Fish Sperm Cryopreservation

for Genetic Improvement and Conservation in Southeast Asia

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Introduction

Fish farmers are beginning to look more often to genetic improvement for gains in production. But improving the genetics of fish species can take a long time. With most fish, for example, it is difficult to keep track of individual males or females and thus the process of developing breeding stocks and improved lines could take a decade or more. At the same time fish managers are looking for ways to protect endangered species. Conservation programs require large populations to ensure biodiversity but for threatened and endangered species the numbers of fish are steadily decreasing. Cryopreservation can help in both of these situations. The availability of frozen sperm is a proven technique for developing, maintaining, and distributing genetic

The Two Questions Most Frequently Asked About Sperm Cryopreservation

Question #1: How long will the samples last?
Cryopreservation is a form of time travel. At cryogenic temperatures such as that of liquid nitrogen (-196 C), all biological activity ceases. That means for the frozen samples, time has stopped. Theoretically, the samples will last indefinitely, however, background radiation could cause damage to the DNA (which would not be repaired because the repair enzymes are not active when frozen). This damage could accumulate and in theory limit the lifetime of samples to thousands of years. In practical terms, the actual lifetime of samples is constrained by the quality and reliability of the storage facility. If the samples are repeatedly removed from liquid nitrogen (for example when someone is looking for particular containers) they can become damaged by warming to temperatures that are still well below 0 C. The bottom line is that with proper handling and security procedures, frozen sperm samples from fish should retain their ability to fertilize eggs long after the humans that froze the samples have died. Through cryopreservation we can truly pass our work on to our descendants.

Question # 2: Sperm is only half of the equation, what about the eggs?
There is a persistent notion that only "half of the genetics" of a species are passed on with sperm. In reality, half of the genetic makeup of *any individual* is passed on from the father (or mother for that matter); essentially all of the genetic variation within a species or population can be passed on through sperm other than the few specific genes associated with sex determination or sex-linked traits in those species where the female gamete determines the sex of the offspring (the so-called "ZZ-ZW" systems seen in birds and snakes and some fishes). The bottom line is that sperm can deliver the genetics necessary for conservation of endangered species, or for genetic improvement in aquaculture. Indeed, milk production in dairy cows is improved by genetic selection on bulls through cryopreserved sperm (i.e., the genetics of the father are used to improve the milk production of the daughter).

In addition, people sometimes feel that cryopreservation of sperm is of limited value if eggs are not available. However, there is no better way to ensure the availability of sperm when needed to fertilize a few precious eggs than by holding it ready by cryopreservation (fresh sperm can sometimes not be collected from males when eggs are available). In addition, in the extreme case where only frozen sperm remains from an *extinct species*, it is possible to inactivate the nucleus (DNA) of an egg from a closely related species and fertilize it with thawed sperm of the extinct species. The application of temperature change or certain chemicals at the specific time of cell division can be used to create offspring that contain only the DNA from the sperm (all-paternal inheritance). This naturally occurring process, called androgenesis, can be used in the hatchery to resurrect the genetics of an extinct species through cryopreserved sperm. This is admittedly a worst-case scenario, but it demonstrates that we should not hold back conservation programs and cryopreservation because of a fear that eggs may not be available. It also points out the importance of further research on the cryopreservation of embryos and larvae, which is currently successful in invertebrates such as oysters.

improvement in livestock, and provides great unexploited potential for fish breeding. In addition the availability of frozen sperm allows conservation programs to make a genetic bank of many males and increases the potential breeding population size to ensure that proper genetic combinations are produced in breeding of endangered species. In this way development of a single technology — cryopreservation — can assist two great needs in Southeast Asia: poverty alleviation through improved aquaculture and conservation of threatened and endangered fish species.

The promise offered by cryopreservation, however, resides in the future, and this raises a number of questions. First, what is cryopreservation? Second, how is it done and how can it be applied in this region? What will be needed to realize the tremendous advantages that it offers? This article will attempt to address some of these questions and provide models and suggestions to assist in the overall process of making the application of cryopreservation to Asian fish a reality.

Initial research success in sperm cryopreservation came more than 50 years ago for aquatic species and livestock. However, since then cryopreserved sperm of livestock has grown into roughly a billion-dollar global industry, while despite work in more than 200 species, cryopreservation of aquatic species sperm remains essentially a research activity with little commercial application. Most aquatic research work has focused on large-bodied aquaculture and commercial fish, such as salmon, carps, and catfish. Other groups such as mollusks, represented by commercially important oyster and abalone species, have received a fair amount of attention as well. However, only a handful of studies have addressed sperm cryopreservation in endangered species, or in small fish, which are becoming increasingly important in biomedical research and in the aquarium trade.

Application of cryopreservation for poverty alleviation?

There is growing evidence that many of the cultured fish stocks in rural Southeast Asia are now significantly degraded because of poor broodstock management practices. Most small-scale farms would benefit from improving their broodstock. In the future it may be impossible to replace or revitalize these fish as wild stocks are likely to be lost through either over exploitation or the destruction of their natural habitats. Bringing in semen is also a simple and relatively inexpensive way to outbreed fish stocks in remote areas once a network is set in place, as shown by the successful case of bull semen industry worldwide.

Overall, this work has yielded techniques that are being applied with varying levels of success around the world. However, barriers to expanded application include a diverse and widely distributed technical literature base, procedural problems, small and uneven sperm volumes, variable results, a general lack of access to the technology, and most importantly, the lack of standardization in practices and reporting. This report will focus on the needs and opportunities for future research and application of cryopreservation in freshwater fish. For the more technically inclined reader several excellent scientific review articles are available that focus on sperm cryopreservation in freshwater fish (**Table 1**). It should be noted here that the term “fish” is an artificial collective of more than 25,000 species characterized more by their differences than by their similarities. To discuss cryopreservation within fish is thus a balancing act of attempting to generalize observations into basic principles while acknowledging the considerable diversity that exists across these organisms.

Table 1. Examples of review articles, books, and special journal issues addressing cryopreservation of large-bodied freshwater fish.

