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Chill Sensitivity and Cryoprotectant Permeability of Dechorionated Zebrafish Embryos, *Brachydanio rerio*

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The zebrafish (*Brachydanio rerio*) was used as a model for basic studies of the chilling sensitivity, permeability and toxicity of cryoprotectants. In both intact and dechorionated embryos, early-stage embryos (1.25, 1.5, 1.75, and 2 h) were more susceptible ($P < 0.05$) to chilling injury at 0°C than late-stage embryos (50, 75, and 100% epiboly and three-somite stage). Moreover, enzymatic removal of the chorion did not alter ($P > 0.05$) this pattern of sensitivity to chilling. Eight-hour zebrafish embryos tolerated short-term exposures to temperatures ranging from 4 to 23°C for 3.5 h with no detrimental developmental effects. The permeability of dechorionated embryos to cryoprotectants was examined by measuring the kinetics of volumetric change at various developmental stages (16 cells to six somites or ca. 1.25 to 14 h postfertilization) at 28.5°C. The dechorionated zebrafish embryo is composed of two complex cellular compartments (i.e., a large yolk and the developing blastoderm). From 40 to 100% epiboly, the volumes of the yolk and blastoderm remained constant, ca. 82 and 18%, respectively. However, these volumes changed rapidly after epiboly. For example, at the six-somite stage, the yolk composed 61% of the total volume, whereas the blastoderm composed 39%. When three- and six-somite embryos were placed in 1.5 and 2.0 *M* cryoprotectants (dimethyl sulfoxide and propylene glycol), osmometric measurement of volume changes indicated no permeation of the cryoprotectants. However, some permeation was observed for six-somite embryos immersed in a 2.0 *M* methanol solution, but not for 3-somite embryos. For up to 30 min at room temperature, these cryoprotectant solutions were not toxic to zebrafish embryos; however, 1.5 *M* glycerol and ethylene glycol solutions were. We conclude that the complex nature of the zebrafish embryo reduces the effectiveness and predictive value of light microscopical measurements for cryoprotectant permeability studies. © 1997 Academic Press

Understanding the osmometric, chill sensitivity, and permeability characteristics of teleost embryos is key to developing a cryopreservation system applicable to a wide array of fish species. Male gamete cryopreservation first succeeded in 1949 when Polge *et al.* (26) successfully froze and thawed human and avian spermatozoa using glycerol as a cryoprotectant. Shortly thereafter, Blaxter (3) applied this approach to teleost gametes and reported success with Atlantic herring spermatozoa, achieving approximately 80% cellular motility upon thawing. Since then, successful sperm cryopreservation (based on offspring produced) has been reported for a host of freshwater and marine teleosts including carp, salmonids, catfish, cichlids, zebrafish, medaka, whitefish, pike, milkfish, grouper, cod, and zebrafish (15, 31). In general, cryopreservation of small-sized, fish spermatozoa is practiced regularly in field and laboratory settings.

Since Blaxter's pioneering experiments more than four decades ago, successful cryopreservation of teleost oocytes and embryos has remained elusive. Five factors are suspected to complicate teleost embryo cryopreservation (27): (i) a large overall size, resulting in a low surface-to-volume ratio, which could retard water and cryoprotectant efflux/influx; (ii) large-sized cells, such as the yolk, which increases the likelihood of membrane disruption by intracellular ice formation (25); (iii) compartments, such as the blastoderm and yolk, with possibly differing osmotic properties; (iv) semipermeable membranes surrounding the embryo (36) which

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may inhibit water and cryoprotectant influx/efflux; and (v) potential susceptibility to chilling injury (31, 41).

The availability of cryopreserved oocytes and embryos, however, could have a profound influence on medical research, aquaculture and conservation biology. The ability to cryopreserve teleost embryos would permit the storage of a diverse gene pool, thus allowing the maintenance of valuable transgenic lines and hybrids. Moreover, the development of frozen, or "insurance" populations would preserve genetic diversity and assist efforts to prevent the extinction of fish species in natural aquatic ecosystems (1, 38, 39).

Our laboratory uses the zebrafish (*Brachydanio rerio*) as a model for developing an understanding of the cryobiological properties of teleost embryos. The objectives of this study were to determine the chill sensitivity and permeability behavior of dechorionated zebrafish embryos using classical osmometric methods. Zebrafish are advantageous because a substantial database on their developmental biology (17, 18, 37), culture *in vitro* (37), and some published information on the permeability and toxicity of cryoprotectants already existed (14, 16, 40–42).

Potentially, one of the main challenges to successfully cryopreserving fish embryos is the chorion. This outer, protective membrane layer consists mostly of glycoproteins (11, 2) and may hinder water and solute movement into and out of the embryo (14). Therefore, throughout most of these studies the chorion was removed. A previous study (41) described the chilling sensitivity of intact zebrafish embryos; however, the chorion is not necessary for proper development in zebrafish and it acts as a potential impedance to movement solutes into the embryo. The removal of the chorion seemed critical for this and future studies; however, it was not clear if the dechorionating enzyme used in the present study would effect the reported (41) chilling sensitivity. Therefore, the chilling sensitivity of a variety of developmental stages was determined for both normal and dechorionated embryos. The dechorionated zebrafish embryo is composed of two complex cellular compartments (i.e., a large yolk and the developing blastoderm) that change throughout development. Information concerning the volume of these compartments is essential for future biophysical modeling. Therefore, the developmental volume change was measured from 40% epiboly to the six-somite stage. As the muscle somites develop, epithelia begin to cover the blastoderm (37), thereby forming another potential barrier. Therefore, these studies were restricted to blastula, gastrula, and early somite developmental stages of the fish embryo. Finally, we determined embryo permeability to cryoprotectants, using optical microscopy to measure the kinetics of volumetric changes in embryos placed in hypertonic solutions, and cryoprotectant toxicity, monitoring their survival after these treatments.

MATERIALS AND METHODS

Maintenance of Animals

Animals were maintained and bred (naturally) according to the procedures described by Westerfield (37). The embryos produced were dechorionated and cultured in embryo medium (which is a modified Hanks' buffer) according procedures described by Westerfield (37). Briefly, 12 to 16 fish were kept in 5-liter aquaria (temp, 28.5°C; pH, 7.0) illuminated with a 12/12 light/dark cycle and fed dry flakes (Aquarian Diet, Mardel, Glendale, IL) and black worms (Tubifecidae). Because the developmental temperature was 28.5°C, all developmental stages described here are similar to those in Westerfield (37).

