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# Advances in the Cryopreservation of Embryos and Prospects for Application to the Conservation of Salmonid Fishes

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## 1. Introduction

Considerable controversy has developed within the conservation community as to the precise magnitude of the biological diversity crisis and the appropriate tactics needed to prevent mass extinctions. Most biologists agree that: (1) an increasing number of species are facing extinction as a result of native habitat loss or fragmentation (Wilson, 1988) and (2) nearly all habitat destruction results from the direct or indirect action of man (Ehrlich and Ehrlich, 1981). Uncertainties about the number of species at risk and the relationship between habitat destruction and species extinction (Mann, 1991) prevent qualitative estimates of what Ehrlich and Wilson (1991) describe as "the epidemic of extinctions now under way." Such uncertainty complicates the formulation of effective public policy directed at preserving biological diversity.

Survival of a species or population in the wild is thought to depend on a secure native habitat that is of sufficient size to support a population meeting certain genetic and demographic criteria (Soulé, 1987). Most of the important requirements are related to the properties and characteristics of the population as a whole, including size, life-history characteristics and the nature of the gene pool. The latter, especially genetic variations within populations or communities of individuals (i.e. polymorphism), represents the key to effective conservation. The ultimate goal of all conservation programs is to preserve genetic variation within the targeted population(s) of a species. The extent of genetic variation determines if the population will maintain sufficient fitness and flexibility to undergo further evolutionary changes (Frankel and Soulé, 1987). Much of the controversy associated with the field of conservation biology is related to the best method to maintain "genetic fitness" for future generations.

Conservation efforts often are classified into: (1) *in situ* programs that protect and manage animal populations within their natural, native habitat; and (2) *ex situ* programs that remove individuals, gametes or embryos from wild populations for controlled breeding and management in captivity. In general, habitat protection is acknowledged as the most efficient approach for conserving bio- and genetic-diversity. For some species, however, *in situ* conservation alone can not be relied upon to ensure the long-term viability of species at risk (Conway, 1988). This is especially true when habitat has undergone extensive change or is likely to be adversely affected by biological, social or political factors. *Ex situ* approaches

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require a higher degree of technological and managerial input, but usually provide better long-term security for maintaining biodiversity. Soulé (1991) proposed an actuarial approach to determine the appropriate mixture of *in situ* and *ex situ* conservation tactics. According to this view, the effectiveness of *in situ* tactics is determined by the "half-life" of native habitats and protected areas (or reserves). Where the expected lifetime of such areas do not meet the demographic requirements for security, Soulé argues that *ex situ* tactics can be used to reach conservation objectives.

In many respects, the current status of some populations of North American and European salmonids may serve as a useful model of the need for dynamic application of Soulé's approach. Native habitats of most threatened salmonid populations have been extensively modified by fisheries, hydroelectric power facilities and/or environmental (e.g., acid rain) policies. The likelihood of continued habitat modification and the critical nature of population declines dictate that *ex situ* management approaches provide the best (or perhaps only) chance for some species. *Ex situ* management offers the only hope for some populations until the current crises can be resolved and sufficient genetic diversity can be preserved for future reintroduction into restored native habitat. Once viable populations are restored in secure reserves, less intensive *in situ* programs can be used to manage the population.

This paper describes the most secure *ex situ* method for preserving the genetic diversity of a population, namely, the cryopreservation, storage and use of animal germ plasm in the form of embryos. The current status of embryo cryopreservation is described, including the theory and practice of the two approaches currently used to cryopreserve embryos and practical obstacles to applying embryo cryopreservation to salmonid fishes.

## 2. Current Status of Embryo Cryopreservation

The cryopreservation and storage of animal germ plasm (spermatozoa, embryos and oocytes) at low temperatures offers unique opportunities for *ex situ* conservation programs (Rall, 1991a; Ballou, 1992). The ability to place germ plasm into a state of suspended animation for any desired period provides a powerful technique to preserve the genetic diversity in a current population. Once preserved, germ plasm banks can be used to "infuse" genetic diversity into future generations at any time. Germ plasm, preserved in the form of embryos, offers advantages for conservation. The most important of these is that the entire genomic constitution is known at the time of cryopreservation. The basic requirements for an effective embryo banking program are: (1) an action plan that integrates the goals and strategies of the *in situ* and *ex situ* components of the conservation program, (2) suitable reproductive and cryopreservation biotechniques for collecting, preserving, storing and using embryos, and (3) systematic sampling schemes for ensuring that most of the genetic diversity in the population is preserved.

The successful cryopreservation and banking of animal germ plasm evolved from Polge et al.'s discovery in 1949 that glycerol protects spermatozoa from the harmful effects of freezing and thawing. Since that time, a wide variety of biological cells have exhibited high survival following controlled freezing and storage at temperatures below -150°C (see Mazur, 1984). Although the first reports of the successful cryopreservation of embryos appeared in the late 1960s, it was Whittingham et al.'s report of normal offspring from cryopreserved mouse embryos in 1972 that marked the breakthrough in embryo cryopreservation. In the twenty years since that report, basic and applied research has yielded similar success with 13 other species of mammals and one species each of insect and marine rotifer (Table 1).

The ability to cryopreserve embryos and store them at low temperatures for any desired period of time has led to important applications. Three examples of practical uses are: (1) Banks

**Table 1.** Reports of Successful Embryo Cryopreservation Leading to Normal Live Offspring.

| | Species | First Report |
|---|---|---|
| Mammalian: | | |
| | Mouse | Whittingham et al., 1972 |
| | Cattle | Wilmut & Rowson, 1973 |
| | Rabbit | Bank & Maurer, 1974 |
| | Sheep | Willadsen et al., 1976 |
| | Rat | Whittingham, 1975 |
| | Goat | Bilton & Moore, 1976 |
| | Horse | Yamamoto et al., 1982 |
| | Eland | Kraemer et al., 1983 |
| | Human | Trounson et al., 1983 |
| | Baboon | Pope et al., 1984 |
| | Marmoset | Summers et al., 1986 |
| | Cynomolgus monkey | Balmaceda et al., 1987 |
| | Cat | Dresser et al., 1988 |
| | Pig | Hayashi et al., 1989 |
| Insect: | | |
| | Fruit fly (*Drosophila melanogaster*) | Steponkus et al., 1990 |
| Sea Urchin: | | |
| | *Hemicentrotus pulcherrimus* | Asahina and Takahashi, 1978 |
| Marine rotifer: | | |
| | *Brachionus plicatilia* | Okamoto et al. 1987 |

of embryos from laboratory mice and rats to ensure the continued availability of rare genotypes and mutants for research. The Jackson Laboratory in Bar Harbor, Maine, maintains 272 strains of genetically-unique, but infrequently used, mice as cryopreserved embryos stored in liquid nitrogen (L.E. Mobraaten, personal communication). (2) Embryo cryopreservation has become an integral component of the cattle embryo transfer industry and is used to transport cattle germ plasm internationally. And (3) embryo cryopreservation is increasingly used as an adjunct of *in vitro* fertilization techniques in the treatment of human infertility.

In the past 20 years, two approaches have been developed to preserve embryos at low temperatures. The first, termed "controlled slow freezing," evolved from Polge et al.'s 1949 discovery of the cryoprotective properties of glycerol and subsequent research during the 1950s and 1960s with microorganisms and mammalian tissue-culture cells. The second approach, termed "vitrification", was originally proposed by Luyet (1937; 1940), but practical application of this approach was achieved 45 years later (Rall and Fahy, 1985; Rall, 1987).

### 3. Controlled Slow-Freezing Procedures

Controlled slow-freezing procedures are characterized by: (1) the addition of molar concentrations of glycerol or another cryoprotectant to the cell suspension; and (2) the use of a controlled rate of freezing to the storage temperature. The basic steps required to preserve embryos by this approach are listed in Table 2. Cryopreservation invariably results in stresses

that lead to a small decrease at least in embryo viability. Therefore, the first step is to ensure that only high quality embryos at the proper developmental stage are processed further. The next step is to suspend the selected embryos in buffered saline containing a cryoprotective solute. For example, mouse embryos usually are collected at the 8-cell stage and then are suspended in buffered saline containing 1.5 molar glycerol or DMSO (see Wood et al., 1987, for detailed protocol). Once the cryoprotectant has partially permeated the blastomeres, the suspension is then cooled and frozen to temperatures below about -120°C. The appropriate cooling and freezing conditions depend upon the specific embryo properties and must be controlled within close tolerances. In our mouse embryo example, it takes approximately 15 min for glycerol to fully permeate into the cells at 20°C. Then, the mouse embryo suspensions are cooled to -7°C and seeded with ice crystals to ensure that ice forms at near equilibrium conditions. Finally, the seeded suspensions are cooled slowly at 0.5°C/min to - 40°C before transfer into liquid nitrogen (-196°C).