Short Title	Citation
Biology, handling and storage of salmonid spermatozoa	Scott and Baynes, 1980
Fish gamete preservation and spermatozoan physiology	Stoss, 1983
Live preservation of fish gametes	Leung and Jamieson, 1991
Cryopreservation of fish spermatozoa	Rana, 1995
Cryopreservation in aquatic species (book)	Tiersch and Mazik (editors), 2000
Cryopreservation of gametes in aquatic species (special issue)	Lahnsteiner (editor), 2000
Cryopreservation of finfish and shellfish gametes	Chao and Liao, 2001
Techniques of genetic resource banking in fish	Billard and Zhang, 2001
Cryopreservation of gametes and embryos of aquatic species	Zhang, 2004
Cryopreservation and short-term storage of sturgeon sperm	Billard et al., 2004
Semen cryopreservation in catfish species	Viveiros, 2005

What is Cryopreservation?

The scientific research of cryobiology and cryopreservation can be traced back to the 1950s after the discovery of the protective qualities of glycerol for freezing of fowl sperm by Polge and colleagues. The first studies of fish sperm cryopreservation were published soon after by Blaxter in 1953, and since then hundreds of scientific papers have been published on research around the world. Cryopreservation is a process where biological materials such as cells and tissues are preserved by cooling to very low temperatures, typically, -196°C (for liquid nitrogen), yet remain viable after later warming to temperatures above 0°C. In essence, cryopreservation involves the removal of excess water from the inside of the cell to the exterior where it can form ice. Successful procedures balance the formation of ice crystals within the cells against excessive dehydration which damages cellular structures. For sperm cryopreservation, this process typically includes a series of steps (described in the sections below): 1) sperm collection and dilution, 2) refrigerated (non-frozen) storage and shipping of samples, 3) examination of sperm quality by microscope, 4) addition of cryoprotectants, 5) packaging of the samples, 6) freezing, 7) frozen storage procedures, 8) thawing, 9) use for fertilization, and 10) production of early life stages for assessment of cryopreservation success. Protocol establishment involves evaluation and optimization of multiple factors at each step (e.g., the type and concentration for each cryoprotectant), and recognition of the interactions among the steps (e.g. between cryoprotectant and cooling rate).

1) Sperm Collection and Dilution

Usually sperm from ripe males can be obtained during the spawning season by either stripping or by crushing of dissected testis. Stripping of sperm involves collection directly from the male into a sterile tube. Care must be taken to avoid contamination of the sperm with dirt, feces, water or urine. Dissection usually involves killing of the male and careful removal of the testis with scissors and forceps. Care must also be taken to avoid contamination with bacteria due to cutting of the intestine. Once removed, the testis is rinsed to remove blood and extra tissues are dissected away. The cleaned testis is weighed and placed in a container with an appropriate amount of extender solution (typically a buffer) before it is crushed. The sperm are released and the solution is filtered to remove pieces of tissue.

Dilution after sperm collection is necessary to maximize the volume for efficient use and for research of various factors. Dilution is usually in the range of one part of sperm to one to four parts of extender solution. Extreme dilution of sperm samples (e.g., 1:50) has been found to reduce sperm motility in mammals, fish and oysters. The term “extender” refers to a solution of salts, sometimes including organic compounds such as sugars that help to maintain sperm viability prior to and during the freezing process (e.g., Hanks’ balanced salt solution). The nature of the effect of extenders is based on the control of pH and salt concentration as well as the supply of energy, and can extend the functional life and fertilizing capability of the sperm. Usually, the extender is a balanced salt buffer of specific pH and osmotic strength. Sometimes other components such as egg yolk and milk are added, but they usually offer little benefit and can interfere with viewing of the samples with a microscope.

Extenders have been developed for many species. Hanks’ balanced salt solution has been used successfully in our laboratory with sperm of more than 50 species, but simple solutions such as 1% unbuffered salt (sodium chloride) have been used with good results. With appropriate testing, extenders can be prepared in large batches and be stored frozen until use. Use of extenders provides increased storage time and dilutes the sperm to a greater volume, making the sperm easier to work with. Extenders can be sterilized by passage through a filter or by autoclaving (if this does not affect the ingredients) and should be refrigerated. This is especially important for extenders that contain sugars. Antibiotics can be added to extenders to reduce the growth of bacteria that reduce sperm viability. Antibiotics at high concentrations can be toxic to sperm cells, therefore, concentrations should be optimized for each species.

The collection and transfer of sperm from fish poses concerns for unintended transfer of microbial organisms. Contamination of samples can occur during collection, processing, storage, and transport. Generally, proper sanitation during collection is essential for limiting the spread and growth of microorganisms such as bacteria, viruses, fungi, mycoplasmas, and parasites. Materials and equipment used to freeze samples should be sterile. Following good practice guidelines for handling and processing of samples is especially important for wild-caught animals where disease-free status cannot be guaranteed.

2) Refrigerated (non-frozen) Storage and Shipping of Samples

Sperm samples can be stored at 4 °C in an ice chest or refrigerator, but care should be taken to avoid accidental freezing of samples. In general, sperm samples are stored in shallow containers with the lids loosely attached to allow oxygenation of the sperm cells during storage.

Supplementation with pure oxygen gas has been shown to increase storage time, but could also be inadvisable depending on the species. Containers should only be partially filled, leaving a large air space between the sperm sample and lid and are inspected daily to provide mixing of the sperm cells. If not mixed, the cells can collect at the bottom and deteriorate.

It is often necessary to ship or transport refrigerated sperm or blood samples for analysis and cryopreservation. Frozen samples can then be shipped back to the original location (e.g., a hatchery) in shipping dewars for use at a later date. Typically it is essential that the samples remain unfrozen prior to cryopreservation. Although this would seem to be simple, experience indicates that samples are often destroyed by improper practices during shipment. There are several precautions that can be used to prevent such losses. The first is to ensure that the samples do not come in direct contact with ice or gel packs in the cooler. A simple cardboard divider can be useful for this (**Figure 1**), as is the precaution of wrapping the gel packs in paper towels. It is important to keep in mind that for warmwater fish we are trying to prevent the samples from becoming heated, not in keeping them as near freezing as possible. For valuable samples, a temperature data logger or a thermometer that records maximum and minimum temperatures can be included.

