New Chapter

Sperm Cryopreservation in Biomedical Research Fish Models

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Introduction

With the continued development of genomic tools conserved and species-specific molecular mechanisms can be identified, and comparative studies among vertebrate species are becoming commonplace for human biomedical research. As the largest class of vertebrates, bony fishes offer almost unlimited versatility for research. Generally, model fish require features such as small body size, high fecundity, ease of culture, and most importantly, specific characteristics for particular research topics. Currently, the most widely used fish models are zebrafish *Danio rerio* (Driever et al. 1994, Kari et al. 2007), medaka *Oryzias latipes* (Wittbrodt et al. 2002), live-bearing fishes of the genus *Xiphophorus* (Walter and Kazianis 2001), mummichog *Fundulus heteroclitus* (Atz 1986, Burnett et al. 2007) and pufferfish *Fugu rubripes* (Elgar et al. 1996). With extensive studies using these fish models, thousands of specific strains and lines have been created, and are currently housed worldwide as live animals in resource centers, such as the Zebrafish International Resource Center (University of Oregon, Eugene) which holds around 1080 inbred, transgenic, knockout and mutant strains; the University of Georgia (Athens), which holds several inbred and transgenic medaka lines, and the *Xiphophorus* Genetic Stock Center (Texas State University, San Marcos), which holds 61 inbred lines (of which most have been maintained for 50 to more than 100 generations) of 24 species. Preservation of the genetic resources of these and other valuable fishes presents significant and urgent challenges. Gamete or embryo cryopreservation is a useful approach to address these challenges.

Ideally, a conservation program should include the preservation of sperm, eggs, embryos and larvae to secure the revival of species or strains. Currently, cryopreservation techniques in fish are mostly applied to sperm. Cryopreservation has not been successful for eggs and early embryos because of their large size, high lipid content, polar organization (Blesbios and Labbe 2003), and membrane impermeability (Hagedorn et al. 1998). Sperm cryopreservation in fish mostly has focused on large-bodied aquaculture species, such as salmonids, carps, and catfishes, and only several studies have addressed aquarium fishes. Due to the small body sizes and limited sperm availability, sperm cryopreservation in aquarium fishes presents challenges, such as in experimental design, gamete collection, and artificial fertilization, especially in live-bearing fishes (Tiersch 2001).

We intend for this review to provide an overview of sperm cryopreservation in zebrafish, medaka, and *Xiphophorus*, the most important biomedical fish models, and hope it can serve as a template for research on other aquarium fishes. These three groups possess some distinct differences. For example, they occur naturally in two habitats: strict freshwater (zebrafish and *Xiphophorus*) (Hawkins et al. 2001) and brackish-water-accommodated freshwater (medaka) (Inoue and Takei 2002), and they have two reproduction modes: external fertilization (zebrafish and medaka) and internal fertilization (*Xiphophorus*).

Yang, H. and T. R. Tiersch. 2011. Sperm Cryopreservation in Biomedical Research Fish Models. In: *Cryopreservation in Aquatic Species*, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 439-454.

Characteristics of Zebrafish, Medaka, and *Xiphophorus* Fishes

Habitat

The natural environment can influence reproductive modes and traits. Zebrafish, a strict freshwater species, naturally occurs in slow or still freshwater systems (rivers, small streams, pools, and rice paddies) in a range extending from Pakistan in the west to Myanmar (Burma) in the east, and from Nepal in the north to the Indian state of Karnataka in the south (Engeszer et al. 2007). Medaka is a euryhaline species, distributed widely in freshwater habitats of China, Japan, and Korea (Naruse 1996, Naruse et al. 1993), and are also found in brackish water (Miyamoto et al. 1986). Unlike zebrafish and *Xiphophorus* fishes, medaka can be acclimated from freshwater to brackish and even sea water, and can reproduce in fresh water and brackish water (Inoue and Takei 2002). Fishes of the genus *Xiphophorus* naturally live in backwaters in Mexico, Guatemala, Belize and Honduras along the Gulf coast of Mexico (Kallman 2001), and are also strict freshwater species.

Reproduction

Zebrafish, medaka, and *Xiphophorus* fishes are all dioecious. Zebrafish reproduce by external fertilization. Eggs and sperm are released into environmental water to fertilize and develop, and fry can hatch at about 24 hr after fertilization at 26-28 ºC (Laale 1977).

In medaka, the male and female participate in mating behavior before spawning, eggs are expelled as a cluster attached to the belly of the female, and become attached to floating aquatic plants. Fry hatch after 7-10 d at 26 ºC (Yamamoto 1975a).

For *Xiphophorus* fishes, the reproduction mode is internal fertilization. Males are distinguished from females by the secondary sexual organ, a modified anal fin called the gonopodium, and other phenotypes such as body color and in some species, a sword-like tail. After copulating with the male, female *Xiphophorus* can store sperm for months and subsequently produce broods at approximately 30-d intervals for 4-5 months without the presence of a male. The intervals between mating and the first brood are irregular and can vary between 26 and 90 d (Tavolga 1949).