Throughout the study, only developmental time points up to the six-somite stage were examined for two reasons. First, during this period, there was little or no epidermis covering the embryo that might act as a diffusion barrier by hindering permeability of water and cryoprotectants. Second, embryos still were relatively round, maintained a reproducible orientation when moved from solution to solution, and were nonmotile, simplifying the optical volumetric measurements. The multicom-

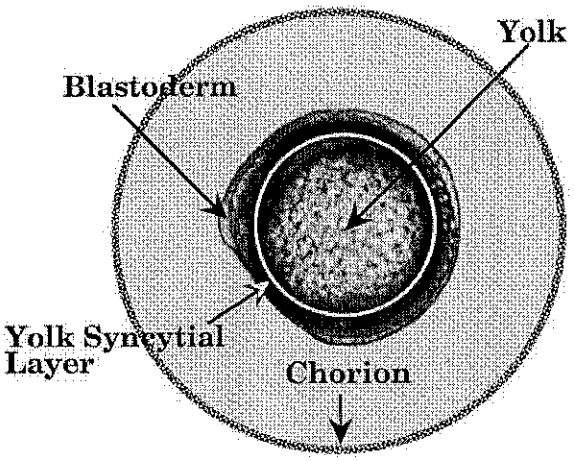

FIG. 1. Image of a six-somite zebrafish embryo that identifies the major compartments (yolk and blastoderm). Although the yolk syncytial layer would not be visible in this image, its position is included for clarity. Additionally, the position of the chorion is indicated; however, this layer is removed in most preparations.

partmental nature of a six-somite embryo is shown in Fig. 1.

Embryo Chilling Sensitivity

Dechorionated zebrafish embryos were prepared by enzymatic digestion (37). Briefly, embryos were collected at 1 h postfertilization (two- to four-cell stage) and transferred with a fire-polished pipet into a sterile, 5-ml Petri dish containing 1.0 ml of embryo medium (EM; 37). All solutions throughout the study were prepared using an EM base. To exclude the possibility that the dechorionating enzyme altered chilling sensitivity, both intact and dechorionated embryos were tested. One embryo group was unmanipulated until chilled, whereas another was dechorionated by adding 200 μl of a pronase solution to the dish (Sigma Chemical Co., St. Louis, MO; 10 mg/ml dissolved in EM). After 5 min, embryos were washed gently three times in 100 ml of EM to remove excess enzyme and then transferred to sterile, agar-coated Petri dishes containing 3 ml of EM. Embryos were dechorionated at the 1 h-stage, because at this time the chorion had not yet hardened.

Chilling sensitivity was determined for embryos at developmental stages corresponding to 1.25, 1.5, and 1.75 h postfertilization (early cell division stages); 2 and 4 h (blastula stages); 6, 8, and 10 h (gastrula stages); and 12 h (three-muscle somite stage). At each developmental stage, flat-bottomed glass vials containing 10 to 56 embryos in 1 ml of EM were placed on wet ice for 10, 20, or 40 min at 0°C. These times represented the intervals for which embryos might experience chilling temperatures during cryopreservation procedures. Temperature was monitored using a control vial with a thermistor (HH23; Omega Engineering, Inc., Stamford, CT). After chilling, vials were removed from the ice and placed into a water bath (28.5°C) for 5 min. Embryos were then transferred into 5-ml Petri dishes containing 3 ml of EM and 30 to 40 μl of streptomycin–penicillin solution (Sigma) and incubated at 28.5°C for 48 h (the beginning of hatching). Surviving embryos were transferred into covered plastic cups with 30 ml of fresh EM for 2 additional days of culture at 28.5°C and then checked for normal development. The assay for normal development was the formation of the swimbladder, present in all normal embryos by Day 4 in culture (i.e., 96 h upright, swimbladder stage) (37; Walker, personal communication). Additionally, formation of fins, tail, pericardium, opercula, and mouth parts were examined as indices of growth and viability. Survival was defined as the percentage of treated embryos with developmentally normal appearance at the 96-h stage.

To determine if embryos could be maintained for short periods at room temperature or lower without adversely affecting development, we examined the effect of embryo exposure to ambient (21 to 23°C) and subambient (4°C) temperatures on normal development. Eight-hour embryos were exposed to one of three treatments: (i) maintenance at 28.5°C (n = 13); (ii) cooled to 21 to 23°C for 3.5 h (n = 13); or (iii) cooled to 4°C for 3.5 h (n = 14). Normal development was monitored

during subsequent culture to Day 4 (as described above).

Morphometric Measurements

Changes in embryo size were measured during development (from 40% epiboly to six-somite stage) using computer-assisted light microscopy. Embryos were examined under a Zeiss compound microscope with a CCD camera (WV-BL200; Panasonic), and images were digitized with a video frame-grabber card (LG3; Scion Corp., Frederick, MD) and stored for later morphometric analysis. Digitized images (100×) were displayed on a high-resolution video monitor and analyzed using a computer-aided morphometry package (Image 1.45, NIH). The outline of each embryo was determined by the computer, or parts of the embryo were outlined manually (with the mouse), and linear, planar, and volumetric parameters of each embryo were calculated. The major and minor axes were used to determine the volume of embryos at all stages using a prolate spheroid formula ($V = 4/3\pi ab^2$), where a and b were the major and minor semi-axes, respectively.

Because zebrafish embryos grew so rapidly during our experiments, we measured the relative volumes of the changing yolk and blastoderm compartments during the developmental stages studied. Images from two embryo groups (10 to 17 embryos/group) were monitored continuously from 40% epiboly to the six-somite stage. At 40% epiboly, much of the spherical yolk was clearly visible, allowing an accurate calculation of the yolk diameter. Because the yolk was partially or entirely obscured by the developing blastoderm, we examined developmentally staged histological material to affirm that the yolk (i) had a spherical volume and (ii) changed little during the subsequent developmental stages. The changing blastoderm volume was calculated from the mean volume of the embryos at each stage minus the mean volume of the yolk calculated at 40% epiboly.

Throughout this paper, data pairs presented as percentages were statistically tested for equality by a χ^2 analysis or a Mann–Whitney U test (30).

