Sperm cryopreservation in fish and shellfish

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Initial success in sperm cryopreservation came at about the same time for aquatic species and livestock. However, in the 50 plus years since then cryopreserved sperm of livestock has grown into a billion-dollar global industry, while despite work in some 200 species with well over 200 published reports, cryopreservation of aquatic species sperm remains essentially a research activity with little commercial application. Most research has focused on large-bodied culture and sport fishes, such as salmonids, carps, and catfishes, and mollusks such as commercially important oyster and abalone species. However, only a handful of studies have addressed sperm cryopreservation in small fishes, such as zebrafish, and in endangered species. Overall, this work has yielded techniques that are being applied with varying levels of success around the world. Barriers to expanded application include a diverse and widely distributed literature base, technical problems, small sperm volumes, variable results, a general lack of access to the technology, and most importantly, the lack of standardization in practices and reporting. The benefits of cryopreservation include at least five levels of improvements for existing industries and for creation of new industries. First, cryopreservation can be used to improve existing hatchery operations by providing sperm on demand and simplifying the timing of induced spawning. Second, frozen sperm can enhance efficient use of facilities and create new opportunities in the hatchery by eliminating the need to maintain live males, potentially freeing resources for use with females and larvae. Third, valuable genetic lineages such as endangered species, research models, or improved farmed strains can be protected by storage of frozen sperm. Fourth, cryopreservation opens the door for rapid genetic improvement. Frozen sperm can be used in breeding programs to create improved lines and shape the genetic resources available for aquaculture. Finally, cryopreserved sperm of aquatic species will at some point become an entirely new industry itself. A successful industry will require integrated practices for sample collection, refrigerated storage, freezing, thawing, rules for use and disposal, transfer agreements, and database development. Indeed the development of this new industry is currently constrained by factors including the technical requirements for scaling-

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up to commercial operations during the transition from research, and the absence of uniform quality control practices, industry standards, marketing and price structures, and appropriate biosecurity safeguards.

Introduction

The scientific underpinnings of cryobiology can be traced back to the 1950s after the discovery of the cryoprotective qualities of glycerol for fowl sperm (Polge, Smith and Parkes, 1949). The first studies of fish sperm cryopreservation were published soon thereafter (Blaxter, 1953), and since then more than 200 species of fish have been studied (Rana, 1995; Tiersch, 2000). Cryopreservation is a process where biological materials such as cells and tissues are preserved by cooling to ultra-low temperatures, typically -196°C (for liquid nitrogen), yet remain viable after subsequent warming to temperatures above 0°C. For sperm cryopreservation, this process typically includes gamete collection, suspension of sperm in an extender, quality assessment, addition of cryoprotectants, equilibration, freezing, thawing and fertilisation, and the development of early life stages for assessment of cryopreservation success (Tiersch, 2000).

Conventional cryopreservation of sperm cells often involves the use of cryoprotectants and slow freezing to produce cellular dehydration and shrinkage. Cryoprotectants are chemicals used to protect cells from damage during freezing and thawing. Although their mechanisms of action are as yet not completely understood, permeating cryoprotectants such as dimethyl sulfoxide are believed to lower the freezing point of the solution, minimise osmotic shock by replacing the water inside the cell, and reduce formation of destructive intracellular ice (Doebbler, 1966; Rowe, 1966; Leung, 1991). Non-permeating cryoprotectants such as sugars and polymers are believed to help stabilise the membrane during cryopreservation (Meryman, 1971). Too little cryoprotectant entering the cell before cooling reduces effectiveness (Taylor, Adams, Boardman and Wallis, 1974), whereas too much causes osmotic swelling and rupture during thawing and dilution. In addition, cryoprotectants are often toxic to cells, and thus choice of cryoprotectant and their optimal concentration (a balance between protection and toxicity) has been the focus for numerous studies. Optimal equilibration before freezing is necessary to allow permeating cryoprotectants the time necessary to penetrate the sperm while minimizing toxicity and can be influenced by temperature.

The choice of optimal cooling rate for freezing has been another major focus of numerous studies of sperm cryopreservation. To be considered as optimal, a rate should be slow enough to minimise the amount of intracellular ice (below a damaging level) and yet be rapid enough to minimise the length of time cells are exposed to what is referred to as the "solution effect" (the concentration and precipitation of solutes that occurs when solubility limits are exceeded during the dehydration caused by ice formation). In general, rapid thawing is preferred to minimise the damage associated with recrystalization (the coalescence of small ice crystals into large crystals during thawing). Numerous studies in sperm cryopreservation have been devoted to optimizing specific components of cryopreservation procedures. However, aside from those factors mentioned above, other factors such as sample density, freezing container, starting temperatures, final temperatures (before plunging into liquid nitrogen), dilution, and cryoprotectant removal after thawing can also affect results (Leibo, 2000). Therefore, cryopreservation procedures must be tailored for each species and cell type, and be based on a thorough understanding of cryobiological properties (e.g., Mauger, Le Bail and Labbe, 2006).

The term "fish" is an artificial collective of more than 25,000 species characterised more by their differences than by their similarities. To broaden this grouping to aquatic species entails further inclusion of invertebrates such as oysters, abalone, and corals (e.g., Hagedorn, Carter,

Steyn, Krupp, Leong, Lang and Tiersch, 2006). A review of cryopreservation within aquatic species is thus a balancing act of attempting to generalise observations into basic principles while acknowledging the considerable diversity that exists across these organisms. For example, consider Figure 1 wherein the relationship of sperm motility to osmotic pressure is illustrated in three groupings of aquatic organisms. Sperm of freshwater species generally become active and increasingly motile in response to a lowering of osmotic pressure (hypotonic to blood plasma). Sperm of marine species behave in an opposite manner with motility increasing with osmotic pressure (hypertonic). The point being that these organisms are adapted to spawn externally at the osmotic pressure of the external environment. A third group displays sperm motility activation in an intermediate range (isotonic). This group, live-bearing fishes of the genus *Xiphophorus*, have evolved internal fertilisation, and as such, the internal anatomy of the female has become the working environment for the gametes with a corresponding change in the osmotic pressure of sperm activation (Huang, Dong, Walter and Tiersch, 2004b; Yang, Hazelwood, Walter and Tiersch, 2006).

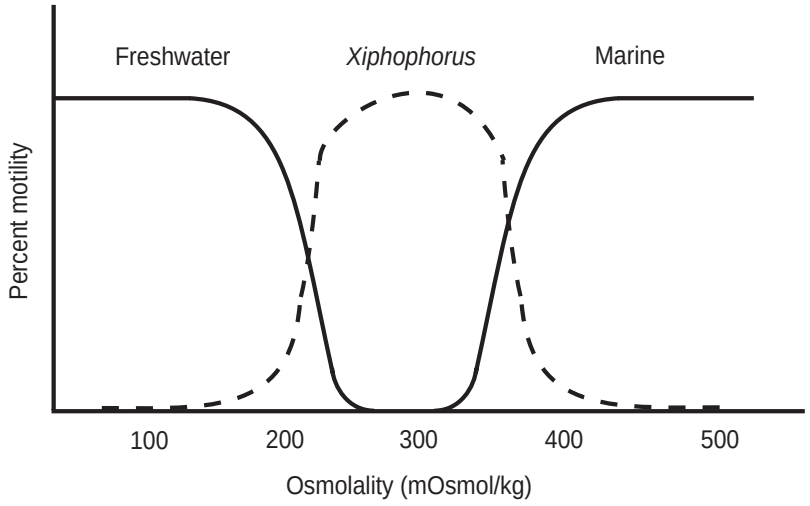

Figure 1. Diagrammatic representation of sperm activation and motility profiles in relation to osmolality for three groupings of aquatic species.