Body Size, Availability of Testis, and Sperm Production

Zebrafish, medaka, and *Xiphophorus* are all small-bodied fishes with lengths of less than 5 cm, and therefore the availability of sperm by stripping is limited to several microliters. Accumulated data on body size, body weight, testis weight, and the sperm production per mg of testis weight (by dissection) for these species are listed in Table 1. Basically, for all of these species correlation analysis has showed that body weight, body length, and testis weight were positively correlated with each other.

Testis Morphology

Gonadal and gametic morphology of fishes have been studied for decades at anatomical or histological levels to identify annual reproduction cycles and length of breeding season, and have been used for analysis of evolution and phylogeny in bony fishes (Jamieson 2009, Parenti and Grier 2004). For zebrafish, medaka, and *Xiphophorus*, testis structure falls into three different types. Zebrafish possess what has been characterized as the anastomosing tubular type (Maack and Segner 2003), which is widely found throughout primitive teleost taxa (Parenti and Grier 2004). Medaka posses the type characterized as the restricted lobular testis (Grier 1976), in

which spermatogonia distribute into the distal ends of lobules. *Xiphophorus* fishes possess the type characterized as the restricted lobular testis (Grier et al. 1980), and the spermatozoa are formed into spermatozeugmata ("sperm bundles"), specialized structures with spermatid nuclei oriented outward toward the Sertoli cells.

Table 1. Basic biological characteristics and sperm production by crushing dissected testis in zebrafish *Danio rerio* (AB lines) from the Zebrafish International Resource Center, medaka *Oryzias latipes* from the University of Georgia (a strain originally derived native to Southern Japan, obtained from Carolina Biological), and four *Xiphophorus* species from the *Xiphophorus* Genetic Stock Center.

Species	Males used	Length (cm)	Weight (g)	Testis (mg)	Sperm /mg of testis (x10^6 cells)	Reference
Zebrafish	45	2.4 ± 0.2	0.295 ± 0.066	3.2 ± 2.0	7.7 ± 2.0	Yang et al. unpublished
Medaka	74	2.6 ± 0.2	0.311 ± 0.052	1.9 ± 0.6	2.0 ± 0.4	(Yang et al. 2010)
X. helleri	45	3.2 ± 0.3	0.630 ± 0.168	9.2 ± 5.5	5.4 ± 2.2	(Huang et al. 2004a)
X. couchianus	66	2.0 ± 0.2	0.184 ± 0.052	3.1 ± 1.0	5.4 ± 2.5	(Yang et al. 2009)
X. maculatus	117	2.5 ± 0.3	0.474 ± 0.149	7.1 ± 3.7	5.8 ± 2.8	Yang et al. unpublished
X. variatus	35	2.5 ± 0.2	0.298 ± 0.096	6.4 ± 3.4	2.4 ± 1.3	Yang et al. unpublished

Sperm Motility Activation

Fish spermatozoa are usually immotile while in the testis and seminal plasma in most species studied (Cosson 2004). Naturally, sperm are activated during spawning to fertilize eggs. For most fishes with external fertilization, osmolality is a dominant factor for activating sperm (Morisawa and Suzuki 1980). In general, for freshwater fish species, motility is initiated by exposure to hypotonic solutions, and for marine species, motility is initiated by exposure to hypertonic solutions (Morisawa and Suzuki 1980). These activation modes match the environment where sperm function during spawning. For example, zebrafish inhabit fresh water, and sperm motility can be activated by exposure to hypotonic solutions (Yang et al. 2007a).

Although they inhabit freshwater, as a euryhaline fish medaka show a mode for activation of sperm motility different from typical freshwater fishes. Sperm motility in medaka could be initiated by distilled water without electrolytes (25 mOsmol/kg) and by Hanks' balanced salt solution with osmolalities spanning from 92 to 686 mOsmol/kg, a range including hypotonic, isotonic, and hypertonic osmolalities (Yang and Tiersch 2009). Upon activation, the sperm could remain continuously motile for as long as one week. These sperm characteristics are distinct from those in most freshwater and marine fishes whether they are external or internal fertilizers, and are potentially representative of other euryhaline fishes.

As viviparous fishes, *Xiphophorus* possess a completely different mechanism for activation of sperm motility. Within the testis, sperm are compacted into spermatozeugmata and are immotile. When suspended in electrolyte solutions isotonic to seminal plasma, the sperm become motile, but in either hypertonic or hypotonic solutions, they are quiescent (Morisawa and Suzuki 1980, Yang et al. 2006). This mode of motility activation has been described in *Xiphophorus* species (Huang et al. 2004a, Yang et al. 2006) and Western mosquitofish *Gambusia affinis* (Morisawa and Suzuki 1980), but may not be representative of all viviparous fishes, such as the redtail splitfin *Xenotoca eiseni* of the family Goodeidae (our unpublished observations). This is because viviparity has emerged independently throughout vertebrate evolution in a

variety of groups (Long et al. 2008), thus presenting a diverse array of adaptations to address the problem of internal fertilization (DeMarais and Oldis 2005, Ryan 1998).