The long-term viability of frozen suspensions is assured only when the storage temperature remains below -130°C. Submersion in the liquid phase of a liquid nitrogen refrigerator is the most practical storage method. At the end of the storage period, the suspensions are warmed and thawed using controlled conditions. For most embryo suspensions, the container is transferred directly into warm water (20 to 37°C) until the suspension thaws. Next, the cryoprotectant is removed from the suspension. The large size and low permeability of most embryos to cryoprotectants usually increases the risk of osmotic shock during this step. Mouse embryos are diluted by one of two methods (Leibo, 1984). In the first, the concentration of cryoprotectant in the suspension is reduced in a series of 4 to 6 equimolar steps by dilution with isotonic saline at 5 min intervals (stepwise dilution). In the second method, embryos are placed in a saline containing sucrose (0.5 to 1 molar) and held until the cryoprotectant leaves the cells (sucrose dilution). This process requires about 10 minutes at 20°C for mouse embryos. Then, the embryos are rehydrated in isotonic saline. Once the cryoprotectant has been removed, the embryos are returned to normal physiological conditions. The development of a successful controlled freezing protocol for embryos (or any other cells) is complicated by many interacting factors (reviewed by Mazur, 1984) that can be divided into two groups. The first is related to the specific intrinsic properties of the embryos in question. These include the permeability characteristics of the cells to water and cryoprotectants, the size of the blastomeres, the presence of any special physiological considerations (e.g. a sensitivity to cold shock or chilling) and any heterogeneity in the properties of individual blastomeres or embryos in the suspension. These cellular properties can vary enormously for different types of cells (e.g. spermatozoa versus embryos) and the same cell type isolated from different species. The second group of factors is related to the procedural steps or conditions that have been selected to prepare and cryopreserve the suspension. Examples include the type of freezing container, the type and concentration of cryoprotectant, and the rates of cooling and warming. Successful cryopreservation is influenced strongly by interactions between these diverse factors. As a result, the steps and conditions of controlled freezing for each type of embryo or cell must be adjusted to minimize the many diverse sources of injury.

The interaction of cellular permeability with other cryobiological factors is one of the most important considerations for developing effective freezing protocols (Mazur, 1970). First, the permeability of the cell membrane to water interacts with the rate of cooling (see third step of controlled freezing, Table 2) and determines the rate at which water leaves the cytoplasm by exosmosis during freezing (Mazur, 1963). Optimum survivals are achieved when the rate of cooling allows loss of most of the cell water. Cells cooled too rapidly or too slowly usually exhibit lower survival. Rapid cooling fails to provide sufficient time for the cells to dehydrate

and avoid the detrimental affects associated with intracellular freezing. In contrast, slow cooling results in excessive dehydration and subjects cells to "solution-effects" injury (Mazur, 1984).

Another interaction between the permeability properties and procedural factors occurs during the second step of freezing (Table 2). The permeability properties of the cell membrane

**Table 2.** Steps of Embryo Cryopreservation by Controlled-Rate Freezing.

| | |
|---|---|
| 1. | Collect and assess embryo quality. |
| 2. | Equilibrate embryos in a solution containing molar concentrations of a cryoprotective solute (e.g., glycerol, DMSO). |
| 3. | Freeze embryo suspension using controlled cooling to temperatures below -130°C. |
| 4. | Low temperature storage at -196°C. |
| 5. | Warm and thaw embryo suspension using controlled conditions. |
| 6. | Remove cryoprotective solute from embryo suspension. |
| 7. | Return embryos to normal physiological conditions. |

to cryoprotectants interact with the conditions (especially time and temperature) selected for equilibration to determine the extent of cryoprotectant permeation into the cytoplasm (Mazur and Miller, 1976). The amount of cryoprotectant permeating into cells before freezing plays an important role in determining the extent of cryoprotection (Taylor et al., 1974). The third interaction occurs when embryos are diluted out of the cryoprotectant solution (see step 6, Table 2). The amount of cryoprotectant in the cell interacts with the dilution procedure to determine the extent of osmotic swelling when the suspending solution is diluted (Levin and Miller, 1981). The most important consequence of these and other interactions is that each step of the freezing process must be optimized to yield high post-thaw survival.

Successful cryopreservation protocols for embryos and cells usually produces a characteristic sequence of changes in the osmotic volume of the cells (Leibo, 1977; Rall, 1991b). The sequence of changes occurring during each step of controlled freezing (Table 2) is shown in Figure 1. No changes in cell volume would be expected during the first step (collection) provided that the embryos are suspended in an isotonic saline. However, during the second step, a transient shrink-swell change in the volume of cells occurs when exposed to a saline containing molar concentrations of a permeable cryoprotectant, such as glycerol. The initial shrinkage results from the exosmosis of water as the cell restores osmotic equilibrium with the hypertonic cryoprotectant solution. Then the cell gradually swells to its original volume as the cryoprotectant permeates the cytoplasm. The time required for a cell to shrink and return to its original volume depends on the permeability of the cell membrane to water and cryoprotectant (Mazur et al., 1974). The permeation properties of cells to cryoprotectants varies widely depending on the type of cell and the size and chemical nature of the molecules. For example, small molecules, such as methanol, usually permeate mammalian embryos very rapidly (<1 min, Rall et al., 1984), whereas large molecules, such as sucrose, do not permeate at all (Leibo, 1984).

During the third step, the cells progressively shrink when frozen by an optimized cooling procedure. This shrinkage is a consequence of a gradual increase in the suspending solution's osmolality when ice forms and gradually grows during cooling. Cells restore osmotic equilibrium with the freeze-concentrated suspending solution by exosmosis of water from the cytoplasm (Mazur, 1970). When the temperature decreases to about -120°C, the freeze-con-

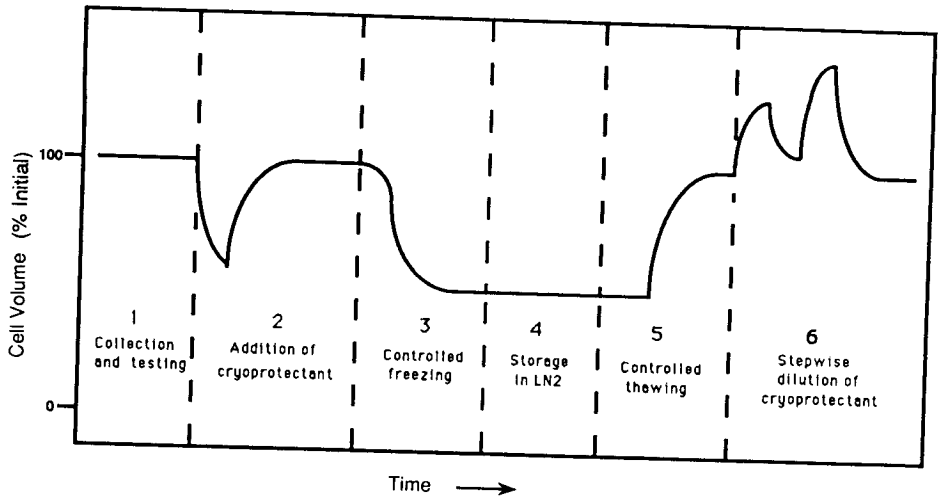



**Figure 1.** Diagrammatic representation of the cell volume changes during each step of successful controlled freezing. These characteristic changes provide a useful guide for optimizing each step of cryopreservation for any type of embryo or cell. See Table 2 and text for details. [Figure reprinted from Rall (1991b) with modifications.]

centrated residual liquid in the extracellular solution and the dehydrated cytoplasm solidify (vitrify) into a glass (Rall et al., 1984). An example of the osmotic behavior of mouse embryos during controlled freezing is shown in Figure 2.

During the fourth step of freezing (low-temperature storage), the cell volume does not change because the entire suspension has solidified completely. However, important osmotic changes occur when cell suspensions are thawed and the cryoprotectant is diluted from the suspension (steps 5 and 6, respectively). Controlled warming results in a gradual decrease in the osmolality of the suspending solution as extracellular ice melts. Cells undergo a gradual increase in volume as water flows into the cytoplasm to restore osmotic equilibrium. It is important that the rate of warming be sufficiently high to prevent further crystallization or recrystallization of ice (Mazur, 1984). In general, embryo suspensions are usually warmed much more rapidly than they are cooled. Rapid warming results in complete thawing of the suspension before significant embryo rehydration. Dilution of a thawed suspension with isotonic saline usually results in a transient osmotic swelling. The initial swelling results from the movement of water into the cells to restore osmotic equilibrium with the hypotonic suspending solution. Once equilibrium is restored, the cells gradually shrink as cryoprotectant leaves the cytoplasm. Excessive osmotic swelling is prevented by reducing the concentration of cryoprotectant in a series of small steps. The appropriate step size and interval between steps is determined by the permeability of the cell membrane to cryoprotectant and water (Schneider and Mazur, 1984).

