New Chapter

Non-Equilibrium Vitrification: an Introduction and Review of Studies Done in Fish

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The Need to Bank Aquatic Germplasm

Among vertebrates, fish represent more than 50% of all known species with a total of 28,800 described species (Diana 2003, Barton 2007). Of the total fish biodiversity, 43% of species are freshwater. Roughly 40% of the freshwater fish species in North America and European are imperiled (Jelks et al. 2008, Kottelat and Freyhof 2007). The main threats are habitat degradation and introduction of non-indigenous species. Overall declines are also occurring in the oceans. Since the 1970s, there has been an 80% decline in coral cover in the Caribbean. Coral reefs are among the most diverse ecosystems on Earth. About 35% of known fish fauna are associated with coral reefs (Barton 2007), yet reef fish density has been declining significantly for more than a decade, at rates ranging from 2.7% to 6% per yr (Paddack et al. 2009). In addition, large predatory fish biomass has been reduced by 90% over the last 50 yr (Myers and Worm 2003). Overfishing, pollution, global warming, ocean acidification, and other ecological impacts have degraded marine ecosystems (Jelks et al. 2008). Furthermore, according to the Red List of the International Union for Conservation of Nature and Natural Resources (IUCN), 65% (940/1452 species) of the ray-finned fishes (Class Actinopterygii) that are listed as imperiled are less than 20 cm in body length. These small fish are typically overlooked in conservation programs. Given this dismal current reality, conservation efforts can no longer be delayed while awaiting more thorough assessments.

Cryopreservation in Fishes

Cryopreservation represents a tool for the protection of genetic resources in aquatic species, and offers many benefits in aquaculture, conservation biology, and medical applications. Benefits include the year-round availability of gametes, reduction of the need for maintaining fish as live populations, protection of valuable genetic lineages, and improvement of genetic lines (Tiersch et al. 2007). Despite these benefits, cryopreservation has only been researched for sperm of some 200 (Tiersch 2000) of the 28,800 fish species described (Barton 2007) with application only beginning, and research on eggs and embryos lagging behind this. As such, less than 1% of species could at present be preserved in “genome resource banks” or “frozen zoos”, with the vast majority remaining unstudied.

Cell Dehydration in Cryopreservation

The goal of cell preservation is to remove as much water as possible from inside the cell without disrupting its integrity (minimum critical volume) (Meryman 1974). The volume of water in almost all animal cells is 70-80%, except for erythrocytes (~50%), and spermatozoa (~50%) (Newton et al. 1999, Petrunkina 2007). There are several processes to dehydrate cells

Cuevas-Uribe, R. and T. R. Tiersch. 2011. Non-Equilibrium Vitrification: an Introduction and Review of Studies Done in Fish. In: *Cryopreservation in Aquatic Species,* 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 309-324.

such as freeze drying (lyophilization) (Kusakabe et al. 2008), evaporative (convective) drying (Biggers 2009), vacuum drying (Meyers et al. 2009), exposure to hypertonic conditions for storage at room temperature (Van Thuan et al. 2005), or cryopreservation (Tiersch et al. 2007). Dehydration by exposure to hypertonic conditions during cryopreservation can be attained by cell exposure to cryoprotectants and extracellular ice crystals (Figure 1).