Collection and shipping of samples often requires more time than expected. It is a good idea to verify the address and phone number of the recipient well before the day of shipping. If storage time is a constraint, collections can be scheduled to finish at the time of shipping, although this requires careful planning. We have found that the best approach is to collect and ship samples in a preliminary rehearsal to identify problems and to avoid loss of valuable samples or data. This rehearsal should be performed using the same procedures and schedule planned for the actual samples, especially if factors such as automobile traffic can interfere with the schedule. The recipient should be notified when the samples are sent and should know when and where the samples will be delivered. Samples should be evaluated before they are shipped, and should be evaluated again immediately upon receipt. With sperm, for example, percent motility and general characteristics (e.g., color, presence of gelling) can be evaluated.

3) Examination of Sperm Quality by Microscope

Most fish spawn by releasing sperm and eggs into the water, a process called external fertilization. Unless certain ions are involved (such as potassium), in freshwater species, sperm motility (swimming) can be activated by reducing the salt concentration of the sperm solution (referred to as a "hypotonic" solution) in comparison to the blood concentration ("isotonic" solution). In marine species, sperm motility can be activated by increasing the salt concentration ("hypertonic" solution). Once activated, the sperm have a short life span (1-2 minutes) of active motility. Thus the sperm need be maintained in an extender with proper salt concentration (usually nearly isotonic to the blood plasma) to inhibit undesired sperm activation during refrigerated storage or cryopreservation.

In some species of fish, sperm remain motile for short periods of time (less than 20 seconds). This makes estimation of motility difficult and samples may need to be evaluated several times to produce an accurate estimate. The use of activating solutions can increase the duration of motility in some species, but complete knowledge of the effects of osmotic activation should be understood for a species before such activation solutions are used. In other species, such as some marine fish, sperm can remain active for as long as 30 min, which simplifies estimation of motility. It is important to ensure that sufficient dilution of sperm is used to elicit maximal activation for each sample.

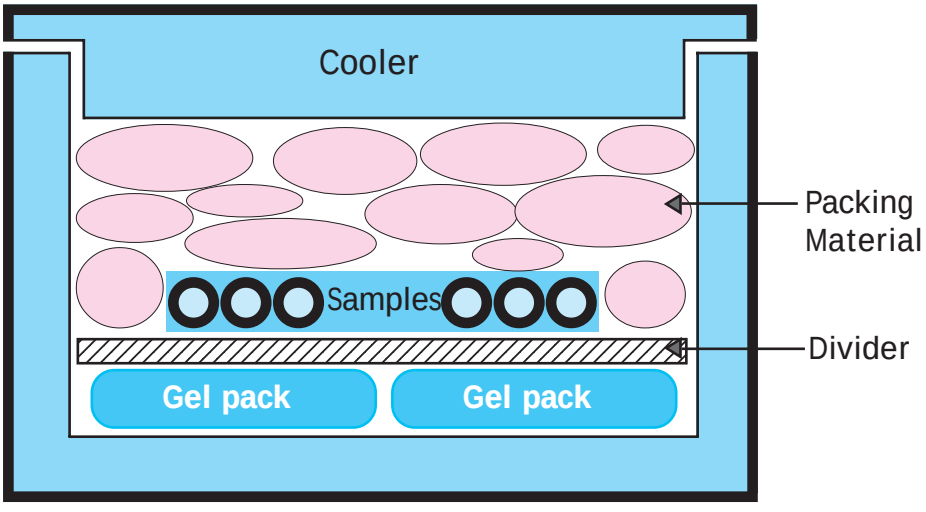

Figure 1. Proper packaging of samples for refrigerated storage during shipment. Samples should be placed in a waterproof bag with protection from freezing during shipment

For estimation of percent motility, only sperm that are actively swimming in a forward motion should be included. Sperm that remain in place with only a vibratory movement should not be included. The procedure can be practiced to ensure that sperm movements are not due to swirling of the activating solution or random movement. Some microscopic organisms (e.g. bacteria) are motile and can be mistaken for sperm by inexperienced observers. The exact procedure used for motility estimation should be reported in sufficient detail to assist in making comparisons among studies.

4) Addition of Cryoprotectants

Typical cryopreservation of sperm cells involves the use of chemicals called cryoprotectants and slow freezing to produce cellular dehydration and shrinkage. Cryoprotectants are chemicals used to protect cells from damage during freezing and thawing, and are classified by whether they penetrate the cell (referred to as "permeating") or remain outside of the cell ("non-permeating"). Although their mechanisms of action are as yet not completely understood, permeating cryoprotectants such as dimethyl sulfoxide are believed to help reduce damage due to the formation of ice crystals within sperm cells. They also help reduce the dehydration damage that occurs when water leaves the cell to become ice in the surrounding solution. Non-permeating cryoprotectants such as sugars and polymers are believed to help stabilize the membrane during cryopreservation. Too little cryoprotectant entering the cell before cooling reduces effectiveness, whereas too much causes swelling and rupture during thawing and dilution.

In addition, cryoprotectants are often toxic to cells, and thus the choice of the types of cryoprotectant and their optimal concentration (a balance between protection and toxicity) has been the focus for numerous studies. After the addition of cryoprotectants to sperm, time is needed for the cryoprotectant to permeate the cells. This is referred to as the equilibration time. For most circumstances it can be set at 15 to 30 min, but it can be varied depending on the type and concentration of cryoprotectant being used. If the necessary concentration of cryoprotectant is toxic to the cells, the equilibration time of rapidly permeating cryoprotectants such as DMSO can be shortened to the minimum time required for filling of straws.

5) Packaging of Samples for Freezing

In cryopreservation, packaging of samples for freezing and storing is important to standardize cooling rate, and to assure sample identification. Various containers have been used for fish sperm, including drinking straws, glass capillary tubes, and plastic cryovials. Plastic French straws have been in use for livestock sperm for decades. These straws come in one-half and one-quarter milliliter volumes, and are produced in over 20 colors. The use of French straws offers the advantages of efficient and reliable sample identification by permanent printing on colored straws, sample safety by complete sealing of the straw, and standardization of the cooling and thawing processes because of their thermal properties and large surface area in relation to their volume.