Permeability and Toxicity of Cryoprotectants

The osmotic volume response of three-somite embryos to five cryoprotectants and survival after stepwise dilution was examined. Embryos were placed in 1.5 M solutions of dimethyl sulfoxide (DMSO), propylene glycol (PG), ethylene glycol (EG), glycerol, or methanol (METH) for 20 min and then measured volumetrically at 2-min intervals (as described above). If the embryo dehydrated (reduced in volume) and then rehydrated (returned to its original volume or slightly larger), it was considered permeable to the cryoprotectant. We also examined the permeability of 2 M solutions of DMSO, PG, and METH using light microscopic volumetric analysis at the three- and six-somite stages (n = 4 to 5 embryos/sample). Embryos were imaged every 2 min over 30 min, and the embryo volume was calculated as described above. Initially, image capture was done every 30 s, but this proved to be unnecessarily repetitious, as no changes were measured at the shorter imaging interval.

To evaluate cryoprotectant toxicity and to determine the optimal dilution method for removal of these components, embryos at the three-somite stage were immersed in each cryoprotectant solution for 20 min (n = 26 to 87), and the cryoprotectant was diluted 1:1 with EM by either a two- or a four-step procedure (10 min each step). Survival of embryos after exposure to the 1.5 M cryoprotectant solutions was determined by assessing normal development after 4 days of culture.

RESULTS

Embryo Chilling Sensitivity

To determine if dechorionation by pronase might alter the chilling sensitivity at 0°C, we compared the chilling sensitivity of intact and dechorionated embryos at various developmental stages (Fig. 2). The 4-h group did show

a reduction in their survival after treatment with pronase. However, when considering all the developmental stages, enzymatic removal of the chorion did not alter ($P > 0.05$) chilling sensitivity of zebrafish embryos. Based on these results, only dechorionated embryos were used in subsequent experiments. Additionally, examining chill sensitivity amongst the various developmental stages revealed that late-staged embryos tolerated cooling to 0°C better ($P < 0.05$) than early-stage embryos. For example, after 40 min at 0°C, none of the intact and dechorionated early-stage embryos (1.25 to 2.0 h) survived, whereas greater than 65% of the late-stage embryos (50% epiboly to three-somite stage) survived.

Embryos are normally cultured in EM at 28.5°C. However, we wished to conduct many experiments at ambient (21 to 23°C) or subambient (4°C) temperatures and wanted to know if this might affect the survival of late-stage embryos. Fortunately, exposure of 8-h embryos (75% epiboly) to ambient or subambient temperatures for 3.5 h had no effect ($P > 0.05$) upon survival (77 and 79%, respectively, compared to 84% survival in controls). These results indicated that 8-h zebrafish embryos tolerate short-term exposures of temperatures ranging from 4 to 23°C with little or no ill effects. After observing hundreds of embryos at various developmental stages, we have observed this same tolerance to exposure to room temperature to hold for all embryos. Therefore, all subsequent experiments were conducted at 21 to 23°C unless indicated otherwise.

Morphometric Measurements

During and beyond epiboly, the blastoderm underwent rapid and marked changes. To follow the change in the blastoderm throughout this period, the mean linear and volumetric parameters of the yolk and blastoderm were measured from 40% epiboly to the six-somite stage in two embryo groups (Fig. 3). At 40% epiboly, the yolk diameter clearly was discernible (646 ± 4 μm, $n = 13$; 633 ± 2 μm, $n = 10$). Although the yolk is continuously consumed by the blastoderm through pinocytosis (36), this process is not measurable by light microscopy at the developmental stages studied; thus a constant yolk volume was used for each developmental stage. During epiboly, the mean volume of the embryo and the blastoderm did not change in the two sample groups ($P > 0.05$; blastoderm = 17 to 22% of the total volume). However, at the three-somite stage, the blastoderm began to increase in size ($P < 0.05$; blastoderm = 30 to 32% total volume) and by the six-somite stage, the volume of the blastoderm had increased to 35 to 39% of the total volume. Linear measurements of the major and minor axes (Table 1) indicated that embryos are relatively spherical during epiboly, but become more prolate around 100% epiboly just before the volume increase at the three-somite stage.

Permeability and Toxicity of Cryoprotectants

Exposure of three-somite embryos to 1.5 *M* glycerol or ethylene glycol was deleterious. Glycerol caused 100% embryo mortality, whereas ethylene glycol caused the blastoderm to dissociate from the yolk within 60 min. Therefore, these cryoprotectants were not tested further. Little or no dehydration (i.e., <3%) was observed when three-somite embryos were placed in 1.5 *M* METH, DMSO, or PG (Fig. 4A). Furthermore, none of the embryos swelled during the 20-min exposure to these later solutions. Nearly all the METH- and DMSO-exposed embryos (95 to 100% and 90 to 95%, respectively) remained viable after cryoprotectant dilution by either a two- or four-step procedure (Fig. 4B). However, embryos exposed to a 1.5 *M* PG solution experienced higher survival (83%) after a four-step dilution than after a two-step dilution (59%; $P < 0.05$).

We reexamined the cryoprotectant permeability using a longer time, higher cryoprotectant concentration, and both three- and six-somite stages (Fig. 5). No osmotic changes consistent with permeation were observed for either stage immersed in 1.5 *M* DMSO or 1.5