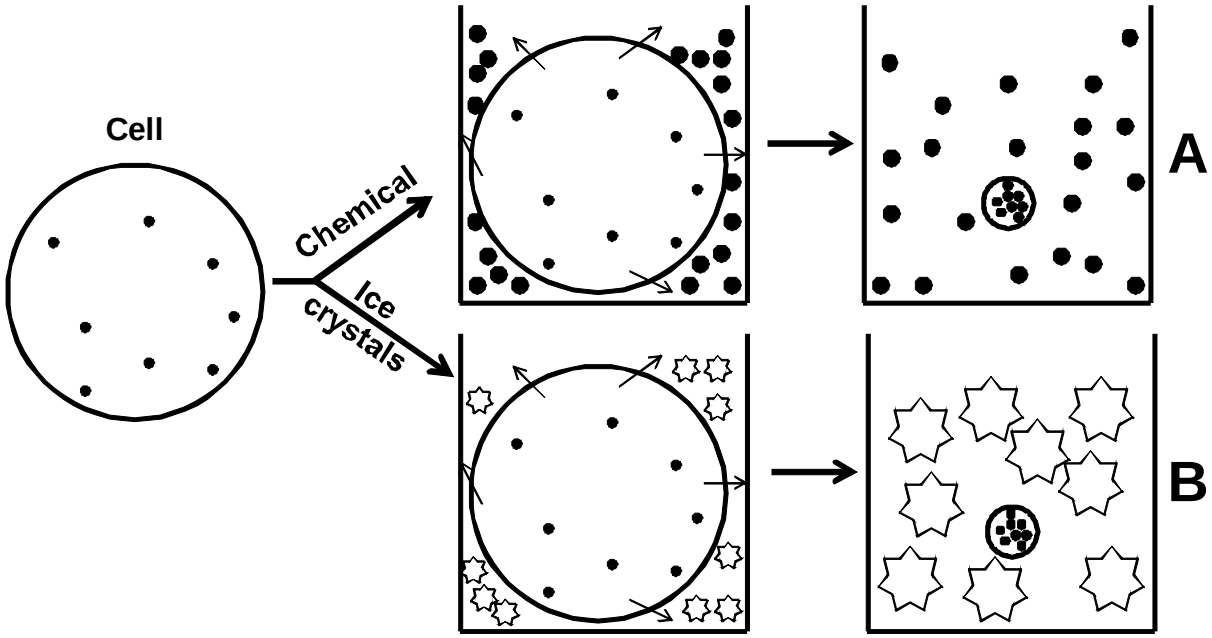

Figure 1. Because cells contain 70 to 80% water by volume, they can dehydrate by osmosis (arrows) until reaching a "minimum volume" known as the osmotically inactive fraction of cell volume. Hypertonic dehydration of the cell can be produced by: (A) non-permeating chemicals (●) and (B) ice crystals (✡). These mechanisms each act to increase osmolality of the extracellular space, which results in cellular dehydration, and thus reduces the chance of intracellular ice crystal formation. Water permeability depends on the cell type (size, membrane composition, shape, developmental stage), exposure temperature (permeability decreases at low temperatures), molecular size and charge (permeable or not), and concentration of molecules (diffusion gradients or differences in osmotic pressure). Note that there is not an increase in the amount of intracellular solutes, rather the cell reaches a minimum volume where the solutes are concentrated and thus the osmolality increases.

Exposure of cells to excessively hypertonic conditions can cause damage by osmotic stress and the solution effect (exposure to the effects of high solute concentrations, including low pH), and this type of injury is related to cell type, temperature, concentration of the suspending solution, and exposure time. The two factors that govern dehydration in cryopreservation are cryoprotectant concentration and cooling rate. When cooling rate is 'too slow', ice crystals will grow in the extracellular space, and the cells will be exposed for a longer period of time to a high hypertonic cold environment. Severe dehydration that leads to cellular disruption is a type of osmotic damage known as the 'solution effect'. When cooling rate is high, the cell will not have enough time to dehydrate because the ice crystals will grow quickly and the cell will become supercooled which will lead to the formation of intracellular ice (Figure 2, next page).