Proper labeling of cryopreserved samples is essential. Usually samples are in storage for weeks, months, or even years before they are thawed. Improperly labeled samples can cause delays in processing, and even worse, could cause genetic contamination of pure stocks of fish. The necessity for proper labeling cannot be overemphasized. The value of samples is directly proportional to the quality of labeling information and record keeping. Unlabeled or poorly labeled samples are essentially worthless and can even be detrimental. At the minimum, straws used for research should be labeled to indicate fish identification number, cryoprotectant, and cryoprotectant concentration. A simple method for labeling is to use straw color for identification of cryoprotectant and a system of marks on the straws to identify fish number and cryoprotectant concentration. If possible, more sophisticated labeling, such as pre-printed straws, should be considered even for research applications. Straws intended for archiving and breeding uses should receive the best labeling possible (e.g., **Figure 2**).

6) Freezing

The choice of optimal cooling rate has been another major focus of numerous studies of sperm cryopreservation. To be considered as optimal, a rate should be slow enough to minimize the amount of ice crystals that form within the sperm cells (below a damaging level) and yet be rapid enough to minimize the length of time cells are exposed to what is referred to as the "solution effect", which is the concentration and precipitation of materials that occurs when solubility limits are exceeded during the dehydration caused by ice formation. There are a number of methods that can be used for freezing. These include the use of computer-controlled freezers which offer precise and reproducible rates, but are usually expensive. Simpler and cheaper freezing is available by suspending samples above liquid nitrogen in a styrofoam cooler, although this is a less reproducible approach. Samples can also be suspended in the neck of a storage dewar. With these latter two methods, the height of the samples above the liquid nitrogen offers control of the temperature and cooling rate.

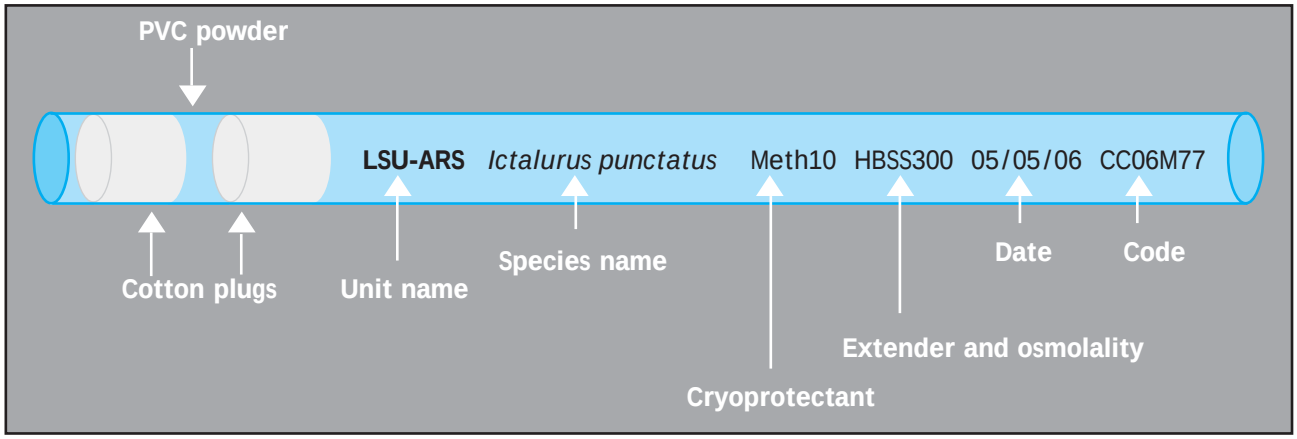

Figure 2. An example of the labeling possible for use with standard French straws. One end comes with a factory plug that is sealed by moistening with sperm suspension. This labeling is produced by a specialized laser printer. In this example, sperm from a channel catfish (*Ictalurus punctatus*) were frozen using 10% methanol ("Meth 10") as the cryoprotectant and Hanks' balanced salt solution prepared at 300 mOsmol/kg ("HBSS300") as the extender. An inventory code is used for identification of individual animals where "CC06M77" refers to "channel catfish 2006 (year) male 77 (adapted from Tiersch and Jenkins, 2003).

Another method is to use a shipping dewar which is a container designed to allow liquid nitrogen to be safely transported. The liquid nitrogen is adsorbed by a filler material within the sides of the dewar allowing maintenance of cryogenic temperatures without the dangers of spills. Nitrogen-vapor shipping dewars were designed to transport cryopreserved materials and to maintain cryogenic temperatures for as long as 3 weeks, but they can also be used to freeze samples in situations where other freezing options are not available (such as in field applications). To freeze in shipping dewars, straws are placed in goblets (plastic cups) and fastened on aluminum canes (holders). The canes are placed in the canister and lowered into the dewar. After 30 minutes the samples should have reached temperatures below -100 °C and can safely be moved to storage dewars. Alternatively, the samples can be left in the shipping dewar if a storage dewar is not available. Cooling rates can be manipulated to some extent by varying container (e.g. straw) size, position within the dewar (top or bottom) or the number of straws per goblet. In addition, straws can also be frozen individually without canes or goblets. The cooling rate can be monitored by use of a thermocouple and recorder. Cooling rates will vary in shipping dewars due to a variety of factors including the time since filling, the number of straws being frozen and the amount of use.

Safety first

Safety precautions are necessary when working with liquid nitrogen. Insulated gloves and safety glasses should always be worn when handling liquid nitrogen. Never place objects cooled by liquid nitrogen against unprotected skin. Use only containers designed for use with liquid nitrogen. Use proper transfer equipment to move and handle samples. Never use hollow rods or tubes as dipsticks, because liquid nitrogen can be driven out of the open end. The opening of any liquid nitrogen container should never be covered or plugged because considerable pressure will build up as the liquid nitrogen vaporizes. In addition, nitrogen gas can collect in closed areas, displacing the air, and create a potential suffocation hazard, so all work should be performed in well-ventilated areas.

7) Frozen Storage Procedures

Storage dewars are designed to safely store cryopreserved samples in liquid nitrogen (-196 C) for extended periods of time. They use a vacuum chamber to provide insulation (**Figure 3**). Liquid nitrogen within the dewar will evaporate over time and must be replaced. The use of alarms on storage dewars is highly desirable. The alarm sounds when the temperature at a probe rises above a certain level, indicating that liquid nitrogen needs to be added to the dewar. The positioning of the sensing probe should take into account the margin of safety required between the sounding of the alarm and the replenishment of liquid nitrogen. For example, would you feel safe with 1 day or 1 week before the uppermost samples began to thaw? The alarm should remain on at all times and be tested regularly. A periodic visual check is also advised in case the alarm should fail. A log book for liquid nitrogen additions can help identify a possible failure of the vacuum seal and assist in estimating storage costs. Care should be taken to ensure that the outer casing of the dewar is not punctured.

