and be transported to a site specializing in cryopreservation. Refrigerated storage allows evaluation of sperm quality and disease screening prior to cryopreservation. Factors such as container size and shape, supplemental oxygenation, storage temperature, addition of antibiotics, osmolality, and extender composition have been studied for several fishes (e.g., Scott and Baynes, 1980; Stoss, 1983; Billard et al., 1995). Dilution rates and osmolality are easily controlled variables that can significantly affect storage time (Bates et al., 1996). Evaporation during storage could be important with the small volumes of sperm from aquarium fishes. Refrigerated storage would likely represent the entry point for inventory and quality control at a cryopreservation center if samples were shipped from other locations. An interesting area of research would be to investigate the natural ability of *Xiphophorus* females to store sperm internally for months (Paaben et al., 1996). Adaptations of the sperm or female reproductive tract could provide opportunities for development of novel methods of sperm storage in this genus.

Freezing

Cryobiology is a developing science with only a rudimentary theoretical framework, and cryopreservation procedures are typically developed through trial and error. The basic principles of cryobiology address the movement of water out of cells during cooling (and its return upon warming) and the effects of cryoprotectant molecules which can act from the inside or outside of cells (Leung, 1991; Rall, 1993; Denniston et al., 2000). Variables of practical importance are the choice of cryoprotectant (and concentration), and the rate of cooling.

Cryoprotectants can be toxic to sperm cells, and it is thus important to establish useful concentrations before performing freezing experiments. Simple observation of motility can determine concentrations that are suitable for

exposure times of 30 minutes or less. This provides sufficient time for the cryoprotectant to enter the cells and to allow for handling and packaging of samples prior to freezing. A useful precaution is to hold back several samples to estimate motility at the start of the freezing process. This simplifies experimental interpretation by providing a method to discriminate between loss of motility before freezing or after thawing and is especially useful for cryoprotectants, such as dimethyl acetamide, that are relatively toxic to sperm cells.

Cooling rate is an important variable in cryopreservation, and single-stage and multiple-stage procedures have been developed (e.g., Leung and Jamieson, 1991; Rall, 1993). It is useful, if possible, to compare slow (e.g., 4°C/min) and fast (e.g., 40°C/min) cooling rates when working with unstudied species. In general, fish sperm do not require complicated freezing protocols, and in our laboratory we have found that a cooling rate of 40°C/min is useful across a broad range of species. After an effective combination of cryoprotectant and cooling rate are determined by research, application can involve simple methods to yield appropriate conditions.

The standard French straws used for cryopreservation of livestock sperm are available in 0.25-ml and 0.5-ml volumes, although the smaller straw would seem most suited for aquarium species. Sperm of larger fishes can be frozen in commercial drinking straws (Wheeler and Thorgaard, 1991). Sperm can also be frozen as pellets by placing droplets on Dry Ice (Stoss, 1983), although this would not be recommended for high-intensity breeding programs because of the difficulty in assuring sample identification and the possibility of contamination of samples with sperm from different males.

An alternative method for freezing in straws is to take advantage of standardized procedures and equipment such as the automated straw fillers and labeling systems available within the bull sperm industry. We have worked with the Louisiana State University Dairy Improvement Center for the past 5 years and have found that technology developed for dairy bull sperm can be used for cryopreservation of sperm from aquatic species (e.g., Roppolo, 2000). This approach provides additional benefits of well-established methods for quality control, inventory, secure storage, and transport.

With the notable exception of the Amazon molly, *Poecilia formosa,* an all-female species that uses sperm cells from males of other species only to activate egg development, sperm cells serve as a carrier for the male haploid genome. As such, we can divide the features that must be preserved by cryopreservation into two components: the head (cargo) and the tail (delivery system) (Figure 2). Live-bearing (viviparous) fishes of the family Poeciliidae possess specific adaptations for internal fertilization, including sperm packets (spermatozeugmata) to facilitate transfer to females (Grier et al., 1981) and an elongate, conical sperm head that may assist packaging in the spermatozeugmata or movement and storage within the female reproductive tract (Kweon et al., 1998). The response of sperm of these fishes

