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A general approach for vitrification of fish sperm

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A GENERAL APPROACH FOR VITRIFICATION OF FISH SPERM

A Dissertation

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In memory of
Herbert Stein and Raymundo Ramirez Delgadillo
who taught me science and motivated me to pursue a Ph.D.

"My duty is to communicate to those who are to become professional thinkers,
the future Doctors of Philosophy, the pure love of Truth."
Father Basile J. Luyet

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There are many people I wish to thank. “Thanks” is a simple word but it can possess a transcendental meaning. This Ph.D. came not just from my effort, but from the support of many others. It took me 5.5 years to finish. This required a lot of patience from my major professor Dr. Tiersch, who was a guide and mentor in my professional and personal life. He always is going to be a good friend and a good example to follow. Most of my learning in cryobiology came by the teaching of Dr. S. P. Leibo who I regard as the “living encyclopedia of cryobiology”. I thank Dr. Leibo for sharing his knowledge, and thank the other members of my Advisory Committee, Dr. Romaire and Dr. Green for their time and for reading this long dissertation. Thanks also to Dr. Walter for serving on my Committee from Texas State University. Also thanks to Dr. Sibel Bargu for accepting at the last minute to serve as Dean’s Representative for my Committee.

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The field of cryobiology, as seen in other scientific fields, started with contributions from Priest-Scientists or men of God and missionaries to science. Father Lazzaro Spallanzani (l'Abate Spallanzani) was the first to freeze and thaw sperm in 1776. In fact, the 'Father of Cryobiology' is Father Basile J. Luyet, from the Order of Missionaries of St. Francis de Sales. St. Francis once said that science, knowledge and wisdom was an eighth sacrament, a way by which divine life was to be dispensed to humankind. During my Ph.D. journey, I realized that the common denominator of these great scientists was a synthesis of their spiritual and professional lives. I have discovered the meaning of "Religioso y estudiante, Religioso por delante" (religious and student, but primarily religious) (St. John of the Cross), and of "*Ora et labora*" (St. Benedict), and working with fish has been "*Piscis assus Christus est Passus*" (St. Augustine). "The solution of the problem of life is life itself. Life is not attained by reason and analysis but first of all by living" (Thomas Merton). When Father Luyet was asked how he reconciled being a scientist and priest, he responded "Truth does not contradict truth." With this I want to give thanks to God for letting me learn to surrender and accept His Will.

Thanks to my family for their love and support. Thanks to my parents who taught me values and principles which are part of my morality. Thanks to my brothers Jorge and Antonio, and my sisters Aurora, Marines, and Fatima for companionship and unconditional help during this journey. Thanks to all of my nieces and nephews, who although too young to understand what a Ph.D. is, have taught me that it is important to enjoy the simple things in life. Thanks to my uncles Antonio, Javier, and Rafael for their support. Thanks to my friends Roberto Rodriguez (who came to my dissertation defense), Ivan, and Molina. Finally, thanks to my sweetheart Elibe Noriega (milagrito de amor), for all her love and support during the writing of this dissertation.

These words were dedicated to me by my father on the day of my dissertation defense:

"Effort and perseverance take us to success.
An objective without efforts is never reached.
Efforts without an objective, it's a waste.

Rafo:
Today, you achieve your Ph.D. objective,
which has demanded your last five years,
in addition to your master's and your bachelor degree.

As the mountain climber,
you reached the top.
For this reason you are very happy.
Congratulations, a lot of congratulations.

But as any activity in life,
when you reach an objective,
immediately you think of another
bigger than your last.
This is your case,
it's your post-doctorate, post-Ph.D.

You will continue the way walking
and climbing the mountains,
which are in front of you:
your personal life and
your professional life.

Remember
that work and studies are part of our lives,
they are not everything.

Finally remember,
give thanks to God,
Who gives you these moments.

With God,
all our objectives can be achieved;
without Him,
we are nothing and
we cannot do anything.

Rafo: Congratulations."
Jorge Luis Cuevas Miguel, August 5, 2011

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List of Abbreviations

Ace	acetamide
AFP	antifreeze proteins
ANOVA	analysis of variance
BD	2,3-butanediol
cm	centimeter
ºC	degree celsius
CPA	cryoprotectant agent
DMSO	dimethyl sulfoxide
DMA	dimethyl acetamide
DNA	deoxyribonucleic acid
EG	ethylene glycol
FL1	fluorescence detector 1
g	gram
Gly	glycerol
h	hour
HBSS	Hanks' balanced salt solution
ICSI	Intracytoplasmic sperm injection
IIF	intracellular ice formation
kg	kilogram
L	liter
m	meter
MB	millions of DNA base pairs
MeOH	methanol
µL	microliter
mg	milligram
MG	methyl glycol
mL	milliliter
mm	millimeter
min	minute
mol	mole
M	molar concentration
mol/L	molar concentration
mOsmol	milliosmoles
MP	1-methoxy-2-propanol
MPD	2-methyl-2,4-pentanediol
MS-222	tricaine methanesulfonate
MW	molecular weight
n	number of observations
ND	not described
nm	nanometers
PEG	polyethylene glycol
PGC	primordial germ cells
PI	propidium iodide
ppm	parts per million
ppt	parts per thousand
PROH	1,2-propanediol
PVA	polyvinyl alcohol
P	calculated probability
r	regression coefficient
SAS	statistical analysis system
sec	second
SD	standard deviation
SOP	standard operating procedures
Tre	trehalose
v	volume
w	weight
X	X-1000™ (proprietary ice blocker)
Z	Z-1000™ (proprietary ice blocker)

Abstract

The goal of this project was to develop streamlined protocols that could be integrated into a standardized approach for vitrification of germplasm for all aquatic species. Vitrification (freezing by formation of "glass" rather than crystalline ice) is simple, fast, inexpensive, can be potentially used to preserve samples in the field, and offers new options for germplasm management especially appropriate for small fishes. Sperm were studied from freshwater fish (channel catfish *Ictalurus punctatus*), viviparous freshwater fish (green swordtail *Xiphophorus hellerii*), and marine fishes (spotted seatrout *Cynoscion nebulosus*, red snapper *Lutjanus campechanus*, red drum *Sciaenops ocellatus*, and southern flounder *Paralichthys lethostigma*). To reduce toxicity, combinations of cryoprotectants at reduced concentrations with incorporation of trehalose and polymers were used to enhance glass formation. For freezing, samples were suspended on 10-µL polystyrene loops and plunged into liquid nitrogen. Thawing was done at 24ºC using Hanks' balanced salt solution at 300 mOsmol/kg for freshwater species, and seawater at 1,020 mOsmol/kg for marine species. Quality after vitrification was evaluated by sperm motility, membrane integrity and when possible fertility. Post-thaw motility of sperm in marine fishes was higher (as high as 70%) than in freshwater fishes (as high as 20%). The percentage of membrane-intact sperm for marine fishes was ~20% except for southern flounder (11%). For freshwater fishes, the percentage of membrane-intact sperm for swordtail was low (<12%) compared to channel catfish (~50%). Adaptations by marine fish to high osmotic pressures could explain the survival in the high cryoprotectant concentrations (40 – 60%) required for vitrification. This research yielded the first successful vitrification of sperm in these fishes and production of offspring from vitrified sperm in channel catfish, green swordtail, and southern flounder. Sperm vitrification offers an alternative approach to conventional cryopreservation for conservation of valuable genetic lineages, such as endangered species, model strains used in research, and improved farmed strains. Furthermore, sperm vitrification could be used to transport cryopreserved sperm from the field to the laboratory to expand genetic resources available for germplasm repositories. This technique could be utilized to reconstitute genetic lines, and as a new option for conservation biology in imperiled aquatic species.

Chapter 1
Foreword

This dissertation offers a general approach rather than a specific method for vitrification of fish sperm. By not focusing on a method, the emphasis was placed on the necessary thinking involved in bringing a new technology, such as vitrification, to a group as broad as fishes. Of the approximately 55,000 species of living vertebrates, more than half are fishes. Despite this, fishes are far less understood than terrestrial vertebrates, and there are few general characterizations that can describe all fishes (Barton 2007). It is not useful to state that fishes do not possess a specific characteristic, because among more than 32,000 species, there are always exceptions. For example, with respect to reproduction, the heterogeneity of fishes include species that can change sex within days, species in which all individuals are females and males are superfluous, species which produce millions of eggs (for example the ocean sunfish *Mola mola* with 28 million eggs per season) or females laying a single egg (longnose skate *Raja rhina*), while other species employ internal fertilization or even viviparity (Paxton and Eschmeyer 2003). Moreover, the process of initiation and activation of sperm motility varies depending on environmental and life history factors. For example, when viewed based on the relation between sperm activation and osmolality, there are three main groupings recognized for fishes (Figure 1.1) (Tiersch et al. 2007). The first grouping is freshwater fishes with external fertilization. Unless certain ions are involved (such as potassium in the case of trout and salmon), sperm of freshwater species generally become active by a lowering of osmotic pressure (hypotonic to blood plasma). The second grouping is marine fishes with external fertilization. Sperm of marine fishes can be activated by an increase of osmotic pressure (hypertonic). The third group contains species with internal fertilization, such as live-bearing fishes, where sperm motility activation is triggered in solutions isotonic to body fluids (Tiersch 2011).

Being the oldest of vertebrates with more than 500 million years of evolution, adaptation has led to the present spectacular diversity of structure and function (Helfman et al. 2009). As such, a general approach is presented in this dissertation because optimum conditions for conventional cryopreservation of fish sperm are highly variable for each species and within individuals from the same species (Scott and Baynes 1980), and vitrification is a completely new

topic for fish sperm. In addition, many factors interact and are difficult or impossible to analyze separately (Leung and Jamieson 1991). Therefore, this dissertation does not try to provide a recipe, or a specific method, on how to perform sperm vitrification. Instead the variables that play a role in vitrification are highlighted and emphasis is placed on the process of adapting and harmonizing these variables in response to a range of specific circumstances. Accordingly, the three groupings of fishes described above (freshwater, marine, and live-bearing) were selected for this project to address a range of sperm activation mechanisms in relation to vitrification.

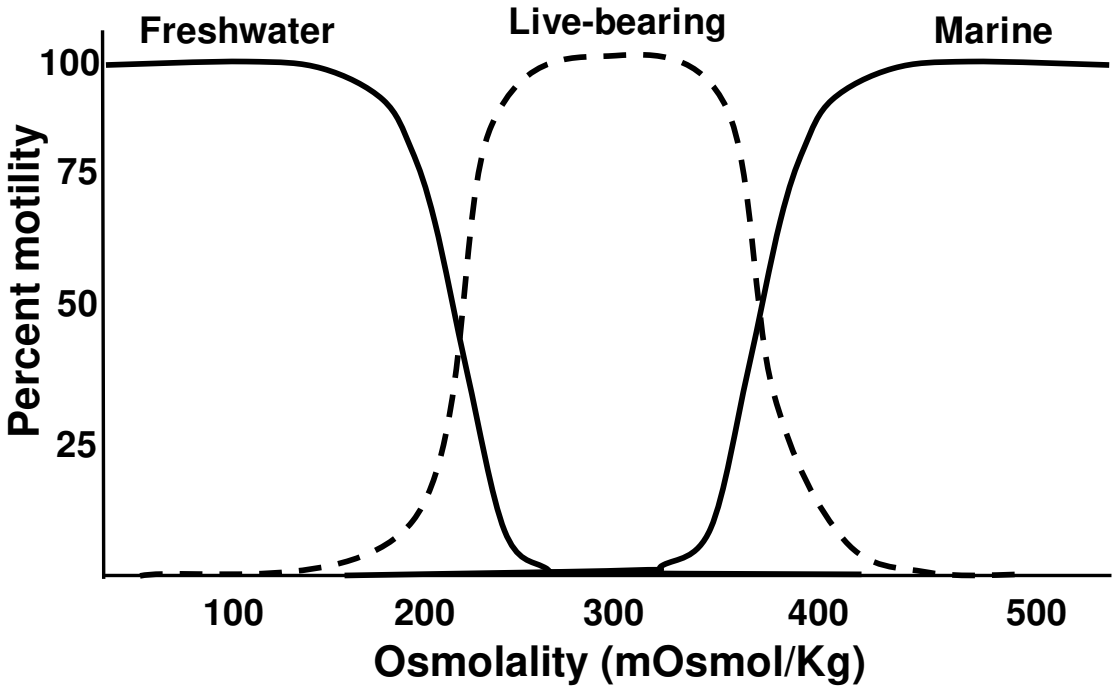

Figure 1.1. The effect of osmolality on sperm activation and motility for three broad groupings of fishes (adapted from Tiersch et al. 2007).