The loss of vacuum will boil off the liquid nitrogen rapidly. Rough handling can cause weakening of the inner neck area, and reduce the working lifetime of the dewar. A roller base will allow safe and easy movement of the dewar.

When removing samples from storage dewars, keep the canister as far down in the dewar as possible to avoid unnecessary thawing of the remaining samples. Remove the samples quickly (an inventory database and proper labeling will reduce searching time) and transfer the samples to liquid nitrogen contained in a styrofoam ice chest. Careless handling of frozen samples can allow them to warm to temperatures that allow formation of intracellular ice crystals, which will damage the cells.

8) Thawing

In general, rapid thawing is preferred to minimize the damage associated with recrystalization (the coalescence of small ice crystals into large crystals during thawing). Samples should be removed from the storage dewar and transferred immediately to a styrofoam ice chest containing liquid nitrogen. This ensures that the samples will not thaw prematurely due to handling. The 0.5-mL straws are held in a 40 to 50 °C water bath (a thermos or small ice chest will work) for ~7 seconds. Specific times and temperatures should to be optimized for the particular species. We have tested a range of thawing temperatures (e.g. from 0 to 60 °C) to optimize protocols for many species. As a rule of thumb, samples are thawed when air bubbles within the straw can move freely within the liquid. The use of transparent or translucent straws will aid in viewing the sample. The samples should be cool to the touch when thawed, not warm.

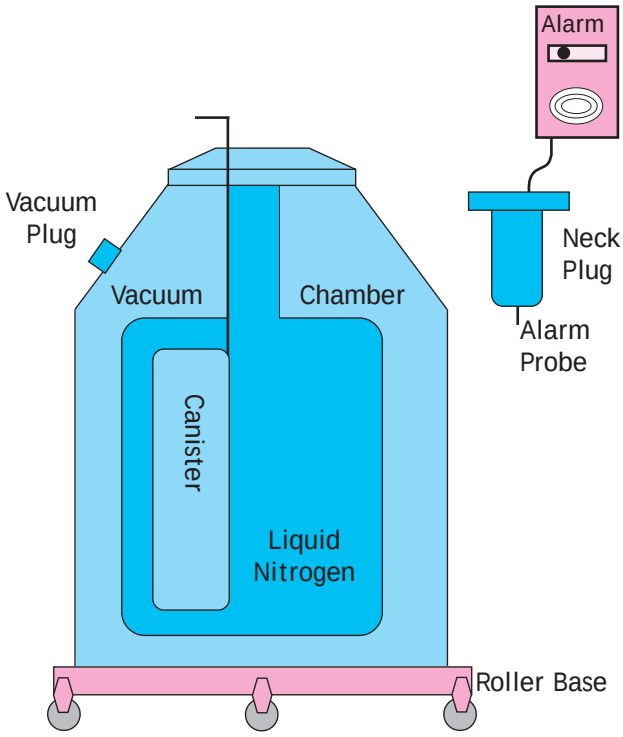

Figure 3. Diagram of a liquid nitrogen storage dewar (with permission from Wayman and Tiersch).

Sperm motility should be estimated after thawing as described above. Qualitative observations of thawed sperm can be quite helpful in evaluating protocols. For example, if the sperm cells are visibly damaged, the cryoprotectant concentration may have been too low, or the cooling rate may have been too rapid. Conversely, if the cell morphology is intact although the sperm are immotile, the concentration of the cryoprotectant may have been too high. This would be confirmed by a reduction in the motility observed immediately before freezing.

9) Use for Fertilization

Artificial spawning involves the collection from females of unfertilized eggs to be combined with sperm. Unless testing has shown otherwise, it is generally best to minimize the time between thawing of sperm and fertilization. After thawing, sperm samples are added to eggs and thoroughly mixed, and the gametes are activated with an appropriate solution (this is called the “dry method”). Other fertilization methods are available and can be evaluated for use with any particular species. Fresh sperm samples can be used to fertilize other batches of eggs to serve as a test for egg quality. After ~5 minutes, additional water is added to water-harden the eggs. Percent fertilization can be determined to evaluate gamete quality. Estimates of sperm concentration can be made by a number of methods, and can be used to calculate the ratio of sperm to eggs.

Artificial spawning in this way allows for a variety of crosses such as the use of one male to fertilize eggs from several females, or for the eggs of one female to be fertilized by sperm from several males. This can lead to a breeding matrix where a group of select males can be mated with a group of select females to develop populations with distinctive traits. By having assayed the parents for genetic markers, breeders can develop broodstocks with enhanced characteristics, such as growth rate or disease resistance. Such a process can also be used to cross two different species to develop hybrids with improved traits, although precautions should be taken to prevent the accidental escape of the hybrid fish into the wild.

10) Production of Early Life Stages for Assessment of Cryopreservation Success

With respect to cryobiology, it is important to note that even simple cells such as sperm have a high degree of internal complexity. The various structures within a sperm

cell represent different functional compartments that can each require different optimal conditions, and thus can each exhibit differential responses to cryopreservation. This can cause a variety of damages and outcomes. For example, damage to the tail could interfere with motility whereas damage to the head could interfere with embryonic development. Sperm quality is a generic term that encompasses proper function of a combination of cellular structures (such as the head, membrane, and tail) that can be superficially evaluated individually by specific assays or in aggregate by examining factors such as the capacity of sperm to fertilize eggs that hatch and develop normally. For this reason it is important to evaluate the quality of thawed sperm by fertilizing eggs and monitoring the development of the offspring.

Current Status of Fish Sperm Cryopreservation

For sperm cryopreservation to become a reliable, cost-effective tool for genetic banking in aquatic species, the overall process needs to be improved, and the approach needs to be integrated into an efficient large-scale platform that links with genetic and biological databases, long-term storage capabilities, inventory management, quality control, sample distribution pathways, biosecurity assurance, utilization and disposal practices, and a sound cryobiological foundation. Numerous studies in sperm cryopreservation have been devoted to optimizing specific components of cryopreservation procedures. However, aside from those factors mentioned above, other factors such as sample density, freezing container, starting temperatures, final temperatures (before plunging into liquid nitrogen), and dilution and cryoprotectant removal after thawing can also affect results. Therefore, procedures must be tailored for each species or population based upon a thorough understanding of cellular properties.