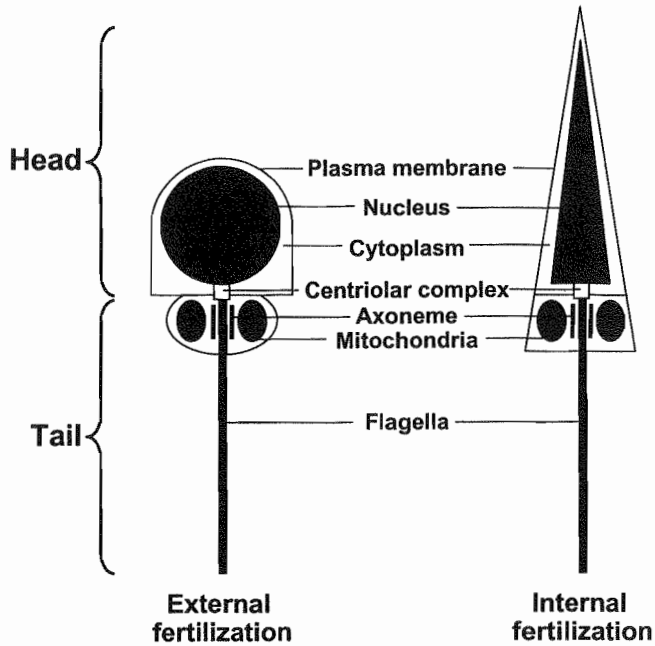

Figure 2. Diagrammatic representation of sperm typical of fishes that employ external fertilization (e.g., zebrafish) or internal fertilization (e.g., live-bearers). Examples of sites that are vulnerable to damage during freezing or thawing are indicated for both types. Based on theory, assuming equality of volume, the sperm cell on the left would require a slower cooling rate because of a smaller ratio of surface area to volume that would curtail outward movement of water and entry of cryoprotectant. The sperm cell on the right could be cooled faster, but its narrow structure could render it more vulnerable to damage by internal ice crystals (although it could present a smaller target to external ice crystals). In each example, it is important to note that the vulnerable sites can each require different optimal conditions and thus there may be differential responses to freezing and thawing in the various components of the head and tail. Damage to the tail could interfere with motility and fertilization, and damage to the head could interfere with embryonic development.

to cryopreservation is untested although some predictions are possible (Figure 2).

Frozen Storage

Storage at temperatures below −130°C is necessary to assure long-term stability of samples (Rall, 1993). Thus, immersion of samples in liquid nitrogen (−196°C) is a standard method for cryogenic storage. The potential for loss of samples during frozen storage is often underappreciated, and thus this section will address some practical concerns not typically found elsewhere. Proper labeling of cryopreserved samples is essential and cannot be overemphasized (Wayman and Tiersch, 2000). Samples can be in storage for months or years before they are thawed. Improperly labeled samples can delay processing, and even worse, could cause genetic contamination of pure stocks. Unlabeled or poorly labeled samples are essentially worthless. If possible, professional labeling, such as preprinted straws from the manufacturer, should be considered.

Straws are often stored in liquid nitrogen in plastic containers called goblets, which are attached to aluminum canes, and are placed in storage canisters. Goblets are manufactured in numerous colors and should be labeled to identify species, date, technician, type of study, and any additional pertinent information. To avoid problems in removing straws, they should be dried before freezing and not packed tightly in the goblets. Canes should be labeled on the top for easy identification. Labeling will decrease searching of Dewar contents and the potential for inadvertent thawing of samples. Careless handling of frozen samples can allow formation of intracellular ice crystals that will damage the cells.

Storage Dewars are designed to hold cryopreserved samples in liquid nitrogen for extended periods. Although a vacuum chamber provides insulation, the liquid nitrogen will evaporate and must be replaced. Alarms on storage Dewars are essential to provide adequate warning for replacement of liquid nitrogen. The outer casing of the Dewar must not be punctured, as the loss of vacuum will disperse the liquid nitrogen rapidly. Rough handling can weaken the inner neck area and reduce the working lifetime of a Dewar. A roller base will allow safe and easy movement of loaded Dewars. When removing samples, the storage canister should be held as far down in the Dewar as possible to avoid unnecessary thawing of the remaining samples. The samples should be removed quickly and transferred to liquid nitrogen contained in a styrofoam ice chest or a shipping Dewar that can be transported safely to the site of thawing.