Because the sample volumes used for vitrification are small (<20 µL), this technique is potentially best suited for use in three areas: biomedical research fish models, genetically improved lines, and endangered species. The overall goal of this project was to develop streamlined protocols that could be integrated into a standardized approach for vitrification of germplasm of aquatic species. The specific objectives were to: 1) identify suitable vitrification solutions by measuring acute toxicity of cryoprotectants at various concentrations; 2) determine

appropriate cooling rates by evaluating apparatus configurations and volumes of samples; 3) determine effective warming methods; 4) evaluate the quality of thawed sperm; 5) evaluate the ability of sperm to fertilize eggs, and 6) develop approaches to integrate vitrification protocols into existing working repository systems for cryopreserved sperm.

Biomedical Research Fish Models

Because many fish species have characteristics such as high fecundity, short generation time, transparent embryos, and easy breeding, they are frequently used as experimental animals or vertebrate model systems. Information from fish model systems has been important to biological research in a broad array of studies such as developmental biology, behavioral ecology, and toxicological research. For example, several transgenic lines of medaka (*Oryzias latipes*) were developed for use as aquatic biomonitors for detection of hazardous substances in water (Winn 2001). In addition, fishes have been used as models for the study of human diseases based on developmental processes, physiological mechanisms, and organ system similarities with mammals. Thousands of fish models have been created to study human diseases. These models include studies of cancer (Patton et al. 2010), apoptosis (Pyati et al. 2007), immune function (Langenau and Zon 2005), and blood or heart disorders (Lam and Gong 2010), to mention just a few. Preservation of genetic resources from aquatic model organisms is vital to the advancement of biomedical research.

Unfortunately, small sperm volumes for fishes such as zebrafish (<5 µL) (Jing et al. 2009) and *Xiphophorus* (<9 µL) (Huang et al. 2004) are characteristic of the majority of research fish models. This limited volume hinders experimental replication and the number of treatments possible without pooling of samples (Tiersch 2001). Because vitrification is most suited for use with microliter volumes of sperm, it fits well with the need to conserve the germplasm from these aquarium fish models. Chapter 4 addresses the potential use of vitrification in live-bearing fishes through study of the green swordtail (*Xiphophorus hellerii*), which is a valuable research model actively used in fields of study including cancer biology, evolution, behavior, physiology, comparative biochemistry, comparative genomics, sex determination, development, endocrinology, behavioral ecology, toxicology, parasitology and immunology (Walter 2011).

Genetically Improved Lines

Techniques such as selective breeding, chromosome set manipulation, sex manipulation, and gene transfer have been used in aquaculture to produce improved lines for various fish species. However, reproductive performance of fishes subjected to some of these techniques has been negatively affected. For example, broodstock males from a selection program for fast growth and late maturation of Atlantic salmon (*Salmo salar*) produced reduced volumes of sperm (<0.1 mL) (Zohar 1996) compared with wild fish (>10 mL) (Kazakov 1981); sex-reversed males from dusky grouper (*Epinephelus marginatus*) also produced reduced volumes of sperm (<400 µL) (Cabrita et al. 2009) as was found for tetraploid loach (*Misgurnus anguillicaudatus*) (Yasui et al. 2010). Cryopreservation can assist in preserving the germplasm of improved lines, including those produced by gene transfer; however, small volumes collected from these lines present a challenge for use by conventional cryopreservation techniques. These genetically improved lines represent enormous investments in time and money, and there is always a risk of accidental loss (Cabrita et al. 2010). Chapter 6 evaluates vitrification as a means to protect and maintain a genetically improved line derived from southern flounder (*Paralichthys lethostigma*).

Endangered Fish Species

Of the total number of fish species described, 43% inhabit freshwater. Among these species, roughly 40% in North America and Europe are considered to be imperiled (i.e., endangered, threatened, or vulnerable) (Jelks et al. 2008; Kottelat and Freyhof 2007). The main threats are habitat degradation and introduction of non-indigenous species. Overall declines are also occurring in the oceans. Since the 1970s, there has been an 80% decline in coral cover in the Caribbean (Gardner et al. 2003). An estimated 19% of the world's coral reefs have been lost and a further 35% are seriously threatened. As a result, one-third of all reef-building corals are considered to be at risk of extinction (Veron et al. 2009). Coral reefs are among the most diverse ecosystems on Earth. About 35% of known fish fauna are associated with coral reefs (Barton 2007), but reef fish density has been declining significantly for more than a decade, at rates ranging from 3% to 6% per year (Paddack et al. 2009). It has been suggested that coral reefs and many of their associated flora and fauna will cease to exist within the next 40 years, causing the

first global extinction of a worldwide ecosystem within history (Veron et al. 2009). In addition, the biomass of large predatory fish has been reduced by 90% during the last 50 years (Myers and Worm 2003), and global marine fisheries are in crisis with as much as 63% of fish stocks worldwide requiring rebuilding (Worm et al. 2009). The global collapse of all taxa currently fished has been speculated to occur by 2048 (Worm et al. 2006). Overfishing, pollution, global climate change, ocean acidification, and other ecological impacts have degraded marine ecosystems (Jelks et al. 2008). Furthermore, according to the Red List of the International Union for Conservation of Nature and Natural Resources (IUCN), 65% (940/1452 species) of the ray-finned fishes (Class Actinopterygii) that are listed as imperiled are less than 20 cm in body length. These small fish are typically overlooked in conservation programs. Given this dismal reality, current conservation efforts can no longer be delayed while awaiting more thorough assessments. Sperm vitrification offers a new option for conservation biology of imperiled aquatic species because no equipment is required, and it can be performed in the field. Chapter 3 evaluates the use of vitrification in freshwater fishes using the channel catfish (*Icatlurus punctatus*) as working model. Chapter 5 further evaluates the use of vitrification in marine fishes.

This research was supported in part by funding from United States Department of Agriculture, the National Institutes of Health National Center for Research Resources, the Louisiana Sea Grant College Program, and the ACRES-LSU Collaborative Research Program. The results of this project represented collaborative efforts among several universities and institutions including the Louisiana State University Agricultural Center Aquaculture Research Station (ARS) in Baton Rouge, Louisiana; the *Xiphophorus* Genetic Stock Center (XGSC) at Texas State University, San Marcos, Texas; the Louisiana University Marine Consortium (LUMCON), Chauvin, Louisiana, and North Carolina State University (NCSU), Raleigh, North Carolina. Optimization of vitrification protocols were developed at the ARS and LUMCON, and fertilization trials were performed at LUMCON, XGSC and NCSU.

During this project, the results of this project have been presented at several scientific meetings (Table 1.1). In addition, three papers related to this project, including Chapter 2 (Cuevas-Uribe and Tiersch 2011a), Chapter 3 (Cuevas-Uribe et al. 2011a), and Chapter 4 (Cuevas-Uribe et al. 2011b) have been published or submitted for publication in peer-reviewed

outlets (Table 1.2). At the time of this writing, Chapters 5 and 6 are intended to be submitted for publication in peer-reviewed journals (Table 1.2). This dissertation also contains Standard Operating Procedures (Appendix A), and the original data reported in the research chapters (Appendix C). Finally, Appendix B which addresses basic issues in sperm quality assessment was published as a book chapter (Cuevas-Uribe and Tiersch 2011b). For internal consistency, all chapters of this dissertation have been prepared in the format of the *North American Journal of Aquaculture* with specific modifications required to meet LSU dissertation format and style.

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Table 1.1. Conference presentations and published abstracts based on the research presented in this dissertation.

Date	Title	Conference	Location
2011	Vitrification in aquatic species: a form of glass, half empty or full?	Aquaculture America[1]	New Orleans, Louisiana
2010	Initial studies in sperm vitrification of marine fishes[2]	Aquaculture 2010[3]	San Diego, California
2010	Sperm vitrification of marine fishes: effect on motility, membrane integrity, and fertilization ability[4]	Louisiana Chapter of the American Fisheries Society	Baton Rouge, Louisiana
2010	Glass from the ocean: sperm from marine fishes are more suited for vitrification	Gulf Coast Conservation Biology Symposium	New Orleans, Louisiana
2009	Sperm vitrification of a live-bearing fish, the green swordtail *Xiphophorus helleri*[2]	Aquaculture America[1]	Seattle, Washington
2009	Sperm vitrification in the live-bearing fish *Xiphophorus helleri*	Annual meeting of Southern Division of the American Fisheries Society	New Orleans, Louisiana
2008	Sperm vitrification of imperiled live-bearing fishes from Central America	Gulf Coast Conservation Biology Symposium	New Orleans, Louisiana
2008	Production of catfish with sperm frozen by ultra-rapid cooling	Aquaculture America[1]	Lake Buena Vista, Florida
2008	Cryopreservation of catfish sperm by rapid non-equilibrium cooling[5]	Louisiana Chapter of the American Fisheries Society	Baton Rouge, Louisiana
2007	Sperm vitrification in aquatic species	Gulf Coast Conservation Biology Symposium	New Orleans, Louisiana
2007	Spectrophotometric estimation of sperm concentration of fish	Aquaculture 2007[3]	San Antonio, Texas
2007	Potential general method to measure sperm concentration of fishes	Louisiana Chapter of the American Fisheries Society	Thibodaux, Louisiana

[1] Annual meeting of U.S. Aquaculture Society.
[2] Award received for Best Abstract from the United States Chapter of the World Aquaculture Society, 2009 and 2010.
[3] Triennial meeting of World Aquaculture Society.
[4] Award received for Best Abstract (2nd place) from the Louisiana Chapter of the American Fisheries Society.
[5] Award received for Best Abstract (3rd place) from the Louisiana Chapter of the American Fisheries Society.

Table 1.2. Published papers and manuscripts in preparation based on the research presented in this dissertation.