Development of Protocols for Sperm Cryopreservation

Cryopreservation is a technique involving a series of steps including sample collection, sperm dilution in extender, cryoprotectant selection, freezing, storage, thawing, and viability detection (Tiersch 2000). Development of protocols for requires suitable choices at each step and consideration of the interactions among the factors. The success of cryopreservation can be demonstrated by fertilization and production of live offspring. Due to the multiple steps and their interactions, errors at each step can accumulate and lead to considerable losses of viable cells. Thus, careful attention should be given to the details at each step, and care should be taken to reduce or eliminate sources of uncontrolled variation (Leibo 2000). Protocols of sperm cryopreservation can vary because of species-specific differences in sperm size, shape, and biochemical characteristics. Development of protocols for sperm cryopreservation for zebrafish, medaka, and *Xiphophorus* fishes are summarized in Table 2 (next page). We review and compare below the current status of sperm cryopreservation in these fishes.

Sperm Collection

Sperm of aquarium fishes can be collected by stripping or by crushing of dissected testis. Due to their small body size, the availability of sperm by stripping is limited to 1-2 µl. For zebrafish and medaka, the stripped samples are composed of highly concentrated single cells. For *Xiphophorus* fishes, stripped sperm samples are a mixture of single sperm cells, and broken and intact bundles (our unpublished observations). Collection by stripping of sperm samples avoids the killing of valuable fish, and individual males can be sampled repeatedly. However, to maximize the volume of sperm available, especially to allow experimental replication, crushing of dissected testis has been used for sperm collection in most published studies.

Extender Selection

Dilution of sperm in extender solution is necessary for cryopreservation. The role of the extender is to retain the functional capability and fertilizing ability of sperm by controlling the pH, osmolality, ion concentration, and in some cases, the supply of energy. An understanding of sperm activation and motility is necessary to formulate extender solutions. Usually, extenders are balanced-salt buffers with specific pH and osmolality to prevent sperm motility activation. Although, many extenders have been reported for sperm cryopreservation, there are not always observed differences in post-thaw motility (Stoss and Holtz 1981).

For zebrafish, osmolality is the dominant factor to control motility activation as stated above. Once activated by hypotonic osmolality, sperm have a short burst of peak motility (30 sec to 2 min) (Yang et al. 2007a). Thus, sperm need to be held in extender that is isotonic to the plasma osmolality (~300 mOsmol/kg) to inhibit activation. In zebrafish, three different extenders (Ginsburg, BSMIS, HBSS) have been reported for use with cryopreservation (Appendix) and all of them functioned well to retain post-thaw fertility (Draper et al. 2004, Harvey et al. 1982, Morris et al. 2003, Yang et al. 2007a). With respect to composition, Ginsburg extender contains a specific brand of powdered skim milk (Draper et al. 2004, Harvey et al. 1982) which is not readily available worldwide, and impedes the observation of sperm motility and morphology,

Table 2. Summary of previous studies in sperm cryopreservation of zebrafish, medaka, and *Xiphorphorus* fishes.

Extender	Cryoprotectant	Packaging	Cooling method	Thawing method	Assessment	Reference
Zebrafish						
Ginsburg	8.3% Methanol	Capillaries	16 ºC/min from 4 to -35 ºC	RT[a]	51 ± 36% fertilization[b]	(Harvey et al. 1982)
BSMIS	10% Dimethyl acetamide	Capillaries	Placement on dry ice for 30 min	Dilution with 20-x volume of RT extender	9-14% fertilization	(Morris et al. 2003)
Ginsburg	8.3% Methanol	Cryovials	Placement on dry ice for 20 min	33 ºC for 8 sec	28 ± 18% fertilization	(Draper et al. 2004)
HBSS	8% Methanol	French straws	10 ºC/min from 5 to -80 ºC	40 ºC for 5 sec	33 ± 20% fertilization	(Yang et al. 2007a)
Medaka						
Fetal bovine serum	10% Dimethyl formamide	Cryovials	Placement in vapor of liquid nitrogen for 10 or 20 min	30 ºC for 0.5-1 min, dilution in 2-x BSS solution	96-100% fertilization	(Aoki et al. 1997)
0.6-M sucrose	10% Dimethyl sulfoxide	Capillaries	Placement on dry ice for 20 min	Holding between fingers	85% fertilization	(Krone and Wittbrodt 1997)
HBSS 350	10% Methanol	French straws	10 ºC/min from 5 to -80 ºC	40 ºC for 5 sec	70 ± 30% hatching	(Yang et al. 2010)
Xiphophorus helleri						
HBSS 240-300	10% Dimethyl sulfoxide	French straws	45 ºC/min from 5 to -80 ºC	40 ºC for 7 sec	29 ± 8% (post-thaw motility)	(Huang et al. 2004a)
HBSS300	14% glycerol	French straws	20-35 ºC/min from 5 to -80 ºC	40 ºC for 7 sec	77 ± 3% (post-thaw motility)	(Huang et al. 2004b)
HBSS310 HBSS500	14% glycerol	French straws	25 ºC/min from 5 to -80 ºC	40 ºC for 5 sec	1-3 of 15 females produced offspring	(Yang et al. 2007b)
Xiphophorus couchianus						
HBSS 240-300	14% glycerol	French straws	25 ºC/min from 5 to -80 ºC	40 ºC for 7 sec	78 ± 3% (post-thaw motility)	(Huang et al. 2004c)
HBSS500	14% glycerol	French straws	25 ºC/min from 5 to -80 ºC	40 ºC for 5 sec	2 of 15 females produced offspring	(Yang et al. 2009)