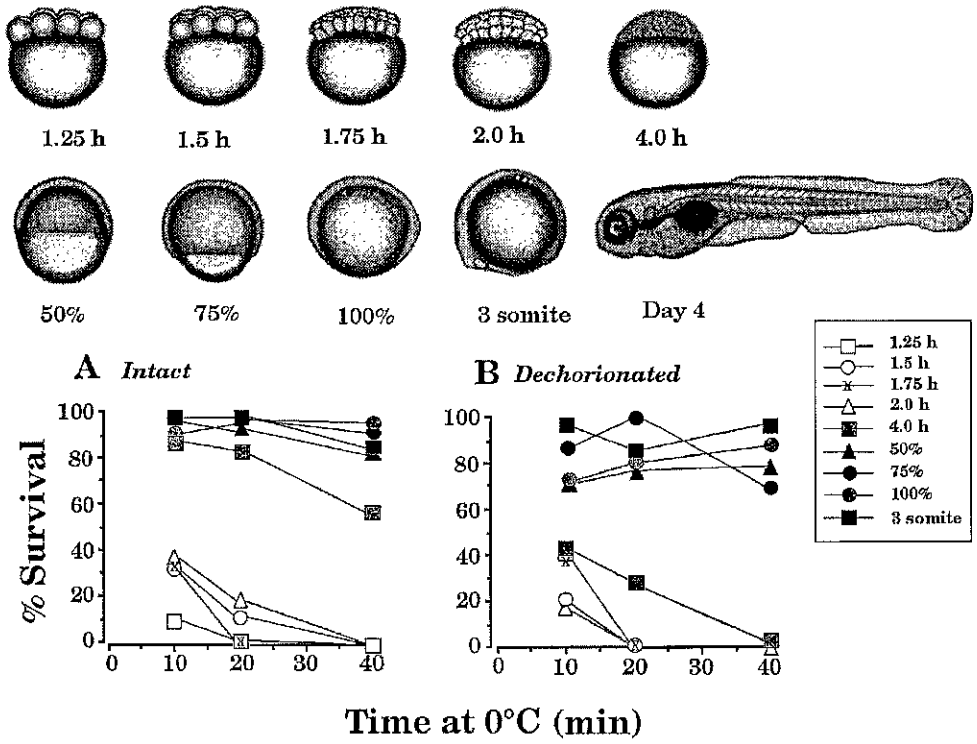

FIG. 2. Line graphs indicate percentage of survival of (A) intact and (B) dechorionated embryos (n = 10 to 56 embryos per datum point) after exposure for up to 40 min at 0°C. Drawings depict the developmental stages used in these studies (modified from Westerfield, 1993). Successful development was based upon the ability to develop to the 96-h upright, swimbladder stage. Both intact and dechorionated embryos expressed similar ($P > 0.05$) chilling sensitivity profiles. Early-staged embryos (1.25 to 4 h) were more susceptible ($P < 0.05$) to chilling injury than late-staged embryos (50% epiboly to three-somite).

and 2.0 M PG. A dehydration (10–13% change in volume) was observed in the 2.0 M DMSO (Figs. 5A and 5B) within 30 min, but no subsequent rehydration. However, six-somite embryos immersed in 2.0 M METH showed clear evidence of osmotic dehydration and rehydration within 20 min [mean dehydration, 8.0 ± 1.7% SEM; mean rehydration, 5.2 ± 1.6% SEM; n = 4 (one embryo was excluded from the analysis because it changed only 1% which is within measurement error); Fig. 5C]. Perhaps embryos immersed in PG and DMSO would have experienced a volume increase if observed for longer than 30 min. However, this was not observed when 100% epiboly embryos (n = 6) were exposed to 3.0 M PG over 2 h (data not shown).

DISCUSSION

Throughout most of these studies, we have used the dechorionated zebrafish embryos as a model for understanding chilling sensitivity, cryoprotectant permeability, and toxicity characteristics. A key to successful cryopreservation lies in understanding these complex and interrelated phenomena (24, 25). The embryos do not need the chorion to grow normally and survive (37), and it is potentially one more permeability barrier. Fortunately, the removal of the chorion using pronase did not significantly alter the chilling sensitivity of the embryos. Additionally, only developmental stages up to the six-somite stage were examined. During this period, there was little or no epithelia covering the embryo that might act as a diffusion barrier to water and cryoprotectants.

We tested chilling sensitivity over a relatively brief period of time (up to 40 min, the expected duration of chilling during a cryopreservation trial), because we were interested

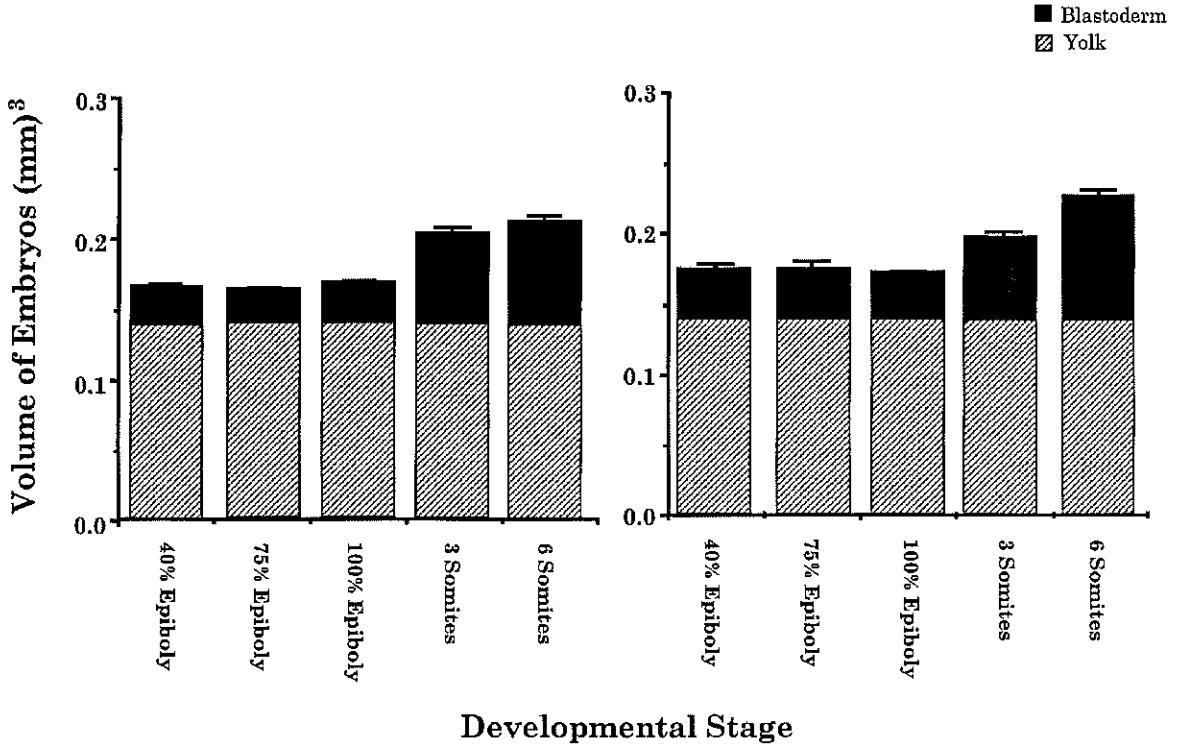

FIG. 3. Mean volume (±SEM) of two groups of normal zebrafish embryos (n = 10 and 17 embryos/group) was measured from epiboly to the six-somite stage. Based on a yolk of constant volume (measured at 40% epiboly), the mean blastoderm volume remained constant throughout epiboly ($P > 0.05$), but increased at the three-somite stage ($P < 0.05$).