Title	Journal / Book	Status	Chapter
Non-equilibrium vitrification: an introduction and review of studies done in fish	*Cryopreservation in Aquatic Species*[1]	Published	2
Production of channel catfish with sperm cryopreserved by rapid non-equilibrium cooling	*Cryobiology*	In press	3
Production of F_1 offspring with vitrified sperm from a live-bearing fish, the green swordtail *Xiphophorus hellerii*	*Zebrafish*	In press	4
Vitrification of sperm from marine fishes: effect on motility and membrane integrity	*Aquaculture*	In preparation	5
Vitrification as alternative approach for sperm cryopreservation in marine fish	*North America Journal of Aquaculture*	In preparation	6
Estimation of fish sperm concentration by use of spectrophotometry	*Cryopreservation in Aquatic Species*[1]	Published	--
Creation of a genome repository for Caribbean and pacific coral[2]	*Biology of Reproduction*	In preparation	--
Development of protocol for sperm cryopreservation of Eastern oyster *Crassostrea virginica* with high-throughput processing[2]	*Aquaculture*	In preparation	--
High-throughput cryopreservation of aquatic spermatozoa: protocol standardization for marine species southern flounder *Paralichthys lethostigma*[2]	*Journal of the World Aquaculture Society*	In preparation	--
Sperm cryopreservation in live-bearing *Xiphophorus* fishes: offspring production from *Xiphophorus variatus* and considerations for establishment of sperm repository[1]	*Zebrafish*	In preparation	--

[1] Tiersch, T.R. and C.C. Green, editors. 2011. *Cryopreservation in Aquatic Species, 2nd Edition*. World Aquaculture Society, Baton Rouge, Louisiana.
[2] Co-author; non-dissertation related.

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Chapter 2*
Introduction

Cryopreservation represents a tool for the protection of genetic resources in aquatic species, and offers many benefits in aquaculture, conservation biology, and medical applications. Benefits include the year-round availability of gametes, reduction of the need for maintaining fish as live populations, protection of valuable genetic lineages, and improvement of genetic lines (Tiersch et al. 2007). Despite these benefits, it has been estimated that cryopreservation has only been researched for sperm of roughly 200 (Tiersch 2011) of the 28,800 fish species described (Barton 2007) with application only beginning, and research on eggs and embryos lagging behind this. As such, currently fewer than 1% of species could be preserved in "genome resource banks" or "frozen zoos", with the vast majority remaining unstudied.

Cell Dehydration in Cryopreservation

To state it simply, the goal of cell preservation is to remove as much water as possible from inside the cell without disrupting its integrity (minimum critical volume) (Meryman 1974). The volume of water in almost all animal cells is 70-80%, except for erythrocytes (~50%), and spermatozoa (~50%) (Newton et al. 1999, Petrunkina 2007). There are several methods to dehydrate cells such as freeze drying (lyophilization) (Kusakabe et al. 2008), evaporative (convective) drying (Biggers 2009), vacuum drying (Meyers et al. 2009), exposure to hypertonic conditions for storage at room temperature (Van Thuan et al. 2005), or cryopreservation (Tiersch et al. 2007). Dehydration by exposure to hypertonic conditions during cryopreservation can be attained by cell exposure to cryoprotectants and extracellular ice crystals (Figure 2.1).

Exposure of cells to excessively hypertonic conditions can cause damage by osmotic stress and the solution effects (exposure to the effects of high solute concentrations, including low pH), and this type of injury is related to cell type, temperature, concentration of the suspending solution, and exposure time. The two factors that govern dehydration in cryopreservation are cryoprotectant concentration and cooling rate. When cooling rate is 'too

*The contents of this chapter were published prior to the completion of this dissertation (Tiersch, T.R. and C.C. Green, editors. 2011. *Cryopreservation in Aquatic Species, 2nd edition*. World Aquaculture Society).

slow', ice crystals will grow in the extracellular space, and the cells will be exposed for a longer period of time to a high hypertonic cold environment. Severe dehydration that leads to cellular disruption is a type of osmotic damage known as the "solution effect." When cooling rate is 'too fast', the cell will not have enough time to dehydrate because the ice crystals will grow quickly and the cell will become supercooled which will lead to the formation of intracellular ice (Figure 2.2).

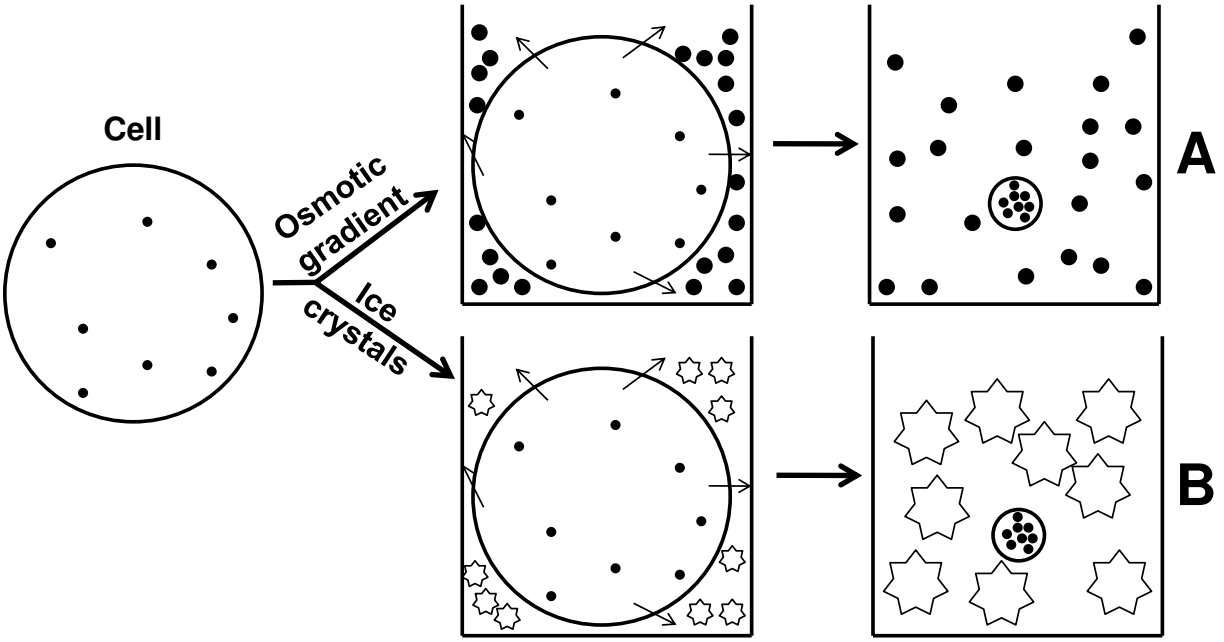

Figure 2.1. Because cells contain 70 to 80% water by volume, they can dehydrate by osmosis (arrows) until reaching a "minimum volume" known as the "osmotically inactive" fraction of cell volume. Hypertonic dehydration of the cell can be produced by osmosis in response to: (A) osmotic gradient created by non-permeating chemicals (●) and (B) extracellular reduction in free water caused by formation of ice crystals (✡). These mechanisms each act to increase osmolality of the extracellular space, which results in cellular dehydration, and thus reduces the chance of intracellular ice crystal formation. Water permeability depends on the cell type (size, membrane composition, shape, developmental stage), exposure temperature (permeability decreases at low temperatures), molecular size and charge (permeable or not), and concentration of molecules (diffusion gradients or differences in osmotic pressure). Note that there is not an increase in the amount of intracellular solutes, rather the cell reaches a minimum volume where the solutes are concentrated and thus the osmolality increases.

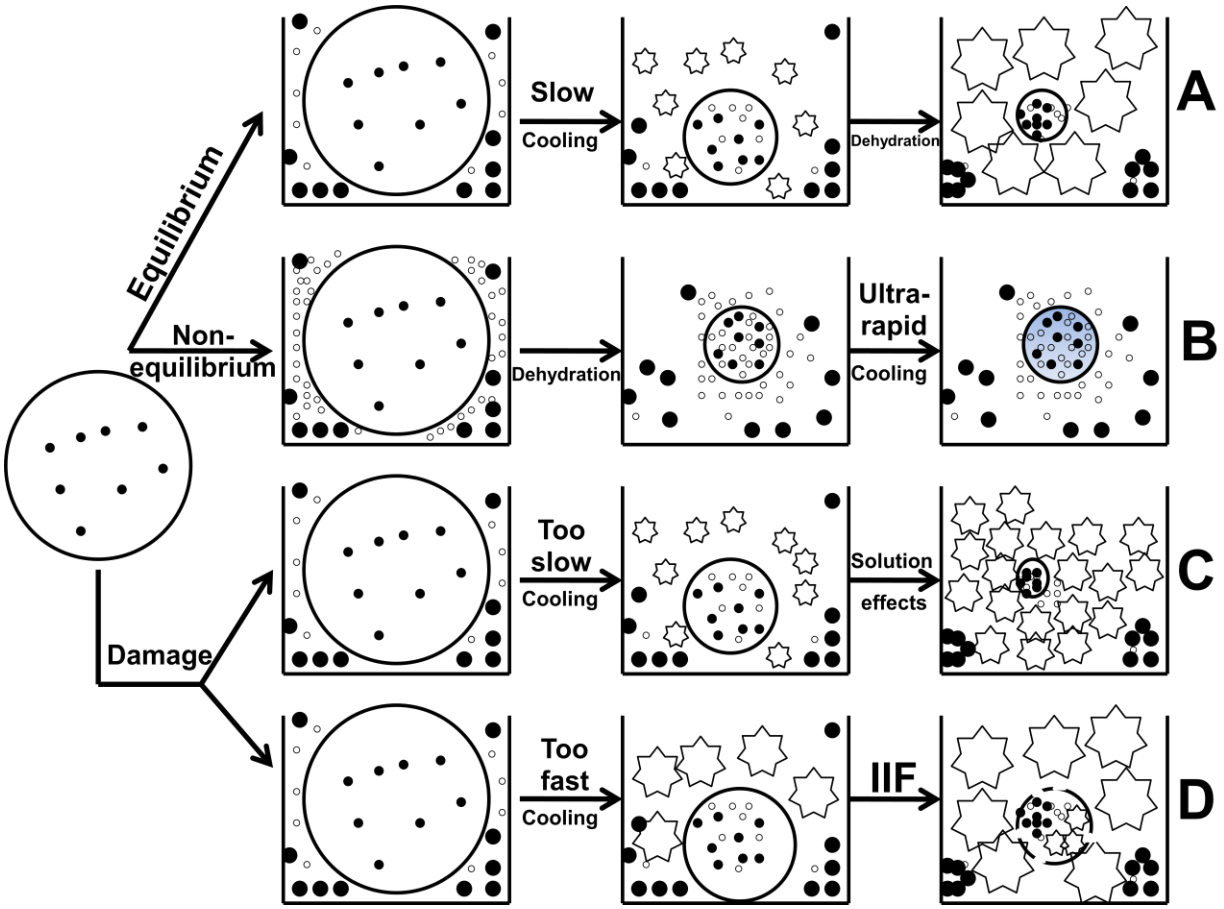

Figure 2.2. A) Equilibrium freezing involves the use of cryoprotectants and "slow freezing" to produce cellular dehydration and shrinkage. Permeating cryoprotectants (○) lower the freezing point of the solution, thereby extending the dehydration time during freezing, and minimizing osmotic shock by avoiding excessive dehydration of the cell. Non-permeating cryoprotectants (●) assist dehydration of the cell, and stabilize the membrane during cryopreservation. Once the first extracellular ice crystals (✡) have formed, as the temperature decreases, water is further incorporated into the growing ice crystals creating a hypertonic condition that produces osmotic dehydration. The combination of increased intracellular solutes and of lowering the temperature increases the viscosity of the solution until the eutectic temperature is reached (-40°C) when the remaining unfrozen solution is solidified (partial vitrification). B) Non-equilibrium vitrification involves the use of high concentrations of cryoprotectants to dehydrate the cell and to replace the intracellular water before the cooling begins. Ultra-rapid cooling prevents the cells and the surrounding medium from undergoing ice crystal formation during cooling. The result is the solidification of the solution into a glass-like state (total vitrification). The two main sources of damage to the cell during cryopreservation are solution effect (C) and intracellular ice formation (IIF). C) Solution effect is due to excessively slow cooling rates, which cause the cell to experience severe volume shrinkage and long-term exposure to high solute concentrations. Excessive shrinkage can cause extrusion of membrane lipids and proteins, and intracellular changes such as reductions in pH that can denature proteins and cause loss of buffering capacity. D) Damage by intracellular ice formation is due to excessively fast cooling rates, which do not allow sufficient egress of intracellular water to maintain equilibrium and the residual supercooled water in the cell undergoes intracellular ice formation which causes damage by disruption of cellular structure and function.

