Research Methods for Cryopreservation of Sperm

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In this section, we have chosen to provide a single integrated set of methods as a starting point for development of protocols and standardization of reports. These methods have been adapted for sperm cryopreservation of more than 30 species of marine and freshwater fishes in our laboratory. The information provided here can be supplemented with methods and information in other chapters to develop new protocols or to work with previously unstudied fishes. There is also a large amount of practical information available for topics such as cryopreservation and freeze-drying methods (Simione and Brown 1991, Day and McClellan 1995), cryopreservation of algae (Morris 1981) and semen of boars (Johnson and Larsson 1985, Johnson and Rath 1991). Sources such as these can be valuable for developing and using cryopreservation protocols even though they are not intended to address aquatic species. Overall, there are several basic components involved in development of cryopreservation protocols. These will be covered individually in the following sections: 1) Sperm collection; 2) Motility estimation; 3) Extenders and refrigerated storage; 4) Labeling of straws, goblets and canes; 5) Addition of cryoprotectants; 6) Filling of straws; 7) Freezing procedures; 8) Storage procedures, and 9) Thawing and fertilization. Each component is important, and just as links form a chain, failure of any single component can lead to failure for the entire project.

Sperm Collection

Stripping

1) Anesthetize fish with MS222 or suitable anesthetic.
2) Blot fish dry with towel to prevent water from mixing with sperm.
3) Gently massage abdomen to expel sperm, being careful not to contaminate sperm with urine or feces.
4) Collect sperm in microhematocrit tubes, syringes, or centrifuge tubes depending on the amount of sperm to be collected.
5) Measure volume of sperm and dilute with extender solution (1:3, 1:5, 1:10, etc. depending on species, concentration, or necessity).

In some species, pressure applied under the pectoral fins can cause release of sperm. This technique minimizes contamination of sperm with urine or feces, which can cause activation of sperm or bacterial contamination. Catherization can reduce contamination of sperm, but it is often impractical. Dilution of samples in extender at the time of collection can counteract the effects of contamination by urine or water. This effect can be tested in studies of refrigerated storage.

Surgical Removal of Testis (for fishes from which sperm cannot be stripped)

1) Anesthetize fish with MS222 or suitable anesthetic.
2) Blot fish dry with towel to prevent water from mixing with sperm.

W. R. Wayman and T. R. Tiersch. 2000. Research Methods for Cryopreservation of Sperm In: *Cryopreservation in Aquatic Species*. Tiersch, T. R. and P. M. Mazik, Editors. World Aquaculture Society, Baton Rogue, Louisiana. Pp. 264-275.

3) Surgically remove testis from fish.
4) Remove excess blood and tissue from testis.
5) Weigh testis.
6) Measure extender solution (10 to 20 mL per g of testis).
7) Crush or slice testis to release sperm.

To crush testis: Place the testis in a resealable plastic bag (e.g. ziplock®), remove air, and add enough extender solution to keep testis in liquid while crushing. Apply pressure to testis in a rolling motion using a small beaker, small bottle, or other round object. Strain smashed testis and extender solution through cell separator (or screen) to remove testicular tissue. Add remaining extender solution to bag to rinse. Add extender from bag to extended sperm.

To slice testis: Place the testis in a resealable plastic bag. Slice the testis into segments to release sperm, using care to avoid cutting the bag. Add half of extender solution to bag and mix. Pour contents of bag through cell separator (or screen) to remove testicular tissue. Add remaining extender to bag to rinse, strain debris and combine the extended sperm.

It is advisable to collect samples of blood plasma or serum, seminal plasma, ovarian fluid and water from the site of fish collection or spawning to measure osmotic pressure. These samples can be frozen in the field.

Motility Estimation

1) Place 2 μL of sperm on microscope slide.
2) Add 20 μL of activating solution, for example, deionized water for freshwater species (20 mOsmol/Kg) or artificial sea water for marine species (800 mOsmol/Kg)
3) Mix thoroughly.
4) Estimate percentage of progressively motile sperm using 100 or 200-X dark-field microscopy.