RT: Room temperature.

This number is not an actual value, it is relative to the control hatch which was 71 ± 5%; thus the actual rate was: 0.71*51 = 36%.

especially after thawing. The other extenders, HBSS and BSMIS, which do not contain milk, are more suitable choices for sperm cryopreservation of zebrafish.

Medaka sperm have a long swimming duration upon activation (Yang and Tiersch 2009). Therefore, the extender is not necessarily required to inhibit sperm motility. Sperm suspended in HBSS at osmolalities ranging from 92 to 373 mOsmol/kg remained continuously motile for 7 d (Yang and Tiersch 2009). Therefore, HBSS can be a candidate extender for medaka sperm across this range of osmolalities. In artificial insemination of medaka eggs, a balanced salt solution (BSS, see Appendix) with an osmolality of 230 mOsmol/kg has been used to retain fertility of the gametes (Iwamatsu 1983, Kinoshita et al. 2009). For sperm cryopreservation, different extenders have been used and have yielded comparable post-thaw motility and fertility, including BSS (Kinoshita et al. 2009), fetal bovine serum (Aoki et al. 1997), 0.6 M sucrose (Krone and Wittbrodt 1997), and HBSS with an osmolality of 350 mOsmol/kg (Yang et al. 2010). Systematic investigation is needed to test whether different osmolalities and composition of extenders can produce differences in sperm viability during sperm cryopreservation.

For *Xiphophorus* fishes, motility can be activated by isotonic solutions (~310 mOsmol/kg), rather than by hypotonic or hypertonic, and upon activation sperm can remain continuously motile for as long as one week (Huang et al. 2004a, Yang et al. 2006). Based on these characteristics, HBSS at 310 mOsmol/kg was used as the extender for sperm cryopreservation, and high motility was obtained after thawing for *X. helleri* (58 ± 7%) (Huang et al. 2004b) and *X. couchianus* (78 ± 3%) (Huang et al. 2004c). Alternatively, HBSS at an osmolality of 500 mOsmol/kg was used as extender for *X. helleri* because sperm immobilized at this osmolality could be re-activated by changing into isotonic osmolality (Yang et al. 2006). Sperm of *X. helleri* extended in HBSS at an osmolality of 500 mOsmol/kg were also cryopreserved, and high motility (~55%) was obtained after thawing (Yang et al. 2006). It was hypothesized that osmotic immobilization of sperm prior to freezing by use of hypertonic HBSS at 500 mOsmol/kg could conserve sperm energy capacity, and consequently could provide an advantage for internal fertilization compared to sperm cryopreserved using isotonic HBSS, especially if the sperm were to be stored prior to cryopreservation or insemination. Through artificial insemination, sperm cryopreserved at 310 and 500 mOsmol/kg each HBSS produced verified (hybrid) offspring (Yang et al. 2007b).

Because of the limited availability of sperm from aquarium fishes, dilution of samples with extender is useful to maximize the volume available for subsequent analysis. Therefore, dilution ratios of testis weight (mg) : HBSS volume (µl) of 1:50, 1:100, 1:200, 1:400, 1:800, and 1:1600 were evaluated with sperm of *X. helleri*. Motility of samples diluted at 1:50, 1:100 and 1:200 was found to be not significantly different before freezing or after thawing, but declined significantly at ratios of higher than 1:200 (Huang et al. 2004b). For zebrafish and medaka, no similar evaluation of dilution ratio has been reported, but ratios of testis weight (mg) : HBSS (µl) of 1: 50-80 were used for sperm cryopreservation, and high percent motility and fertility were obtained in thawed sperm (Yang et al. 2007b). Extreme dilution of samples has been found to reduce sperm motility in animals such as mammals (Harrison et al. 1978), rainbow trout *Oncorhynchus mykiss* (Billard 1983, Scott and Baynes 1980) and eastern oysters *Crassostrea virginica* (Paniagua-Chavez et al. 1998), and remains a topic for study in aquarium fishes.