An alternative dilution approach (not shown in Figure 1) is the so-called sucrose procedure (Leibo, 1984). Cells are placed into a saline containing a 0.25 to 1 molar concentration of an impermeable solute (usually sucrose). The absence of cryoprotectant in the suspending solution allows rapid efflux of cryoprotectant from the cytoplasm. The impermeable solute prevents (or reduces) the transient increase in cell volume because it increases the osmolality of the suspending solution. As the cryoprotectant leaves the cytoplasm, the cell progressively shrinks due to the hypertonic extracellular solution. Once the cryoprotectant leaves the cells, the suspending solution is replaced with isotonic saline.

## 4. Vitrification Approaches

The most important difference between vitrification and controlled rate freezing is the method used to dehydrate cells before low-temperature storage (Rall, 1987). Vitrification approaches produce osmotic dehydration by placing cells in a highly concentrated solution of cryoprotectant (>6 molar) *prior to cooling*. Then the entire cell suspension is transformed from the liquid state into a glassy solid by cooling to the storage temperature (-196°C). Vitrification offers considerable promise for simplifying and improving the cryopreservation of cells because potential injury associated with ice formation in the suspension is eliminated (Rall and Fahy, 1985). Vitrification has been applied to a wide variety of embryos (Table 3). Most of the reports have been for mammalian systems that have been successfully cryopreserved by controlled freezing methods. However, in one case, *Drosophila* embryos, vitrification has succeeded for cryopreservation, where controlled freezing has, so far, failed (Steponkus et al., 1990).

Despite differences in the method used to produce osmotic dehydration, the basic steps of vitrification (Table 4) are similar to those of controlled rate freezing (Table 1). In fact, the development of a successful vitrification protocol is complicated, in large part, by the same factors that influence controlled freezing methods (see above). Most of the differences are related to the need for a vitrification solution consisting of one or several cryoprotectants. The greatest challenge in developing a successful vitrification protocol is to formulate a vitrification solution that satisfies two requirements (Fahy et al., 1984; Rall, 1987). The first is related to the physical-chemical properties of the vitrification solution; it must be sufficiently concentrated to avoid crystallization during cooling and to vitrify into a glassy solid. The second is to match the choice of cryoprotectants with the intrinsic permeability and toxicity properties of the cells in question. Ideally, at least one of the cryoprotectants in the vitrification solution should permeate the cytoplasm, but the overall composition must not produce excessive osmotic stress or chemical toxicity (Rall, 1987). Inappropriate cryoprotectants are those that permeate very rapidly, or very slowly, or that produce toxic injury at high concentration. A vitrification solution that is appropriate for an embryo of one species and developmental stage may be inappropriate for embryos of other species or developmental stages, primarily because of differences in cellular permeability characteristics.

**Table 3.** Reports of the Successful Vitrification of Embryos.

| Species | Reference |
|---|---|
| Mammalian embryos: | |
| Mouse* | Rall and Fahy, 1985 |
| Cattle* | Massip et al., 1986 |
| Hamster oocytes | Critser et al., 1986 |
| Rat* | Kono et al., 1988 |
| Rabbit* | Smorag et al., 1989 |
| Goat* | Yuswiati and Holtz, 1990 |
| Sheep* | Schiewe et al., 1991 |
| Cat | Rall et al., unpubl. |
| Plant somatic embryos: | |
| *Asparagus officinalis** | Uragami et al., 1989 |
| Insect embryos: | " |
| *Drosophila melanogaster** | Steponkus et al., 1990 |

*Normal live offspring reported from vitrified embryos.

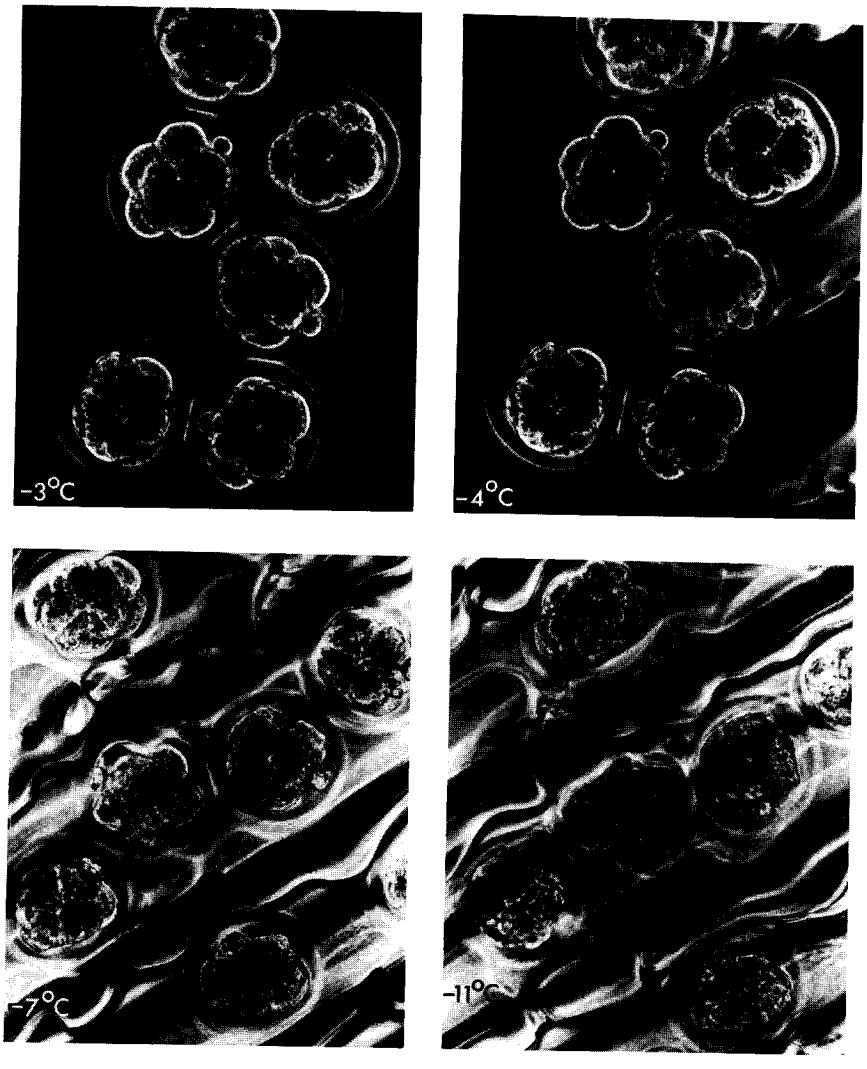
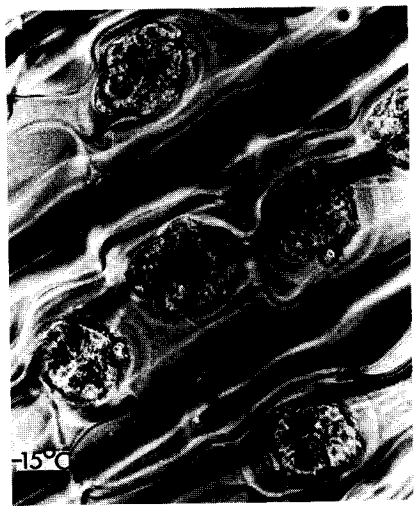



**Figure 2.** Microscopical appearance of six 8-cell mouse embryos during controlled slow freezing. Briefly, embryos were equilibrated in 1.5 molar glycerol in saline until the glycerol fully permeated into the cells. Embryos were then placed onto the stage of a cryomicroscope and frozen at 0.5°C/min to -31°C and photographs were taken at the indicated temperatures. During controlled slow freezing ice began to grow around the embryos at -4°C. The embryos gradually shrank to approximately half their original diameter as the suspending solution gradually crystallized. The embryos were then cooled rapidly to about -147°C. Each embryo is approximately 75 μm in diameter. [Figure reprinted from Rall and Polge (1984) with modifications.]