Initial research success in sperm cryopreservation came more than 50 years ago for aquatic species and livestock. However, since then cryopreserved sperm of livestock has grown into an annual billion-dollar global industry, while cryopreservation of aquatic species sperm remains essentially a research activity with little commercial application. Most research work has focused on large-bodied culture and sport fishes, such as salmonids, carps, and catfishes. Other groups such as mollusks, represented by commercially important oyster and abalone species, have received a fair amount of attention as well. However, only a handful of studies have addressed sperm cryopreservation in aquarium fishes, such as zebrafish (*Danio rerio*), which are becoming increasingly important in biomedical research (Tiersch, 2001), and in endangered species. Overall, this work has yielded techniques that are being applied with varying levels of success around the world. However, the barriers to expanded application include a diverse and widely distributed literature base, technical problems, small sperm volumes, variable results, a general lack of access to the technology, and most importantly, the lack of standardization in practices and reporting.

This review will focus on the needs and opportunities for future research and application of cryopreservation in aquatic species. To do this we will follow the general thematic approach afforded by the groupings shown in Figure 1. First, because several excellent review articles are already available that focus on sperm cryopreservation in freshwater fishes (primarily large-bodied commercially important species) we will direct the reader elsewhere for this information (Table 1). We will use the two other groupings to serve as models for aquatic species in general. Accordingly, second, we will present an overview of aquatic species cryopreservation research through examination of live-bearing fishes of the genus *Xiphophorus*, as representatives of the neglected but important aquarium fishes. Third, we will present the need for standardization by an overview of the existing oyster literature (a marine invertebrate; marine fishes have been reviewed by Suquet, Dreanno, Fauvel, Cosson and Billard, 2000). We will finish with a look to the future of application of cryopreservation in aquatic species.

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

Short Title	Citation
Biology, handling and storage of salmonid spermatozoa	Scott and Baynes, 1980
Fish gamete preservation and spermatozoan physiology	Stoss, 1983
Live preservation of fish gametes	Leung and Jamieson, 1991
Cryopreservation of fish spermatozoa	Rana, 1995
Cryopreservation of finfish and shellfish gametes	Chao, Lin, Chen, Hsu and Liao, 1997
Cryopreservation in aquatic species (book)	Tiersch and Mazik (editors), 2000
Cryopreservation of gametes in aquatic species (special issue)	Lahnsteiner (editor), 2000
Techniques of genetic resource banking in fish	Billard and Zhang, 2001
Cryopreservation of gametes and embryos of aquatic species	Zhang, 2004
Cryopreservation and short-term storage of sturgeon sperm	Billard, Cosson, Noveiri and Pourkazemi, 2004
Semen cryopreservation in catfish species	Viveiros, 2005

Sperm cryopreservation in live-bearing fishes of the genus *Xiphophorus*

Viviparous fishes of the genus *Xiphophorus* (family Poecilliidae) are valuable for biomedical research because hybrid crosses between certain species within this genus have provided well-studied spontaneous and induced tumor models (Anders, 1967; Schartl, 1995; Walter and Kazianis, 2001). In 1928, a melanoma model for *Xiphophorus* was established by Gordon and Kosswig, demonstrating that certain cancers were inherited diseases (Anders, 1991). Since then, numerous studies have used *Xiphophorus* in medical research. Specific strains have been developed (Kazianis and Walter, 2002) and inbred lines have been created for study of gene structure and expression (e.g., David, Mitchell and Walter, 2004; Heater, Oehlers, Rains and Walter, 2004; Kazianis, Khanolkar, Naim, Rains, Trono, Garcia, Williams and Walter, 2004; Meierjohann, Schartl and Volff, 2004; Oehlers, Heater, Rains, Wells, David and Walter, 2004). Additionally, ornamental strains with specific body coloration have been developed by commercial farmers (e.g, www.ekkwill.com).

Although of great importance for conservation of valuable species and lines, sperm cryopreservation was essentially unexplored in *Xiphophorus* or in live-bearing fishes as a group before 2004 (Tiersch, 2001). The genus *Xiphophorus* can serve as a representative of both viviparous fish (with reproduction by internal fertilisation) and aquarium fish. As for the aquarium fishes, sperm cryopreservation research has been limited to zebrafish (Harvey, Norman and Ashwood-Smith, 1982; Morris, Berghmans, Zahrieh, Neuberg, Kanki and Look, 2003; Draper,

McCallum, Scout, Slade and Moens, 2004) and medaka (*Oryzias latipes*) (Aoki, Okamoto, Tatsumi and Ishikawa, 1997) which are also medical research models. Internal fertilisation is an atypical mode of reproduction in fishes and has involved specialised adaptations in morphology, physiology, and biochemistry in males and females. These features of *Xiphophorus* fishes, in combination with their small body size (2-5 cm) and associated small testis (5-12 mg), make sperm cryopreservation considerably more challenging than in fish species with external fertilisation.

Sperm characteristics of fishes from the genus Xiphophorus

Due to viviparity, sperm from *Xiphophorus* fishes possess specialised characteristics distinct from those found in sperm of fishes with external fertilisation (Fig. 2): 1) mature sperm have a cylindrical nucleus, a short, thickened midpiece, and an elongated axoneme, and within the testis sperm form discrete head-head agglutination bundles (Jonas-Davies, Winfrey and Olson, 1983); 2) after insemination, the spermatozoa remain in the head-head agglutination bundles within the female reproductive tract (Hoar, 1969; Grier, 1975); 3) inseminated sperm can be stored live for months in the female reproductive tract before fertilisation of mature oocytes, and females may produce broods at approximately 30-d intervals over 4-5 months after a single insemination (Tavolga, 1949); 4) motility can be activated by suspension of sperm obtained from crushed bundles in buffer isotonic to *Xiphophorus* plasma (Yang *et al.*, 2006); 5) suspended sperm can remain continuously motile for 4-7 d of storage at 4°C (Huang *et al.*, 2004b), and 6) sperm possess glycolytic activity comparable to mammalian species (Kallman, 1975; Gardiner, 1978). In the following section, recent studies of sperm cryopreservation in *Xiphophorus* are reviewed. These studies could assist research in other viviparous fishes. Also, due to internal fertilisation, this group could serve as a model for studies of sperm cryopreservation and reproduction in other taxa including mammals.