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

Equilibrium vs. Non-equilibrium Cryopreservation

Cryopreservation can be produced by two approaches: slow equilibrium cryopreservation (standard method) and rapid non-equilibrium vitrification. The main difference between these methods is that standard cryopreservation allows extracellular ice crystal formation while in vitrification ice crystal formation is suppressed. As stated above, dehydration of the cells during slow equilibrium freezing takes places during cooling, while dehydration in non-equilibrium vitrification takes places before cooling (Figure 2.2). To dehydrate the cell before cooling, high concentrations of cryoprotectants (40 – 60%) are normally used. To achieve the vitreous state before ice crystals have the chance to form (Kuleshova et al. 2007), it is necessary to rapidly cool through a specific temperature zone (-5 to -40°C) (Shaw and Jones 2003) of potential crystallization. The resultant glass retains the random molecular arrangement of a liquid but has

the mechanical properties of a solid (Taylor et al. 2004). This ultra-rapid cooling is typically done by plunging the samples directly into the liquid nitrogen (Fahy et al. 1984). Neither high cryoprotectant concentration nor increased cooling rates are essential for vitrification to occur. Partial (usually) or total intracellular vitrification can occur incidentally during traditional slow equilibrium cooling, and may help to ensure survival of some portion of cryopreserved samples (Vajta et al. 2009).

The goal of equilibrium freezing and non-equilibrium vitrification is to prevent intracellular ice crystal formation and to protect cells from damage. Vitrification is typically achieved by partial replacement of intracellular water via permeating cryoprotectants, which readily form glass, and by drawing out the intracellular water via non-permeating cryoprotectants (Figure 2.2). As a result, by combination of permeating and non-permeating cryoprotectants, the net concentration of the permeating cryoprotectant is increased in the intracellular space and their combined effect enhances the overall viscosity of the cell (Jain and Paulson 2006). In practice, the exposure to cryoprotectants is usually performed at room temperature (Kuleshova et al. 2007). The assessment of glass formation for cryoprotectants is relatively straightforward. Crystallization can be distinguished by the observance of a milky appearance after plunging samples into liquid nitrogen, while glass formation appears as transparent (Figure 2.3) (Ali and Shelton 2007).

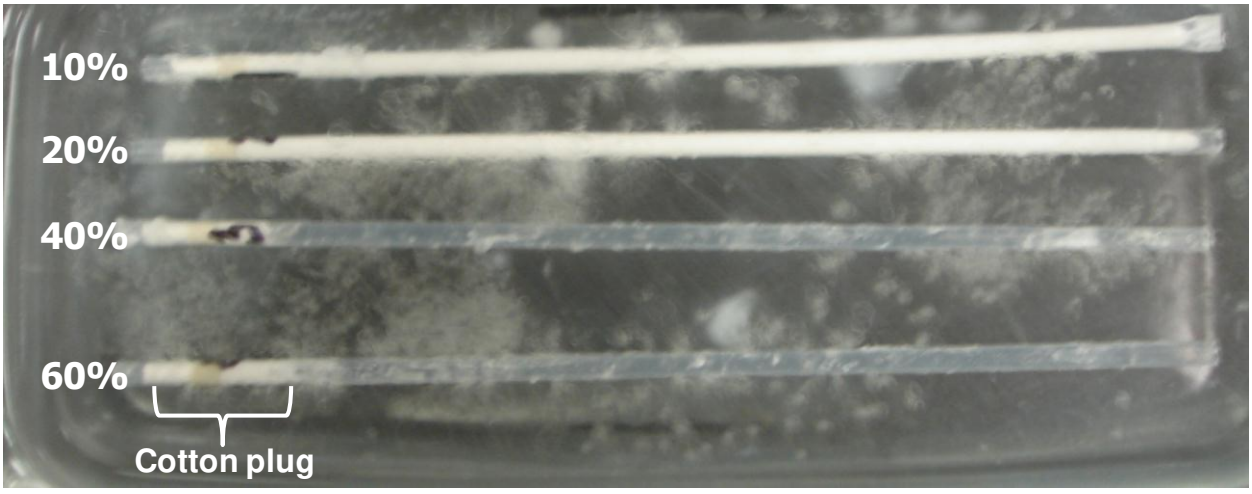

Figure 2.3. Visual observation of crystallization and vitrification of ethylene glycol at different concentrations. French straws (0.25-mL) with a cotton plug at one end were filled with 10, 20, 40 or 60% of ethylene glycol and plunged into liquid nitrogen. Straws with 10 and 20% had a milky appearance indicating ice formation while straws with 40 and 60% remained transparent indicating glass formation.

Cryoprotectants in Vitrification

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

The second type used in vitrification is known as the non-permeating cryoprotectants. The two main functions of these cryoprotectants are to dehydrate the cell during cooling by increasing the osmolality of the extracellular space, and to prevent excessive osmotic swelling during warming. Non-permeating cryoprotectants have high molecular weights ($\geq$ 342 daltons) and can be monosaccharide sugars (MW $\approx$ 180 daltons), disaccharide sugars, polysaccharides, and macromolecules (Swain and Smith 2010). Adding sugars to the vitrification solution can increase the dehydration rate before cooling and enhance viscosity (Varghese et al. 2009). Sugars, especially disaccharides such as sucrose and trehalose, are effective in enhancing glass formation (Fuller 2004). Adding other agents such as polymers can facilitate vitrification and reduce the concentration of permeating cryoprotectants necessary to form glass (Fahy et al. 1984). Low molecular weight copolymers such as polyvinyl alcohol can inhibit ice formation and prevent the formation of ice crystals during warming (Wowk 2005). Another ice blocker is

antifreeze proteins (AFP) that control the growth of ice crystals. Antifreeze proteins act by adsorbing to the surface of small ice crystals, inhibiting their growth. Antifreeze proteins could be used in vitrification to inhibit ice growth during warming (Fuller 2004).

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

Devices Used in Vitrification

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

Sources of Variation in Vitrification

There are many variables that influence the success of vitrification including the: 1) effects of exposure time to cryoprotectant solutions, and their concentration and temperature; 2) number of steps in which the cryoprotectant is added and removed; 3) type of device used for vitrification (which as stated above influences the size of the vapor coat and cooling rate); 4) quality and the developmental stage of the cells tested (Liebermann et al. 2002); 5) viscosity and volume of the sample (Yavin and Arav 2007); 6) absolute pressure (higher hydrostatic pressures decrease the homogeneous nucleation temperature and increase the glass transition temperature) (Rabin and Steif 2009), and 7) warming process (ice crystal formation can occur during sub-optimal warming) (Leibo 2000). Overall, the high concentrations of cryoprotectants required are near the maximum tolerable limit of cells. As such, there is an inverse relationship between cooling rate and cryoprotectant concentration, i.e. the higher the cooling rate, the lower the concentration needed and *vice versa* (Mazur et al. 2008).

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

Advantages of Vitrification

Vitrification is considered an attractive alternative to standard cryopreservation for specific applications and it has been used for the cryopreservation of spermatozoa, embryos, oocytes, stem cells, and organs in a variety of taxa (Tucker and Liebermann 2007). Vitrification does not require expensive equipment, is simple, achieves cryopreservation in sec, and can be

used to preserve samples in the field. In addition, it offers perhaps the greatest potential for success in overcoming the challenges for preservation of fish eggs and embryos.

Sperm Vitrification

The first report of sperm vitrification was by Luyet and Hodapp (1938) who used sucrose to dehydrate frog sperm prior to immersion in liquid air (-192°C) (Table 2.1). During the same year another report on cryoprotectant-free human sperm vitrification was published (Jahnel 1938). After the discovery of glycerol as cryoprotectant (Polge et al. 1949), studies on vitrification were sporadic and reported results varied, mainly low viability after vitrification (Table 2.1). It was not until the 1980s that the interest of vitrification resumed when mouse embryos were vitrified using high concentrations (e.g. 36%) of cryoprotectants (Rall and Fahy 1985). But these high concentrations of cryoprotectants needed to achieve vitrification were believed to be extremely toxic to the sperm (Watson 1995) and no further studies of sperm vitrification were published until 2000s. A major breakthrough came in 2001, when Nawroth et al. managed to successfully vitrify human sperm without any conventional cryoprotectants when it was cooled in very thin films in copper cryoloops (Nawroth et al. 2002). Since then, several other attempts have been successfully vitrified sperm with and without cryoprotectant in mammals (Table 2.1). Intracellular vitrification can be easy to achieve in cells such as spermatozoa because of their small size, low water content of spermatozoa (e.g. osmotically inactive volume ~60%) (Dinnyes et al. 2007), and high content of soluble macromolecules compared with oocytes and embryos (Isachenko et al. 2003). Due to all these characteristics, it is unlikely that intracellular ice would be present in spermatozoa at any cooling rate (Morris 2006, Morris et al. 2007). The sperm is damaged during cryopreservation due the combinations of different factors such as high rates of efflux of water (Henry et al. 1993), and the osmotic imbalance encountered during thawing (Morris 2006).

Table 2.1. Literature review on the studies of sperm vitrification.

Species	Cryoprotectant	Container	Summary of finding	References
Frog	Sucrose	Mica sheet of 100 micra	60% motility after immersion in liquid air	Luyet and Hodapp 1938
Human	None	Glass tubes	Some sperm resumed motility	Jahnel 1938
Human	None	Thin-walled capillary tubes (internal diameter 0.2 mm)	As high as 10% motility after immersion in liquid nitrogen	Shettles 1940
Rat	Sucrose	Mica sheet of 100 micra	No survival	Luyet and Gehenio 1940
Human, rat, mouse, guinea pig, rabbit and bull	None, sucrose or ringer solution with double amount of calcium	Capillary pipette, wire loops or smearing sperm on mica or cellophane	As high as 50% human sperm revival after liquid nitrogen immersion and 0.1% recoverable motility of sperm from bull and rabbit	Hoagland and Pincus 1942
Human	None	Capillary tubes and platinum loops	Abundant sperm revived using capillaries tubes after immersion in liquid nitrogen	Parkes 1945
Human, rabbit and fowl	Gly, propylene glycol, EG	Capillary tubes	No survival of undiluted fowl sperm after “quick freezing”. No description of liquid nitrogen usage.	Polge et al. 1949
Rabbit, ram, bull, horse and boar	None	Pellets (0.05-0.10 mL) directly into liquid nitrogen	From 1947 to 1950 Smirnov vitrified sperm and obtained rabbits after insemination with vitrified sperm	Cited by: Salamon and Maxwell 1995
Horse, rabbit and guinea pig	Gly	Capillary tubes	No survival of rabbit and guinea pig sperm after “rapid freezing. No mention of liquid nitrogen.	Smith and Polge 1950
Human	Gly	12 mm x 75 mm test tubes	23% motility and 15% viability based on eosin-nigrosin after immersion in liquid nitrogen	Sherman and Bunge 1953
Human	Gly	10 mm x 75 mm test tubes	30% post-thaw survival based on eosin-nigrosin test	Sherman 1954
Fowl	Laevulose	Thin films or capillary tubes	No survival	Smith 1961
Ram	Egg yolk-citrate-lactose	Pellets (0.03-mL) directly into liquid nitrogen	9% motile sperm after pelleting directly into liquid nitrogen	Salamon 1968
Bull	Polyols, EG, Gly, inositol	Pellets (0.015-mL) directly into liquid nitrogen	As high as 40% motility	Nagase and Tomizuka 1968

Table 2.1. Continued.