A current problem in cryopreservation research is the lack of standardization within the scientific literature for aquatic species in each step involved in the process. Comparisons among different studies are difficult to perform and could be invalid in most cases due to the procedural and reporting variations across studies. Optimization of protocols without standardization offers little value for the improvement of existing methods and results, especially for the future development of commercial application. Controversy and inconsistency would be reduced if more congruent approaches were utilized and results among various studies could be directly compared. Suggestions for improvement include the creation and widespread acceptance of standard references to assist in harmonizing terminology, and the development and utilization of standardized educational programs. Standardization of research practices and reporting could be accomplished through establishment of guidelines for publication of results. Once in place the guidelines could be made available to journal editors and reviewers to assist in evaluation of research reports.

The Future Prospects and Models for Application of Cryopreservation in Fish

Cryopreservation research and application each require consideration of an interconnected series of activities and this involves more than simple freezing of samples. A successful program involves integrated practices for sample collection, refrigerated storage, freezing, thawing, rules for use and disposal, transfer agreements, and database development. This concept is usually described within the activities of a germplasm repository at a single facility (**Figure 4**). The application of cryopreservation offers many benefits. With respect to commercialization, the benefits of cryopreservation include at least five levels of improvements that address existing industries and the creation of new industries.

1) Cryopreservation, at a minimum, can be used to improve existing hatchery operations by providing sperm on demand and greatly simplifying the timing of induced spawning. This prevents the problem, for example, of collecting ripe eggs, but not having sperm available to fertilize them.

2) Frozen sperm can greatly enhance efficient use of facilities and create new opportunities in the hatchery by eliminating the need to maintain live males. Potentially all of the resources in a hatchery, which are typically limited, could be diverted to use for females and larvae.

3) Valuable genetic lineages that currently exist, such as endangered species, research models or improved farmed strains can be protected by storage of frozen sperm. This could be very important for species such as shellfish in which valuable broodstocks must be stored in natural waters.

4) Cryopreservation opens the door for rapid genetic improvement. Frozen sperm can be used in breeding programs to create new improved lines and shape the genetic resources available for aquaculture operations. A dramatic example of this potential opportunity is provided by the dairy industry, which relies almost entirely upon cryopreserved sperm to produce improvements in milk yields.

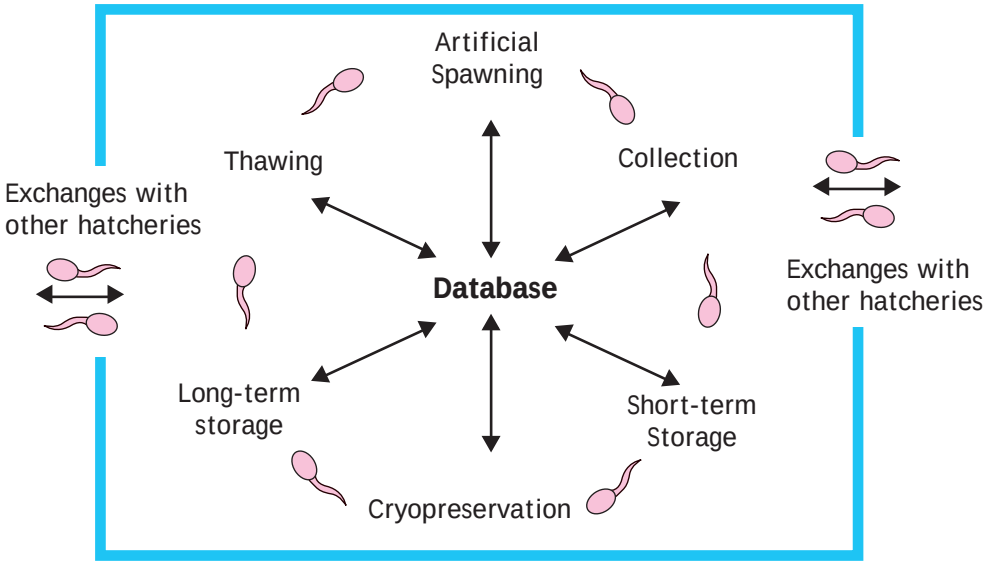

Figure 4. Activities of fish sperm cryopreservation at a single location (linked in a circle). Consecutive components are shown as a clockwise flow of sperm and two-way arrows are used to indicate maintenance of a centralized database for information on motility, quality control points (shown as sperm), fertilization, and inventory. Refrigerated (right side) and frozen sperm (left) can leave the facility (with permission from Caffey and Tiersch).

5) Cryopreserved sperm of aquatic species will at some point, likely within the coming decade, become an entirely new industry itself. The global market for dairy bull sperm is around 1 billion dollars each year. Large, highly valuable global markets for cryopreserved sperm of aquatic species are now on the horizon.

Sperm cryopreservation in aquatic species is only beginning to find application on a commercial scale. The development of this new industry is constrained by a number of factors including the technical requirements for scaling-up to commercial operations during the transition from research. This problem has been addressed by research in our laboratory over the past 10 years that documents the feasibility of utilizing commercial dairy cryopreservation facilities to provide a jumpstart for cryopreservation in aquatic species such as catfish and oysters. The dairy bull industry provides a business model for developing commercial application for cryopreserved sperm of fish (**Figure 5**). In addition, industries such as this can provide equipment, protocols, facilities, and distribution networks that can be adopted for use with fish sperm.