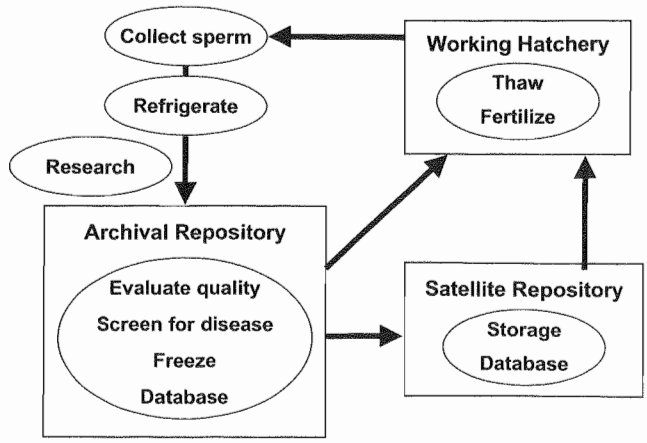

Figure 3. A scheme for a simple germplasm repository system based on three physical locations (shown in squares) providing archival storage, duplicative storage (satellite repository), and production and use of sperm. Activities are shown in ovals.

A successful cryopreservation program requires planning (e.g., Cloud and Thorgaard, 1993; Harvey et al., 1998; Tiersch, 2000). The development of a repository system offers maximum security and efficiency. In such a system, sites can be designated for specific activities and, at a minimum, should include an archival repository and a satellite repository (preferably geographically separated for protection against natural disasters) and a working hatchery or laboratory that utilizes frozen samples and provides broodstock or samples to go back into the system (Figure 3). Regional, national, and international regulations, treaties, and agreements are variously applied to transport of cryopreserved sperm (Jenkins, 2000a).

Thawing

Theoretically, the kinds of damage that can occur during freezing also can occur during thawing (Leung and Jamieson, 1991; Denniston et al., 2000). Hence, it is practical to thaw samples rapidly to minimize the period of vulnerability (Stoss, 1983). For warmwater fishes, a 7-second exposure to 40°C in a waterbath is useful (Wayman and Tiersch, 2000), although we typically test a range of thawing temperatures (e.g., from 0 to 60°C) to optimize protocols for each species (Wayman et al., 1998). Unless shown to be otherwise, it is advisable to use sperm for fertilization or motility analysis immediately after thawing, as these abilities can diminish rapidly (Stoss, 1983).

Fertilization

Fertilization of eggs of externally fertilizing fishes is well studied and can be carefully controlled (Billard et al., 1995;

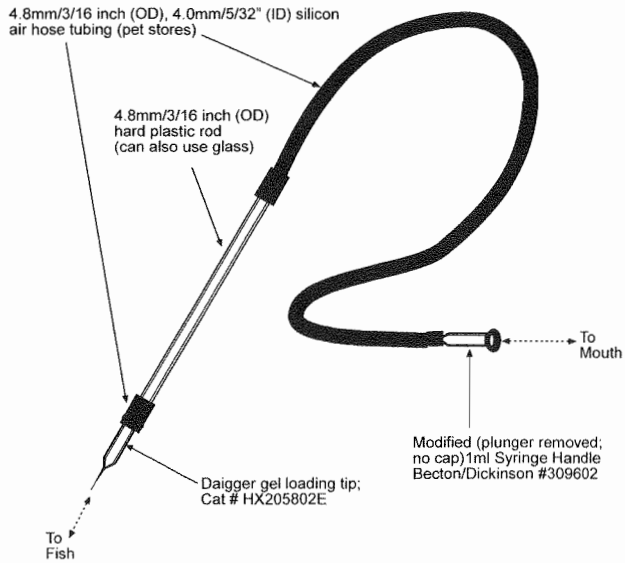

Figure 4. A simple device that can be used to overcome the difficult problem of fertilizing live-bearing fishes. Sperm can be transferred from the pipet into the recipient female (figure provided by Steven Kazianis, Southwest Texas State University).

Lahnsteiner et al., 1996). After development of initial techniques for cryopreservation, it will be essential to maximize the fertilizing ability of the limited numbers of sperm available from aquarium fishes. This can include optimization of cryopreservation procedures to improve motility and fertilization, as well as careful consideration of sperm-to-egg ratios (e.g., Lahnsteiner et al., 1996).

Work with internally fertilizing fishes will be more difficult. First, a specialized device and some skill is required to inseminate the females (Figure 4). Second, embryonic development occurs within the female and is not easily monitored as for external eggs. Experiments could utilize the percentage of gravid females within a population as an endpoint for fertilization, or employ the killing of females after insemination to identify the percentage of developing embryos. Neither method is straightforward, however, given that the females can store sperm (virgins are essential for these experiments) prior to fertilization of eggs, which complicates the choice of a sampling time after insemination. In addition, oocytes are not all fertilized at the same time. Multiple, developmentally separated broods exist simultaneously within females and can complicate the evaluation of within-female fertilization percentages.