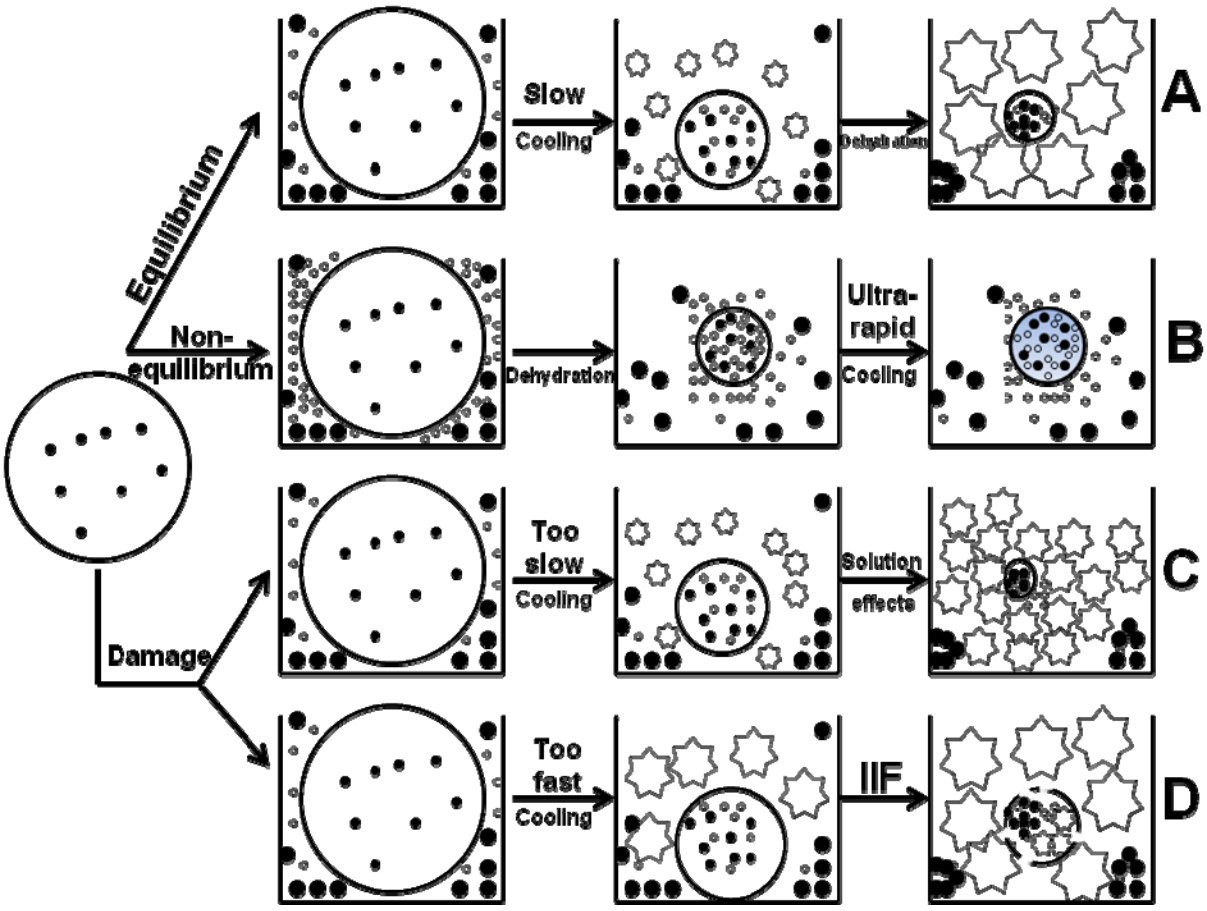

Figure 2. A) Equilibrium freezing involves the use of cryoprotectants and "slow freezing" to produce cellular dehydration and shrinkage. Permeating cryoprotectants (○) lower the freezing point of the solution, and minimize osmotic shock by replacing the water inside the cell. Non-permeating cryoprotectants (●) assist dehydration of the cell, and stabilize the membrane during cryopreservation. Once the first extracellular ice crystals (✡) have formed, as the temperature decreases, water is further incorporated into the growing ice crystals creating a hypertonic condition that produces osmotic dehydration. The combination of increased intracellular solutes and of lowering the temperature increases the viscosity of the solution until the eutectic temperature is reached (-40 °C) when the remaining unfrozen solution is solidified (partial vitrification). B) Non-equilibrium vitrification involves the use of high concentrations of cryoprotectants to dehydrate the cell and to replace the intracellular water before the cooling begins. Ultra-rapid cooling prevents the cells and the surrounding medium from undergoing ice crystal formation during cooling. The result is the solidification of the solution into a glass-like state (total vitrification). The two main sources of damage to the cell during cryopreservation are solution effect (C) and intracellular ice formation (IIF). C) Solution effect is due to excessively slow cooling rates, which cause the cell to experience severe volume shrinkage and long-term exposure to high solute concentrations. Excessive shrinkage can cause extrusion of membrane lipids and proteins, and intracellular changes such as reductions in pH that can denature proteins and cause loss of buffering capacity. D) Damage by intracellular ice formation is due to excessively fast cooling rates, which do not allow sufficient egress of intracellular water to maintain equilibrium and the residual supercooled water in the cell undergoes intracellular ice formation which causes damage by disruption of cellular structure and function.

Traditional cryopreservation seeks a happy medium between the time it takes for the cell to dehydrate without causing the solution effect, and a cooling rate that will not cause substantial intracellular ice formation. There is another procedure used in cryopreservation that consists of dehydrating the cell before cooling begins. This approach is known as 'rapid non-equilibrium vitrification'. This is different from slow equilibrium cooling protocols in that dehydration and cryoprotectant permeation take places before the cooling begins. In addition, the cells are exposed to an ultra-rapid cooling rate (typically >1,000°C/min) (Mazur et al. 1972, Leibo 1989, Mazur 2004). During this rapid cooling, the viscosity increases and the water molecules do not have time to arrange themselves into a crystalline structure, and therefore form an amorphous solid (vitrified) water. The resultant solid retains the random molecular arrangement of a liquid but has the mechanical properties of a solid (a "snapshot" of the liquid state). The temperature at which the sample is no longer a liquid but rather in the amorphous glassy solid (vitreous or non-crystalline ice), is known as the glass-transition temperature (-130 °C). The glass transition temperature can be raised by the addition of cryoprotectants (Fahy 1988). The purpose of vitrification is to reach the glass transition temperature as fast as possible through rapid cooling and by increasing the concentration of cryoprotectants. The goals of this chapter are to provide background on vitrification in general and to review the recent studies done in fish vitrification.