Species	Cryoprotectant	Container	Summary of finding	References
Ram	Egg yolk-citrate-lactose	Pellets (0.03-mL)	17% motility after pelleting at -150ºC and 5% motility after pelleting into liquid nitrogen	Salamon 1970
Ram	Raffinose, glucose and Gly	Pellets (0.03-mL) directly into liquid nitrogen	15% motility after submersion of the pellet into liquid nitrogen	Visser 1974
Ram	Gly	0.5-mL straws	No survival after plunged into liquid nitrogen	Fiser and Fairfull 1984
Mammalian	----	----	Review on the cryobiology of sperm vitrification	Rall 1991
Human	Gly	0.25-mL straw	Cooling of 800ºC/min but thawing of 400ºC/min yielded 20% motility, 50% membrane intact	Henry et al. 1993
Human	Not described	1-mL vials	21.5% motile sperm recovered	Ziegler and Chapitis 1998
Human	None or Gly	Copper loops (5 mm diameter) or 0.25-mL straw	50% post-thaw motility using loops and 2% in straws. 38% viable (acrosome-reacted cells)	Nawroth et al. 2002
Human	Gly, trehalose, EG	Nylon cryoloops (5 µL)	45% post-thaw motility after ultra-rapid freezing	Schuster et al. 2003
Human	Gly	Cylindrical tubes or 0.25 straw	Motility depended on cooling rates from 1-78%	Grischenko et al. 2003
Human	----	----	Review on studies on sperm vitrification	Isachenko et al. 2003
Human	None or Gly	Cooper loops (5 mm diameter)	52% post-thaw motility and 85% undamaged DNA	Isachenko et al. 2004a
Human	None	Cooper loops (5 mm diameter)	55% post-thaw motility and 79% fertilization ability	Isachenko et al. 2004b
Human & bull	Gly	Nylon cryoloops (1 mm)	65% post-thaw motility and 48% fertilization	Desai et al. 2004
Human	None	Loops, droplets and straws	40% reduction of sperm motility after vitrification	Isachenko et al. 2005
Human	None	Fibreplug and CVM1 kit	As high as 57% survival by eosin exclusion	Irving and Harrison 2006
Human	None	Pellets (30 µL) in liquid nitrogen	54% motility and 63% mitochondrial intact	Schulz et al. 2006
Human	----	----	Review on vitrification including sperm	Katkov et al. 2006
Human	Gly	1.8-mL cryovials	The cryovials were not plunged into liquid nitrogen	Peirouvi et al. 2007
Human	----	----	Book chapter that review sperm vitrification	Isachenko et al. 2007
Human	Sucrose	Pellets (30 µL) in liquid nitrogen	57% motility and 65% mitochondrial intact	Isachenko et al. 2008
Human	Not described	Cryoloop	25% sperm apoptosis	Khalili et al. 2010
Human	Trehalose and Gly	0.25 mL straw	54% motility after solid surface vitrification	Vutyavanich et al. 2010

There were no publications on vitrification of fish sperm at the start of the studies done in this dissertation. During the writing of this dissertation, two reports addressing basic research questions only were published on vitrification in fish sperm (Andreev et al. 2009, Merino et al. 2011). These studies did not overlap with the purpose of this dissertation which was to develop streamlined protocols that could be integrated into a standardized approach for vitrification of aquatic species germplasm.

Vitrification of Fish Eggs and Embryos

Studies in fish vitrification date back to 1938 when Basile Luyet attempted to vitrify juvenile goldfish (*Carassius auratus*) (40 mm standard length) by plunging the fish into liquid air (-194°C) (Luyet 1938). Since then, there have been more than 30 publications addressing embryo vitrification in 11 species of fish (most within the past 10 years) (Table 2.2). Zebrafish (*Danio rerio*) is the most widely studied for vitrification, accounting for 50% of the studies. The results from vitrification studies had been controversial with documentation of "survival", but there has been a controversial lack of reproducibility with these studies (e.g., Edashige et al. 2006). One of the main problems has been a lack of standardization in the methods and terminology used. For example, the term "survival" has been applied to "intact" embryos, hatched embryos, movement and twitching of embryos, and dye exclusion (such as trypan blue). Standardization of the terminology plays a key factor for reproducibility and validation. At least four studies have reported larvae hatched from vitrified embryos (Table 2.2), but none of these studies has been reproduced (Edashige et al. 2006). The limited success in fish embryo vitrification could be due to their large size (> 1 mm in diameter), low surface-to-volume ratio, the complexity of the multi-cellular embryo, and low permeability which could inhibit the entrance of cryoprotectants into the various embryonic compartments (Zhang 2004, Robles et al. 2009).

Table 2.2. Most studies on vitrification in fishes have occurred within the past 10 years, and have addressed zebrafish embryos.

Species	Vitrified material	Summary of finding	Reference
Carassius auratus	40- mm fingerling	No survival	Luyet 1938
Not reported	embryos	Intracellular blacking appeared	Wang et al. 1987
Clupea pallasi	eggs	Normal chorion and micropyles	Pillai et al. 1994
Clarias gariepinus	eggs	None survived	Magyary et al. 1995
Danio rerio	6 somite and heartbeat stages	≤ 32% intact morphology	Zhang and Rawson 1996
Danio rerio	Heartbeat stage	Two live embryos	Chao et al. 1997
Danio rerio	1 cell to prim-6 stages	≤ 80% intact morphology	Liu et al. 1998
Danio rerio	100% epiboly	All died	Hagedorn et al. 1998
Danio rerio	100% epiboly	Fell apart	Janik et al. 2000
Clarias gariepinus	eggs and embryos	No embryonic development	Urbanyi et al. 2000
Lateolabrax japonicus	neurula to prehatch	One embryo hatched	Tian et al. 2003
Scophthalmus maximus	tail bud and tail bud free	≤ 49% intact morphology	Robles et al. 2003
Scophthalmus maximus	tail bud and tail bud free	≤ 54% enzymatic activity	Robles et al. 2004
Danio rerio	high blastula and 5-somite stages	≤ 12% enzymatic activity	Robles et al. 2004
Danio rerio	high blastula and 5-somite stages	Differences of viable cells, SYBR[a]	Martinez et al. 2005
Pseudopleuronectes[b]	gastrula to starting pigment	1% continued development	Robles et al. 2005
Paralichthys olivaceus	20 somites to body movement	Seven embryos hatched	Zhao et al. 2005
Paralichthys olivaceus	neurula to hatching	Fourteen larvae hatched	Chen and Tian 2005
Paralichthys olivaceus	tail bud stage	No embryos survived	Edashige et al. 2006
Sparus aurata	tail bud and tail bud free stages	≤ 28% intact morphology	Cabrita et al. 2006
Danio rerio	caudal fin	≤ 63% attachment rate	Cardona-Costa et al. 2006
Danio rerio	blastomeres	≤ 20% survival, trypan blue	Cardona-Costa et al. 2007
Pagrus major	heartbeat stage	≤ 78% intact morphology	Ding et al. 2007
Prochilodus lineatus	morula to 6 somites	None viable	Ninhaus-Silveira et al. 2008
Carassius auratus	caudal fin	No cell outgrowth	Moritz and Labbe 2008
Danio rerio	5 somites	≤50% viable cells, SYBR	Martinez-Paramo et al. 2009
Tinca tinca	23 and 29 h	No living embryos	El-Battawy and Linhart 2009
Danio rerio	testicular tissue	94% cell survival, trypan blue	Bono-Mestre et al. 2009
Danio rerio	blastomeres	90% survival and 20% recovery	Cardona-Costa et al. 2009
Danio rerio	64-cell to 20-somite stage	25% PGC[c] survival	Higaki et al. 2009
Danio rerio	14-20 somites	30% PGC survival	Higaki et al. 2010b
Danio rerio	14-18 somites	≤83% PGC survival	Higaki et al. 2010a
Danio rerio	stage III oocytes	≤69% survival, trypan blue	Guan et al. 2010

[a]SYBR: SYBR-14 and propidium iodide. [b]*Pseudopleuronectes americanus*. [c]PGC: primordial germ cells.

Several approaches have been used to improve embryo vitrification in the studies listed in Table 2.2 by enhancing permeation of cryoprotectants. These include embryo dechorionation, enzymatic permeabilization (using the enzyme pronase), partial removal of yolk, microinjection of cryoprotectants, artificial expression of aquaporin-3, addition of AFP and polymers to inhibit ice formation and enhance glass formation, the use of cold-tolerant fish species (e.g., winter flounder *Pseudopleuronectes americanus* which produces AFP), assisted hatching techniques (e.g. piercing the egg), evaluation of different apparatuses and warming temperatures, and evaluating embryos at different developmental stages. Although some of these approaches have increased permeability, the concentrations necessary within the embryo for vitrification have not been achieved (Robles et al. 2009).

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

New Strategies for Application of Cell Vitrification

Because cryopreservation of fish eggs and embryos has been unreliable, new technologies have been developed to conserve paternal and maternal genetic information. These technologies use surrogate production through transplantation of blastomeres, testicular cells (e.g., spermatogonial stem cells), or primordial germ cells (PGC) (Yamaha et al. 2007). Vitrification has been applied to cryopreserved blastomeres (Cardona-Costa and Garcia-Ximenez 2007, Cardona-Costa et al. 2009), testicular cells (Bono-Mestre et al. 2009), and PGC (Higaki et al. 2009, Higaki et al. 2010b) (Table 2.2). In fact, zebrafish (striped-type) were produced from surrogate zebrafish (golden-type, germ-line chimeras) that were generated through transplantation of vitrified germ cells (PGC from striped-type) (Higaki et al. 2010a). This is a

breakthrough in cryopreservation because it presents alternative strategies to preserve fish genomes.

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

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Chapter 3*

Production of Channel Catfish with Sperm Cryopreserved by Rapid Non-Equilibrium Cooling

Vitrification is the solidification of water into a glass-like state (non-crystalline ice). To achieve this state, it is usually necessary to cool water rapidly through the temperature region of potential crystallization (i.e. –5 to -40°C) (Shaw and Jones 2003) and to reach an amorphous glassy state before ice crystals have the opportunity to form (Kuleshova et al. 2007). The resultant glass retains the random molecular arrangement of a liquid (i.e. an "arrested liquid") but has the mechanical properties of a solid (Taylor et al. 2009). Vitrification of biological samples normally involves high concentrations of cryoprotectants, and the ultra-rapid cooling required for glass formation is typically achieved by plunging samples directly into liquid nitrogen (Fahy et al. 1984).