An interesting situation exists for the mangrove fish, *Rivulus marmoratus,* which is the only known vertebrate to act as a natural functional hermaphrodite with internal self-fertilization. This fish is oviparous (produces eggs that hatch outside of the body) and would present the problems described above for artificial insemination and possibly others related to the fate of unfertilized eggs prior to shedding. The sperm of this fish has the typical morphology of externally fertilizing species. This, coupled with the observation that other members of the family Rivulidae and some individual *Rivulus marmoratus* practice external fertilization, suggest that internal fertilization developed recently in this species (Kweon et al., 1998). Sperm cryopreservation is unstudied in this family as well as in the live-bearers.

Evaluation

Evaluation of the success of cryopreservation can range from estimation of first cleavage to the fecundity of fish produced from cryopreserved sperm. Various end points for research include blastulation, gastrulation, neurulation, development of eye pigmentation, hatching, yolksac absorption, and initiation of first feeding. Due to a lack of standardization, each of these appears in the literature as estimators of percent fertilization, which complicates comparisons among studies. As stated previously, damage to sperm from cryopreservation can be manifested at different times during development, and the potential for confounding influences increases with age. For example, differential exposure to temperature, aeration, or stocking densities can cause spurious differences among treatment groups. It is therefore important to exercise rigid experimental design and control when assessing the effects of cryopreservation. A few studies have compared survival and growth beyond hatching of fish produced with fresh and thawed sperm (e.g., Kerby, 1983; Tiersch et al., 1994). Such experiments will be easily accomplished with aquarium fishes and would be a useful precursor to establishing germplasm repositories.

Future Applications and Links to Other Technologies

The true value of cryopreservation can be derived when it is integrated into existing programs (e.g., Tiersch et al., 1998). Often cryopreservation can make other technologies available. The following examples illustrate some possible applications of cryopreservation with aquarium fishes.

Carrier Lines for Cryopreserved Storage of Valuable Genes

Inbred lines of aquarium fishes bearing specific mutations or transferred genes can be of significant research value. It should be possible to develop specific inbred lines of aquarium fishes with little male-to-male variation in selected traits useful to cryopreservation, such as year-round production of large volumes of high-quality sperm that respond well to cryopreservation. Breeding of these fish with mutant lines could combine the desired mutation with desired cryopreservation traits. These carrier lines would also provide useful founder stocks for transgenic fishes.

Inbred Lines for Cryopreservation Research

Inbred lines of aquarium fishes that vary in their response to cryopreservation could provide an extremely valuable tool to dissect the responses of sperm cells, eggs, and embryos to freezing and thawing. Other research could address the effects of environmental manipulations, such as temperature, diet composition (e.g., Labbe et al., 1995) and water chemistry on the response to cryopreservation. Systematic screening could identify mutants of value to cryopreservation research.

Use of Tetraploid Broodstocks

Chromosome set alterations yielding three or four sets of chromosomes (triploidy or tetraploidy) occur naturally in fishes and are well studied (Ihssen et al., 1990). Typically, production of tetraploidy is experimentally accomplished by interference with the first mitotic division by temperature shock or application of pressure or chemicals, which yields a single undivided cell containing the equivalent of two nuclei. Upon maturity, these fish produce diploid sperm capable of fertilizing normal haploid eggs and producing offspring with three sets of chromosomes, a condition termed interploid triploidy. Such fish are functionally sterile due to aberrant meiotic segregation of the three sets of chromosomes. This raises the potential for distribution of cryopreserved sperm from tetraploid males to protect proprietary research lines. Sterile mutants could be produced from the cryopreserved sperm, which would replace the need to ship live fish for research or as broodstock. In addition, triploid transgenic fish offer a barrier to reproduction in the wild should fish escape, and would allow greater accessibility of research lines in areas where transport of live transgenic fishes is prohibited.

Utilization of Androgenesis

Androgenesis is another form of chromosome set manipulation that is well studied in fishes (Stoss, 1983; Ihssen et al., 1990). This natural phenomenon results in offspring that carry only two sets of paternal chromosomes. Androgenesis is induced in a manner similar to tetraploidy, except that maternal (egg) chromosomes are inactivated by irradiation (typically gamma or ultraviolet), which after doubling of the paternal (sperm) nucleus at first mitosis yields complete homozygosity. This process raises the possibility of recovering lost research lines or extinct species (Thorgaard et al., 2000). If cryopreserved sperm was available from the line or species, it could be used to produce homozygous offspring by androgenesis and irradiated eggs from another line or species.