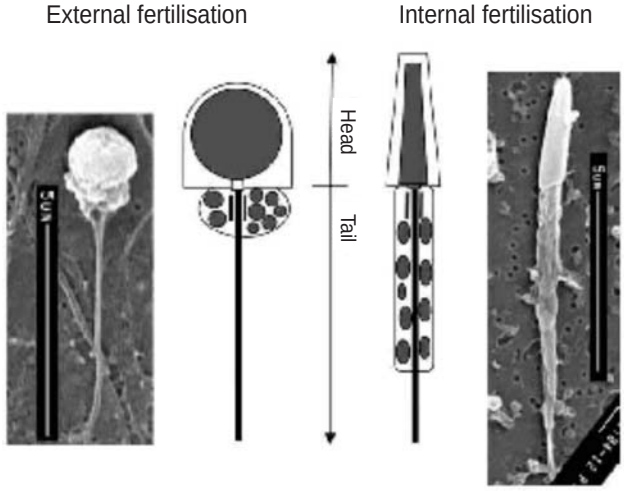

Figure 2. Representative scanning electron micrographs and diagrammatic views of sperm cells typical of fishes that employ external fertilisation (left, Nile tilapia, *Oreochromis niloticus*) or internal fertilisation (right, green swordtail *Xiphophorus helleri*). Figure reprinted with permission from Huang *et al.* (2004c).

Development of protocols for sperm cryopreservation in Xiphophorus

Generally, sperm cryopreservation comprises a series of steps including: 1) sperm collection; 2) extension of sperm in extender; 3) addition of cryoprotectant; 4) packing of sperm sample; 5) freezing; 6) thawing, and 7) estimation of fertility (Tiersch, 2000). Protocol establishment involves evaluation and optimization of multiple factors at each step (e.g., the type and concentration for each cryoprotectant), and recognition of the interactions among the steps (e.g., between cryoprotectant and cooling rate).

Sperm collection and dilution

Usually fish sperm can be obtained by either stripping or crushing of dissected testis. Because of the small body size of *Xiphophorus*, the availability of sperm by stripping is limited to ~1 microliter. To maximise the volume of available sperm, crushing of dissected testis was chosen for sperm collection in the following studies (Huang, Dong and Tiersch, 2004a; Huang *et al.*, 2004b; Huang, Dong, Walter and Tiersch, 2004c; Yang *et al.*, 2006). Dilution after sperm collection was necessary to maximise the volume for subsequent experiments of various factor optimizations. However, extreme dilution of sperm samples has been found to reduce sperm motility in mammals, fishes, and oysters (e.g., Paniagua-Chavez, Buchanan and Tiersch, 1998). Thus, dilution ratios of 1:50, 1:100, 1:200, 1:400, 1:800, and 1:1600 (v:v) were evaluated with sperm of *X. helleri* (Huang *et al.*, 2004b). Motility of samples diluted at 1:50, 1:100 and 1:200 was not significantly different before freezing and after thawing, but the motility declined significantly at ratios of higher than 1:200.

Evaluation of extender solutions

The term "extender" refers to a solution of salts, sometimes including organic compounds such as sugars that help to maintain sperm viability prior to and during the freezing process (e.g., Hanks' balanced salt solution). The use of extenders is based on the control of pH and osmolality as well as the supply of energy, and can extend the functional life and fertilising capability of sperm. As stated above, in most fishes with external fertilisation, unless controlled by the concentration of specific ions, sperm motility can be activated by either hypotonic media (for freshwater fish) or hypertonic media (marine species), and have a short life span (1-2 min) upon being activated (Morisawa and Suzuki, 1980; Morisawa, Suzuki, Shimizu, Morisawa and Yasuda, 1983). Thus, sperm typically need to be maintained in an extender with proper osmolality (usually nearly isotonic to the plasma osmolality) to inhibit undesired sperm activation during refrigerated storage or cryopreservation (Bates, Wayman and Tiersch, 1996).

In contrast to fishes with external fertilisation, the sperm of *Xiphophorus* fishes exhibit different behaviours (Yang *et al.*, 2006). When sperm of *X. helleri* were suspended at 11 different osmotic pressures (24–500 mOsmol/kg) of Hanks' balanced salt solution (HBSS), motility was observed between 116 and 425 mOsmol/kg, with highest motility observed for samples suspended in HBSS at 310 mOsmol/kg, which was similar to that of the blood plasma (~320 mOsmol/kg). Sperm were not motile when the osmolality was lower than 116 or higher than 425 mOsmol/kg. Motility of the immobilised (non-motile) sperm could be activated by changing the osmotic pressure to 291–316 mOsmol/kg, and motility of immobilised sperm from hypertonic HBSS (> 425 mOsmol/kg) was significantly higher than that from hypotonic HBSS (< 145 mOsmol/kg) after 48 h of storage.

Based in part on these observations, HBSS at 310 mOsmol/kg was chosen as an initial extender for sperm cryopreservation and motility as high as 70% was obtained in thawed sperm

after cryopreservation (Huang *et al.*, 2004a,b,c). Further study revealed that fresh sperm immobilised in HBSS 500 retained motility during refrigerated (non-frozen) storage significantly longer than did sperm stored in a motile (non-immobilised) state in isotonic HBSS (Yang *et al.*, 2006). The immobilised sperm suspended at 500 mOsmol/kg in HBSS were also cryopreserved, and high motility (~55%) was obtained after thawing. Subsequent parallel testing of cryopreservation of sperm from individual fish showed that motile sperm (in isotonic extender) and immobilised sperm (in hypertonic extender) showed the same motility after thawing with activation in isotonic HBSS (Yang *et al.*, 2006). By immobilising sperm prior to freezing, the use of hypertonic HBSS was hypothesised to conserve energy capacity, and consequently to provide an advantage for artificial insemination compared to sperm cryopreserved using isotonic HBSS.

Cryoprotectant selection and equilibration

Cryoprotectants used in screening for cryopreservation in *Xiphophorus* include dimethyl sulfoxide (DMSO), N-dimethyl formamide, N-dimethyl acetamide, glycerol, propylene glycol, methanol, and sucrose. These are commonly used cryoprotectants for all aquatic species. Each was evaluated with final concentrations of 6% and 10% (v/v). Results with *X. helleri* indicated that DMSO and glycerol were suitable cryoprotectants, and further evaluation with different concentrations showed that glycerol was better than DMSO in retaining motility and prolonging storage time of sperm after thawing. The most effective concentration for glycerol was 14% for non-immobilised sperm of *X. helleri* (Huang *et al.*, 2004b,c). Similarly, 14% glycerol showed the best effect in cryopreservation for another species, *X. couchianus* (Huang *et al.*, 2004a), and in immobilised sperm of *Xiphophorus helleri* with concentrations of 10% and 15% (Yang *et al.*, 2006).

Prior to freezing, an equilibration period is necessary to allow permeating cryoprotectants to penetrate the sperm. Equilibration times of 10, 20, 30, 60, and 120 min at 4 ºC before freezing were evaluated for sperm of *X. helleri* and *X. couchianus*. An equilibration time less than 30 min yielded the highest post-thaw motility in both species, but there were no consistent differences across the range of 10 to 120 min. Thus, equilibration time was considered to be not critical when glycerol was used as the cryoprotectant for *Xiphophorus* fishes.