Equilibrium vs. Non-equilibrium Cryopreservation

Cryopreservation can be produced by two approaches: slow equilibrium cryopreservation (standard method) and rapid non-equilibrium vitrification. The main difference between these methods is that standard cryopreservation allows extracellular ice crystal formation while in vitrification ice crystal formation is suppressed. As stated above, dehydration of the cells during slow equilibrium freezing takes places during cooling, while dehydration in non-equilibrium vitrification takes places before cooling (Figure 2). To dehydrate the cell before cooling, high concentrations of cryoprotectants (40 – 60%) are normally used. To achieve the vitreous state before ice crystals have the chance to form (Kuleshova et al. 2007), it is necessary to rapidly cool through a specific temperature zone (-5 to -40 °C) (Shaw and Jones 2003) of potential crystallization. The resultant glass retains the random molecular arrangement of a liquid but has the mechanical properties of a solid (Taylor et al. 2004). This ultra-rapid cooling is typically done by plunging the samples directly into the liquid nitrogen (Fahy et al. 1984). Neither high cryoprotectant concentration nor increased cooling rates are essential for vitrification to occur. Partial (usually) or total intracellular vitrification can occur incidentally during traditional slow equilibrium cooling, and may help to ensure survival of some portion of cryopreserved samples (Vajta et al. 2009).

The goal of equilibrium freezing and non-equilibrium vitrification is to prevent intracellular ice crystal formation and to protect cells from damage. Vitrification is typically achieved by partial replacement of intracellular water via permeating cryoprotectants, which readily form glass, and by drawing out the intracellular water via non-permeating cryoprotectants (Figure 2). As a result, by combination of permeating and non-permeating cryoprotectants, the net concentration of the permeating cryoprotectant is increased in the intracellular space and their combined effect enhances the overall viscosity of the cell (Jain and Paulson 2006). In practice, the exposure to cryoprotectants is usually performed at room temperature (Kuleshova et al. 2007). The assessment of glass formation for cryoprotectants is relatively straightforward.

Crystallization can be distinguished by the observance of a milky appearance after plunging samples into liquid nitrogen, while glass formation appears as transparent (Figure 3) (Ali and Shelton 2007).

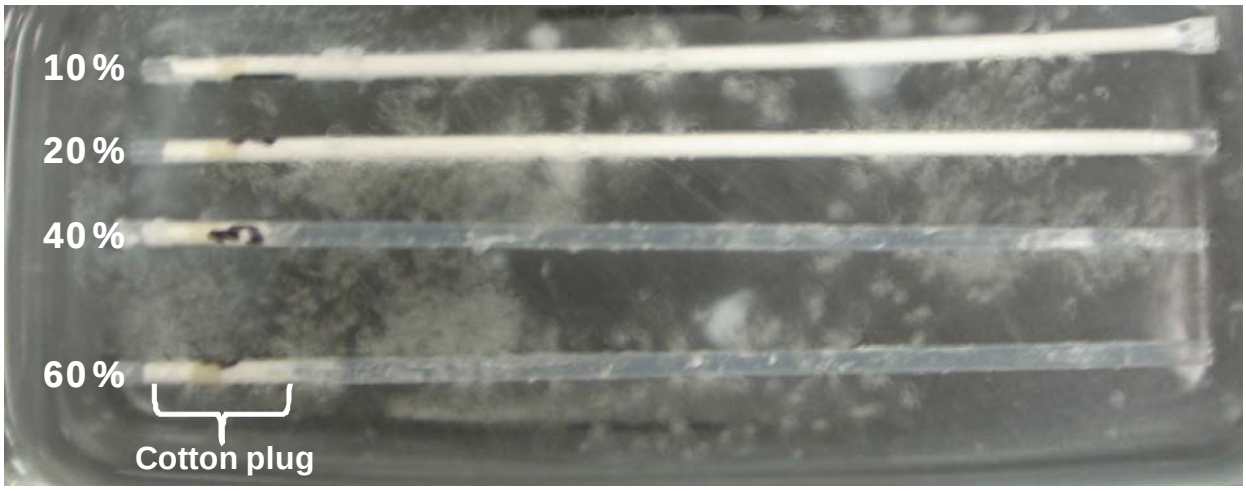

Figure 3. Visual observation of crystallization and vitrification of ethylene glycol at different concentrations. French straws (0.25-ml) with a cotton plug at one endwere filled with 10, 20, 40 or 60% of ethylene glycol and plunged into liquid nitrogen. Straws with 10 and 20% had a milky appearance indicating ice formation while straws with 40 and 60% remained transparent indicating glass formation.