-15°C


-20°C


-25°C


-31°C


-147°C

**Table 4.** Steps of Embryo Vitrification.

| | |
|---|---|
| 1. | Collect and assess embryo quality. |
| 2. | Equilibrate and dehydrate embryos in a concentrated, but nontoxic, solution of cryoprotectants (Vitrification Solution). |
| 3. | Vitrify embryo suspension by cooling to temperatures below -130°C. |
| 4. | Low temperature storage (-196°C). |
| 5. | Warming and softening of glassy solution into the liquid state. |
| 6. | Immediate removal of cryoprotective solutes from embryo suspension. |
| 7. | Return embryos to normal physiological conditions. |

**Table 5.** Examples of Vitrification Solutions for Embryo Cryopreservation.

| Solution Name | Cryoprotectant | | Reference |
|---|---|---|---|
| | Permeating (Molar) | Nonpermeating (% Wt./Vol.) | |
| VS1 | DMSO (2.62 M)<br>Acetamide (2.62 M)<br>Propylene glycol (1.3 M) | PEG (6%) | Rall, 1987 |
| VS2 | Propylene glycol (5.5 M) | PEG or BSA (6%) | Rall, 1987 |
| VS3 | Glycerol (6.5 M) | PEG or BSA (6%) | Rall, 1987 |
| Massip's VS | Glycerol (2.2 M)<br>Propylene glycol (3.2 M) | BSA (0.4%) | Massip et al., 1986 |
| Kasai's EFS | Ethylene glycol (7.2 M) | Ficoll (18%)<br>Sucrose (0.3 M) | Kasai et al., 1990 |

Each solution also contains an isotonic saline.
Key: DMSO = dimethyl sulfoxide; PEG = polyethylene glycol (8000 MW); BSA = bovine serum albumin; Ficoll = polymer of sucrose (70,000 MW).
See Rall (1987), Massip et al. (1986) and Kasai et al. (1990) for details.

### 4.1. Vitrification Solutions

Vitrification solutions for embryos (Table 5) have three common features. First, each contains a mixture of low and high molecular weight cryoprotectants. The partial permeation of low molecular weight solutes into the cells protects cells from the potentially harmful effects of cellular dehydration and ensures that the cytoplasm vitrifies during cooling. High molecular-weight polymers protect cells by stabilizing cell membranes and increasing the ability of the solution to vitrify (Fahy et al., 1984; Rall, 1987). Second, the total concentration of solutes in each solution is high. This ensures that the solution vitrifies during cooling to the storage temperature and avoids crystallization (devitrification) during subsequent warming. The concentration required to prevent crystallization depends on the rates of cooling and warming (Fahy et al., 1984). The first three solutions listed in Table 5 were formulated to vitrify when cooled at rates of 20°C/min and avoid devitrification when warmed at rates greater than 100°C/min (Rall, 1987). The remaining solutions require much higher rates of cooling and

warming to avoid crystallization (about 1,000 and 2,000°C/min, respectively). And third, each vitrification solution contains an isotonic saline component to provide normal levels of extracellular electrolytes.

### 4.2. Osmotic Consequences of Vitrification

Successful cryopreservation by vitrification requires careful control of the conditions used to equilibrate embryos in the vitrification solution. The appropriate choice of conditions depends upon the permeability properties of the cells to water and the cryoprotectants in the solution, the toxic sensitivities of the cells, and the effect of temperature on permeability and toxicity. The ultimate aim of the equilibration procedure is to produce a concentrated, dehydrated cytoplasm while controlling the extent of cryoprotectant permeation, osmotic stresses and chemical toxicity.

Optimized vitrification procedures result in a characteristic sequence of changes in the osmotic volume of cells (Rall, 1991b). The sequence of changes in cell volume occurring during each step of the vitrification procedure (Fig. 3) closely parallel those during controlled-rate freezing (Fig. 1). The most important differences occur during the equilibration and cooling steps. Figure 3 illustrates the use of a three-step equilibration procedure to achieve the necessary

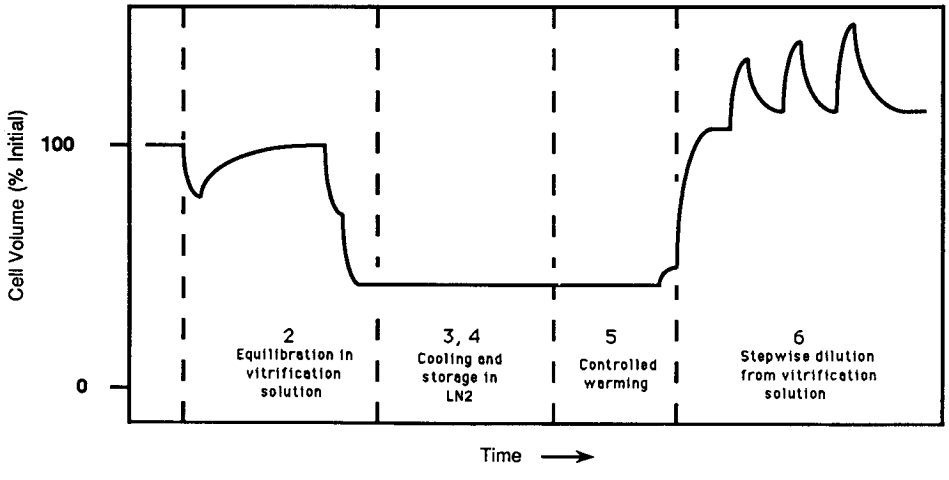



**Figure 3.** Diagrammatic representation of the cell volume changes during each step of successful vitrification. These characteristic changes provide a useful guide for optimizing each step of vitrification for any type of embryo or cell. See Table 4 and text for details. [Figure reprinted from Rall (1991b) with modifications.]

degree of intracellular dehydration for vitrification. The first step is identical to that used for freezing; embryos are transferred into a saline containing about 1.5 to 2 molar concentration of the same cryoprotectants in the final vitrification solution. The embryo suspension is then held at room temperature until the cryoprotectants permeate into the cells. This results in a shrink-swell change in cell volume. Then the embryos are transferred into the final vitrification solution in two short steps to limit further permeation of cryoprotectants and produce osmotic dehydration. The appropriate conditions for equilibration depend on the permeability of the cells to the cryoprotectants in the vitrification solution. For example, 8-cell mouse embryos require about 20 min for 1.6 molar glycerol to fully permeate into the cells at 22°C. Then,

dehydration of the cytoplasm is accomplished by one minute exposures to 4.2 molar glycerol and vitrification solution VS3a at 22°C.

Once the cytoplasm has dehydrated, the entire cell suspension is vitrified by cooling to temperatures below -130°C. Therefore, no change in cell volume occurs during cooling and storage in liquid nitrogen. Vitrified suspensions usually require rapid warming to prevent crystallization from the physical-chemical process called devitrification (Rall, 1987). There is no change in cell volume during warming until about 0°C. Once thawed, the cryoprotectants in the suspension must be diluted immediately to prevent further permeation and reduce the likelihood of toxicity (Rall and Fahy, 1985). Figure 3 shows the effects of stepwise dilution of the suspension immediately after warming. As in the case of controlled-rate freezing (Fig. 1), embryos undergo a transient osmotic swelling when the concentration of cryoprotectants is reduced. The reasons for cellular swelling during stepwise dilution are the same as those described earlier during controlled-rate freezing. Cells diluted from vitrification solutions often require more steps to prevent excessive swelling due to the presence of higher amounts of cryoprotectants in their cytoplasm (Rall, 1987). The use of sucrose dilution procedures for vitrified embryos has been very effective (Rall, 1987; Massip et al., 1986; Schiewe et al., 1991).

## 5. Cryopreservation of Fruit Fly (*Drosophila melanogaster*) Embryos by Vitrification

A recent report on the vitrification of fruit fly embryos is an example of some of the inherent species-specific problems that can be encountered (Steponkus et al., 1990). The approaches used to overcome impediments to cryopreserving large-sized insect embryos may provide useful insights for attempts to cryopreserve salmonid embryos.