Packing of samples for freezing

In cryopreservation, packing of samples for freezing and storing is important to standardise cooling and thawing rates, and to assure sample identification. Because of the small volume of sperm available, glass capillary tubes or cryovials have been employed in cryopreservation in aquarium fish (Harvey *et al.*, 1982; Aoki *et al.*, 1997; Morris *et al.*, 2003; Draper *et al.*, 2004). For *Xiphophorus* fishes, standard French straws (0.25 ml), used for cryopreservation of livestock sperm, were chosen for sperm packaging. The use of French straws offers the advantages of efficient and reliable sample identification by permanent printing on colored straws, sample safety by complete sealing of the straws, and standardization of the cooling and thawing processes.

Determination of cooling rate for freezing

Cooling rate is an important factor in sperm cryopreservation. Theoretically, it should be fast enough to minimise the exposure time of sperm to concentrated extracellular solutions and yet

it should be slow enough to minimise, by cellular dehydration, the formation of intracellular ice to below a damaging level. The optimum cooling rates vary with different cryoprotectants and species, and can be determined empirically by experimentation, or by theoretical calculation using techniques such as differential scanning calorimetry, a technique used to measure two heat releases from the same cell suspension during freezing of live cells and dead cells (Devireddy, Raha and Bischof, 1998). For *Xiphophorus*, rates of 5, 15, 20, 25, 30, 35 and 45ºC per min were tested to cool sperm from 5ºC to –80ºC before plunging into liquid nitrogen. The results showed that 20-30ºC per min were best when sperm were cryopreserved with 14% glycerol (Huang *et al.*, 2004a,b). The prediction by differential scanning calorimetry agreed closely with this result in *X. helleri* (Thirumala, Huang, Dong, Tiersch and Devireddy, 2005) but not in *X. maculatus* (Pinisetty, Huang, Dong, Tiersch and Devireddy, 2005).

Overall, using post-thaw motility as an indicator of overall gamete quality, the optimised protocol for sperm cryopreservation in *X. helleri* and *X. couchianus* is as follows: collection of sperm by crushing of dissected testis in a volume of HBSS equal to 20-100 times the testis weight (yielding a sperm concentration of $\sim 10^8$ cells per ml) at an osmolality of 310 or 500 mOsmol/kg, holding on ice, the addition of chilled HBSS-glycerol as cryoprotectant to the sperm suspension to yield a final concentration of 14%, equilibration for 10-120 min, loading into 250-ml French straws, freezing with a cooing rate of 20-25°C per minute from 5°C to -80°C, plunging of the samples into liquid nitrogen for storage, and thawing at 40°C for 7 seconds before use for insemination and motility estimation.

Artificial insemination

After insemination, sperm of *Xiphophorus* fishes can reside within the female reproductive tract for months prior to fertilisation (Tavolga, 1949). This creates significant problems for monitoring the fate of sperm after insemination and for evaluating fertility. In initial studies, despite high motility in thawed samples, artificial insemination with cryopreserved sperm failed to produce live young. This resulted in the development of several hypotheses, including: 1) sperm have limited energy stores, and in the process of cryopreservation, motility in the extender could shorten the storage time of sperm in the female reproductive tract prior to fertilisation; 2) in the process of cryopreservation, the suspension of sperm in extender reduces the sperm density below that necessary for fertilisation, or 3) the toxicity of cryoprotectants used in cryopreservation may shorten the sperm lifetime, or affect inseminated females, and consequently reduce fertilisation (Yang *et al.*, 2006). To address these hypotheses, immobilization of sperm by hypertonic HBSS was used to maintain potential energy stores; centrifugation was used to increase the concentration of sperm density (Yang *et al.*, 2006), and washing of cryoprotectant from thawed sperm was also accomplished by centrifugation, which improved retention of motility to 72 hours. The ionic composition of the extender, and addition of glucose and fetal bovine serum were also studied for the purposes of increasing sperm motility after thawing and improving effectiveness with the female reproductive tract for internal fertilisation (Dong, Huang and Tiersch, 2006b).

Based on these efforts, successful fertilisation and offspring production by cryopreserved sperm has been obtained in *X. helleri* (Yang *et al.*, in review). This claim was supported by use of artificial insemination between two species that yield distinct hybrid offspring to verify paternity via cryopreserved sperm by both phenotype and genotype. Virgin females of *X. maculatus* were used for insemination with cryopreserved sperm from *X. helleri*. Motile and osmotically immobilised sperm each produced confirmed offspring. In addition, washed and non-washed sperm each produced confirmed offspring, suggesting that the toxicity of the glycerol concentrations tested did not affect production of offspring.

Overall, the optimised protocol to prepare sperm samples after thawing for artificial insemination is as follows: transfer of thawed samples into 1.5-ml microcentrifuge tubes, centrifugation at 1000 x g for 5 minutes, decanting of most of the supernatant, and re-suspension of the sperm pellet into 15 µl of fresh HBSS before use for insemination. Care was taken in these trials to ensure that a concentration of at least 10^9 sperm cells per ml was attained before insemination. These protocols represent the first report of successful fertilisation and offspring production by cryopreserved sperm in a live-bearing fish. Future studies will focus on the refinement of current protocols, and their application to other species of live-bearing fishes.

The need for standardization

For sperm cryopreservation to become a reliable, cost-effective tool for genetic banking in aquatic species, the overall process needs to be improved, and the approach needs to be integrated into an efficient large-scale platform that links with genetic and biological databases, long-term storage capabilities, inventory management, quality control, sample distribution pathways, biosecurity assurance, utilization and disposal practices, and a sound cryobiological foundation (Tiersch and Mazik, 2000). At present, a student of the aquatic species cryopreservation literature would conclude that the available procedures are plagued with extraordinary variability in results and reporting (Tiersch, 2000). The sources of this underlying variability derive from the empirical approach used for protocol development, the diversity of training background and experience of the researchers involved, a lack of standardization and definition of procedures (e.g., Leibo, 2000), and unexplainable results in terms of classical cryobiological principles.

The importance of estimating sperm concentration

It is likely that a lack of control of sperm concentration is one of the main reasons for the inconsistency observed among various studies, especially in relation to the concentration of cryoprotectant that offers best protection. This is because most cryopreservation studies in aquatic species do not standardise sperm concentrations prior to freezing, and instead have used methods such as dilution ratios based on volume. For example, in a review of the most studied group of aquatic organisms, the salmonid fishes, examination of 27 published reports (between 1978 and 2003) revealed the use of dilution ratios (mainly 1:3 of sperm-to-extender) for all studies (Dong and Tiersch, unpublished). The variations in sperm concentration that are possible by use of a set dilution ratio for a population of males are sufficient to span across and beyond the effective range of the available concentration of cryoprotectant. This effectively acts as a significant uncontrolled variable within otherwise well designed studies and can produce considerable variability in results.