Cryoprotectants in Vitrification

There are basically two types of cryoprotectants used in vitrification. The first type is the permeating cryoprotectants that are generally low molecular weight, non-electrolytes with high solubility in water. Permeating cryoprotectants have differential toxicity depending on the type, concentration, temperature, and time of exposure. The purpose of permeating cryoprotectants is to replace water from inside the cell with cryoprotectant. In this way the cell does not shrink beyond a minimum volume during cooling and these cryoprotectants decrease the freezing point while increasing the probability to form glass inside the cell. The rate of penetration depends on the chemical composition (e.g., molecular weight, hydrogen bonding capability, number and orientation of hydroxyl, amide, and sulfoxide groups) of the cryoprotectant as well as the properties of cell membranes (Leibo 2008). For vitrification purposes, each cryoprotectant forms a vitreous state at different concentrations. For example a strong glass former such as propylene glycol (MW = 76) produces glass at concentrations of ≥4 mol/L (30%), but methanol (MW = 32), which is one of the most permeable of cryoprotectants, is a weak glass former and will not vitrify even at high concentrations (crystallizes at 99.8%) (Ali and Shelton 2007). Due to the glass-formation properties and toxicity of the cryoprotectants to the cell, a mixture of cryoprotectants is often used for vitrification. Generally the mixture of cryoprotectants has a lower aggregate toxicity to the cell because it vitrifies at lower concentrations, and they combine the cumulative properties of each cryoprotectant such as permeability and glass formation.

The second type of cryoprotectants used in vitrification is known as the non-permeating cryoprotectants. The two main functions of these cryoprotectants are to dehydrate the cell during cooling by increasing the osmolality of the extracellular space, and to prevent excessive osmotic swelling during warming. Non-permeating cryoprotectants have high molecular weights (≥ 342 daltons) and can be monosaccharide sugars, disaccharide sugars, polysaccharides, and

macromolecules (Swain and Smith 2010). Adding sugars to the vitrification solution can increase the dehydration rate before cooling and enhance viscosity (Varghese et al. 2009). Sugars, especially disaccharides such as sucrose and trehalose, are effective in enhancing glass formation (Fuller 2004). Adding other agents such as polymers can facilitate vitrification and reduce the concentration of permeating cryoprotectants necessary to form glass (Fahy et al. 1984). Low molecular weight copolymers such as polyvinyl alcohol can inhibit ice formation and prevent the formation of ice crystals during warming (Wowk 2005). Another ice blocker is antifreeze proteins (AFP) that control the growth of ice crystals. Antifreeze proteins act by adsorbing to the surface of small ice crystals, inhibiting their growth. Antifreeze proteins could be used in vitrification to inhibit ice growth during warming (Fuller 2004).

Most cryoprotectants tend to have toxic and hypertonic effects when used at concentrations that are effective for successful vitrification (Yavin 2007). There are a number of other ways to reduce the concentration of individual cryoprotectants required for vitrification, such as: applying high hydrostatic pressure, step-wise addition of cryoprotectants, and limiting exposure time at higher concentrations to a minimum (Fahy et al. 1984). In addition, the toxicity of cryoprotectants can be counteracted by the use of 'toxicity neutralizers' such as formamide or urea (Fahy 2010).

Devices Used in Vitrification

After the addition of cryoprotectants, the cells are ultra-rapidly cooled, usually performed in a single step in which the sample is plunged directly into liquid nitrogen. One hindrance to attaining rapid cooling by immersion in liquid nitrogen is the formation of a gas layer on the surface of the sample. Because liquid nitrogen is at its boiling point, heat withdrawn from the sample will vaporize the adjacent nitrogen, forming an insulating gaseous layer that retards the rate of heat transfer. But the rate of cooling can be increased by adding a thin insulation on the surface of the device (e.g., coating with talc) (Meryman 2007), or by application of a vacuum above the liquid nitrogen (nitrogen slush) (Shaw and Jones 2003, Yavin and Arav 2007). To achieve highest cooling rates and to prevent heterogeneous nucleation (formation of ice nuclei triggered by surfaces or impurities), the volume of the vitrification solution should be minimized (Dinnyes et al. 2007). To minimize the volume, special devices are used including: 0.25-ml French straws (45-µl sample volume, estimated cooling rate of 2,500 °C/min), 0.25-ml French straws (25-µl sample, 4,460 °C/min), open-pulled straw (1-µl sample, 16,700 °C/min), cryotop (0.1-µl sample, 23,000 °C/min), cryotip (1-µl sample, 12,000 °C/min), and hemi-straw (0.5-µl sample, 1,600 °C/min) (Chen and Yang 2007, Quinn 2010). In addition to cooling, the type of device used to vitrify influences the warming rate because its size and composition determine thermal conductance (Watson and Fuller 2001). Special attention is necessary when handling small-volume samples because of potential crystallization during storage or warming.