Variables that can influence the success of vitrification include the: 1) effects of exposure time to cryoprotectant solution, cryoprotectant concentration, and temperature; 2) number of steps in which the cryoprotectant is added and removed; 3) type of device used for vitrification (which influences the size of the vapor coat and cooling rate); 4) quality and the developmental stage of the cells tested (Liebermann et al. 2002); 5) viscosity and volume of the sample (Yavin and Arav 2007); 6) absolute pressure (higher hydrostatic pressures decrease the homogeneous nucleation temperature and increase the glass transition temperature) (Rabin and Steif 2009), and 7) warming process (ice crystal formation can occur during sub-optimal warming) (Leibo 2000). Overall, the high concentrations of cryoprotectants required are near the maximum tolerable limits of cells. As such, there is an inverse relationship between cooling rate and cryoprotectant concentration, i.e. the higher the cooling rate, the lower the concentration needed and *vice versa* (Mazur et al. 2008). To attain the highest cooling rates, the volume of the sample needs to be minimized.

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Studies in fish vitrification can be traced back to 1938 when Basile Luyet attempted to vitrify juvenile goldfish, *Carassius auratus*, (40 mm, standard length) by plunging the fish into liquid air (-194°C) (Luyet 1938). The first success came in 1938 when Luyet and Hodapp successfully vitrified frog sperm (60% moving) (Luyet and Hodapp 1938), and in the same year Jahnel vitrified human sperm and was able to observe a "vivid motility pattern" after warming (Jahnel 1938). These early successes were achieved without the benefit of penetrating cryoprotectants such as glycerol. Although Bernstein and Petropavlovski used glycerol to "freeze" bull, ram, stallion, boar and rabbit spermatozoa to a temperature of -2°C in 1937 (Bernstein and Petropavlovsky 1937), it was not until the 1940s when Rostand (frog; glycerol; -6°C) (Rostand 1946), Polge et al. (human, rabbit and fowl; glycerol, propylene glycol, ethylene glycol; -79°C) (Polge et al. 1949), and Smirnov (ram; glycerol; -78°C) (Smirnov 1949) discovered the beneficial effects of cryoprotectants for deep cooling of biological samples. As stated above, vitrification usually requires high concentrations of cryoprotectants (40 to 60% by volume) and high cooling rates (>1,000°C/min). As such, a misconception arose at the time of these early experiments that high concentrations of cryoprotectants would be toxic and further attempts were not made to vitrify sperm (Holt 1997, Watson 1995). In addition, Parkes reported in 1945 that "spermatozoa do not survive when minute amounts of semen are frozen as films or in fine capillary tubes" (Parkes 1945). Thus after a 40-year hiatus, the field of vitrification was re-opened in 1985 when mouse embryos were vitrified using high concentrations (e.g. 36%) of cryoprotectants (Rall and Fahy 1985). In an attempt to derive vitrification methods that would not require high concentrations, human spermatozoa were vitrified in 2002 using small volumes (20 µL) without the use of cryoprotectants (Nawroth et al. 2002). This use of small sample size allowed ultra-rapid cooling (estimated to be as high as 720,000°C/min). Since then, several other studies have reported successful vitrification of human and bovine sperm with and without cryoprotectants (Desai et al. 2004, Isachenko et al. 2004a, Isachenko et al. 2008, Isachenko et al. 2004b, Isachenko et al. 2005, Schuster et al. 2003).

Vitrification is considered to be an attractive alternative to standard cryopreservation and has been used in mammals for the cryopreservation of spermatozoa, embryos, oocytes, stem cells, and organs (Tucker and Liebermann 2007). The advantages of vitrification are that it does not require expensive equipment, it is simple and requires sec for freezing, and it can be used to

preserve samples in the field. In addition, it offers perhaps the greatest potential in overcoming the challenges for preservation of fish embryos (Hagedorn and Kleinhans 2011). At present, there have been two reports addressing vitrification (for basic research purposes) in fish sperm (Andreev et al. 2009, Merino et al. 2011). In addition, partial vitrification is likely to have occurred coincidentally in other studies addressing conventional cryopreservation (i.e., equilibrium cooling) (Table 3.1). Based on the advances in mammalian sperm vitrification, the discovery of cryoprotectant-free vitrification, and the finding that a wide range of cooling rates (160 – 250°C/min) can attain sperm vitrification (Isachenko et al. 2004b), we evaluated vitrification as an option to cryopreserve fish sperm. To develop a vitrification protocol, the first step was to identify suitable vitrification solutions by measuring the toxicity of cryoprotectants at various concentrations, exposure times, and temperatures. Cryoprotectants are not equally effective for vitrification, and do not have equivalent toxicity or osmotic effects (Pegg 2005). The typical aim in vitrification protocols is to increase the speed of temperature change while keeping the concentration of cryoprotectants (although high) as low as possible (Nawroth et al. 2005). Accordingly, the second step was to select a vitrification device that would minimize sample volume, and allow ultra-rapid cooling. This would allow the use of less concentrated cryoprotectants, and prevent heterogenous ice formation (Vajta and Nagy 2006). Thus, vitrification should be performed in a kinetic way, reconciling concentration of the vitrificant with the rates of cooling and warming (Katkov et al. 2006).

The overall goal of the project was to develop streamlined protocols that could be integrated into a standardized approach for vitrification of aquatic species germplasm. The objectives of the present study were to: (1) evaluate the acute toxicity of 5, 10, 20 and 30% methanol, n,n-dimethyl acetamide, dimethyl sulfoxide, 1,2-propanediol, and methyl glycol; (2) evaluate a range of apparatus commonly used for cryopreservation and vitrification of mammalian sperm; (3) compare vitrification with and without cryoprotectants; (4) evaluate the post-thaw membrane integrity of sperm vitrified in different cryoprotectant solutions, and (5) evaluate the ability of vitrified sperm to fertilize eggs. We report the first successful sperm vitrification and production of offspring in fish from vitrified sperm of channel catfish. Although the fertilization values were low, the feasibility of using vitrification for fish sperm was demonstrated.

Table 3.1. Previous studies in fish sperm that attempted to cryopreserve samples by plunging into liquid nitrogen. The cryoprotectants (CPA) used were dimethyl sulfoxide (DMSO), glycerol (Gly), ethylene glycol (EG), propanediol (PROH), and methanol (MeOH). Despite the potentially rapid cooling rate, none of these studies likely resulted in substantial vitrification because of low CPA concentrations, large sample volumes, or poor heat transfer of containers.

Scientific name	Cryoprotectant	CPA (%)	Exposure time	Container	Thawing (°C)	Assessment	Reference
Salmo salar	DMSO, Gly, EG	2.5 – 27.5	30min to 8 h	1-mL ampoule and aluminium packet	5	No motility or fertility	Hoyle et al. 1968
Salmo salar	EG	5	1 h	Aluminium packet	3	No motility or fertility	Hoyle & Idler 1968
Salmo salar	DMSO, Gly, EG	5 – 40	30 min to 6 h	1-mL ampoule	ND[a]	Completely destroyed	Truscott et al. 1968
Mugil cephalus	DMSO, Gly	3.3 – 15	<30 min	Glass vial (0.2-0.3 mL)	20 – 37	"Some degree of fertility"	Hwang et al. 1972
Mugil cephalus	DMSO, Gly	6 – 30	ND	Glass vial (20 x 30 mm)	20 – 24	"Some degree of fertility"	Chao et al. 1975
Ictalutus punctatus	DMSO, Gly, PROH	5 – 20	1 to 24 h	5-mL vials	4	"Unsuccessful"	Guest et al. 1976
Cyprinus carpio	DMSO, Gly, EG	2.5 – 15	5 s to 6 min	Ampoules in basket	0 – 60	Coagulation of sperm	Moczarski 1977
Salmo salar	DMSO, Gly	12.5	ND	2-mL ampoules	38	7% motile, 80% fertilization	Mounib 1978
Gadus morhua	DMSO, Gly	12.5	ND	2-mL ampoules	38	7% motile, 60% fertilization	Mounib 1978
Morone saxatilis	DMSO, Gly, EG, PROH	10 – 25	ND	0.5-mL plastic tubes	ND	No fertilization	Kerby 1983
Clarias gariepinus	DMSO, Gly, MeOH	5 & 12.5	20 min	Straws and vials	20	No motility	Steyn et al. 1985
Hydrocynus forskahlii	DMSO, Gly	7 & 11	10 min	1-mL cryotubes	25	No motility	Steyn et al. 1991
Clupea pallasi	DMSO, Gly, EG	5 – 15	10 min	1.8-mL microcentrifuge	4 – 37	≤95% fertilization	Pillai et al. 1994
Xyrauchen texanus	MeOH	10	1.5 – 30 min	0.5-mL straws	20 – 40	Straws burst at thawing	Tiersch et al. 1998
Dicentrarchus labrax	DMSO	10	None	1.5-mL cryovials[b]	50	≤70% fertilization	Fauvel et al. 1998
Salvelinus fontinalis	DMSO, Gly	5	1 – 20 min	5-mL straws[c]	35 – 40	39% fertilization	Lahnsteiner 2000
Salvelinus alpinus	DMSO, Gly	5	1 – 20 min	5-mL straws[c]	35 – 40	39% fertilization	Lahnsteiner 2000
Oncorhynchus mykiss	DMSO	7	15 min	5-mL macrotubes	25 – 80	≤73% fertilization	Cabrita et al. 2001
Xiphophorus helleri	Gly	14	10 min	0.25-mL French straws	40	Minimal motility <1%	Huang et al. 2004
Thunnus orientalis	DMSO, Gly, MeOH	10 – 30	5 – 20 min	0.25-mL straw	20	No motility	Gwo et al. 2005
Cyprinus carpio	DMSO	10	10 min	0.25-mL straw	70	No motility, jelly form	Irawan et al. 2010
Oncorhynchus mykiss	DMSO, lipids, sugars	10	ND	Glass cell layer 0.1 mm	34 – 36	90% fertilization vs. fresh sperm control	Andreev et al. 2009
Acipenser gueldenstaedtii	DMSO, lipids, sugars	10	ND	Glass cell layer 0.1 mm	34 – 36	90% fertilization vs. fresh sperm control	Andreev et al. 2009

[a] ND: not described.
[b] Cryovials immersed for 15 sec were laid on a tray 2 cm above liquid nitrogen for 15 min and dropped into liquid nitrogen.
[c] Straws were cooled at 0 to 1 cm above the level of the liquid nitrogen.

The channel catfish (*Ictalurus punctatus*) was selected for this research because it is reasonably well studied in the field of cryopreservation. Work with channel catfish began in the 1970s (Guest et al. 1976) and subsequent research has refined protocols and moved towards standardization for high-throughput commercial-scale sperm cryopreservation for the closely related blue catfish (*I. furcatus*) (Hu et al. 2011). In addition, early out-of-season induced spawning of channel catfish is an established technique that allows extended production of eggs and fry for research projects outside of the natural (1 – 2 month) spawning season (Lang and Tiersch 2007, Pawiroredjo et al. 2008), and synchronized conditioning of broodstock enables a predictable spawning schedule (Wolters and Tiersch 2004). Furthermore, catfish is the largest foodfish aquaculture industry in the United States (USDA-NASS 2005). Although the small sperm volumes used in vitrification are not practical for commercial aquaculture production, catfish provided a useful working model for this work in terms of reproductive availability and control, and can serve as a model for small-bodied fishes (< 5 cm) which have minute sperm volumes such as the endangered Neosho madtom (*Noturus placidus*) (Jelks et al. 2008), which is in the same taxonomic family (Ictaluridae), and important biomedical research models such as zebrafish (*Danio rerio*).