In some species of fish (e.g. razorback sucker *Xyrauchen texanus*), sperm remain motile for short periods of time (<10 sec). This makes estimation of motility difficult and samples may need to be evaluated several times to yield an accurate estimate. The use of activating solutions of ~140 mOsmol/Kg 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, especially marine fishes, sperm can remain active for as long as 30 min, which simplifies estimation of motility. Ensure that sufficient dilution of sperm (e.g. > 1:10 with activating solution) is used to elicit maximal activation for each sample.

For estimation of percent motility, include only sperm that are actively swimming in a forward motion. Sperm that remain in place with only a vibratory movement should not be included. Practice the procedure to ensure that sperm movements are not due to swirling of the activating solution or Brownian movement. Some microscopic organisms (e.g. bacteria) are motile and can be mistaken for sperm by inexperienced observers

(Jenkins and Tiersch 1997). Report the exact procedure used for motility estimation in sufficient detail to assist in making comparisons among studies.

Extenders and Refrigerated Storage

Extenders

Extenders have been defined as "a solution of salts, sometimes including organic compounds, which helps maintain viability of cells during refrigeration" (Graybill and Horton 1969). Extenders have been developed for many species. Hanks' balanced salt solution (Table 1) has been used successfully in our laboratory with sperm of several species including channel catfish *Ictalurus punctatus* (Tiersch et al. 1994) and simple solutions such as 1% unbuffered NaCl have been used with good results (e.g. Gwo et al. 1991).

Table 1. Ingredients of Hanks' balanced salt solution.

Ingredient	g/L
NaCl	8.00
KCl	0.40
$CaCl_2 \cdot 2H_2O$	0.16
$MgSO_4 \cdot 7H_2O$	0.20
Na_2HPO_4	0.06
KH_2PO_4	0.06
$NaHCO_3$	0.35
$C_6H_{12}O_6$ (glucose)	1.00

Extenders are formulated at an osmolality that will maintain sperm cells in a non-activated state (e.g. 280 to 300 mOsmol/Kg for freshwater species and 200 to 300 mOsmol/Kg for marine species). Report the osmotic pressure of extender solutions if possible. Before use verify that the extender solution does not activate sperm (Bates et al. 1996). With appropriate testing it is likely that particular 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. Specific dilution ratios should be optimized for each species.

Extenders should 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. At a minimum, sterilize the water used in preparing extenders. Bacteria such as *Pseudomonas* are ubiquitous in aquatic environments and can rapidly cause deterioration of extended sperm (Jenkins and Tiersch 1997). Antibiotics can be added to extenders to reduce the growth of bacteria that reduce sperm viability (Stoss 1983). Antibiotics at high concentrations can be toxic to sperm cells, therefore, concentrations should be optimized for each species (e.g. Christensen and Tiersch 1996).

Refrigerated Storage

Sperm samples should be stored at 4 °C in an ice chest or refrigerator and care should be taken to avoid freezing the samples. In general, sperm samples should be 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 (Brown and Brown, pp. 130-137, this volume). Containers should only be partially filled, leaving a large air space between the sperm sample and lid and should be inspected daily to provide mixing of the sperm cells. If not mixed, the cells can collect at the bottom and deteriorate.

Labeling of Straws, Goblets and Canes

Proper labeling of cryopreserved samples is essential. Usually samples are in storage for days, 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 fishes. 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.

Labeling of Straws

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. Marking could consist of a series of dashes and dots, in which dashes represent the number five and dots represent the number one. Dashes and dots would be summed to yield the final values. For example, fish number can be identified at the top of the straw (near the factory plugging). Cryoprotectant concentration can be marked lower with sufficient space between it and the fish number to allow easy recognition (Figure 1). If possible, more sophisticated labelling, such as pre-printed straws, should be considered even for research applications. Straws intended for archiving and breeding uses should receive the best labelling possible.

Labeling of Goblets

Straws can be stored in LN_2 in plastic containers called goblets. 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 frozen straws, do not pack them too tightly in the goblets

Labeling of Canes

Goblets are usually attached to aluminum canes for storage in LN_2. Canes should be labeled on the top for easy identification. Labeling will decrease excessive searching of dewar contents for necessary straws. This helps to protect the straws from warming during handling.