Oysters, the most studied group of invertebrates, offer a relatively self-contained literature base that is representative of aquatic species in general (reviewed by Dong 2005). Currently, there are approximately 26 reports directly related to sperm cryopreservation in oysters since the first study some 35 years ago (Lannan, 1971). These reports include 16 peer-reviewed journal articles, 1 abstract, 2 book chapters, 2 conference proceedings, 1 thesis, 1 dissertation, 1 technical report, and 2 review articles. Nineteen of the 24 research reports (~80%) were produced for sperm from a single species, the Pacific oyster, *Crassostrea gigas*. A review of this literature identified the failure of sperm concentration standardization in 75% of the 16 peer-reviewed journal articles. These studies in particular were characterised by inconsistent or conflicting results even with the same cryoprotectants used at the same nominal concentra-

tions. In other words it is difficult to find agreement in results with particular cryoprotectants or other variables in any two studies within a single species.

Based on thess observations, a recent study was performed to illustrate the need for standardization of sperm concentration by research of sperm agglutination in Pacific oysters (Dong *et al.*, 2006c). Sperm agglutination after thawing is a relatively frequent phenomenon observed for aquatic species, especially when sub-optimal cryopreservation protocols are used. The study evaluated various factors affecting sperm agglutination of thawed samples from diploid and tetraploid oysters. It was found that agglutination in thawed samples was mainly due to the lack of sufficient cryoprotectant for a specific sperm concentration. The exact mechanism of sperm agglutination remains unclear. However, morphological examination of cross sections indicated at least two forms of agglutination (formed with and without cryoprotectant) that could be used as an important tool to understand the cryopreservation process within the micro-environment of the straw. Furthermore, the fact that the level of sperm agglutination was directly determined by sperm concentration, in addition to the type of cryoprotectant, cryoprotectant concentration, and cooling and thawing methods emphasised the importance of procedural standardization and systematic optimization and integration of protocols involving multiple factors. The results presented in this study call attention to the requirement to standardise sperm concentrations prior to cryopreservation; otherwise the reporting of cryoprotectant concentration or molarity offers little value and can be misleading. Sperm concentrations can be estimated readily by use of spectrophotometric methods (e.g., Dong, Huang and Tiersch, 2006c).

Other areas for standardization

The need for standardization extends elsewhere, as inconsistency exists in other components of cryopreservation technology among and within studies, such as in initial sperm quality, gamete collection methods, extender formulation, cryoprotectant choice, cooling rate and method, thawing rate and method, insemination protocols, and evaluation of post-thaw sperm quality (Rana, 1995; Gwo, 2000; Tiersch, 2000). Again we can look to the oyster literature review described above as an illustrative example for other aquatic species. Cryopreservation of oyster sperm involves variables ranging from broodstock condition to larval development, and for each step, various procedures have been used among different studies. For gamete collection, the most commonly used methods were dry stripping (which kills the oyster), aspiration using a pipette or syringe of gonad material through a hole drilled in the shell (without killing the oyster), or induced spawning. Few studies indicated what part of the gonad was sampled, although a recommendation has been made that no more than half of the gonad volume be extracted to avoid including immature or nutritive cells (McFadzen, 1995). Milt from individual males or pooled milt from several males has been used for various studies. Fewer than half of the reports indicated a sperm quality assessment prior to freezing, and when quality was assessed, motility was the sole criterion used. A similar state of within and among study variability exists for most factors that are studied. This lack of standardization places a substantial burden on the aquatic cryopreservation research community.

Furthermore, with respect to cryobiology, it is important to note that even simple cells such as sperm have a high degree of internal complexity. The various structures within a sperm cell represent different functional compartments that can each require different optimal conditions, and thus can each exhibit differential responses to cryopreservation. This can cause a variety of damages and outcomes. For example, damage to the tail could interfere with motility whereas damage to the head could interfere with embryonic development. Sperm quality is a generic term that encompasses proper function of a combination of cellular structures (such as the

nucleus, plasma membrane, or axoneme) that can be evaluated individually by specific assays such as viability staining combined with flow cytometry (e.g., DeBaulny, LeVern, Kerboeuf and Maisse, 1997; Segovia, Jenkins, Paniagua-Chavez and Tiersch, 2000; Paniagua-Chavez, Jenkins, Segovia and Tiersch, 2006), motility estimates, Comet assay (e.g., Zilli, Schiavone, Zonno, Storelli and Vilella, 2003), computer-assisted sperm analysis (e.g., Kime, Van Look, McAllister, Huyskens, Rurangwa and Ollevier, 2001), or in aggregate by examining factors such as the capacity of sperm to fertilise eggs that hatch and develop normally. Sperm quality can be affected by a number of factors, and damage can occur to multiple structures. These would not all be detectable with a single assay (such as motility estimates). Accordingly, it is important to realise that it will be necessary to integrate several parameters of sperm quality into screening panels. As indicated above, several assays already exist for fish, but no comprehensive screening exists for aquatic species. Research is necessary not only to identify and further develop assays, but also to understand the principles and mechanisms that underlie results.

Misinterpretation of osmotic effects

A final example of systematic error that a lack of standardization can introduce into cryopreservation research in aquatic species, especially marine organisms, comes again from oysters. In another recent study (Dong, Huang, Henk and Tiersch, 2006a) spermatozoa from Pacific oysters were examined after non-isotonic fixation. Morphological anomalies, such as membrane rupture, detached tails, and the formation of tail vesicles (typically associated with damage attributable to procedures such as cryopreservation) were observed. Osmolalities within the range of 800 to 1,086 mOsmol/kg were found to be functionally isotonic to sperm of Pacific oysters, but osmolalities below or above this caused severe cell damage. Although the damage associated with non-isotonic fixation was evident in all parts of the cells, the most vulnerable locations were the plasma membrane and flagellar motor apparatus. Hypotonic fixation was associated with the formation of tail vesicles (Fig. 3). Oyster sperm became swollen in hypotonic fixative, and bending or coiling of the axoneme within the tail vesicles led to the appearance of multiple axonemal structures in cross sections when observed by transmission electron microscopy. This phenomenon might be generally misinterpreted as the presence of double tails. This and other fixation artifacts can lead to the misinterpretation of damage caused by cryopreservation in ultrastructure studies of sperm of aquatic species. These findings indicate that caution is required in the interpretation of previously published results addressing ultrastructural changes in relation to cryopreservation. Future studies should ensure that samples are prepared with isotonic fixation, especially in marine species, and that the formation of tail vesicles be scrutinised objectively before claims are made linking them to treatment effects (e.g., cryopreservation) or biological variation (e.g., double tails).