Sources of Variation in Vitrification

Some variables that influence the success of vitrification are: 1) the effects of exposure time to cryoprotectant solutions, and their concentration and temperature; 2) the number of steps in which the cryoprotectant is added and removed; 3) the type of device used for vitrification (which as stated above influences the size of the vapor coat and cooling rate); 4) the quality and the developmental stage of the cells tested (Liebermann et al. 2002); 5) the viscosity and volume of the sample (Yavin and Arav 2007); 6) the absolute pressure (higher hydrostatic pressures

decrease the homogeneous nucleation temperature and increase the glass transition temperature) (Rabin and Steif 2009), and 7) the warming process (ice crystal formation can occur during sub-optimal warming) (Leibo 2000). Overall, the high concentrations of cryoprotectants required are near the maximum tolerable limit of cells. As such, there is an inverse relationship between cooling rate and cryoprotectant concentration, i.e. the higher the cooling rate, the lower the concentration needed and *vice versa* (Mazur et al. 2008).

Thus to develop a vitrification protocol, the first step is to identify suitable vitrification solutions by measuring the toxicity of cryoprotectants at various concentrations, exposure times, and pre-freeze exposure temperatures. The second step is to select a vitrification device that will minimize the volume of the sample, and allow ultra-rapid cooling. Minimum volume methods allow the use of less concentrated cryoprotectants, and prevent heterogeneous ice formation (Vajta and Nagy 2006). The aim in any vitrification protocol is to increase the speed of temperature change while keeping the concentration of cryoprotectants (although high) as low as possible (Nawroth et al. 2005). Thus, vitrification should be performed in a kinetic way ('fast enough'), balancing concentration of the vitrificant and the rates of cooling and warming (Katkov et al. 2006).

Advantages of Vitrification

Vitrification is considered an attractive alternative to standard cryopreservation for specific applications and it has been used for the cryopreservation of spermatozoa, embryos, oocytes, stem cells, and organs in a variety of taxa (Tucker and Liebermann 2007). Vitrification does not require expensive equipment, is simple, achieves cryopreservation in sec, and can be used to preserve samples in the field. In addition, it offers perhaps the greatest potential for success in overcoming the challenges for preservation of fish eggs and embryos.

Vitrification of Fish Eggs and Embryos

Studies in fish vitrification can be dated back to 1938 when Basile Luyet attempted to vitrify juvenile goldfish *Carassius auratus* (40 mm, standard length) by plunging the fish into liquid air (-194°C) (Luyet 1938). Since then, there have been more than 30 publications dealing with embryo vitrification in 11 species of fish (most within the past 10 yr) (Table 1). Zebrafish *Danio rerio* is the most widely studied for vitrification, accounting for 50% of the studies. The results from vitrification studies had been controversial with documentation of "survival", but there has been a lack of reproducibility with these studies (e.g., Edashige et al. 2006). One of the main problems has been a lack of standardization in the methods and terminology used. For example, the term "survival" has been applied to "intact" embryos, hatched embryos, movement and twitching of embryos, and dye exclusion (such as trypan blue). Standardization of the terminology plays a key factor for reproducibility and validation. At least four studies have reported larvae hatched from vitrified embryos (Table 1), but none of these studies have been reproduced (Edashige et al. 2006). The limited success in fish embryo vitrification could be due to their large size (> 1 mm in diameter), low surface-to-volume ratio, the complexity of the multi-cellular embryo, and low permeability which could inhibit the entrance of cryoprotectants into the various embryonic compartments (Zhang 2004, Robles et al. 2009).

Table 1. Most studies on vitrification in fishes have occurred within the past 10 yr, and have addressed zebrafish embryos.