in knowing if some developmental stages should be excluded from future trials. We found that early-stage embryos (1.25 to 2 h) were more chill-sensitive than late-stage (50% epiboly to three-somite) embryos. Examining the data in Fig. 2 at the 10-min time point revealed that less than 40% of the intact early-stage embryos (1.25 to 2 h) survived chilling at 0°C; however, greater than 85% of the intact late-stage embryos (50% epiboly to three-somite stage) survived. Zhang and Rawson (41) also examined the chilling sensitivity at 0°C of intact zebrafish embryos. After 12 min at 0°C, they reported that only 20% of the blastula-stage embryos (2 h) survived; whereas, 100% of the later-stage embryos (50 to 100% epiboly) survived. Stage-dependent chill sensitivity has been reported for many other spe-

TABLE 1
Dimensions of Developing Zebrafish Embryos

Developmental stage	Mean size (μm ± SEM)		Volume (mm^3 ± SEM)	n
	Major axis	Minor axis		
40% Epiboly	714.8 ± 2.8	691.5 ± 2.7	0.174 ± 0.002	17
75% Epiboly	711.1 ± 3.4	694.2 ± 3.7	0.176 ± 0.003	15
100% Epiboly	740.4 ± 5.5	673.9 ± 2.8	0.172 ± 0.002	16
Three-somite	781.1 ± 4.0	701.7 ± 4.8	0.197 ± 0.003	15
Six-somite	796.7 ± 5.1	745.5 ± 4.3	0.227 ± 0.003	11

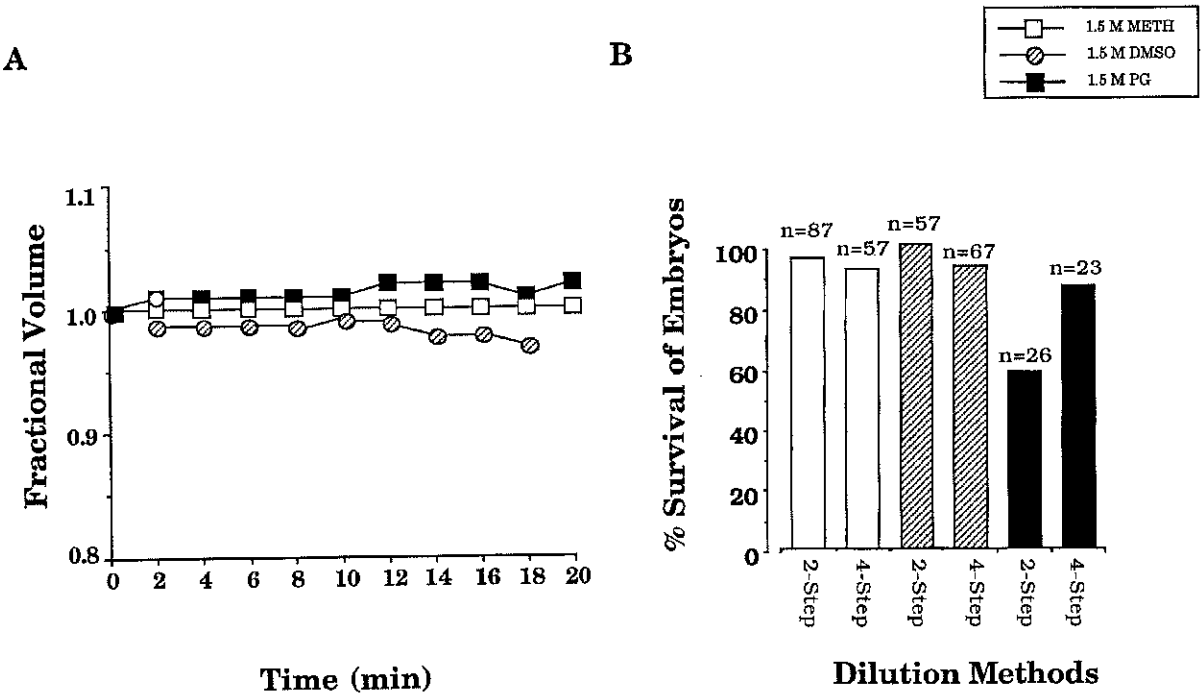

FIG. 4. Permeability (A) and survivorship (B) of three-somite embryos after exposure to one of three cryoprotectants for 20 min (22°C). Methanol (METH) produced no volume change, and 95 to 100% survival by both dilution methods. DMSO caused no dehydration and no permeation with 90 to 95% survival for both dilution methods. Propylene glycol (PG) caused a slight increase in volume, and the four-step dilution yielded higher ($P > 0.05$) survival (83%) than the two-step dilution (59%).

cies of fish embryos by Maddock (23, for brown trout), Haga (8, for rainbow trout), Cloud *et al.* (5, for fathead minnows), and Lubzens *et al.* (21, for Japanese carp). Additionally, 8-h embryos experienced no deleterious effect on the development after short (ca. 3 h) exposures to ambient and sub-ambient temperatures. However, prolonged exposure to subambient temperatures can severely affect the development of the zebrafish. Embryos (before 16-cell stage) cultured at subambient temperatures (18°C) did not complete epiboly due to partial depolymerization of microtubules which are important for cellular movements and reorganization (20). This sensitivity of prolonged exposure to subambient temperatures may be species-dependent, because no deleterious effect on development was observed in a more temperate species, medaka, cultured at 18°C (personal communication, R. Fluck).