Cryoprotectant Selection

In sperm cryopreservation, cryoprotectants are additives necessary for protection against freezing damage due to intracellular ice crystal formation and excessive dehydration. Usually

cryoprotectants are grouped into two categories: permeating cryoprotectants (e.g., dimethyl sulfoxide (DMSO), methanol, and glycerol) and non-permeating cryoprotectants (e.g., egg yolk, milk, and proteins). A variety of cryoprotectants have been evaluated for different species (Fuller 2004), and selections are usually determined experimentally. Theoretically, the higher the concentrations of cryoprotectant, the better the protection to sperm cells should be during cryopreservation. However, high concentrations of cryoprotectants can be toxic or lethal to sperm cells. The optimum concentration should be a value which balances these two effects. Usually, concentrations of 5 to 20% were chosen for experiments with sperm cryopreservation in aquarium fish.

For zebrafish, the toxicity of DMSO, N,N-dimethyl acetamide (DMA), methanol, and glycerol at concentrations of 5, 10, and 15% were evaluated with sperm cells. Glycerol was the most toxic, and was eliminated from further consideration. The other three chemicals were used for sperm cryopreservation, and analysis of post-thaw motility showed that methanol at a concentration of 8% was the best choice (Yang et al. 2007a). This was also the choice in two earlier studies (Draper et al. 2004, Harvey et al. 1982). In addition, DMA (10%) was used as a cryoprotectant for zebrafish sperm (Morris et al. 2003), but the fertilization level after thawing (9-14%) was lower than that observed (28-51%) when methanol was used as cryoprotectant (Draper et al. 2004, Harvey et al. 1982, Yang et al. 2007a).

For medaka, 10% DMSO and 10% dimethyl formamide (DMF) were used to cryopreserve sperm, and after thawing yielded good motility (78-100%) and hatching (82-100%) (Aoki et al. 1997, Krone and Wittbrodt 1997). A recent study systematically evaluated cryoprotectant and cooling rate for medaka sperm cryopreservation (Yang et al. 2010) and compared them with the two previous publications. The evaluation of acute toxicity of six cryoprotectants, methanol, 2-methoxyethanol (ME), DMSO, DMA, DMF, and glycerol showed that methanol and ME (5% and 10%) did not change the sperm motility after 30 min; DMSO, DMA, and DMF (10% and 15%) and glycerol (5%, 10% and 15%) significantly decreased the motility of sperm within 1 min after mixing. Based on these results, methanol and ME were selected as cryoprotectants (10%) to evaluate with different cooling rates (from 5 to 25 °C/min) and were compared in parallel to DMSO and DMF (10%) because of their use as cryoprotectants in previous publications (Aoki et al. 1997, Krone and Wittbrodt 1997). The highest post-thaw motility (50 ± 10%) was observed at a cooling rate of 10 °C/min with methanol as cryoprotectant. Comparable post-thaw motility (37 ± 12%) was obtained at a cooling rate of 15°C/min with ME as cryoprotectant. With DMF, post-thaw motility at all cooling rates was ≤ 10% which was significantly lower than that of methanol and ME. With DMSO, post-thaw motilities were ≤ 1% at all cooling rates, and significantly lower compared to the other three cryoprotectants. Fertility testing of thawed sperm cryopreserved with 10% methanol at a rate of 10 °C/min showed average hatching of 70 ± 30% which was comparable to that of fresh sperm (86 ± 15%).

For *Xiphophorus*, DMSO, DMF, DMA, glycerol, propylene glycol, methanol, and sucrose were evaluated as cryoprotectants, each with final concentrations of 6% and 10% (v/v). The results indicated that DMSO and glycerol were suitable cryoprotectants, and further evaluation of these two cryoprotectants at different concentrations showed that glycerol was better than DMSO in retaining motility and prolonging storage time for *X. helleri* sperm after thawing, and the effective concentration for glycerol was 14% (Huang et al. 2004b). Also, glycerol showed the best results for cryopreservation of *X. couchianus* sperm with a

concentration of 14% (Huang et al. 2004c), and for osmotically immobilized sperm from *X. helleri* (Yang et al. 2006, Yang et al. 2007b).

After mixing with sperm, cryoprotectants require time for equilibration to penetrate the cells. This is a dynamic process depending on the permeability of sperm cells, cryoprotectants, and their concentrations. For zebrafish, based on toxicity analysis, a 10- to 20-min equilibration time was chosen (Yang et al. 2007a). For medaka, based on the toxicity analysis an equilibration time of less than 30 min was used (Yang et al. 2010). For *Xiphophorus*, equilibration times of 10, 20, 30, 60, and 120 min were evaluated for sperm of *X. helleri* and *X. couchianus*. An equilibration time of less than 30 min yielded the highest post-thaw motility in each species, but there was no consistent difference across equilibration times ranging from 10 to 120 min (Huang et al. 2004b, Huang et al. 2004c).