Species	Vitrified material	Summary of finding	Reference
Carassius auratus	40- mm fingerling	No survival	Luyet 1938
ND[a]	embryos	Intracellular blacking appeared	Wang et al. 1987
Clupea pallasi	eggs	Normal chorion and micropyles	Pillai et al. 1994
Clarias gariepinus	eggs	None survived	Magyary et al. 1995
Danio rerio	6 somite and heartbeat stages	≤ 32% intact morphology	Zhang and Rawson 1996
Danio rerio	Heartbeat stage	Two live embryos	Chao et al. 1997
Danio rerio	1 cell to prim-6 stages	≤ 80% intact morphology	Liu et al. 1998
Danio rerio	100% epiboly	All died	Hagedorn et al. 1998
Danio rerio	100% epiboly	Fell apart	Janik et al. 2000
Clarias gariepinus	eggs and embryos	No embryonic development	Urbanyi et al. 2000
Lateolabrax japonicus	neurula to prehatch	One embryo hatched	Tian et al. 2003
Scophthalmus maximus	tail bud and tail bud free	≤ 49% intact morphology	Robles et al. 2003
Scophthalmus maximus	tail bud and tail bud free	≤ 54% enzymatic activity	Robles et al. 2004
Danio rerio	high blastula and 5-somite stages	≤ 12% enzymatic activity	Robles et al. 2004
Danio rerio	high blastula and 5-somite stages	Differences of viable cells, SYBR[b]	Martinez et al. 2005
Pseudopleuronectes[c]	gastrula to starting pigment	1% continued development	Robles et al. 2005
Paralichthys olivaceus	20 somites to body movement	Seven embryos hatched	Zhao et al. 2005
Paralichthys olivaceus	neurula to hatching	Fourteen larvae hatched	Chen and Tian 2005
Paralichthys olivaceus	tail bud stage	No embryos survived	Edashige et al. 2006
Sparus aurata	tail bud and tail bud free stages	≤ 28% intact morphology	Cabrita et al. 2006
Danio rerio	caudal fin	≤ 63% attachment rate	Cardona-Costa et al. 2006
Danio rerio	blastomeres	≤ 20% survival, trypan blue	Cardona-Costa et al. 2007
Pagrus major	heartbeat stage	≤ 78% intact morphology	Ding et al. 2007
Prochilodus lineatus	morula to 6 somites	None viable	Ninhaus-Silveira et al. 2008
Carassius auratus	caudal fin	No cell outgrowth	Moritz and Labbe 2008
Danio rerio	5 somites	≤50% viable cells, SYBR	Martinez-Paramo et al. 2009
Tinca tinca	23 and 29 hr	No living embryos	El-Battawy and Linhart 2009
Danio rerio	testicular tissue	94% cell survival, trypan blue	Bono-Mestre et al. 2009
Danio rerio	blastomeres	90% survival and 20% recovery	Cardona-Costa et al. 2009
Danio rerio	64-cell to 20-somite stage	25% PGC[d] survival	Higaki et al. 2009
Danio rerio	14-20 somites	30% PGC survival	Higaki et al. 2010b
Danio rerio	14-18 somites	≤83% PGC survival	Higaki et al. 2010a
Danio rerio	stage III oocytes	≤69% survival, trypan blue	Guan et al. 2010

[a]ND: not described. [b]SYBR: SYBR-14 and propidium iodide. [c]*Pseudopleuronectes americanus*. [d]PGC: primordial germ cells.

Several approaches have been used to improve embryo vitrification in the studies listed in Table 1 by enhancing permeation of cryoprotectants. These include embryo dechorionation, enzymatic permeabilization (using the enzyme pronase), partial removal of yolk, microinjection of cryoprotectants, artificial expression of aquaporin-3, addition of AFP and polymers to inhibit ice formation and enhance glass formation, the use of cold-tolerant fish species (e.g., winter flounder *Pseudopleuronectes americanus* which produces AFP), assisted hatching techniques (e.g. piercing the egg), evaluation of different apparatuses and warming temperatures, and evaluating embryos at different developmental stages. Although some of these approaches have increased permeability, the concentrations necessary within the embryo for vitrification have not been achieved (Robles et al. 2009).

Future research on embryo vitrification could focus on neutralization of cryoprotectant toxicity (Fahy 2010), use of biopolymer-mediated intracellular sugars (Lynch et al. 2010), induction of suspended animation-like states before cooling (such as anhydrobiosis) (Blackstone et al. 2005), application of laser pulses (Kohli et al. 2007) and ultrasound (Wang et al. 2008, Silakes and Bart 2010) to increase permeability to cryoprotectants, and the use of innovative technologies such as magnetic field freezers (Kaku et al. 2010), and vacuum equilibration methods (Gwo et al. 2009).