The efficacy of cryoprotectant permeation and equilibration is crucial to formulating successful cryopreservation procedures. Harvey *et al.* (16) examined the permeability of 5.3 h (50% epiboly) zebrafish embryos to isotopically labeled glycerol and DMSO and found these solutes permeated into dechorionated and intact embryos. Unfortunately, the amount and location (i.e., yolk versus blastoderm) of these permeating cryoprotectants were unclear. Subsequently, Harvey (13) reported that glycerol protected only the blastoderm, and neither cryoprotectant effectively preserved the entire embryo at −196°C. Recently, Suzuki *et al.* (32; for carp, medaka, and rainbow trout) and Lubzens *et al.* (21; for carp) reported an uptake of DMSO solution into the perivitelline space and some tissues, but the permeation level was insufficient for cryopreservation. Zhang and Rawson (42) describe cryoprotectant (i.e., a variety of cryoprotectant combinations) permeation into intact six-somite and 27-h embryos by measuring the vol-

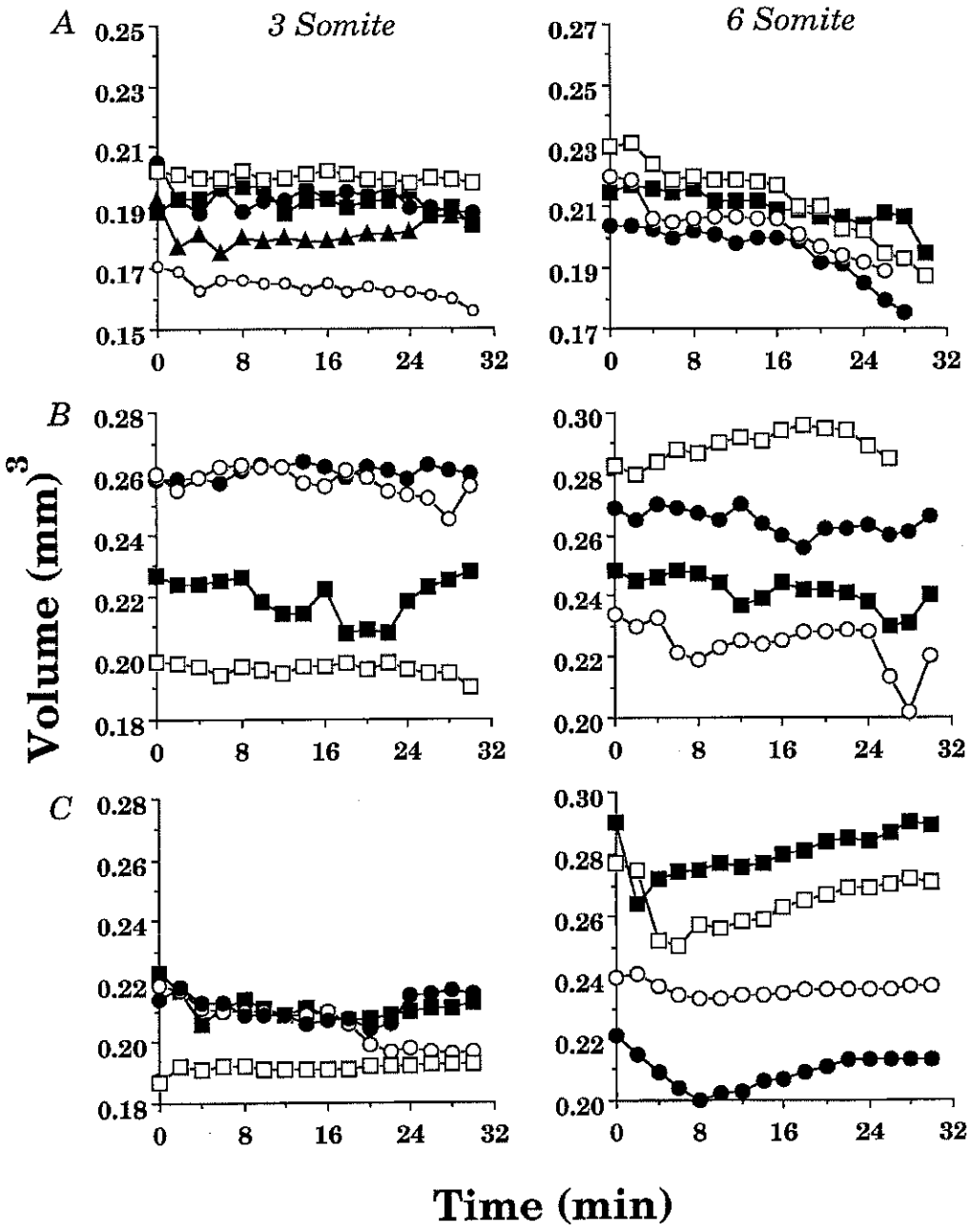

FIG. 5. Volumes of three- and six-somite embryos (each symbol represents one individual) exposed to 2.0 *M* cryoprotectant solutions over 30 min. Embryos were immersed in the cryoprotectant, and the first images were digitized at ca. $t = 1$ min. (A) DMSO: no clear change was observed in volume of the three-somite embryos; however, ca. 10 to 13% dehydration was observed in six-somite embryos after 15 min. (B) PG: no clear change was observed in volume of the three- or six-somite embryos. (C) METH: no change was observed in three-somite embryos; however, a 1 to 10% dehydration and rehydration volume changes were observed in the six-somite embryos after 6 to 12 min. This suggested that METH permeated the six-somite embryos.

ume changes in the outer chorion. Clearly, cryoprotectants permeated the chorion into the perivitelline space. However, this method provides no information concerning the cryoprotectant permeation into the yolk or the blastoderm.

In Fig. 4 and 5, no observable shrinkage was detected when the embryos were im-

mersed in a 1.5 or 2.0 *M* PG solution for 30 min. However, a 10 to 13% dehydration was observed in 2.0 *M* DMSO at the six-somite stage. Another nonpermeable solute, 0.35 *M* NaCl, shows a similar pattern to the DMSO (ca. 10–15% volume change within 30 min) (10). A major difference between the salt and cryoprotectant solutions is that the 2.0 *M* DMSO and PG solutions have an osmolality approximately four times that of the 0.35 *M* NaCl solution (683 mosm). Clearly, other processes besides osmotic forces are at work, because the rate of dehydration for the 2.0 *M* DMSO is similar to the 0.35 *M* NaCl solution, whereas the 2.0 *M* PG shows no dehydration. Most likely, the DMSO and PG have modified the membranes, thus altering the membrane permeability (Lp). In fact, the Lp for the yolk of the zebrafish embryo decreased by 10-fold in the presence of 3.0 *M* PG (10). There are a number of studies that report a change in the Lp in the presence of cryoprotectants (7, 22, 35, 33, 34). Hammerstedt *et al.* (12) have reviewed a number of possible interactions that cryoprotectants might have on membrane bilayers, including direct alteration of membrane bilayers and stearic interactions with bound proteins on the external surface. Although the Lp is altered by the presence of PG, the mechanisms for this change are unknown. Most importantly, it is the Lp determined under experimental conditions (i.e., when cryoprotectants are in the solution) that must be considered when designing cryopreservation trials.