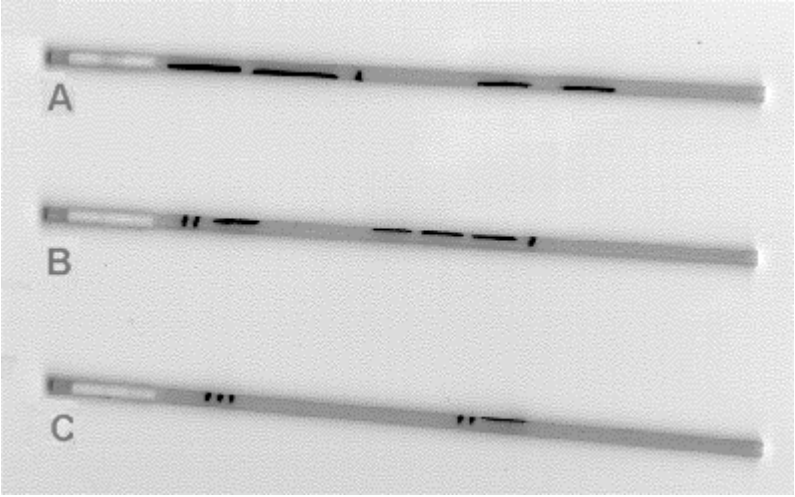

Figure 1. A labeling system for straws used for research. Straw A: fish # 11 and cryoprotectant concentration of 10%, straw B: fish # 7 and cryoprotectant concentration of 16%, straw C: fish # 3 and a cryoprotectant concentration of 7%.

Addition of Cryoprotectants

Background

Cryoprotectants are chemicals that allow cells to survive freezing protocols. They are grouped into two broad categories: those that are able to permeate cells and those that are not. Examples of permeating cryoprotectants include dimethyl sulfoxide (DMSO), methanol and glycerol. Examples of non-permeating cryoprotectants include sugars such as glucose or sucrose, polymers such as dextran, milk and egg proteins and antifreeze proteins such as those found in polar fishes. Use reagent-grade chemicals and report the manufacturers. Record all available information on specific lots of reagents.

Cryoprotectant Toxicity

Dependent on dose, most cryoprotectants are toxic to cells. To minimize toxic effects by cryoprotectants, they can be added slowly, at cool temperatures and should be diluted with extender solutions (e.g. 1:1) prior to addition to sperm. Some cryoprotectants can cause exothermic reactions when mixed with extenders. Care should be taken to make sure that mixtures are cool before addition to sperm. Always report cryoprotectant concentration clearly in molarity or percent (or both) and be sure to indicate if the reported concentration is before or after final dilution with extended sperm.

Equilibration Time

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. Always define and report equilibration time.

Equilibration Motility

To determine the approximate percentage of viable sperm after equilibration (before freezing), motility can be estimated at the start of the freezing procedure. Loss of motility can be attributed to toxic effects of the cryoprotectants. This information can help to optimize the freezing protocol by changing cryoprotectants, adjusting cryoprotectant concentration, or by adjusting equilibration time.

Filling of Straws

Background

French straws are designed to hold a specific volume (e.g. 0.5 or 0.25 mL). Manufacturers place a sealing powder surrounded by cotton plugs at one end of the straw. This area seals the straw when the sperm mixture is drawn into the powder.

Filling Individual Straws

To fill straws individually, a syringe (1 to 3-mL) can be fitted with a piece of tubing or a special adapter. This attaches to the straw and creates an air-tight seal. The straw is filled by drawing up on the syringe plunger. When the sperm mixture is ~ 0.5 cm from the bottom of the cotton plug, the straw is removed from the mixture (Figure 2). The sperm in the straw is drawn into the sealant powder between the cotton plugs. This seals the straw at the top and creates an air space within the straw. This space is necessary for safe and effective cryopreservation. The use of transparent or translucent straws greatly assists the filling process.