Other challenges for commercial development include disease concerns for sample transfers, pricing structures, and product quality control issues. The presence of a cooperative framework across species can assist finding

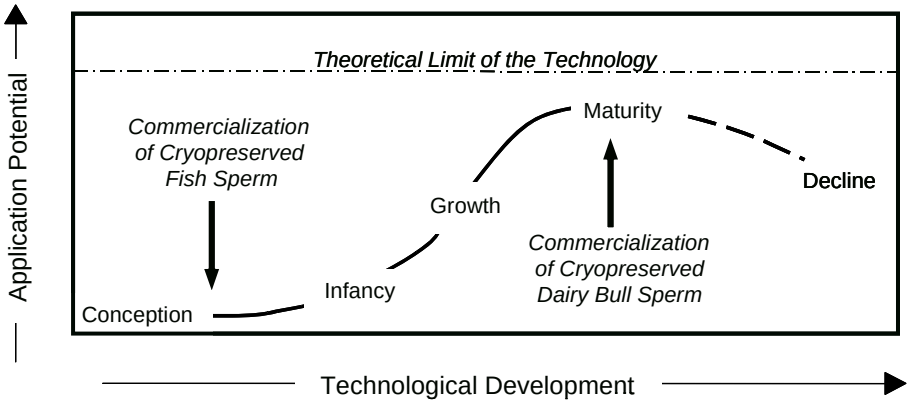

Figure 5. Conceptualized market life cycle for application of cryopreservation technology. Cryopreservation of dairy bull sperm is an example of a mature technology, while the commercialization of fish sperm cryopreservation is currently somewhere between conception and infancy (with permission from Caffey and Tiersch).

solutions for problems such as these. A model for developing multi-species repositories for genetic resources comes from the newly formed National Animal Germplasm Program (NAGP) of the United States Department of Agriculture (USDA). The NAGP is patterned after the well-established USDA National Plant Germplasm System. The NAGP has committees for beef and dairy cattle, swine, goats and sheep, poultry, and aquatic species. The Aquatic Species Committee brings together members from universities, industry, and federal agencies. A structure such as this could assist development of repositories within and among Southeast Asian countries. Indeed, a useful model for aquatic species everywhere is the development of an integrated repository system that incorporates a single or a few well-equipped, experienced central facilities that carry out most of the cryopreservation work using samples or broodstock sent to the facility. Other facilities can serve as satellite repositories to protect backup samples, and as user endpoints for the samples such as hatcheries (**Figure 6**).

The cryopreservation research in our laboratory at the LSU Agricultural Center is intended to assist the transition from cryopreservation research to application through work on protocol standardization, gamete quality, sample labeling, and database development to provide a repository to protect genetic resources, including endangered species, and to assist in developing existing and future industries for culture of aquatic species. Work such as this needs to be done in Southeast Asia as well. Overall, beyond the initial development of facilities, procedures, and training of personnel, the largest practical constraints to realization of a cryopreservation industry for fish is at present the absence of uniform quality control practices, industry standards, and appropriate disease transfer safeguards. The control of the movement of pathogens into and out of a facility or area is referred to as biosecurity. This topic will assume great importance in the future for use and transfer of frozen samples.

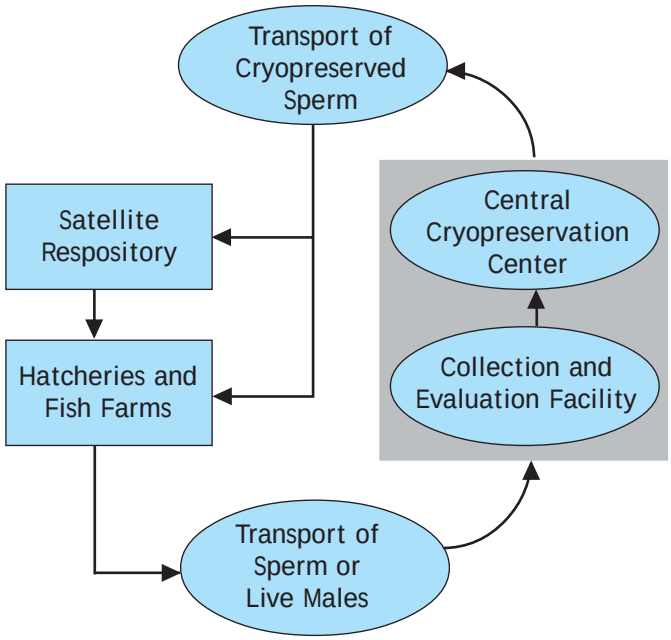

Figure 6. A model for a simple germplasm repository system based on three physical locations (shown in rectangles) providing a quarantine area for incoming samples, archival storage (central repository), backup storage (satellite repository), and production and use of sperm (working hatchery). Activities are shown in ovals, and transfers of samples are shown by arrows.

Caveats for application of cryopreservation to threatened and endangered (T & E) species

1) Technology is seductive and not always necessary.
2) The techniques of cryopreservation are unrefined or non-existent for most species.
3) Cryopreservation is a tool, not a final solution.
4) Cryopreservation does not protect habitat.
5) Cryopreservation does not replace existing management plans.
6) Cryopreservation can buy time or be a waste of resources.
7) There are few ethical guidelines for the application of cryopreservation to conservation.

In order for cryopreservation to assume a functioning role in assisting aquaculture production and aquatic species conservation it has to proceed beyond development of the initial technical requirements. A series of activities are required to be in place for full-scale application. These activities have not yet been fully implemented anywhere for any aquatic species. There are problems and barriers at each step, but getting started is usually the hardest part. The entry-level requirements for equipment, facilities, and training are high and force potential users to focus on technology development and technical problems. However after this hurdle is passed, the focus can shift to coordination of activities and realization of the great opportunities provided by cryopreservation. A final phase of maturation in application would see cooperation and connections forming among governmental agencies, non-governmental organizations, academic institutions, and private companies.

The major activities in this potential pathway for application are listed below:

1) Development of *technical capabilities and facilities* at well-funded and secure locations.
2) Establishment of *training programs* for procedural efficiency, and recruiting of personnel.
3) Development of *appropriate biosecurity safeguards* to control movement of pathogens in and out of facilities.
4) Development of a *functioning storage repository*, with rules for use and disposal of samples, and with appropriate security for basic services (e.g., electricity, liquid nitrogen, refrigeration capabilities, and aeration for aquaria and holding tanks)
5) Implementation of archival *labeling of samples and the creation of robust databases* capable of handling biological information concerning samples (including geographical information system (GIS) data on collections), and maintaining correct inventory and identification of sample locations.
6) Further development of capabilities *computing and information transfer* including the ability to interact and exchange information with other databases.
7) Increasing of the sample processing capabilities to enable *high throughput of samples*. This would include installation and use of automated or semi-automated equipment for labeling, filling, and sealing of straws, and the procurement of commercial-scale freezing and storage capabilities.
8) After central facilities have developed strong operational capabilities, a sustained effort should be made to develop *cooperation with other organizations* and facilities. These relationships can include sharing of samples, capabilities and expertise. Efforts should be made to link cryopreservation with existing or planned activities such as fish sampling programs or cooperation with specialized hatcheries during spawning seasons.
9) To assist interactions among organizations, basic arrangements should be discussed and put forth as *formal transfer agreements* that can be negotiated and put in place to describe things such as responsibilities, rights, and ownership of samples.
10) Because different facilities will have different approaches there should be establishment of *quality control protocols and standardization* of labeling, terminology, reporting of results, and databases.
11) Essentially, individual repositories can at this point be linked by *establishment of a full repository system*, and end users of cryopreserved sperm, such as both small-scale and commercial hatcheries and farms can interact with this system.
12) *Coordinated regional activities* can take place in individual countries or be administered across borders to encompass river systems or ecosystems.