Materials and Methods

Fish Source and Care

Healthy, mature (3 - 4 year-old) male channel catfish (1.3 - 2.6 kg) of current commercial stocks were obtained from Baxter Land Company Inc. (Arkansas City, Arkansas) and maintained in aerated earthen ponds at the Louisiana State University Agricultural Center, Aquaculture Research Station. Fish were fed daily to satiation with a commercial diet (Aquaxcel, Cargill™, 45% protein), and were routinely screened for disease by the Louisiana Aquatic Animal Disease Diagnostic Laboratory at the LSU School of Veterinary Medicine. Temperature and dissolved oxygen were monitored twice daily, and dissolved oxygen was maintained around 5 ppm. Experiments were conducted during Spring (February to May in southern Louisiana) each year from 2007 to 2010. Guidelines from the Institutional Animal Care and Use Committees of Louisiana State University were followed for animal care and use.

Acute Toxicity of Cryoprotectants

Sperm cannot be collected by stripping from ictalurid catfishes (Viveiros 2003) and thus large, mature males must be killed for collection of testis by dissection (Tiersch et al. 1994). Three males were killed with a blow to the head and their testes were removed. Adherent tissues were dissected away, and testes were blotted dry and weighed (5.3 – 9.5 g). Testes were crushed in Hanks' balanced salt solution (Tiersch et al. 1994) prepared at 300 mOsmol/kg (HBSS300) measured with a vapor pressure osmometer (model 5500 Wescor, Inc., Logan, UT) to provide an initial dilution ratio of 1 g of testis per 3 mL of HBSS300. Sperm suspensions from individual males were strained through a series of filters, progressing from a sieve (mesh size of 0.5 mm) to a nylon screen with a final pore size of 200 µm. Sperm concentration was estimated by use of a hemacytometer (Hausser Scientific, Horsham, PA) and diluted to a final concentration of 5×10^8 sperm/mL with HBSS300.

Five cryoprotectants, methanol (MeOH; Fisher Scientific, Fair Lawn, NJ), n,n-dimethyl acetamide (DMA; Sigma-Aldrich, St Louis, MO), dimethyl sulfoxide (DMSO; OmniSolv, France), 1,2-propanediol (PROH; Sigma-Aldrich), and methyl glycol (2-methoxyethanol, MG; Sigma-Aldrich) were used at final concentrations of 5, 10, 20 and 30% (v/v) (Table 3.2). Cryoprotectant solutions were prepared in HBSS300 at double the final concentration and kept cold (4°C) before being added at that temperature to the sperm suspension at a ratio of 1:1 (200 µL of cryoprotectant solution: 200 µL of sperm suspension). The samples were held on ice during the acute toxicity experiment.

Sperm motility was estimated using dark-field microscopy (Optiphot-2, Nikon, Garden City, NY) at 200-X magnification within 30 sec of dilution with cryoprotectant solution. Catfish sperm (as for most freshwater fishes) is immobile in the testis and becomes activated when exposed to hypotonic solutions. The resultant peak motility is rapid and transient (< 1 min). The addition of 20 µL of distilled water was used to activate 1 µL of sperm suspension placed on a glass slide without the use of a coverslip. The percentage of sperm swimming actively in a forward direction was estimated immediately (within 5 sec) after addition of distilled water, then at 5 min intervals for 30 min, and finally at 60 min.

Table 3.2. Cryoprotectant concentrations expressed as percent (volume/volume), molarity, and the osmolality used for the acute toxicity experiment. The cryoprotectants were diluted in Hanks' balanced salt solution at 300 mOsmol/kg for osmolality measurement.

Cryoprotectant	Percent (v/v)	Molarity (mol/L)	Osmolality (mOsmol/kg)
Methanol	5	1.24	278
	10	2.47	271
	20	4.94	261
	30	7.41	234
Dimethyl acetamide	5	0.54	478
	10	1.08	828
	20	2.15	1413
	30	3.23	1507
Dimethyl sulfoxide	5	0.70	1193
	10	1.41	2015
	20	2.82	3349
	30	4.22	5118
Propanediol	5	0.68	1202
	10	1.36	2000
	20	2.72	2445
	30	4.08	2946
Methyl glycol	5	0.63	236
	10	1.27	281
	20	2.54	279
	30	3.81	258

Vitrification Device Configurations

A pilot study was carried out prior to the experiments to test eight vitrification apparatus (Figure 3.1): 20-µm mounted cryoloop™ (0.5 – 0.7 mm) (Hampton Research, Aliso Viejo, CA), capillary tubes (70 µL, Fisherbrand, Pittsburgh, PA), gel-loading pipette tips (Dot Scientific, Burton MI), 0.25-mL French straws (IMV international, Minneapolis, Minnesota), cut standard straws (0.25-mL French straw with a ~20° bevel cut at the end), 10-µL polystyrene loops (Nunc™, Roskilde, Denmark), and 5-mm nichrome loops (~ 15 µL) (Cole-Parmer, Vernon Hills, IL). The parameters that were evaluated were: efficiency of loading and unloading of samples, sample storage, sample volume, speed of cooling and warming, visualization of glass formation, sample labeling, and cost per sample.

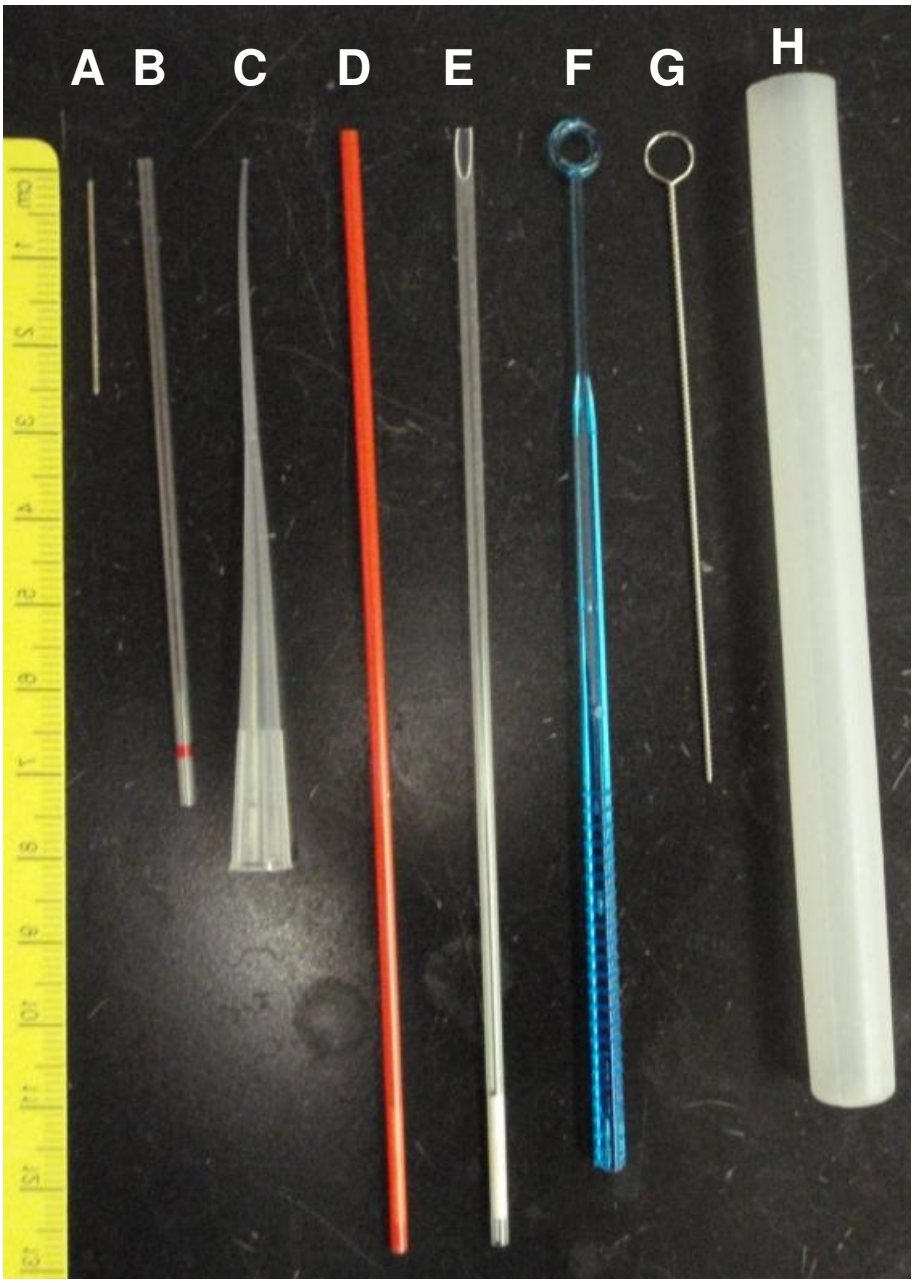

Figure 3.1. Different apparatuses were screened to choose the most suitable device for further experiments. The parameters evaluated were: ease of handling, loading and unloading of sample, storage, volume, speed of cooling and warming, visualization of glass formation, labeling, and cost. The apparatus were: (A) 20-µm cryoloop, (B) capillary tube, (C) gel-loading tip, (D) 0.25-mL straw, (E) cut standard straw, (F) 10-µL polystyrene loop, (G) 5-mm nichrome loop. The apparatus were stored in 12-cm goblets (H).

Procedures for Spawning, Thawing, and Fertilization

In the hatchery, fish are often injected with hormones to induce the final stages of oocyte maturation and to synchronize the readiness of multiple females for efficient stripping of eggs (Mylonas and Zohar 2007). Female channel catfish were obtained from Baxter Land Company Inc. and maintained at the Aquaculture Research Station. During 2007 the females were induced to ovulate using standard procedures (Lang and Tiersch 2007) by the intraperitoneal injection of 10 mg/kg of carp pituitary extract (lot numbers: 031109, 032209, 033109, Stoller Fisheries, Spirit Lake, IA) (Bosworth et al. 2005). From 2008 to 2010, ovulation was induced by intraperitoneal injection of 100 µg/kg of luteinizing hormone-releasing hormone analog (2008, 2009: Syndel International Inc., Canada; 2010: Argent Laboratories, Redmond, WA) (Bosworth et al. 2005). Final maturation of oocytes and time of ovulation was monitored by ultrasound (Novelo et al. 2011). Eggs were stripped by gentle abdominal pressure. Eggs from single females were separated into aliquots by placing a monolayer in a 100-mL tri-pour beaker (Fisher Scientific). Because egg size can vary, these aliquots had an average of 114 (± 23) eggs in 2007, 167 (± 15) eggs in 2008, 148 (± 19) eggs in 2009, and 146 (± 22) eggs in 2010.