### 5.1. Features of Drosophila Embryos That Complicate Cryopreservation

Three intrinsic properties of *Drosophila* embryos complicate cryopreservation. First,

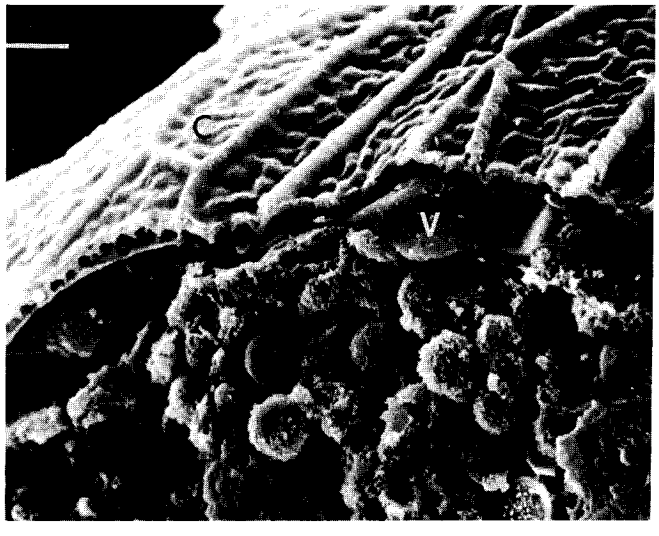



**Figure 4.** Scanning electron micrograph of freeze-fractured *Drosophila melanogaster* embryo. The major membrane systems, chorion (C), vitelline membrane (V) and intracellular yolk spheres (Y), are visible. The length of the scale bar is 5 μm. [Figure reprinted from Fullilove et al., 1978.]

they are large ovoids, approximately 0.5 mm long and 0.15 mm wide, with a flattened dorsal surface and slightly convex ventral surface. One consequence of this large size is a low surface area to volume ratio compared to mammalian embryos. This reduces the rate at which water and cryoprotectants can flow into and out of the embryo during the steps of cryopreservation (Mazur, 1984). For example, theoretical calculations suggest that *Drosophila* embryos require very low rates of controlled freezing (<0.5°C/min) to ensure adequate osmotic dehydration and avoid the deleterious effects of intra-cellular freezing (Lin et al., 1989).

Second, a complex membrane system surrounds and isolates embryos from their environment from fertilization to hatching 24 h later, (Fig. 4). The membranes of native embryos are impermeable to solutes but allow gas exchange and limited movement of water vapor. The outer membrane, called the eggcase or chorion, is a tough, opaque barrier that surrounds an inner membrane, called the vitelline membrane. The vitelline membrane is covered with a waxy layer which is thought to be the primary barrier to water and solute permeation. Native embryos tolerate exposure to solutions of formaldehyde, methanol and

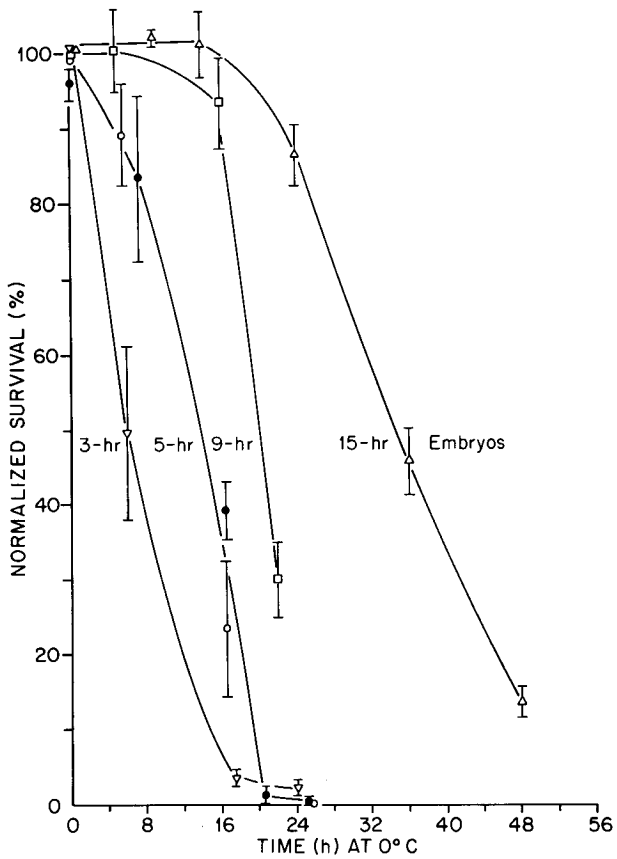



**Figure 5.** Effect of exposure to 0°C on the survival of 3, 5, 9 and 15 hour-old *Drosophila melanogaster* embryos. Embryos were cooled at 5°C/min from room temperature to 0°C. Survival is based on the percentage of treated embryos that develop to hatched larvae, and were normalized to room temperature controls, which averaged 81%. [Data reprinted from Mazur et al., 1992.]

chloroform with no ill effects (Limbourg and Zalokar, 1978). These permeability barriers must be removed before cryopreservation is possible.

Third, *Drosophila* embryos usually are killed when exposed to low environmental temperature (Myers et al., 1988). The sensitivity of *Drosophila* embryos to chilling injury depends upon their developmental stage. Mazur and colleagues (1992) report that the earlier the developmental stage, the more rapidly embryos die when held at 0°C (Fig. 5). Approximately half of 3, 5, 9 and 15 h old embryos die, respectively, after 6, 14, 20 and 34 h of exposure to 0°C. Although 15 h old embryos tolerate up to 18 h of exposure to 0°C with no ill effects, exposure to subzero temperatures greatly increases the rate of death (Fig. 6). For example, cooling to -30°C for less than 1 minute results in 100% mortality.

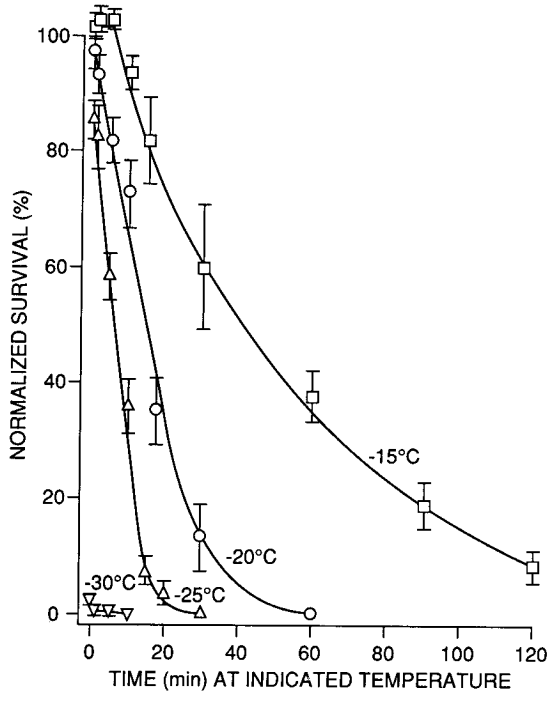



**Figure 6.** Effect of exposure to subzero temperatures on the survival of 12 to 15 hour-old *Drosophila melanogaster* embryos. Embryos were cooled rapidly to the indicated temperatures, held for the appropriate time and then warmed rapidly to room temperature. Survival is based on the percentage of treated embryos that develop to hatched larvae, and were normalized to room temperature controls, which averaged 82%. [Data reprinted from Mazur et al., 1992.]

### 5.2. Removal of Permeability Barriers

Perhaps the most important property of *Drosophila* embryos that limits current cryopreservation techniques is their permeability barriers. The chorion can be removed relatively easily (Hill, 1945), but removal of the waxy layers of the vitelline membrane requires controlled exposure to organic solvents (Widmer and Gehring, 1974; Limbourg and Zalokar, 1978).

Lynch et al. (1989) examined many factors related to the permeabilization of *Drosophila* embryos and developed an optimized procedure for cryopreservation. The steps of their procedure are:

1. Solubilize chorion by suspending embryos in 2.6% sodium hypochlorite (50% bleach) for 2 min and then rinse embryos with distilled water.
2. Extract waxy layers by sequential extraction with organic solvents:
   a. isopropanol (20 sec).
   b. n-hexane (30 sec).
   c. rinse with *Drosophila* Ringer's solution.
3. Suspend embryos in isotonic saline during subsequent steps.

Most embryos (80-90%) develop normally to hatched larvae when permeabilized by this procedure, and about 80-90% of the embryos are permeable to water and cryoprotectants. It is important to note that these two populations often do not coincide (i.e. some permeabilized embryos may not be viable and some viable embryos may not be permeabilized). Mazur et al. (1991) recently reported that the repeatability and efficacy of permeabilization can be improved by controlling the amount of alcohol carried from step 2a to 2b. Mazur et al.'s modifications of step 2 are: (a) rinse embryos with isopropanol for 20 seconds; (b) remove all adhering alcohol from embryos by air-drying; (c) rinse embryos in n-heptane containing 0.3% 1-butanol for 90 seconds; and (d) remove residual alkane/alcohol by air drying and rinsing with isotonic saline.