To summarise, a lack of standardization is typically observed within the scientific literature for aquatic species in each step involved in the cryopreservation process. Comparisons among different studies are difficult to perform and could be invalid in most cases due to the procedural and reporting variations across studies. The points made in the section above call attention to the requirement for researchers to standardise sperm concentration in particular and protocols in general during cryopreservation research and reporting. Optimization of protocols without standardization offers little value for the improvement of existing methods and results, especially for the future development of commercial application. Controversy and inconsistency would be reduced if more congruent approaches were utilised and results among various studies could be directly compared. Suggestions for improvement include the creation and

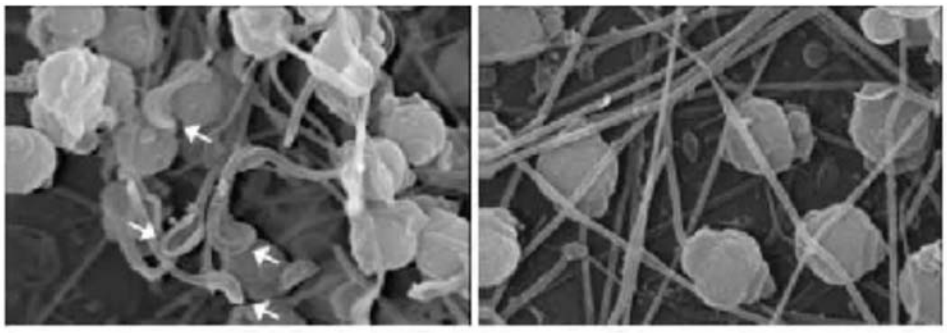

Figure 3. Scanning electron micrographs of spermatozoa from Pacific oysters. Left panel: Samples fixed in 4% glutaraldehyde at 356 mOsmol/kg (hypotonic). Right: Samples fixed in 4% glutaraldehyde supplemented with sufficient sucrose to bring the final osmolality to 967 mOsmol/kg (isotonic). Arrows indicate tail vesicles formed after hypotonic fixation. Figure adapted with permission from Dong *et al.* (2006a).

widespread acceptance of standard references to assist in harmonizing terminology (e.g., Tiersch and Mazik, 2000; Fuller, Lane and Benson, 2004) and the development and utilization of standardised educational programs. Standardization of research practices and reporting could be accomplished through establishment of guidelines for publication of results. These guidelines could be developed and approved by societies and organizations that are involved in cryopreservation, and they could be applied gradually in phases to allow researchers to adjust methodologies. Once in place the guidelines could be made available to journal editors and reviewers to assist in evaluation of research reports.

The future prospects for application of cryopreservation in aquatic species

With respect to commercialization, the benefits of cryopreservation include at least five levels of improvements that address existing industries and the creation of new industries. First, cryopreservation, at a minimum, can be used to improve existing hatchery operations by providing sperm on demand, and greatly simplifying the timing of induced spawning. This prevents the problem, for example, of collecting ripe eggs, but not having sperm available to fertilise them. Second, frozen sperm can greatly enhance efficient use of facilities and create new opportunities in the hatchery by eliminating the need to maintain live males. Potentially all of the resources in a hatchery, which are typically limited, could be diverted to use for females and larvae. Third, valuable genetic lineages that currently exist, such as endangered species, research models, or improved farmed strains can be protected by storage of frozen sperm. This could be very important for marine species such as shellfish in which valuable broodstocks must be stored in natural waters. Fourth, cryopreservation opens the door for rapid genetic improvement. Frozen sperm can be used in breeding programs to create new improved lines and shape the genetic resources available for aquaculture operations. A dramatic example of this potential opportunity is provided by the dairy industry, which relies almost entirely upon cryopreserved sperm to produce improvements in milk yields. Finally, cryopreserved sperm of aquatic species will at some point, likely within the coming decade, become an entirely new industry itself. The global market for livestock sperm is around a billion dollars each year. Large, highly valuable global markets for cryopreserved sperm of aquatic species are now on the horizon.

Most of the technological innovations that have advanced the field of germplasm cryopreservation arose from a sound understanding of the mechanisms of cryodamage and cryoprotection (Mazur, 1970; Mazur, 1984). Successful cryopreservation of germplasm must address intrinsic biophysical properties (e.g., osmotic tolerance limits), and cryopreservation procedures based on these biophysical properties are necessary to minimise cryodamage and maximise survival (Rall, 1993). The sections above call attention to the importance of the cumulative effects arising from all activities in cryopreservation and the importance of standardization for the future potential commercialization of cryopreserved sperm in aquatic species.

Cryopreservation research and application each require consideration of an interconnected series of activities and this involves more than simple freezing of samples. A successful program involves integrated practices for sample collection, refrigerated storage, freezing, thawing, rules for use and disposal, transfer agreements, and database development. Sperm cryopreservation in aquatic species is only beginning to find application on a commercial scale. The development of this new industry is constrained by a number of factors including the technical requirements for scaling-up to commercial operations during the transition from research. This problem has been addressed by previous research that documents the feasibility of utilizing commercial dairy cryopreservation facilities to provide a jumpstart for cryopreservation in aquatic species such as catfish (Lang, Riley, Chandler and Tiersch, 2003) and oysters (e.g., Dong, Endeline, Huang, Allen and Tiersch, 2005). Although market price structures for cryopreserved sperm are yet to emerge for aquatic species, they will be resolved as the industry develops in response to the existing strong demand for genetic resources (Caffey and Tiersch, 2000). Thus, overall, the largest practical constraint to realization of a cryopreservation industry for aquaculture is at present the absence of uniform quality control practices, industry standards, and appropriate biosecurity safeguards. The lack of quality control and biosecurity (Tiersch and Jenkins, 2003) cast a dark shadow over industry development and could discourage use of cryopreservation technology in general.

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References

Anders, F. (1967). Tumour formation in platyfish-swordtail hybrids as a problem of gene regulation. *Experientia* **23**: 1-10

Anders, F. (1991). Contributions of the Gordon-Kosswig melanoma system to the present concept of neoplasia. *Pigment Cellular Research* **4**: 7-29

Aoki, K., Okamoto, M., Tatsumi, K. and Ishikawa, Y. (1997). Cryopreservation of medaka spermatozoa. *Zoological Science* **14**: 641-644

Bates, M.C., Wayman, W.R. and Tiersch, T.R. (1996). Effect of osmotic pressure on the activation and storage of channel catfish sperm. *Transactions of the American Fisheries Society* **125**: 798-802

Billard, R., Cosson, J., Noveiri, S.B. and Pourkazemi, M. (2004). Cryopreservation and short-term storage of sturgeon sperm, a review. *Aquaculture* **236**: 1-9

Billard, R. and Zhang, T. (2001). Techniques of genetic resource banking in fish. In: *Cryobanking the Genetic Resource. Wildlife Conservation for the Future?* Edited by Watson, P.F. and Holt, W.V., Taylor and Francis, London, pp. 156-170

Blaxter, J.H.S. (1953). Sperm storage and cross-fertilization of spring and autumn spawning herring. *Nature* **172**: 1189-1190

Caffey, R.H. and Tiersch, R.T. (2000). Economics and marketing of cryopreserved fish sperm. In:

Cryopreservation in Aquatic Species. Edited by Tiersch T.R. and Mazik, P.M., World Aquaculture Society, Baton Rouge, LA, pp. 388-408

Chao, N.H., Lin, T.T., Chen, Y.J., Hsu, H.W. and Liao, H.W. (1997). Cryopreservation of late embryos and early larvae in the oyster and hard clam. *Aquaculture* **155**: 31-44

David, W.M., Mitchell, D.L. and Walter, R.B. (2004). DNA repair in hybrid fish of the genus *Xiphophorus*. *Comparative Biochemistry and Physiology C* **138**: 301-309

DeBaulny, B.O., LeVern, Y., Kerboeuf, D. and Maisse, G. (1997). Flow cytometric evaluation of mitochondrial activity and membrane integrity in fresh and cryopreserved rainbow trout (*Oncorhynchus mykiss*) spermatozoa. *Cryobiology* **34**: 141-149

Devireddy, R.V., Raha, D. and Bischof, J.C. (1998). Measurement of water transport during freezing in cell suspensions using a differential scanning calorimeter. *Cryobiology* **36**: 124-155

Doebbler, G.F. (1966). Cryoprotective compounds - review and discussion of structure and function. *Cryobiology* **3**: 2-11

Dong, Q. (2005). *Comparative studies of sperm cryopreservation of diploid and tetraploid Pacific oysters*. PhD thesis, Louisiana State University, Baton Rouge, Louisiana.