One possible explanation for the lack of osmotic dehydration and permeation observed during cryoprotectant permeation was that the cryoprotectant permeated rapidly at a rate comparable to that of the water, thus canceling large volume changes (28). Another explanation was that permeation was a very slow process, and, therefore, not observed within the experimental period. However, the longer-duration PG data presented did not support this hypothesis. An alternative explanation is that some sort of volume regulation (osmoregulation) process is at work that confounds the experimental results. The long time course of the experiments clearly provides enough time for such processes to occur. Future experiments are planned to investigate volume regulation in the zebrafish embryo.

In most of our experiments, light microscopy failed to provide us with a clear understanding of cryoprotectant permeability. In our studies, of the five cryoprotectants tested, only METH permeated the six-somite embryo; however, the volume changes were small and inconsistent between replicates. This was also reported by Zhang and Rawson (42) in similar experiments. However, METH usually exhibits a high permeability that may reduce the efficacy of determining volume changes by light microscopy. Rall *et al.* (28) successfully cryopreserved mouse embryos in 3 *M* METH, after an equilibration period of 10 min, but observed no change in the volume of embryos over 180 min. Moreover, when we considered the potential errors in the system, such as reproducibility errors in determining the volume from an image (up to 2 to 4%) or slight movements of the embryos leading to large changes in the measurement of the major or minor axis, the small dehydration (ca. 8%) and rehydration events (5%) observed with METH are close to the noise level. Finally, DMSO and PG can permeate the blastoderm but not the yolk (9). However, no rehydration event was observed using light microscopy. This may be due to the relatively small contribution that the blastoderm renders to the entire embryo volume at the three- and six-somite stages. We conclude that the complex nature of the zebrafish embryo reduces the effectiveness and predictive value of light microscopical measurements for cryoprotectant permeability studies.

To obtain a better understanding of permeability, we recently used magnetic resonance (MR) microscopy to study solute flux in live zebrafish embryos (9). This allows real time visualization of kinetic processes, including the permeation of solutes across membranes

and the accurate quantification of cellular components, such as lipids and cellular water. Although the resolution of MR microscopy cannot compare with high-resolution light and electron microscopy, it has several advantages. It is noninvasive and can detect and characterize the distribution of specific molecules, can be sensitized to molecular dynamics, and allows spatial resolution in a live, developing animal (4). MR microscopy has revealed that methanol, DMSO, and PG permeated the blastoderm, but only methanol is distributed throughout the embryo (9). These results may explain the difficulty in observing any permeation of DMSO and PG using light microscopy. Even if the smaller-volume blastoderm did go through a dehydration and rehydration event, it would have been masked by the dehydration of the larger-volume yolk. All the data on cryoprotectant permeation into the zebrafish embryo suggests that the yolk syncytial layer (a single cell that surrounds the yolk) blocks the movement of some cryoprotectants into the yolk (9).

Although there are many complex factors involved in cryopreservation, we predict that understanding the physiology of the yolk syncytial layer will be a key element to the future successful cryopreservation of zebrafish embryos. Developing techniques for freezing the sperm, eggs, and embryos of fish for inclusion in a genetic bank is a critical, worldwide need. Within the next 25 to 50 years, it is predicted that sensitive aquatic ecosystems will experience massive extinctions of endemic species (19, 29). Already many freshwater communities in developing regions, including Lake Victoria, the rivers of Madagascar and the Atlantic rivers of Brazil are critically affected by human encroachment. In North America and Europe, salmon stocks are threatened by competition with hatchery stocks, habitat degradation from deforestation, and air pollution-induced acidification (6). Systematic germplasm banking from natural communities would maintain species diversity, prevent species extinction, and allow time to rehabilitate the native environment to support species reintroduction.

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REFERENCES

1. Ballou, J. D. Potential contribution of cryopreserved germ plasm to the preservation of genetic diversity and conservation of endangered species in captivity. *Cryobiology* **29,** 19–25 (1992).
2. Begovac, P. C., and Wallace, R. C. Vitelline envelop proteins in the pipefish. *J. Morphol.* **193,** 117–133 (1986).
3. Blaxter, J. H. S. Sperm storage and cross-fertilization of spring and autumn spawning herring. *Nature* **172,** 1189–1190 (1953).
4. Callaghan, P. T. "Principles of Nuclear Magnetic Resonance Microscopy," Claredon Press, Oxford, 1991.
5. Cloud, J. G., Erdahl, A. L., and Graham, E. F. Survival and continued normal development of fish embryos after incubation at reduced temperatures. *Trans. Am. Fish. Soc.* **117,** 503–506 (1988).
6. Gausen, D. The Norwegian gene bank program for Atlantic salmon (*Salmo salar*). *In* "Genetic Conservation of Salmonid Fishes" (J. G. Cloud and G. H. Thorgaard, Eds.), pp. 181–187. Plenum, New York, 1993.
7. Gilmore, J. A., McGann, L. E., Liu, J., Gao, D. Y., Peter, A. T., Kleinhans, F. W., and Critser, J. K. Effect of cryoprotectant solutes on water permeability of human spermatozoa. *Biol. Reprod.* **53,** 985–995 (1995).
8. Haga, Y. On the subzero temperature preservation of fertilized eggs of rainbow trout. *Bull. Jpn. Soc. Sci. Fish.* **48,** 1569–1572 (1982).
9. Hagedorn, M., Hsu, E., Pilatus, U., Wildt, D. E., Rall, W. F., and Blackband, S. J. Magnetic resonance microscopy and spectroscopy reveal kinetics of cryoprotectant permeation in a multicompartmen-

tal biological system. *Proc. Natl. Acad. Sci. USA* **93,** 7454–7459 (1996).