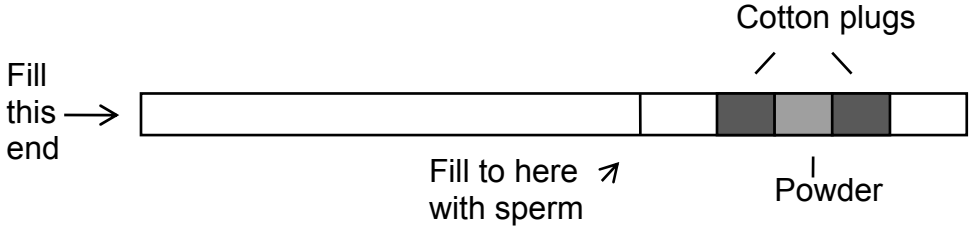

Figure 2. Diagram of standard French straw used for cryopreservation.

Filling Multiple Straws

To fill multiple straws in batches of 15 or more, a manifold of appropriate size (commercially available) is attached to a vacuum pump hose. The amount of suction is regulated by use of a valve connected to the vacuum hose by use of a "T" fitting. The straws are held together with a clamp and attached to the manifold. The flow of extended sperm can be regulated by holding the straws vertically. Filling can be completed by slowly lowering the manifold away from vertical. Once the straws are filled to within 0.5 cm from the cotton plugs they are removed from the sperm mixture. The straws are then lowered to horizontal to seal the factory-plugged end.

Sealing Straws

To seal with PVC powder: Tap the open end of the straws in PVC powder to force it into the straw. Place the straws in extender solution to gel the powder. After a short period of time, the straws can be removed from the extender and cleaned. Press the end of the straw against a flat surface to force the gel plug further into the straw (this also helps to remove excess powder from the sides) and wipe the outside of the straw.

To seal with balls: Each straw is sealed by individually forcing an appropriate-sized glass or metal ball (commercially available) into the end of the straw. The ball must be forced entirely into the straw because insufficient sealing can allow LN_2 to leak into the straw during freezing and storage. *Upon thawing, this LN_2 will vaporize and cause the straw to explode or the ball to be expelled from the straw at a high rate of speed. Both situations are very dangerous. As an additional precaution, always point the end sealed with a ball in a safe direction (usually down) and wear eye protection during thawing.*

Handling of Straws

Straws should be handled from the factory-plugged end and should be wiped dry to keep them from sticking together when frozen. Straws sealed with balls will occupy more space in the goblets than will straws sealed with powder.

Freezing Procedures in the Field

Nitrogen-vapor Shipping Dewars

A shipping dewar is a device that allows LN_2 to be safely transported. The LN_2 is adsorbed by a filler material within the sides of the dewar (Figure 3) allowing use of cryogenic temperatures without the dangers of LN_2 spills. Nitrogen-vapor shipping dewars were designed to transport cryopreserved materials, but they can also be used to freeze samples in situations where other freezing options are not available (such as in field applications). Shipping dewars were designed to maintain cryogenic temperatures for as long as 3 wk, making these dewars useful for cryopreservation and storage in the field (e.g. Wayman et al. 1996).

Filling Nitrogen-vapor Shipping Dewars

Dispense LN_2 into the dewar until it is filled to just below the top. Allow ~30 min for the LN2 to be adsorbed in the dewar wall. Refill the dewar to the previous level. Repeat these steps until LN_2 remains in the dewar after 30 min. Pour off excess LN_2 before use. *Use caution when disposing of the excess LN_2 because it will freeze whatever it comes in contact with (e.g. human skin, floor tiles or drain systems).*

Freezing in Nitrogen-vapor Shipping Dewars

To freeze in shipping dewars, place the straws in goblets and fasten the goblets on canes. Place the canes in the canister and lower it into the dewar. Replace the shipping dewar top. After ~30 min 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 be frozen individually without canes or goblets. Be sure to monitor the cooling rate, for example by use of a thermocouple and recorder, and be aware that cooling rates will vary in shipping dewars due to a variety of factors including time since filling, the number of straws being frozen and the amount of use. Report the conditions used for freezing in dewars in sufficient detail for others to repeat the procedure.