Dong, Q., Eudeline, B., Huang, C., Allen, J. and Tiersch, T.R. (2005). Commercial-scale sperm cryopreservation of diploid and tetraploid Pacific oysters, *Crassostrea gigas*. *Cryobiology* **50**: 1-16

Dong, Q., Huang, C., Henk, M.C. and Tiersch, T.R. (2006a). Fixation methods can produce misleading artifacts in sperm cell ultrastructure of diploid and tetraploid Pacific oysters, *Crassostrea gigas*. *Cell and Tissue Research* **324**: 335-345

Dong, Q., Huang, C. and Tiersch, T.R. (2006b). Post-thaw amendment of cryopreserved sperm for use in artificial insemination of a viviparous fish, the green swordtail *Xiphophorus helleri*. *Aquaculture* (in press)

Dong, Q., Huang, C. and Tiersch, R.T. (2006c). Control of sperm concentration is necessary for standardization of sperm cryopreservation in aquatic species: evidence from sperm agglutination in oysters. *Cryobiology* (in press)

Draper, B.W., McCallum, C.M., Scout, J.L., Slade, A.J. and Moens, C.B. (2004). A high-throughput method for identifying *N*-ethyl-*N*-nitrosourea (ENU)-induced point mutations in zebrafish. In: *The Zebrafish Genomics, and Informatics. Methods in Cell Biology*, volume 77. Edited by Detrich III, H.W., Westerfield, M. and Zon, L.I., Elsevier Press, San Diego, pp. 91-112

Fuller, B.J., Lane, N. and Benson, E.E. (2004). *Life in the Frozen State*. CRC Press, New York, pp. 1-672

Gardiner, D.M. (1978). Utilization of extracellular glucose by spermatozoa of 2 viviparous fishes. *Comparative biochemistry and physiology A - Physiology* **59**: 165-168

Grier, H.J. (1975). Aspects of germinal cyst and sperm development in *Poecilia-latipinna* (Teleostei-Poeciliidae). *Journal of Morphology* **146**: 229-249

Gwo, J.C. (2000). Cryopreservation of aquatic invertebrate semen: a review. *Aquaculture Research* **31**: 259-271

Hagedorn, M., Carter, V.L., Steyn, R.A., Krupp, D., Leong, J.C., Lang, R.P. and Tiersch, T.R. (2006). Preliminary studies of sperm cryopreservation in the mushroom coral, *Fungia scutaria*. *Cryobiology* **52**: 454-458

Harvey, B., Norman, K.R. and Ashwood-Smith, M.J. (1982). Cryopreservation of zebrafish spermatozoa using methanol. *Canadian Journal of Zoology* **60**: 1867-1870

Heater, S.J., Oehlers, L.P., Rains, J.D. and Walter, R.B. (2004). DNA polymerase mRNA and protein expression in *Xiphophorus* fish. *Comparative Biochemistry and Physiology C* **138**: 325-334

Hoar, W.S. (1969). Reproduction. In: *Fish Physiology*. Edited by Hoar, W.S. and Randall, D.J., Academic Press, New York, pp. 1-72

Huang, C., Dong, Q. and Tiersch, T.R. (2004a). Sperm cryopreservation of a live-bearing fish, the platyfish *Xiphophorus couchianus*. *Theriogenology* **62**: 971-989

Huang, C., Dong, Q., Walter, R.B. and Tiersch, T.R. (2004b). Initial studies on sperm cryopreservation of a live-bearing fish, the green swordtail *Xiphophorus helleri*. *Theriogenology* **62**: 179-194

Huang, C., Dong, Q., Walter, R.B. and Tiersch, T.R. (2004c). Sperm cryopreservation of green swordtail *Xiphophorus helleri*, a fish with internal fertilization. *Cryobiology* **48**: 295-308

Jonas-Davies, J.A.C., Winfrey, V. and Olson, G.E. (1983). Plasma-membrane structure in spermatogenic cells of the swordtail (Teleostei, *Xiphophorus helleri*). *Gamete Research* **7**: 309-324

Kallman, K.D. (1975). The platyfish, *Xiphophorus maculatus*. In: *Handbook of Genetics*. Edited by King, R.C., Plenum Publishing Corporation, New York, pp. 81-132

Kazianis, S., Khanolkar, V.A., Naim, R.S., Rains, J.D., Trono, D., Garcia, R., Williams, E.L. and Walter, R.B. (2004). Structural organization, mapping, characterization and evolutionary relationships of CDKN2 gene family members in *Xiphophorus* fishes. *Comparative Biochemistry and Physiology C* **138**: 291-299

Kazianis, S. and Walter, R.B. (2002). Use of platyfishes and swordtails in biological research. *Laboratory Animal* **31**: 46-52

Kime, D.E., Van Look, K.J.W., McAllister, B.G., Huyskens, G., Rurangwa, E. and Ollevier, F. (2001). Computer-assisted sperm analysis (CASA) as a tool for monitoring sperm quality in fish. *Comparative Biochemistry and Physiology C-Toxicology & Pharmacology* **130**: 425-433

Lahnsteiner, F. (2000). Introduction to the special issue on 'Cryopreservation of gametes in aquatic species'. *Aquaculture Research* **31**: 229-229

Lang, R.P., Riley, K.L., Chandler, J.E. and Tiersch, T.R. (2003). The use of dairy protocols for sperm

cryopreservation of blue catfish *Ictalurus furcatus*. *Journal of the World Aquaculture Society* **34**: 66-75

Lannan, J.E. (1971). Experimental self-fertilization of Pacific oyster, *Crassostrea-gigas*, utilizing cryopreserved sperm. *Genetics* **68**: 599-601

Leibo, S.P. (2000). Sources of variation is cryopreservation. In: *Cryopreservation in Aquatic Species*. Edited by Tiersch, R.T. and Mazik, P.M., World Aquaculture Society, Baton Rouge, Louisiana, pp. 75-83

Leung, L.K.P. (1991). Principle of biological cryopreservation. In: *Fish Evolution and Systmatics: Evidence form Spermatozoa*. Authored by Jamieson, B.G.M., Cambridge University Press, Cambridge, UK, pp. 231-244

Leung, L.K.P. and Jamieson, B.G.M. (1991). Live preservation of fish gametes. In: *Fish Evolution and Systmatics: Evidence form Spermatozoa*. Authored by Jamieson, B.G.M., Cambridge University Press, Cambridge, UK, pp. 245-269