Packaging of Samples for Freezing

In sperm cryopreservation, packaging of samples for freezing and storage is important to standardize the cooling rate, and to assure sample identification. Currently, several different kinds of containers have been used for aquarium fishes such as plastic cryovials, glass tubes and ampules, and plastic straws. The different materials and shapes of these containers result in different heat transfer properties during freezing and thawing. Even for the same style of container, differences can exist with products from different manufacturers, which can result in variation of cooling or thawing rates. Therefore, it is necessary to standardize the packaging method to ensure that protocols will be repeatable especially in different laboratories.

For the aquarium fishes addressed in this review, the small volumes of sperm available limit the choices for sample packaging. In zebrafish and medaka, glass capillary tubes or cryovials were first employed in sperm cryopreservation (Aoki et al. 1997, Draper et al. 2004, Harvey et al. 1982, Krone and Wittbrodt 1997, Morris et al. 2003). Recently, to standardize protocols with potential for automation at high throughput, French straws were chosen for sperm packaging with the smallest commercially available volume (0.25 ml), and a more standardized protocol was developed with results comparable to previous studies (Yang et al. 2007a, Yang et al. 2010). For *Xiphophorus* fishes, French straws (also 0.25 ml) were used for sample packaging in all studies (Huang et al. 2004a, Huang et al. 2004b, Huang et al. 2004c, Yang et al. 2006, Yang et al. 2007b, Yang et al. 2009). Compared to capillary tubes or cryovials, the use of French straws has the following advantages: potential for use with automated filling and sealing equipment, sample identification by permanent printing of alpha-numeric labels or barcodes, sample biosecurity by complete sealing, and by virtue of thin high surface area-to-volume ratio, standardization of the cooling and thawing processes.

Cooling Rate Selection

Cooling rate is a crucial factor in sperm cryopreservation because it affects the osmotic and pH balance of intracellular and extracellular solutions during freezing. Theoretically, with an excessively slow cooling rate, osmotic equilibrium is maintained, and much of the freezable water leaves the cell resulting in excessive dehydration; with an excessively fast cooling rate, little or no freezable water leaves the cell, and thus large intracellular crystals can form, causing damage to the cell. Ideally, a balanced situation allows survival when the cooling rate is fast enough to minimize the time of exposure to concentrated solutions and yet is slow enough to minimize the amount of intracellular ice formation. Optimum cooling rates vary with different cryoprotectants and the physiology of sperm cells from different species, and can be determined

empirically by experimentation, or predicted by theoretical calculation using techniques such as differential scanning calorimetry (DSC). This technique can be used to estimate water permeability (Lp) at subzero temperatures and the activation energy of that process, and these values can be used to compute the amount of water loss in cells as a function of cooling rate and temperature, and predict the optimum cooling rate from such plots (Devireddy et al. 1998). The cooling process for aquarium fishes has been accomplished by use of the following methods: placement on dry ice, suspension in liquid nitrogen vapor, and controlled cooling with a programmable freezer. Dry ice and liquid nitrogen vapor are inexpensive and can be used in field situations, but the cooling rates are difficult to quantify and control. In contrast, programmable freezers offer high levels of control and reproducibility, but are expensive and difficult to use in the field.

For zebrafish, the cooling methods that have been reported are placement on dry ice (Draper et al. 2004, Harvey et al. 1982, Morris et al. 2003) and a programmable freezer (Yang et al. 2007a). With 8% methanol as the cryoprotectant and HBSS as extender in 0.25-ml straws, the suitable cooling rate was identified as 10 ºC/min (Yang et al. 2007a), and with Ginsburg buffer plus powdered milk as extender in 2-ml cryotubes 16 ºC/min was selected (Harvey et al. 1982).

For medaka, cooling was provided by use of liquid nitrogen vapor (Aoki et al. 1997) or dry ice (Krone and Wittbrodt 1997) without quantification. In our study, cooling rate, controlled by a programmable freezer, was found to be a sensitive factor for determining post-thaw motility of medaka sperm. A change of as small as 5 ºC/min in the cooling rate resulted in a significant change in post-thaw motility, and a cooling rate of 10 ºC/min was identified when 10% methanol was used as cryoprotectant in 0.25-ml straws (Yang et al. 2010).

For *Xiphophorus*, cooling rate was controlled by use of a programmable freezer. The results showed that 20 to 30 ºC per min was optimum when sperm were cryopreserved with 14% glycerol in 0.25-ml straws (Huang et al. 2004b, Huang et al. 2004c). The optimal value of cooling rate predicted by DSC agreed with the empirical results in *X. helleri* (Huang et al. 2004b, Thirumala et al. 2005), but not in *X. maculatus* for which the cooling rate was predicted as 47 ºC/min (Pinisetty et al. 2005).