Ethics discussion topics for application of cryopreservation to conservation

1) Cryopreservation is a form of time travel.
2) We cannot be certain of how frozen samples will be used in the future.
3) Use and disposal of cryopreserved samples will require rules.
4) Quality control and quality assurance will be required.
5) The value of frozen samples is determined by record-keeping and database management.
6) Cryopreserved material can be used for genetic reconstitution instead of production.
7) Genetic management can be intentional or unintentional.
8) Organisms considered to be desirable or innocuous today, may not be so in the future.
9) Diseases of today could be worse when reintroduced in the future.
10) We cannot predict the interactions of today's organisms with those of the future.
11) Species do not live in isolation: it takes a community.
12) Resource allocation is necessary for cryopreservation.
13) Cryopreservation could be viewed as mitigation for development activity.
14) Private sector involvement is possible for cryopreservation of T & E species.
15) Saving a species can transform it.

Acknowledgements

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Regional Training on the Cryopreservation of Freshwater Fish Semen

From 7 to 12 August 2006, SEAFDEC held a one week training aiming at introducing the principles and main applications of cryopreservation of aquatic species in the Southeast Asian region. Researchers from the different SEAFDEC member countries were invited to participate to a seminar which was followed by a hands-on workshop hosted by the Thai Department of Fisheries at the Aquatic Animal Genetic Research and Development Institute (AAGRDI) located in Pathumthani, which has state of the art facilities. The author was invited in the capacity of resource person and shared his experience and expertise acquired at LSU Agricultural Center. Other experts from the region were also invited to share their experience with the researchers from the region, notably Dr. Amrit Bart from the Asian Institute of Technology and Dr. Veerapong Vuthiphandchai from Burapha University.

The training aimed at being a first step toward a more widespread use of cryopreservation as an important tool for the promotion of aquaculture and the conservation of endangered fish species, with a special focus on freshwater species. While the activities had a strong focus on technology, a discussion was initiated on what could be the applications of cryopreservation of aquatic species in Southeast Asia. Benefits were identified during the dialogue between regional participants both for commercial and small-scale fish farming, as well as for the conservation of endangered freshwater species. Such a regional exchange of ideas was found essential and the participants emphasized that it should be continued in the future. Possibilities for doing so under a similar SEAFDEC initiative will be explored.

Regional Training on the Cryopreservation of Freshwater Fish Semen

Readings

A comprehensive reading offering a good overview and insights:

Tiersch TR and Mazik PM (editors). 2000. Cryopreservation in Aquatic Species. World Aquaculture Society. Baton Rouge, Louisiana, USA. 439 pages.

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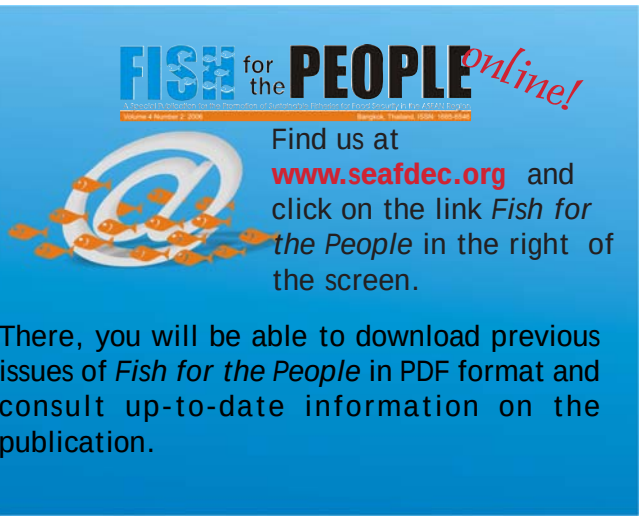
Dr. Terrence Tiersch is a Professor of aquaculture genetics, and has worked at the Aquaculture Research Station of the Louisiana State University Agricultural Center in Baton Rouge since 1992. In 2000 he was the lead editor of the book entitled Cryopreservation in Aquatic Species which is the only comprehensive reference work in the field. He serves as Chairman of the Aquatic Species Committee of the USDA National Animal Germplasm Program. The author can be reached at *TTiersch@agcenter.lsu.edu.*



INVITATION TO CORRESPONDING WRITERS

With many issues of *Fish for the People* already published, we hope that we have given you a good idea of the aims and general tone of the publication. Recently, we have been publishing more articles from external contributors. We are further inviting contributions from writers interested in promoting relevant issues on fisheries in developing countries. While the publication will continue to focus on the Southeast Asian region, future issues can address relevant issues from other tropical regions.

Fish for the People is a policy-orientated publication. It is not a forum for publication of research findings, nor is it intended to provide detailed technical information. The publication targets not only experts or scientists, but also other traditionally less technically-oriented fisheries stakeholders, such as policy-makers, donors, government staff, managers, and more generally, an informed lay public with an interest in how our fisheries are managed.

Readable, accessible articles that address the various issues discussed at the ASEAN-SEAFDEC Millennium Conference are most desired. Articles should focus on newly emerging issues relevant to sustainable regional or tropical fisheries management. They should present important issues with clear regional messages, emphases, thrusts, problem areas, and propositions for improving current situations.

Through *Fish for the People*, we hope that authors will gain the attention and consideration of targeted fisheries stakeholders, and contribute to the future achievement of more sustainable fisheries.

Correspondence related to editorial matters should be sent to *fish@seafdec.org*