Mauger, R.E., Le Bail, P.Y. and Labbe, C. (2006). Cryobanking of fish somatic cells: optimizations of fin explant culture and fin cell cryopreservation. *Comparative Biochemistry and Physiology B* **144**: 29-37

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

Mazur, P. (1984). Freezing of living cells - mechanisms and implications. *American Journal of Physiology* **247**: C125-C142

McFadzen, I.R.B. (1995). Cryopreservation of the sperm of Pacific oyster *Crassostrea gigas*. In: *Methods in Molecular Biology: Cryopreservation and Freeze-Drying Protocols*. Edited by Day, J.G. and McCellan M.R., Humana Press, Totowa, pp. 145-149

Meierjohann, S., Schartl, M. and Volff, J.N. (2004). Genetic, biochemical and evolutionary facets of Xmrk-induced melanoma formation in the fish *Xiphophorus*. *Comparative Biochemistry and Physiology C* **138**: 281-289

Meryman, H.T. (1971). Cryoprotective agents. *Cryobiology* **8**: 173-183

Morisawa, M. and Suzuki, K. (1980). Osmolality and potassium ion - their roles in initiation of sperm motility in teleosts. *Science* **210**: 1145-1147

Morisawa, M., Suzuki, K., Shimizu, H., Morisawa, S. and Yasuda, K. (1983). Effects of osmolality and potassium on motility of spermatozoa from fresh water cyprinid fishes. *Journal of Experimental Biology* **107**: 95-103

Morris, J.P., Berghmans, S., Zahrieh, D., Neuberg, D.S., Kanki, J.P. and Look, A.T. (2003). Zebrafish sperm cryopreservation with N,N-dimethylacetamide. *Biotechniques* **35**: 956-968

Oehlers, L.P., Heater, S.J., Rains, J.D., Wells, M.C., David, W.M. and Walter, R.B. (2004). Gene structure, purification and characterization of DNA polymerase beta from *Xiphophorus maculatus*. *Comparative Biochemistry and Physiology C* **138**: 311-324

Paniagua-Chavez, C.G., Buchanan, J.T. and Tiersch, T.R. (1998). Effect of extender solutions and dilution on motility and fertilizing ability of Eastern oyster sperm. *Journal of Shellfish Research* **17**: 231-237

Paniagua-Chavez, C.G., Jenkins, J., Segovia, M. and Tiersch, T.R. (2006). Assessment of gamete quality for the eastern oyster (*Crassostrea virginica*) by use of fluorescent dyes. *Cryobiology* **53**: 128-138

Pinisetty, D., Huang, C., Dong, Q., Tiersch, T.R. and Devireddy, R.V. (2005). Subzero water permeability parameters and optimal freezing rates for sperm cells of the southern platyfish, *Xiphophorus maculatus*. *Cryobiology* **50**: 250-263

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

Rall, W.F. (1993). Advances in the cryopreservation of embryos and prospects for the application to the conservation of salmonid fishes. In: *Genetic Conservation of Salmonid Fishes*. Edited by Thorgaard, G.H. and Cloud, J.G., Plenum Press, New York, pp. 137-158

Rana, K.J. (1995). Cryopreservation of fish spermatozoa. In: *Methods in Molecular Biology: Cryopreservation and Freeze-Drying Protocols*. Edited by Day, J.G. and McCellan M.R., Humana Press, Totowa, pp. 151-165

Rowe, A.W. (1966). Biochemical aspects of cryoprotective agents in freezing and thawing. *Cryobiology* **3**: 12-18

Schartl, M. (1995). Platyfish and swordtails - a genetic system for the analysis of molecular mechanisms in tumor-formation. *Trends Genetics* **11**: 185-189

Scott, A.P. and Baynes, S.M. (1980). A review of the biology, handling and storage of Salmonid spermatozoa. *Journal of Fish Biology* **17**: 707-739

Segovia, M., Jenkins, J.A., Paniagua-Chavez, C. and Tiersch, T.R. (2000). Flow cytometric evaluation of antibiotic effects on viability and mitochondrial, function of refrigerated spermatozoa of Nile tilapia. *Theriogenology* **53**: 1489-1499

Stoss, J. (1983). Fish gamete preservation and spermatozoan physiology. In: *Fish Physiology, Volume IX, Part B, Behavior and Fertility Control*. Edited by Hoar, W.S., Randall, D.J. and Donaldson, E.M., Academic Press, Orlando, FL, pp. 305-350

Suquet, M., Dreanno, C., Fauvel, C., Cosson, J. and Billard, R. (2000). Cryopreservation of sperm in marine fish. *Aquaculture Research* **31**: 231-243

Tavolga, W.N. (1949). Embryonic development of the platyfish (*Platypoecilus*), the swordtail (*Xiphophorus*) and their hybrids. *Bulletin of the American Museum of Natural History* **94**: 161-230

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

Thirumala, S., Huang, C.J., Dong, Q.X., Tiersch, T.R. and Devireddy, R.V. (2005). A theoretically estimated optimal cooling rate for the cryopreservation of sperm cells from a live-bearing fish, the green swordtail *Xiphophorus helleri*. *Theriogenology* **63**:

2395-2415

Tiersch, T.R. (2000). Introduction. In: *Cryopreservation in Aquatic Species*. Edited by Tiersch T.R. and Mazik, P.M., World Aquaculture Society, Baton Rouge, LA, pp. xix-xxvi

Tiersch, T.R. (2001). Cryopreservation in aquarium fishes. *Marine Biotechnology* **3**: S212-S223

Tiersch, T.R. and Jenkins, J.A. (2003). Biosecurity considerations for cryopreserved gametes and early life stages of aquatic species. In: *Biosecurity in Aquaculture Production Systems: Exclusion of Pathogens and Other Undesirables*. Edited by Lee, C.-S. and O'Bryen, P.J., World Aquaculture Society, Baton Rouge, Louisiana, pp. 171-198

Tiersch, T.R. and Mazik, P.M. (2000). *Cryopreservation in Aquatic Species*. World Aquaculture Society, Baton Rouge, LA, pp. 1-439

Viveiros, A.T.M. (2005). Semen cryopreservation in catfish species, with particular emphasis on the African catfish. *Animal Breeding Abstracts* **73**: 1N-9N

Walter, R.B. and Kazianis, S. (2001). *Xiphophorus* interspecies hybrids as genetic models of induced neoplasia. *Journal of Institute for Laboratory Animal Research* **42**: 299-322

Yang, H., Hazelwood, L., Walter, R.B. and Tiersch, T.R. (2006). Effect of osmotic immobilization on refrigerated storage and cryopreservation of sperm from a viviparous fish, the green swordtail *Xiphophorus helleri*. *Cryobiology* **2**: 209-218

Zhang, T. (2004). Cryopreservation of gametes and embryos of aquatic species. In: *Life in the Frozen State*. Edited by Fuller, B., Lane, N. and Benson, E., CRC Press, New York, pp. 415-435

Zilli, L., Schiavone, R., Zonno, V., Storelli, C. and Vilella, S. (2003). Evaluation of DNA damage in *Dicentrarchus labrax* sperm following cryopreservation. *Cryobiology* **47**: 227-235