Storage of Frozen Samples

Holding of frozen samples in liquid nitrogen (-196 ºC) in a storage dewar is a standard method for cryogenic storage of samples from aquarium fishes. During storage, the important considerations are sample identification, potential contamination, and inventory of frozen samples. The use of plastic or French straws for packaging, especially newer forms with high safety and durability, offer the advantages of permanent labeling by printer, and complete sealing which minimizes or prevents transfer of materials (e.g., sperm cells or bacteria) among samples stored in the same dewar (Morris 2005).

Thawing of Frozen Samples

Theoretically, the process of thawing is the reverse of freezing, and thus the damage that can occur during cooling can also occur during warming, primarily through formation of intracellular ice crystallization between -40 °C and 0 °C (Leung and Jamieson 1991). Thus, it is usually desirable to thaw cryopreserved samples rapidly to minimize the period of crystal propagation (termed “recrystallization”). Currently, systematic evaluation of thawing rates has not been reported for sperm cryopreservation of zebrafish, medaka, or *Xiphophorus*, probably because of the limited sample volumes. Generally, for frozen samples packaged in 0.25-ml

French straws, a 5-sec exposure within a 40 ºC water bath is practical and yields suitable motility and fertility after thawing in zebrafish and *Xiphophorus* fishes (Huang et al. 2004a, Huang et al. 2004b, Huang et al. 2004c, Yang et al. 2007a, Yang et al. 2006, Yang et al. 2007b, Yang et al. 2009, Yang et al. 2010). For frozen samples packaged in capillary tubes or cryovials in zebrafish and medaka, the thawing process has been performed by leaving samples at room temperature (Harvey et al. 1982), diluting with room temperature buffer (Aoki et al. 1997, Morris et al. 2003), holding within a 33 ºC water bath (Draper et al. 2004), or holding within the fingers (Krone and Wittbrodt 1997).

Viability Analysis of Cryopreserved Sperm

The purpose of cryopreservation is to obtain viable sperm which retain their fertility. Examination of the viability of cryopreserved sperm can include evaluation of morphology, membrane integrity, motility, ability to bind oocytes, and fertilization. Motility is the most widely used assay, but fertilization is considered to be the most informative.

Artificial insemination is necessary to test the fertility of cryopreserved sperm. This process includes a series of steps: egg collection, holding of eggs prior to fertilization, thawing of cryopreserved sperm, mixing of the sperm and eggs, activation of the gametes, fertilization confirmation, hatching of fertilized eggs, and offspring harvest and identification. For species with internal fertilization such as the *Xiphophorus* fishes, this process involves more complicated techniques such as the injection of sperm (2-4 µl) into the female reproductive tract and pregnancy confirmation. Factors related to females such as egg quality can also determine fertilization success. Therefore, development of standardized protocols for fertilization assays including collection and holding of eggs and sperm need be included in protocol development for sperm cryopreservation.

For zebrafish and medaka, artificial fertilization protocols have been established with fresh sperm, and can be directly modified to provide fertilization analysis of cryopreserved sperm (Westerfield 2005, Yamamoto 1975b). Eggs can be collected daily by squeezing of females or dissection (for medaka), held in isotonic buffer to retain fertility, and be mixed with a sperm suspension for fertilization. For zebrafish, after mixing of sperm and eggs, fresh water needs to be added to activate gametes for fertilization, but for medaka, this is not necessary because fertilization can occur in isotonic buffer as stated above. Fertilization and hatching are determined by assessing the percentage of developing embryos or hatched fry.

For *Xiphophorus* fishes, artificial insemination with cryopreserved sperm must consider points such as the use of virgin females (because female *Xiphophorus* can store sperm for successive broods), proper injection volume and technique, pregnancy monitoring for as long as 90 d, and confirmation of paternal contribution to offspring. Previous work has shown that centrifugation did not reduce post-thaw motility (Dong et al. 2006), and thus removal of the cryoprotectant from thawed sperm by washing is feasible although it may not be necessary (Yang et al. 2007b). Also, social interactions among females are another factor to be considered because they can influence maturation and brood timing (Earley 2006). Thus, based on these considerations, hybrid offspring produced through artificial insemination with cryopreserved sperm from *X. helleri*, were used for verification of paternity in the first report for viviparous fishes (Yang et al. 2007b). Following the same protocols, live young have been produced with cryopreserved sperm from *X. couchianus* (Yang et al. 2009).

Future Research Topics

With the expanding use of aquarium fishes as research models, new mutants, transgenic individuals, strains, and lines are being continually created. To protect and maintain this burgeoning number of valuable individuals, strains and lines, it is essential to develop a germplasm repository program with gamete and embryo cryopreservation. Sperm cryopreservation will be the main focus based on current technologies and approaches until the development of protocols for egg and embryo cryopreservation in fish. At present, protocols for sperm cryopreservation are available for use with zebrafish, medaka, and *Xiphophorus* fishes, and live offspring have been produced with cryopreserved sperm. Future research needs include the following topics.