New Strategies for Application of Cell Vitrification

Because cryopreservation of fish eggs and embryos has been unreliable, new technologies have been developed to conserve paternal and maternal genetic information. These technologies use surrogate production through transplantation of blastomeres, testicular cells (e.g., spermatogonial stem cells), or primordial germ cells (PGC) (Yamaha et al. 2007). Vitrification has been applied to cryopreserved blastomeres (Cardona-Costa and Garcia-Ximenez 2007, Cardona-Costa et al. 2009), testicular cells (Bono-Mestre et al. 2009), and PGC (Higaki et al. 2009, Higaki et al. 2010b) (Table 1). In fact, zebrafish (striped-type) were produced from surrogate zebrafish (golden-type, germ-line chimeras) that were generated through transplantation of vitrified germ cells (PGC from striped-type) (Higaki et al. 2010a). This is a breakthrough in cryopreservation because it presents alternative strategies to preserve fish genomes.

As part of biodiversity conservation strategies and to improve the genetic diversity through cryobanking of somatic tissues, vitrification has been applied to cryopreserve caudal fin cells from zebrafish (Cardona-Costa et al. 2006) and goldfish (Moritz and Labbe 2008). After cell culture of vitrified fin pieces, somatic cells were produced. Somatic cells should be considered for cryobanking of valuable or endangered fishes. In addition, somatic cells can be used to regenerate fish by nuclear transfer or somatic cloning technology (Siripattarapravat et al. 2009, Bail et al. 2010). Another method to regenerate fish is by androgenesis (all-paternal inheritance). Fertilization of irradiated eggs could be done by using: cryopreserved diploid sperm (e.g., from a tetraploid male) (e.g., Yasui et al. 2010), two cryopreserved sperm (dispermic androgenesis) (e.g., Grunina et al. 2006), or one cryopreserved sperm followed by suppression of first cleavage (Babiak et al. 2002). Another way to produce dispermic androgenesis that remains unexploited is by intracytoplasmic sperm injection (ICSI) of two cryopreserved sperm (Poleo et al. 2005a). ICSI has been used in fishes such as Nile tilapia *Oreochromis niloticus* (Poleo et al. 2005b), zebrafish (Poleo et al. 2008), and medaka *Oryzias latipes* (Otani et al. 2009).

Preservation of large sperm volumes is not necessary to reconstitute lines by production of founder populations. Vitrification could play a key role in the cryopreservation of small sperm volumes. Recently, vitrification of human spermatozoa was reported using small volumes (20 µL) (Nawroth et al. 2002). Several attempts have been made to plunge fish sperm samples into liquid nitrogen but none of these reports made specific reference to vitrification (e.g., Guest et al. 1976, Huang et al. 2004, Gwo et al. 2005). The combination of large volumes (> 250 µL), and low cryoprotectant concentration (<15%) make it unlikely that total vitrification occurred within these samples. Our recent studies have addressed development of generalized protocols for sperm vitrification in fish. Offspring were produced from vitrified sperm samples of channel catfish *Ictalurus punctatus*, green swordtail *Xiphophorus helleri*, and southern flounder *Paralichthys lethostigma* (Cuevas-Uribe et al. unpublished). From the recent work in sperm vitrification (our unpublished studies) it seems that marine fish sperm had higher survival after non-equilibrium vitrification, perhaps because they are adapted to deal with higher osmotic pressures (sea water > 1,000 mOsmol/Kg). Because the volumes used for sperm vitrification are small (~20 µL), this technique is currently best suited for use with aquarium fishes including endangered species. Sperm vitrification can be used to reconstitute lines from valuable biomedical models (such as zebrafish or *Xiphophorus* spp.), conserve mutants for development of novel lines for ornamental aquaculture, and transport frozen sperm from the field to the laboratory to expand the genetic resources available for germplasm repositories (Cuevas-Uribe and Tiersch unpublished).

Thus at present vitrification is most suited for use with microliter volumes of sperm, and other single-cell applications. It also offers potential for embryos and tissues if studies can be standardized and repeated among laboratories. More research needs to be done to evaluate the possible genetic consequences of using high concentrations of cryoprotectants (Tatone et al. 2010), and to evaluate the levels of microbial and viral contamination possible when samples are directly plunged into liquid nitrogen (Bielanski and Vajta 2009).

Acknowledgments

This work was supported in part by funding from ACRES-LSU Collaborative Research Program, NIH National Center for Research Resources, and Louisiana Sea Grant College Program. We thank S. P. Leibo for considerable discussion and guidance, N. Novelo for editing the manuscript, K. S. Da Costa for translation of reference, and J. Atilano for assistance with the literature review. This manuscript was approved for publication by the Director the Louisiana Agricultural Experiment Station as number 2010-244-5258.

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