### 5.3. Cryopreservation Strategy

Initial attempts to cryopreserve permeabilized embryos by controlled freezing methods were unsuccessful (Leibo et al., 1988). Embryos did not survive cooling below -35°C at the low rates (0.2 to 0.5°C/min) required to ensure osmotic dehydration. The low rates of cooling resulted in long periods of exposure to subzero temperatures, and the lack of survival presumably resulted from the high sensitivity of *Drosophila* embryos to chilling injury (see Fig. 6). The inability to prevent this injury during controlled-rate freezing precluded this approach for cryopreservation. Vitrification approaches were examined in the hope that rapid cooling would preclude the kinetic processes associated with chilling injury (Steponkus et al., 1990).

Fortunately, chilling injury could be prevented by using ultra-rapid cooling (≥4,000°C/min) in combination with vitrification. After examining a range of developmental stages, cryoprotectants, equilibration procedures, cooling and warming conditions, vitrification solutions and dilution procedures, a successful vitrification procedure was identified (Steponkus et al., 1990). The specific conditions and procedures are listed in Table 6.

The ability of embryos to survive and develop to hatched larvae following various steps of the vitrification procedure is shown in Table 7. Control replicates indicate that permeabilization and equilibration in 2.125 molar ethylene glycol do not significantly reduce the rate of development of embryos to hatched larvae (88% and 80% survival, respectively). However, these steps in combination with dehydration in the vitrification solution resulted in a significant decline in survival (55%). An even smaller proportion of embryos (6.8%) developed to larvae after dehydration and cooling at 4,000°C/min in liquid nitrogen. Higher survivals were obtained when embryos were dehydrated and cooled rapidly in either liquid propane or partially-solidified liquid nitrogen (9.4% and 18.3%, respectively). The high level of variation in survival observed in these experiments probably reflects the difficulty in producing embryo

suspensions with the same permeability characteristics before cryopreservation (Mazur et al., 1991).

The process used to develop an effective cryopreservation procedure for *Drosophila* embryos provides important lessons for other biological materials. First, innocuous chemical and/or physical modifications of membrane systems may eliminate intrinsic permeability barriers that limit cryoprotectant permeation or osmotic dehydration. Second, the entire life cycle of an animal must be examined to determine the most appropriate developmental stage for cryopreservation. In this case, 12 to 15 h *Drosophila* embryos were selected for two reasons: (a) embryos at this stage exhibited the lowest sensitivity to chilling injury; and (b) embryos begin to develop a new membrane system (cuticle) at about 16 h that limits permeation of cryoprotectants and water. The last important lesson is that vitrification, when used in

**Table 6.** Cryopreservation of *Drosophila melanogaster* Embryos by Vitrification.

1. Age of embryos: 13 to 15 hours.
2. Vitrification solution: 8.5 M ethylene glycol plus 6% (wt/vol) bovine serum albumin in *Drosophila* medium.
3. Permeabilization of embryos by method listed in text.
4. Stepwise equilibration in vitrification solution:
   a. Place embryos into 2.125 molar ethylene glycol plus 6% BSA in *Drosophila* medium for 20 min at 22°C.
   b. Place embryos in vitrification solution for 8 min at 0°C.
5. Ultra-rapid cooling of about 25 embryos on an electron microscope grid to -196°C (4,000 to 25,000°C/min).
6. Storage in liquid nitrogen (-196°C).
7. Rapid warming (1,000°C/min) and rapid dilution of cryoprotectant at 22°C.

**Table 7.** Survival of *Drosophila melanogaster* Embryos after Various Steps of the Vitrification Procedure.

| Step* | Treatment Description | Survival** (Mean ± SD) |
|---|---|---|
| 3 | Permeabilization control | 88.0% ± 5.2 |
| 4a | Equilibration in 2.125 molar control | 80.0% ± 7.7 |
| 4b | Dehydration in vitrification solution control | 55.0% ± 13.3 |
| 5 | Vitrification in liquid nitrogen (-196°C) | 6.8% ± 4.4 |
| 5 | Vitrification in liquid propane (-196°C) | 9.4% ± 7.5 |
| 5 | Vitrification in liquid nitrogen slush (-204°C) | 18.3% ± 8.9 |

*See Table VI and text for details.

**Values represent the means of at least 30 replicates of ≥100 embryos per replicate. (Data from Steponkus et al., 1990)

combination with ultra-rapid cooling, may provide unique opportunities to avoid the deleterious effects of chilling injury.

## 6. Factors Complicating the Application of Embryo Cryopreservation to Salmonid Fishes

Little progress has been made to date in applying embryo cryopreservation procedures to salmonids (see Stoss, 1983 for review). This probably reflects four specific features of salmonid fish embryos that present serious obstacles to cryopreservation. First, salmonids, like most teleosts, have a complex membrane system that plays an important role in controlling the immediate environment of the embryo (Groot and Alderdice, 1985). Immediately after shedding, salmonid eggs are flaccid and surrounded by a proteinaceous membrane, the chorion or egg case. When transferred into water, the egg undergoes a series of changes independent of fertilization, called water activation or hardening (Hayes, 1949). Water activation results in the release of cortical granules and colloidal materials from the cortical alveoli into the future perivitelline space. The perivitelline fluid is produced when water flows across the chorion in response to an osmotic pressure gradient between the external water and the colloids. Within about 3 h, net flow of water across the chorion ceases due to a counteracting hydrostatic pressure within the perivitelline space. The intra-embryonic pressure of salmonids after water hardening varies from 20-50 mm Hg (Eddy, 1974; Alderdice et al., 1984) and yields turgid embryos that resist crushing when covered by gravel (Hayes, 1949). The presence of a chorion ranging in salmonids from 28 to 61.6 μm thick (Groot and Alderdice, 1985), large amounts of colloidal material in the perivitelline fluid (42% solids by weight; Eddy, 1974), and membranes surrounding both the embryo and yolk constitute a complex system for developing effective procedures for cryoprotectant permeation and osmotic dehydration.

Second, the embryos of salmonid fishes are very large ellipsoids. Groot and Alderdice (1985) report that the equivalent spherical diameters of five species of Pacific salmon range from 5.96 mm for sockeye (*Oncorhynchus nerka*) to 8.67 mm for chinook (*O. tshawytscha*). When compared to *Drosophila* embryos, the diameter and volume of salmonid embryos are, respectively, approximately 20-fold and $10^3$-$10^4$ fold greater. This large size results in a much lower surface area to volume ratio when compared to *Drosophila* or mammalian embryos. One consequence of such a low ratio is a reduction in the rate at which water and cryoprotectants can move into and out of the embryo during the steps of cryopreservation (Mazur, 1984).

A third complicating feature is the presence of a large quantity of yolk. Yolk provides all nutrients for embryonic development in a high density form (41% solids by weight; Blaxter, 1969) and comprises at least 90% of the dry weight of salmonid embryos prior to hatching (Marr, 1966). During the steps of cryopreservation, yolk probably acts as an independent compartment and responds osmotically in a manner analogous to the cellular cytoplasm. The development of a single effective protocol for cryoprotectant permeation and osmotic dehydration of the yolk and cell compartments may be difficult due to known large differences in their volume and water contents, and the likelihood that their membranes have different permeability characteristics.

A fourth potentially complicating factor is a sensitivity to chilling injury. At present it is not known if salmonid embryos are injured by subzero temperature exposure in an manner analogous to *Drosophila*. The only relevant data are for unfertilized eggs of *Salmo gairdneri* which reportedly undergo "normal" water hardening following super-cooling or freezing to -20°C (Erdahl and Graham, 1980; Harvey and Ashwood-Smith, 1982). These reports and the

fact that salmonid embryos normally develop in cold water (2-15°C) suggests that chilling injury may not be a major problem.

### 6.1. Strategies for Cryopreservation

It should be emphasized that none of the intrinsic features listed above present insurmountable obstacles to the application of cryopreservation to salmonid embryos. However, considerable basic and applied research is required to investigate the cryobiological implications of each of these features. The first step is to establish a model teleost system to evaluate inherent properties of fish embryos and develop protocols. The characteristics of an ideal model system include: (1) small adult size; (2) short generation interval; (3) the ability to maintain fish and embryos *in vitro* throughout the entire life cycle; (4) the reproductive physiology, embryology and developmental genetics must be well-characterized; (5) eggs, embryos and spermatozoa must be available daily (i.e. nonseasonal breeder); and (6) appropriate reproductive biotechniques must be available (e.g.,oocyte, semen and embryo collection, embryo micromanipulation and survival surgery). Two candidate teleosts, zebrafish (*Brachydanio rerio*) and medaka (*Oryzias latipes*), exhibit many of these features (Laale, 1977; Yamamoto, 1975; Winfield and Nelson, 1991; Grady et al., 1991) and have been the subject of previous cryobiological studies (Harvey and Chamberlain, 1982; Harvey et al., 1983; Harvey, 1983; Arii et al., 1987).