Utilization of Intracytoplasmic Sperm Injection

Research on intracytoplasmic sperm injection (ICSI) in vertebrates began during the late 1970s when sperm injected into hamster eggs progressed to pronuclei and early cleavage was observed (Uehara and Yanagimachi, 1976). Since then, microinjection of sperm has become an important tool in basic and applied science. In mammals and amphibians, ICSI has been used to produce transgenic animals, and presently it is used to overcome problems of male infertility in humans and domestic livestock (Perry et al., 1997). There are a variety of applications for direct sperm injection into eggs of aquatic organisms, including the production of hybrids not possible by other methods, study of fertilization and embryonic development, preservation of endangered species, and the production of polyploidy. The technique has recently been evaluated for application in fishes (Poleo et al., 2001). Microinjection of single sperm into the animal pole by use of a pipet inserted through the egg micropyle (Figure 5) yielded fertilization, larval development, and hatching of zebrafish.

Because cryopreservation is not yet perfected or even attempted in most aquarium fishes, the use of low-quality samples including nonmotile sperm should be considered. Techniques such as ICSI can allow fertilization that otherwise would not be possible. In addition, germplasm repositories based on cryogenic storage in liquid nitrogen might fail due to technical problems, accidents, loss of key personnel, political pressures, or programmatic changes. These problems would be compounded in developing countries where the expense of liquid nitrogen could inhibit reposi-

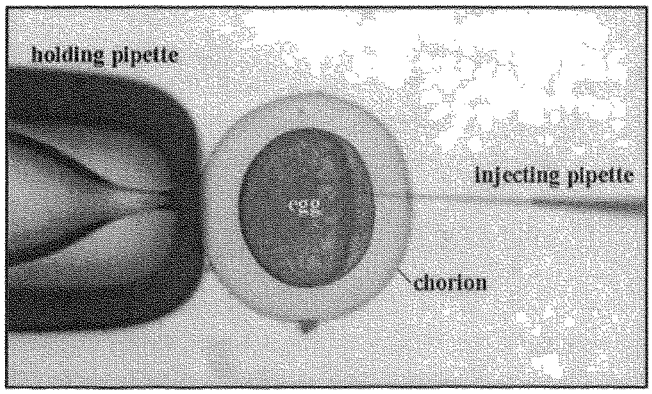

Figure 5. Intracytoplasmic sperm injection (ICSI) through the micropyle into the animal pole of a zebrafish egg. Normal larval development can result from this procedure leading to hatching, yolk sac absorption, and feeding (Poleo et al., 2001).

tory maintenance. Efforts should be made to identify methods that complement cryogenic storage. Development of techniques such as ICSI for use in aquatic species thus could not only reclaim damaged sperm, but would also open the door to use of other less costly methods of storage, such as freeze-drying or preservation in alcohol (Tateno et al., 1998; Tiersch, 2000).

A real benefit of this procedure would be in the conservation of endangered species. Well-developed ICSI procedures could utilize nonmotile sperm to produce founder populations for restoration that have the necessary genetic variation required for release into nature in recovery programs. Sperm injection can also be used to produce heterozygous androgenesis (Thorgaard et al., 2000). By injection of two (or three) sperm cells into an irradiated egg, non-homozygous androgenetic fish could be produced with fewer homozygous lethal alleles, yielding increased survival.

Summary

The ease of control of spawning and environmental variables in aquarium fishes provides numerous research benefits, hence their popularity as research models. This can facilitate the integration of cryopreservation studies with other research, such as the analysis of genetic mutations. The availability of research tools, such as inbred lines, can position aquarium fishes as research models for the study of cryopreservation and cryobiology. In fact, production of inbred lines can be assisted given the potential for self-fertilization by use of thawed sperm in sequential hermaphrodites. Cryopreservation in aquarium fishes can also assist development of research lines by enabling breeding schemes based on genetic analysis of sperm samples (e.g., Pittman-Cooley and Tiersch, 2000). It can be used to assist protection of genetic resources and endangered species through germplasm repositories and to increase availability of research lines through shipment of frozen sperm. Most importantly, the research infrastructure already in place for aquarium fishes could rapidly lead to its application and linkage with related technologies and provide a platform for the much-needed standardization of techniques. Thus, although little studied at present, cryopreservation in aquarium fishes could serve as a model to provide the missing connection between research and application of cryopreservation for all aquatic species.

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