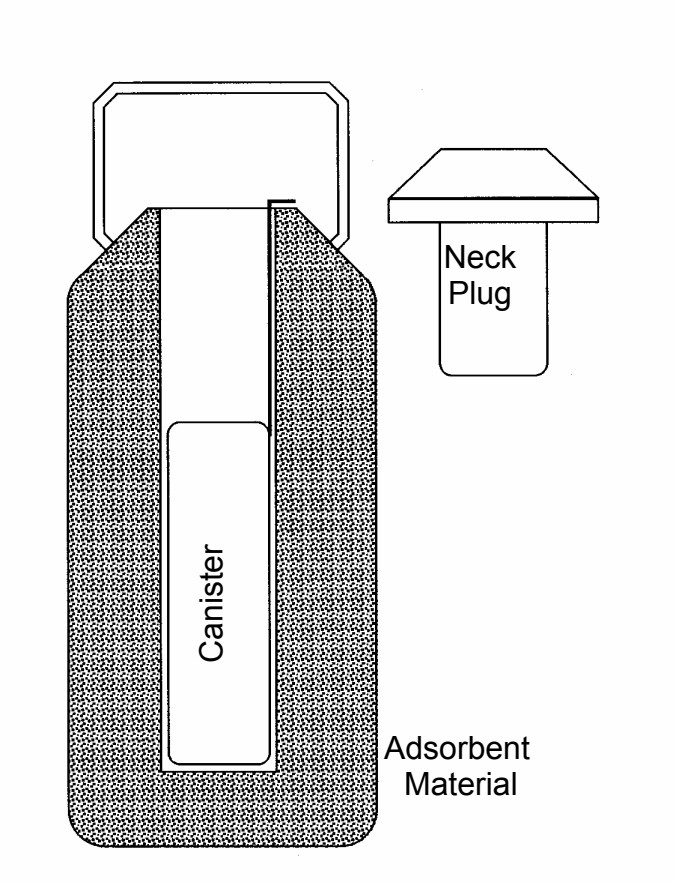

Figure 3. Diagram of a shipping dewar.

Freezing Procedures in the Laboratory

Freezing in Computer-controlled Freezers

Computer-controlled freezers are manufactured by several companies (e.g. Planer, Cryomed) and cool samples by metering of cryogenic nitrogen vapor into a freezing chamber. The rate of freezing is controlled by a microprocessor. There are many different cooling protocols (some use two or more cooling rates within a program), although a simple freezing protocol with only one rate can be effective (Leung and Jamieson 1991).

1) Connect LN_2 to freezer.
2) Turn on freezer and select cooling rate.
3) Start program.
4) Place canes in freezing chamber when prompted.

5) Remove canes from freezing chamber when prompted.
6) Immediately place canes in storage dewar.
7) Allow freezer to return to start temperature.
8) Run another program or turn off freezer.
9) Remove freezer lid to allow condensation to evaporate (this is important in humid locations).

Most computer-controlled freezers allow programming of steps for insertion and removal of straws. If the freezer does not, samples should be held at the equilibration temperature or at 4 °C until the beginning of the freezing steps. If the freezer does not prompt the user to remove samples, the program should allow the samples to be held at the final temperature (e.g. -80 °C) before removal to ensure proper freezing and to allow sufficient time to transfer samples to LN_2.

Storage Procedures

General Considerations

Storage dewars are designed to store cryopreserved samples in LN_2 for extended periods of time. They use a vacuum chamber to provide insulation (Figure 4). Liquid nitrogen (LN_2) within the dewar will evaporate over time and must be replaced. The use of alarms on storage dewars is essential. The alarm sounds when the temperature at a probe raises above a certain level, indicating that LN_2 needs to be added to the dewar. Some alarms require installation and positioning of the probe in relation to the level of LN_2. This positioning should take into account the margin of safety required between the sounding of the alarm and the replenishment of LN_2. For example, would you feel safe with 1 d or 1 wk before the uppermost samples began to thaw? Be certain that the alarm remains on at all times and is tested daily. A periodic visual check of the LN_2 level is also advised in case the alarm should fail. A log book for LN_2 additions can help identify a possible failure of the vacuum seal and assist in estimating storage costs. Ensure that the outer casing of the dewar is not punctured. The loss of vacuum will boil off the LN_2 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.

Removing Samples

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 (proper labeling will reduce searching time) and transfer the samples to LN_2 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.