The first specific study is to determine the permeability characteristics during development from eggs and fertilized zygotes to hatching. The permeation of water and a range of potential cryoprotective solutes should be examined, including small molecules (e.g. methanol) and larger solutes (e.g. sugars). Previous reports of water "exchange" in teleosts (Loeffler and Lovtrup, 1970; Harvey and Chamberlain, 1982) and cryoprotectant permeation (Harvey and Ashwood-Smith, 1982; Harvey et al., 1983) greatly underestimated these properties. That is because the osmotically inactive volumes of perivitelline fluid, yolk and cytoplasm were not taken into account in estimating the intraembryonic volume available for water and solutes. As noted above, the solids content of yolk is very high and cytoplasm is somewhat lower (respectively, 41 and 15% by volume). Therefore, the "unavailable volume" should be determined by measuring the Boyle van't Hoff relationship (Leibo, 1980) for each compartment of the embryo.

Another important area for study concerns the membrane systems surrounding the embryo. It is likely that success will require modification or removal of the chorion and perivitelline fluid to speed the permeation of cryoprotectants and water (Harvey, 1983). Current studies of chimera formation, transgenesis and other genetic/embryological manipulations in teleosts may provide useful procedures and approaches. Those studies often require the insertion of micropipets or other instruments through the chorion (Maclean et al., 1987). Alternative approaches, such as disassociation of membranes with proteolytic enzymes or chemicals, have potential for simplifying procedures (Iwamatsu, 1983). A less attractive alternative to the cryopreservation of whole embryos is to isolate embryonic cells from developing embryos for cryopreservation and eventual thawing and transfer into recipient embryos. Under ideal conditions the resulting chimeric embryos will yield chimeric adults that produce viable gametes from the transferred cells. If genetic or developmental barriers prevent the recipient embryo from forming viable gametes (e.g. triploids, Utter et al., 1983; or perhaps homozygous clones, Streisinger et al., 1981), the transplanted cells may serve as the sole source of gametes.

A third area of study is related to potential chilling injury. Studies should be designed to determine the inherent sensitivity of teleost embryos to low temperatures. Studies involving mammalian embryos suggest that a high sensitivity to chilling injury is associated with large amounts of intraembryonic lipids (Polge et al., 1974). Therefore, the sensitivity of the yolk and cell compartments to low temperature should be examined.

## 7. Conclusions

In conclusion, the development of an effective cryopreservation procedure for teleost embryos has great potential for facilitating the conservation of endangered populations of salmonids. The formation of salmonid embryo banking programs to preserve unique genetic diversity within populations can offer benefits as an integral part of *ex situ* and *in situ* conservation efforts. A concerted effort to systematically examine the feasibility of applying cryobiological procedures to fishes is needed to provide the necessary information for making this possible.

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## References

Alderdice, D.F., J.O.T. Jensen, and F.P.J. Velsen. 1984. Measurement of hydrostatic pressure in salmonid eggs. *Can. J. Zool.* 62:1977-1987.

Arii, N., K. Namai, F. Gomi, and T. Nakazawa. 1987. Cryoprotection of medaka embryos during development. *Zool. Sci.* 4:813-818.

Asahina, E. and T. Takahashi. 1978. Freezing tolerance in embryos and spermatozoa of the sea urchin. *Cryobiology* 15:122-127.

Ballou, J. D. 1992. Potential contribution of cryopreserved germ plasm to the preservation of genetic diversity and conservation of endangered species in captivity. *Cryobiology* 29:19-25.

Blaxter, J.H.S. 1969. Development: Eggs and larvae, in: "Fish Physiology," W. S. Hoar and D. J. Randall, eds., vol. 3, pp. 178-252, Academic Press, New York.

Conway, W. 1988. Can technology aid species preservation?, in: "Biodiversity," E. O. Wilson, ed., pp. 263-268, National Academy Press, Washington, DC.

Critser, J., B.W. Arneson, D.V. Aaker, and G.D. Ball. 1986. Cryopreservation of hamster oocytes: Effects of vitrification or freezing on human sperm penetration of zona-free hamster oocytes. *Fertil. Steril.* 46:277-284.

Eddy, F.B. 1974. Osmotic properties of the perivitelline fluid and some properties of the chorion of Atlantic salmon eggs (*Salmo salar*). *J. Zool. Lond.* 174:237-243.

Ehrlich, P.R. and A.H. Ehrlich. 1981. "Extinction: The Causes and Consequences of the Disappearance of Species," Random Press, New York.

Ehrlich, P.R. and E.O. Wilson. 1991. Biodiversity studies: Science and policy. *Science* 253:758-762.

Erdahl, D.A. and E.F. Graham. 1980. Preservation of gametes of freshwater fish, in: "Proc. 9th International Congress on Animal Reproduction and Artificial Insemination, RT-H-2," pp. 317-326.

Fahy, G.M., D.R. MacFarlane, C.A. Angell, and H.T. Meryman. 1984. Vitrification as an approach to cryopreservation. *Cryobiology* 21:407-426.

Frankel, O.H. and M.E. Soulé. 1981. "Conservation and Evolution." Cambridge University Press, Cambridge.

Fullilove, S.L., A.G. Jacobson, and F.R. Turner. 1978. Embryonic development: Descriptive, in: *"The Genetics and Biology of Drosophila,"* M. Ashburner and T. R. F. Wright, eds., vol. 2C, p. 151, Academic Press, New York.

Grady, A.W., I.E. Greer, and R.M. McLaughlin. 1991. Laboratory management and husbandry of the Japanese medaka. *Lab Anim.* 20:22-28.

Groot, E.P. and D.F. Alderdice. 1985. Fine structure of the external egg membrane of five species of Pacific salmon and steelhead trout. *Can. J. Zool.* 63:552-566.

Hayashi, S., K. Kobayashi, J. Mizuno, K. Saitoh, and S. Hirano. 1989. Birth of piglets from frozen embryos. *Vet. Record* 125:43-44.

Harvey, B. 1983. Cooling embryonic cells, isolated blastoderms and intact embryos of the zebra fish *Brachydanio rerio* to -196°C. *Cryobiology* 20:440-447.

Harvey, B. and M.J. Ashwood-Smith. 1982. Cryoprotectant penetration and supercooling in the eggs of salmonid fishes. *Cryobiology* 19:29-40.

Harvey, B. and J.B. Chamberlain. 1982. Water permeability in the developing embryo of the zebrafish, *Brachydanio rerio. Can. J. Zool.* 60:268-270.

Harvey, B., R.N. Kelley, and M.J. Ashwood-Smith. 1983. Permeability of intact and dechorionated zebra fish embryos to glycerol and dimethyl sulfoxide. *Cryobiology* 20:432-439.

Hayes, F.R. 1949. The growth, general chemistry and temperature relations of salmonid eggs. *Q. Rev. Biol.* 24:281-308.

Hill, D.L. 1945. Chemical removal of the chorion from *Drosophila* eggs. *Drosoph. Inform. Serv.* 19:62.

Iwamatsu, T. 1983. New techniques for dechorionation and observations on the development of the naked egg in *Oryzias latipes. J. Exp. Zool.* 228:83-89.

Kono, T., O. Suzuki, and Y. Tsunoda. 1988. Cryopreservation of rat blastocysts by vitrification. *Cryobiology* 25:170-173.

Laale, H.W. 1977. The biology and use of the zebra fish *Brachydanio rerio* in fisheries research. *J. Fish Biol.* 10:121-173.

Leibo, S.P. 1977. Fundamental cryobiology of mouse ova and embryos, in: 'The Freezing of Mammalian Embryos," K. Elliott and J. Whelan, eds., pp. 69-92, Elsevier, Amsterdam.

Leibo, S.P. 1980. Water permeability and its activation energy of fertilized and unfertilized mouse ova. *J. Membr. Biol.* 53:179-188.

Leibo, S.P. 1984. A one-step method for direct nonsurgical transfer of frozen-thawed bovine embryos. *Theriogenology* 21:767-790.

Leibo, S.P., S.P. Myers, and P.L. Steponkus. 1988. Survival of *Drosophila melanogaster* embryos cooled to subzero temperatures. *Cryobiology* 25:545-546.

Levin, R.L. and T.W. Miller. 1981. An optimum method for the introduction and removal of permeable cryoprotectants. *Cryobiology* 18:32-48.