Standardization of the Procedures for Sperm Cryopreservation

To enable application of protocols developed for sperm cryopreservation across different laboratories, procedural standardization is necessary, especially for determination and choice of sperm concentration, sample packaging, and labeling. Standardization is also necessary for terminology (e.g., strict definition of terms such as fertilization and motility) and in defining the essential parameters necessary for complete reporting of results. Sperm concentration is an extremely important factor in sample preparation for cryopreservation, and can directly influence results (Dong et al. 2007). Because most studies of aquatic species cryopreservation do not control or report sperm concentrations, it is likely that this is the single largest uncontrolled variable in this research area (Tiersch et al. 2007). Also, due to the limited sperm availability in aquarium fishes, maximized use of cryopreserved samples for strain or line recovery requires control of sperm concentration and determination of suitable sample loading in each container (e.g., French straw). Hemocytometer counts and spectrophotometry are commonly used methods to measure sperm concentration. For aquarium fishes, specialized spectrophotometers with a 2-µl sample size are desirable to avoid sample waste (Tan et al. 2010).

Evaluation of Gamete Quality

Considerable variation in characteristics of post-thaw sperm is generally the rule in most species studied (Mazur et al. 2008), and has been found in zebrafish (Yang et al. 2007a), and *X. couchianus* (Yang et al. 2009). The reasons for this variability, for example a range in post-thaw sperm fertility of from 5 to 81% in samples with the same initial motility in zebrafish (Yang et al. 2007a), have not been identified. A more comprehensive understanding of sperm quality needs to be obtained for assessing within-species variation in the susceptibility of spermatozoa to damage during cryopreservation, including factors such as membrane integrity and mitochondria function.

A genetic basis for variation in post-thaw semen viability has been suggested (Thurston et al. 2002), and it has been proposed that certain molecular markers could be identified to link with the genes influencing this variation. Currently, estimation of sperm quality before cryopreservation provides an opportunity to predict the outcome of sperm cryopreservation, but it will be necessary to develop a functional and molecular understanding of the factors that influence sperm cryopreservation. Sperm quality estimation could include: analysis of the relationship of sperm performance with body condition and nutrition, the use of flow cytometry for measuring membrane integrity and mitochondrial integrity, the use of computer-assisted

sperm analysis (Cosson 2004) for quantifying motility characteristics, and detection of changes in protein profiles in sperm after cryopreservation for predictive biomarkers.

Establishment of a Working Repository System for Cryopreserved Sperm

After cryopreservation, frozen samples stored in liquid nitrogen require permanent and clear identification. The use of plastic straws for packaging of sperm samples offers the advantage of permanent labeling by direct printing and the use of automated barcode readers. A labeling and coding system for each cryopreserved sample needs to be developed, including information on biology, genetics, intellectual property rights, source and ownership, sample collection, and handling. Integrated databases need to be developed with the existing genetic databases for these fishes, inventory procedures need be established to allow easy access to cryopreserved samples from a specific species and strain, and biosecurity procedures need be established to minimize or prevent transfer of pathogenic contaminants with cryopreserved samples (Tiersch and Jenkins 2003).

Acknowledgments

We thank R. Walter of the *Xiphophorus* Genetic Stock Center, Z. Varga and C. Carmichael of the Zebrafish International Resource Center, and R. Winn and M. Norris of the Aquatic Biotechnology and Environmental Laboratory of the University of Georgia for providing fish, technical help, and valuable discussion. This review was supported by funding from R24RR024790 and R24RR023998 from the NIH National Center for Research Resources, the U.S. Department of Agriculture, and the National and Louisiana Sea Grant College Programs. This manuscript has been approved for publication by the Director of the Louisiana Agricultural Experiment Station as number 2010-244-5260.

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Appendix

1. BSMIS (Morris et al. 2003):
   75 mM NaCl, 70 mM KCl, 2mM $CaCl_2$, 1mM $MgSO_4$ and 20 mM Tris, pH 8.0, store at 4 ºC.
2. BSS (balanced salt solution) (Iwamatsu 1983):
   Solution A: 0.111 M NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.81 mM $MgSO_4$-$7H_2O$
   Solution B: 0.6 M $NaHCO_3$. Working solution: adjust solution A with solution B to bring the pH to 7.4
3. Ginsberg buffer (Ginsburg 1963):
   0.111 M NaCl, 3.4 mM KCl, 2.7 mM $CaCl_2$-$2H_2O$, 2.4 mM $NaHCO_3$. Note: the order of addition is important to prevent precipitation. Freezing medium (see: www.zfin.org): 9 mL Ginsburg buffer, 1 mL methanol, and 1.5 g powdered skim milk. The order of ingredients is important to prevent precipitation of the milk, and this medium needs to be used within 3 hr.
4. HBSS (Hanks' balanced salt solution) (Hanks 1975, Tiersch et al. 1994):
   0.137 M NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, and 5.55 mM glucose, pH = 7.8.