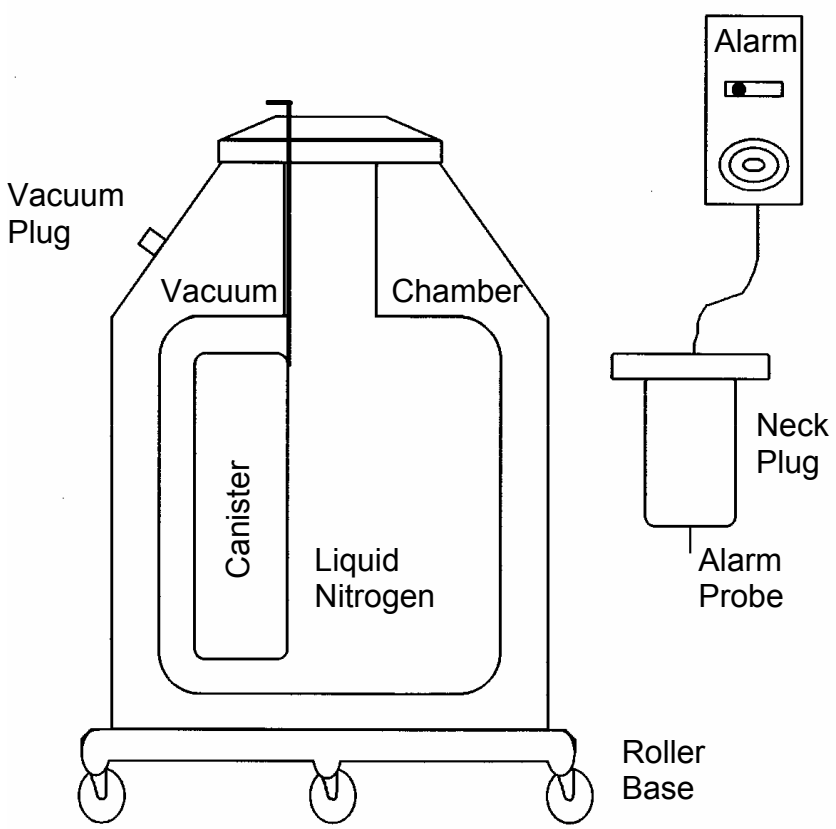

Figure 4. Diagram of a LN_2 storage dewar.

Liquid Nitrogen Safety Precautions

Always wear insulated gloves and safety glasses when handling LN_2. Never place objects cooled by LN_2 against unprotected skin. Use only containers designed for use with LN_2. Use proper transfer equipment to move and handle samples. Never use hollow rods or tubes as dipsticks, because LN_2 can be driven out of the open end.

Never cover or plug the opening of any LN_2 container. Considerable pressure will build up as the LN_2 vaporizes. Always allow proper venting. In addition, nitrogen gas can collect in closed areas, displacing the air, and creating a potential suffocation hazard. Work in well-ventilated areas.

Thawing and Fertilization

Thawing

Samples should be removed from the storage dewar and transferred immediately to a styrofoam ice chest containing LN_2. This ensures that the samples will not thaw prematurely due to handling. Hold 0.5-mL straws in a 40 to 50 °C water bath (a thermos or small ice chest will work) for ~7 sec. Specific times and temperatures should to be optimized for the particular species. We test a range of thawing temperatures (e.g. from 0 to 60 °C) to optimize protocols for each species (Wayman et al. 1998). 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. If the straw is not transparent, the samples should be cool to the touch when thawed.

Sperm motility should be estimated 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 equilibration motility.

Fertilization

Thawed sperm samples should be added to eggs and thoroughly mixed, and the gametes should be activated with an appropriate solution (the "dry method"). Other fertilization methods are available and should be evaluated (Urbanyi et al., pp. 286-287, this volume). Fresh sperm samples should be used to fertilize other batches of eggs to serve as a control for egg quality. After ~5 min, water should be added to water-harden the eggs. Percent fertilization should 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, which should always be reported if possible.

Acknowledgements

We thank M. Bates, M. Christensen, D. Glenn, P. Lang, C. Paniagua, L. Pittman-Cooley and G. Roppolo for technical assistance. This work supported in part by funding from the United States Department of Agriculture, United States Fish and Wildlife Service, Louisiana Sea Grant College Program, and the Louisiana Catfish Promotion and Research Board. Approved for publication by the Director of the Louisiana Agricultural Experiment Station as manuscript number 99-66-0355.

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