Limbourg, B. and M. Zalokar. 1978. Permeabilization of *Drosophila* embryos. *Dev. Biol.* 35:382-387.

Lin, T.-T., R.E. Pitt, and P.L. Steponkus. 1989. Osmometric behavior of *Drosophila melanogaster* embryos. *Cryobiology* 26:453-471.

Loeffler, C.A. and S. Lovtrup. 1970. Water balance in the salmon egg. *J. Exp. Biol.* 52:291-298.

Luyet, B.J. 1937. The vitrification of organic colloids and of protoplasm. *Biodynamica* 1(39):1-14.

Luyet, B.J. and P.M. Gehenio. 1940. Life and Death at Low Temperatures, Biodynamica Press, Normandy, Missouri.

Lynch, D.V., T.-T. Lin, S.P. Myers, S.P. Leibo, R.J. MacIntyre, R.E. Pitt, and P.L. Steponkus. 1989. A two-step method for permeabilization of *Drosophila* embryos. *Cryobiology* 26:445-452.

Maclean, N., D. Penman, and Z. Zhu. 1987. Introduction of novel genes into fish. *Bio/Technol.* 5:257-261.

Mann, C.C. 1991. Extinction: Are ecologists crying wolf. *Science* 253:736-738.

Marr, D.H.A. 1966. Influence of temperature on the efficiency of growth of salmonid embryos. *Nature* 212:957-959.

Massip, A., P. van der Zwalmen, and F. Ectors. 1986. Pregnancies following transfer of cattle embryos preserved by vitrification. *Cryo-Letters* 7:270-273.

Mazur, P. 1963. Kinetics of water loss from cells at subzero temperatures and the likelihood of intracellular freezing. *J. Gen. Physiol.* 47:347-369.

Mazur, P. 1970. Cryobiology: The freezing of biological systems. *Science* 168:939-949.

Mazur, P. 1984. Freezing of living cells: Mechanisms and implications. *Amer. J. Physiol.* 247C:125-142.

Mazur, P., K.W. Cole, and A.P. Mahowald. 1991. Critical role of alcohol in the permeabilization of 12-hr *Drosophila* embryos by alkanes. *Cryobiology* 28:524.

Mazur, P., K.W. Cole, P.D. Schreuders, and A.P. Mahowald. 1991. Confirmation of the ability of permeabilized 12-hr *Drosophila* embryos to survive cooling to -200°C. *Cryobiology* 28:524-525.

Mazur, P., R.H. Miller, and S.P. Leibo. 1974. Survival of frozen-thawed bovine red cells as a function of the permeation of glycerol and sucrose. *J. Membr. Biology* 15:137-158.

Mazur, P. and R.H. Miller. 1976. Survival of frozen-thawed human red cells as a function of the permeation of glycerol and sucrose. *Cryobiology* 13:523-536.

Mazur, P., U. Schneider, and A.P. Mahowald. 1992. Characteristics and kinetics of subzero chilling injury in *Drosophila* embryos. *Cryobiology* 29:39-68.

Myers, S.P., D.V. Lynch, D.C. Knipple, S.P. Leibo, and P.L. Steponkus. 1988. Low-temperature sensitivity of *Drosophila melanogaster* embryos. *Cryobiology* 25:544-545.

Okamoto, S., M. Tanaka, H. Kurokura, and S. Kasahara. 1987. Cryopreservation of parthenogenic eggs of the rotifer *Brachionus plicatilia*. *Nippon Suisan Gakkaishi* 53:2093.

Polge, C., A.U. Smith, and A.S. Parkes. 1949. Revival of spermatozoa after vitrification and dehydration at low temperatures. *Nature* 164:666.

Polge, C., I. Wilmut, and L.E.A. Rowson. 1974. The low temperature preservation of cow, sheep, and pig embryos. *Cryobiology* 11:560.

Rall, W.F. 1987. Factors affecting the survival of vitrified mouse embryos. *Cryobiology* 24:387-402.

Rall, W.F. 1991a. Guidelines for establishing animal genetic resource banks: Biological materials, management, and facility considerations, in: "Proceedings of the Wild Cattle Symposium," D. L. Armstrong and T. S. Gross, eds., pp. 96-106, Henry Doorly Zoo, Omaha, Nebraska.

Rall, W.F. 1991b. Prospects for the cryopreservation of mammalian spermatozoa by vitrification, in: 'Reproduction in Domestic Animals, Supplement 1, Proceedings of the 2nd

International Conference on Boar Semen Cryopreservation," L. A. Johnson and D. Roth, eds., pp. 65-80, Paul Parey Scientific Publishers, Hamburg.

Rall, W.F. and G.M. Fahy. 1985. Ice-free cryopreservation of mouse embryos at -196°C by vitrification. *Nature* 313:573-575.

Rall, W.F. and C. Polge. 1984. Effect of warming rate on mouse embryos frozen and thawed in glyceol. *J. Reprod. Fertil.* 70:285-292.

Rall, W.F., D.S. Reid, and C. Polge. 1984. Analysis of slow-warming injury of mouse embryos by cryomicroscopical and physio-chemical methods. *Cryobiology* 21:106-121.

Schiewe, M.C., W.F. Rall, L.D. Stuart, and D.E. Wildt. 1991. Analysis of cryoprotectant, cooling rate and in situ dilution using conventional freezing or vitrification for cryopreserving sheep embryos. *Theriogenology* 36:279-283.

Schneider, U. and P. Mazur. 1984. Osmotic consequences of cryoprotectant permeability and its relation to the survival of frozen-thawed embryos. *Theriogenology* 21:68-79.

Smorag, V., B. Gajda, B. Wieczorek, and J. Jura. 1989. Stage-dependence viability of vitrified rabbit embryos. *Theriogenology* 31:1227-1231.

Soulé, M.E. 1987. "Viable Populations for Conservation," Cambridge University Press, Cambridge, U.K.

Soulé, M.E. 1991. Conservation: Tactics for a constant crisis. *Science* 253:744-750.

Steponkus, P.L., S.P. Myers, D.V. Lynch, L. Gardner, V. Bronshteyn, S.P. Leibo, W.F. Rall, R.E. Pitt, T.-T. Lin, and R.J. MacIntyre. 1990. Cryopreservation of *Drosophila melanogaster* embryos. *Nature* 345:170-172.

Stoss, J. 1983. Fish gamete preservation and spermatozoan physiology, in: "Fish Physiology," W.S. Hoar, D.J. Randall, and E.M. Donaldson, eds., vol. 9B, pp. 305-350, Academic Press, New York.

Streisinger, G., C. Walker, N. Dover, D. Knauber, and F. Singer. 1981. Production of clones of homozygous diploid zebra fish (*Brachydanio rerio*). *Nature* 291:293-296.

Taylor, R., G.D.J. Adams, C.F.B. Boardman, and R.G. Wallis. 1974. Cryoprotective-permeant vs nonpermeant additives. *Cryobiology* 11:430-438.

Uragami, A., A. Sakai, N. Nagai, and T. Takahashi. 1989. Survival of cultured cells and somatic embryos of *Asparagus officinalis* cryopreserved by vitrification. *Plant Cell Rpts.* 8:418-421.

Utter, F.M., O.W. Johnson, G.H. Thorgaard, and P.S. Rabinovitch. 1983. Measurement and potential applications of induced triploidy in Pacific salmon. *Aquaculture* 33:329-354.

Whittingham, D.G., S.P. Leibo, and P. Mazur. 1972. Survival of mouse embryos frozen to -196 and -269°C. *Science* 178:411-414.

Widmer, B. and W.J. Gehring. 1974. A method for permeabilization of *Drosophila* eggs. *Drosoph. Inform. Serv.* 51:149.

Wilson, E.O. 1988. The current status of biological diversity, in: "Biodiversity," E. O. Wilson, ed., pp. 3-18, National Academy Press, Washington, DC.

Winfield, I.J. and J.S. Nelson. 1991. "Cyprinid Fishes: Systematics, Biology and Exploitation," Chapman and Hall, London.

Wood, M.J., D.G. Whittingham, and W.F. Rall. 1987. The low temperature preservation of mouse oocytes and embryos, in: "Mammalian Development," M. Monk, ed., pp. 255-280, IRL Press, Practical Approach Series, Oxford.

Yamamoto, T. 1975. "Medaka (Killifish) Biology and Strains," Keigaku Publ. Co., Tokyo.

Yuswiati, E. and W. Holtz. 1990. Successful transfer of vitrified goat embryos. *Theriogenology* 34:629-631.