10. Hagedorn, M., Kleinhans, F. W., Freitas, R., Liu, J., Hsu, E., Wildt, D. E., and Rall, W. F. Water distribution and permeability of zebrafish embryos, *Brachydanio rerio.* In press (1997).
11. Hamazaki, T., Iuchi, I., and Yamagami, K. A spawning female-specific substance reactive to antichorion (egg envelope) glycoprotein antibody in the teleost, *Oryzias latipes. J. Exp. Zool.* **235,** 269–279 (1985).
12. Hammerstedt, R. H., Graham, J. K., and Nolan, J. P. Cryopreservation of mammalian sperm: What we ask them to survive. *J. Androl.* **11**(3), 73–88 (1990).
13. Harvey, B. Cooling of embryonic cells, isolated blastoderms, and intact embryos of the zebra fish *Brachydanio rerio* to minus 196 Celsius. *Cryobiology* **20,** 440–447 (1983).
14. Harvey, B., and Chamberlain, J. B. Water permeability in the developing embryo of the zebrafish, *Brachydanio rerio. Can. J. Zool.* **60,** 268–270 (1982).
15. Harvey, B., Kelley, R. N., and Ashwood-Smith, M. J. Cryopreservation of zebrafish spermatozoa using methanol. *Can. J. Zool.* **60,** 1867–1870 (1982).
16. Harvey, B., Kelley, R. N., and Ashwood-Smith, M. J. Permeability of intact and dechorionated zebra fish (*Brachydanio rerio*) embryos to glycerol and dimethyl sulfoxide. *Cryobiology* **20,** 432–439 (1983).
17. Hisaoka, K. K., and Battle, H. I. The normal developmental stages of the zebrafish, *Brachydanio rerio* (Hamilton-Buchanan). *J. Morphol.* **102,** 311–328 (1958).
18. Hisaoka, K. K., and Firllit, C. F. Further studies on the embryonic development of the zebrafish, *Brachydanio rerio* (Hamilton-Buchanan). *J. Morphol.* **107,** 205–255 (1960).
19. Janzen, D. H. The most endangered tropical ecosystem. *In* "Biodiversity" (E. O. Wilson and F. M. Peter, Eds.), pp. 130–137. National Academy Press, Washington, 1988.
20. Jesuthasan, S. Cold-sensitive microtubules are required during early cleavage stages for dorsoventral specification, epiboly and cytokinesis in the zebrafish embryo. *Zebrafish Dev. Genet.* **8** (1996).
21. Lubzens, E., Amos, A., and Magnus, Y. Steps towards cryopreservation of Japanese ornamental carp embryos: Sensitivity to low temperatures and permeation of ^{3}H-dimethylsulfoxide (DMSO). Submitted for publication.
22. Macy, R. I. Transport of water and urea in red blood cells. *Am. J. Physiol.* **246,** C195–C203 (1984).
23. Maddock, B. G. A technique to prolong the incubation period of brown trout ova. *Prog. Fish Cult.* **36,** 219–222 (1974).
24. Mazur, P. Cryobiology: The freezing of biological systems. *Science* **168,** 939–949 (1970).
25. Mazur, P. Freezing of living cells: Mechanisms and implications. *Am. J. Physiol.* **247,** C125–C142 (1984).
26. Polge, C., Smith, A. U., and Parkes, A. S. Revival of spermatozoa after vitrification and dehydration at low temperatures. *Nature* **164,** 666 (1949).
27. Rall, W. F. Recent advances in the cryopreservation of salmonid fishes. *In* "Genetic Conservation of Salmonid Fishes (J. G. Cloud and G. H. Thorgaard, Eds.), pp. 137–158. Plenum, New York, 1993.
28. Rall, W. F., Czlonkowski, C., Barton, S. C., and Polge, C. Cryoprotection of Day 4 mouse embryos by methanol. *J. Reprod. Fertil.* **70,** 293–300 (1984).
29. Raven, P. H. Our diminishing tropical forests. *In* "Biodiversity" (E. O. Wilson and F. M. Peter, Eds.), pp. 119–122. National Academy Press, Washington, 1988.
30. Sokal, R. S., and Rohlf, F. J. "Biometry." Freeman, San Francisco, 1969.
31. Stoss, J. Fish gamete preservation and spermatozoan physiology. *In* "Fish Physiology" (W. S. Hoar, D. J. Randall, and E. M. Donaldson, Eds.), Vol. IX, pp. 305–350. Academic Press, New York, 1983.
32. Suzuki, T., Komada, H., Takai, R., Arii, K., and Kozima, T. T. Relation between toxicity of cryoprotectant DMSO and its concentration in several fish embryos. *Fish. Sci.* **61,** 193–197 (1995).
33. Toon, M. R., and Solomon, A. K. Modulation of water transport in human red cells: Effect of urea. *Biochim. Biophys. Acta* **940,** 266–274 (1988).
34. Toon, M. R., and Solomon, A. K. Transport parameters in the human red cell membrane: Solute-membrane interactions of hydrophilic alcohols and their effect on permeation. *Biochim. Biophys. Acta* **1022,** 57–71 (1990).
35. van Hoek, A. N., de Jong, M. D., and van Os, C. H. Affects of dimethylsulfoxide and mercurial sulfhydryl reagents on water and solute permeability of rat kidney brush border membranes. *Biochim. Biophys. Acta* **1030,** 203–210 (1990).
36. Wallace, R. A., and Selman, K. Ultrastructural aspects of oogenesis and oocyte growth in fish and amphibians. *J. Electron Microsc. Tech* **16,** 175–201 (1990).
37. Westerfield, M. "The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (*Brachydanio rerio*)." University of Oregon Press, Eugene, 1993.
38. Wildt, D. E. Genetic resource banking for conserving wildlife species: Justification, examples and be-

coming organized on a global basis. *Anim. Reprod. Sci.* **28,** 247–257 (1992).

39. Wildt, D. E., Seal, U. S., and Rall, W. F. Genetic resource banks and reproductive technology for wildlife conservation. *In* "Genetic Conservation of Salmonid Fishes" (J. G. Cloud and G. H. Thorgaard, Eds.), pp. 159–173. Plenum, New York, 1993.
40. Zhang, T., Rawson, D. M., and Morris, G. J. Cryopreservation of pre-hatch embryos of zebrafish (*Brachydanio rerio*) embryos. *Aqua. Liv. Res.* **6,** 145–153 (1993).
41. Zhang, T., and Rawson, D. M. Studies on chilling sensitivity of on zebrafish (*Brachydanio rerio*) embryos. *Cryobiology* **32,** 239–246 (1995).
42. Zhang, T., and Rawson, D. M. Feasibility studies on vitrification of intact zebrafish (*Brachydanio rerio*) embryos. *Cryobiology* **33,** 1–13 (1996).