Goblets containing cut standard straws or loops were removed from the liquid nitrogen canister and liquid nitrogen was allowed to drain from the goblets. Samples from Experiments 1 to 5 were thawed into conical tubes (15-mL, Corning, NY) containing 5 mL of HBSS300 warmed in a water bath at 40ºC. Samples from Experiment 6 were thawed into 1.5-mL microcentrifuge tubes containing 1 mL of HBSS300 warmed in a water bath at 40ºC (Appendix A, SOP-4). Tubes containing the straws or loops were gently agitated in the water bath for 10 sec and the suspensions were mixed with egg aliquots. Water from the hatching system (10 mL for Experiments 1-5, or 5 mL for Experiment 6) was added at the same time as the sperm suspension to activate the gametes and avoid heat shock. Egg quality was evaluated by using fresh sperm collected that day from males of the same population for fertilization of replicate egg batches (egg quality control). To evaluate the occurrence of gynogenesis (a form of parthenogenesis or uniparental reproduction), vitrification solution or warm HBSS300 (40°C) without sperm was added to aliquots of eggs in duplicate. The purpose of the gynogenetic controls was to be sure that chemical shock (vitrification solutions) or temperature shock (warm HBSS300) did not activate embryogenesis in unfertilized eggs by retention of the second polar body (Komen and

Thorgaard 2007). The percentage of embryos to reach neurulation (~30 h at 25°C) was used as a conservative index of fertilization (Tiersch et al. 1994). Neurulation corresponded to the optic-cup stage to pectoral fin-bud stage (Saksena et al. 1961) or early stage V (organogenesis) (Makeeva and Emel'yanova 1993). The neurulated embryos were counted by viewing with the naked eye using back illumination, and fertilization rate was expressed as the percentage of neurulated embryos in relation to the total number of eggs (referred to as "neurulation").

Vitrification Procedures

Experiment 1. Evaluation of Cryoprotectant-free Vitrification:

Two apparatuses were chosen: nichrome loops and cut standard straws. Sperm were collected from four males as described above and diluted to 1 x 10^9 sperm/mL with HBSS300. For vitrification with cut standard straws, 20 µL of sperm suspension were loaded into the cut end of five straws by use of a micropipette. No equilibration time was needed because there were no cryoprotectants involved. After loading, straws were placed inside a goblet (10-mm Visotube, IMV, L'Aigle, France) attached to a cane, and submerged in liquid nitrogen. The time for this process was ~2 min. For vitrification using nichrome loops, a film (~ 15 µL) of sperm sample was suspended inside the loop and individual loops were plunged into liquid nitrogen. The time required for loading and freezing for each loop was ~50 sec. After the film was frozen, the loop was placed in a goblet attached to a cane (five nichrome loops per goblet), and stored in liquid nitrogen.

Experiment 2. Evaluation of Two Apparatuses with a Single Cryoprotectant:

The apparatuses selected were polystyrene loops and cut standard straws, and MG was used as the cryoprotectant. Sperm were collected from four males as described above and diluted to 1 x 10^9 sperm/mL with HBSS300. Sperm suspensions were diluted 3:2 with the cryoprotectant solution to achieve final concentrations of 20% methyl glycol and 1.2 x 10^7 cells/mL for cut standard straws and 6 x 10^6 cells/mL for loops. Equilibration time was held to a minimum (i.e. <5 min). For these studies, we defined equilibration time as having two components: 1) time of exposure to cryoprotectants of the sperm samples before freezing, and 2) processing time from the first addition of cryoprotectant until plunging into liquid nitrogen. If a step-wise addition of cryoprotectants was used, exposure time could vary in each step. Cut standard straws (containing

20 µL of sample) and polystyrene loops (containing a thin film ~10 µL of sample) were submerged in liquid nitrogen as described above within 5 min of dilution with cryoprotectant. The samples in cut standard straws (five per goblet) and polystyrene loops (four per goblet) were stored in liquid nitrogen.

Experiment 3. Evaluation of Different Concentrations Using a Single Cryoprotectant:

Methanol was tested at final concentrations of 10% and 20% in HBSS300 using cut standard straws. Sperm suspensions from four individual males were vitrified using the procedure described above for MG and cut standard straws. The difference from Experiment 2 was that for 10% MeOH the sperm suspensions were diluted 4:1 with cryoprotectant solution.

Experiment 4. Evaluation of Higher Cryoprotectant Concentrations by Combination of Cryoprotectants in a Three-step Addition:

A combination of 5% MeOH, 10% MG, and 20% PROH was tested using nichrome loops. Sperm were collected from three males as described above and adjusted to a concentration of 1 x 10^9 sperm/mL with HBSS300. Dilutions of MeOH, MG and PROH in HBSS300 were prepared individually to yield a concentration of 40% each. The sperm suspension was diluted 1:1 with 40% MeOH and the exposure for this first addition was 2 min. Methyl glycol was added at a ratio of 1:1, and after 1 min PROH was added at a ratio of 1:1. A thin film of sample was suspended in individual nichrome loops, which were submerged into liquid nitrogen within 2 min of the addition of PROH (total equilibration time from the first addition was ~ 5 min). Loops were stored in goblets (five per goblet) attached to canes in liquid nitrogen.

Experiment 5. Evaluation of Higher Cryoprotectant Concentrations by Combining Cryoprotectants in a Two-step Addition:

Methanol at 10% in combination with 20% MG, and the proprietary glass formation enhancers 1% X-1000™, and 1% Z-1000™ (21^{st} Century Medicine, Fontana, CA) were tested in polystyrene loops and cut standard straws. Sperm were collected from three males as described above, and adjusted to 1 x 10^9 sperm/mL with HBSS300. Methanol was diluted in HBSS300 to yield a concentration of 40%. A double-strength cryoprotectant solution containing 40% MG, 2% X-1000™, and 2% Z-1000™ was prepared in HBSS300. The sperm suspensions were

diluted 1:1 with the 40% MeOH solution and the exposure for this first addition was 5 min. The cryoprotectant solution containing 40% MG, 2% X-1000™, and 2% Z-1000™ was added to the sperm suspension at a ratio of 1:1. Samples were immediately loaded into cut standard straws or polystyrene loops and individually submerged into liquid nitrogen within 1 min (~ 50 sec) of the addition of the cryoprotectant solution (total equilibration time from the first addition was ~ 6 min). Samples were stored in goblets (five cut standard straws or three loops per goblet) as described above.

Experiment 6. Evaluation of Vitrification Solutions in One-step Addition:

Three vitrification solutions were tested: 20% MeOH + 10% MG + 10% PROH; 20% MeOH + 20% MG; and 20% MeOH + 20% MG + 0.25 M trehalose. Sperm were collected from three males as described above and adjusted to 1 x 10^9 sperm/mL with HBSS300. Double-strength cryoprotectant solutions were prepared in HBSS300 and diluted with sperm suspension at a ratio of 1:1 (Appendix A, SOP-1). Samples were immediately loaded (within 15 sec) into polystyrene loops, and submerged in liquid nitrogen within 1 min (~ 50 sec) after the addition of the vitrification solutions (equilibration time, ~ 1 min) (Appendix A, SOP-2). Glass formation was assessed by observing the appearance of the vitrified sample (a milky appearance indicated ice crystal formation). Although direct visualization of vitrification resulted in a transparent form, some microscopic ice crystals could have been present. Loops were stored in goblets (three per goblet) in liquid nitrogen.

Assessment of Membrane Integrity

Sperm samples from three males were vitrified using two apparatuses: polystyrene loops, and 0.25-mL straws. Six treatments were evaluated: 1) no cryoprotectant, 2) 10% MeOH, 3) 20% MeOH, 4) 20% MG, 5) 20% MeOH + 10% MG +10% PROH, and 6) 20% MeOH + 20% MG. Cryoprotectant solutions were prepared at double-strength in HBSS300 and diluted 1:1 with sperm at 1 x 10^9 sperm/mL. Individual loops and straws were submerged into liquid nitrogen, within 1 min of addition of cryoprotectant solutions (equilibration time, ~ 1 min). The samples were stored in liquid nitrogen for 20 days before flow cytometry analysis. To thaw the sperm, each loop was warmed directly in 495 µL of HBSS300 at room temperature (24°C), and straws were thawed at 40°C for 5 sec. Thawed sperm from the straws was further diluted by adding 5

µL of sperm suspension to 495 µL of HBSS300. The sperm concentration for the diluted thawed samples was held between 5 x 10^6 to 1 x10^7 cells/mL.

To evaluate membrane integrity by flow cytometry, fresh and thawed sperm were filtered through 35-µm nylon mesh and duplicate aliquots of 250 µL were stained with the fluorescent dyes SYBR-14 and propidium iodide (PI) (live/dead sperm viability kit, Molecular Probes, Eugene, OR). Final concentrations of the fluorescent dyes were 100 nM SYBR-14 and 12 µM PI, and samples were incubated in the dark for 10 min at room temperature prior to analysis. Flow cytometry was performed using an instrument (C6 Accuri Cytometers Inc., Ann Arbor, MI) equipped with a 488-nm, 50-mW solid-state laser. Flow cytometer performance was assessed using fluorescent validation beads (Spherotech, Accuri Cytometers Inc.) to ensure that coefficient of variation values were < 3.0% (calculated based on full peak height) for the fluorescence detectors (FL1, FL2, FL3, and FL4). Each microcentrifuge tube was flicked gently 3 times with a finger prior to analysis to ensure suspension of the cells, and 10 µL of sample were analysed at a flow rate of 35 µL/min using CFlow® software (version 1.0.202.1, Accuri Cytometers Inc.). Green fluorescence (SYBR 14) was detected with a 530 ± 15 nm bandpass filter (FL1), and red fluorescence (PI) was detected with a >670 nm longpass filter (FL3). Events were viewed on forward-scatter (FSC) vs. side-scatter (SSC) plots, and a gate (used to define target cells within the total event population) was drawn around the sperm population to exclude non-sperm events. Gated events were viewed on a scatter plot showing FL1 vs. FL3 with fluorescence compensation based on the computed median fluorescence values of the different populations to reduce spectral overlap. Sperm that stained with SYBR 14 alone were considered to have an intact membrane, and those that stained with both SYBR 14 and PI or PI alone were considered to be membrane-compromised.

Statistical Analysis

The fixed treatment variables were: cryoprotectant (T), concentration (C), and incubation time (I). Acute toxicity data were analyzed as a factorial (5T x 4C x 8I) randomized block design. The channel catfish males were grouped in a block to remove variation among individual motility from the error term. The dependant variable was sperm motility (%). The control (fresh sperm) was excluded from the model, but was used as a reference to ensure sperm viability. Analysis

was conducted using a mixed ANOVA procedure for all interactions among T, C, and I. Membrane integrity data were analyzed using a mixed ANOVA procedure with apparatus and cryoprotectants as fixed treatments and membrane intact (%) as a dependent variable. The control (fresh sperm) was excluded from the model, but was used as a reference for viable sperm. Statistical differences were determined at an $\alpha = 0.05$ level using Tukey's adjustment. Statistical analyses were performed using SAS software (Statistical Analysis System Inc., version 9.1; SAS Institute, Cary, NC).

Results

Acute Toxicity of Cryoprotectants

When low concentrations (5%) of cryoprotectants were used, there was no difference ($P = 0.52$) in the type of cryoprotectant, and the sperm motility remained high (40-60%) (Figure 3.2). When the concentrations were increased to 10%, two cryoprotectants (MeOH and MG) were the least toxic with no difference between them ($P = 0.25$), and motility remained high (~50%). Time played a key role in the toxicity of cryoprotectants. When 10% was used, motility did not change ($P = 0.96$) within the first 10 min of exposure for each cryoprotectant used. After 10 min, two groups of cryoprotectants could be distinguished. The first group (MeOH and MG) was the least toxic (~50% motility), while the second group (DMSO and PROH) was more toxic (<20% motility). After the cryoprotectant was increased to 20% (around half of the concentration needed for vitrification) motility was reduced in all treatments (Figure 3.2). At 5 min after the addition of 20% of cryoprotectants, the average motility for MeOH was 33 ± 3%, followed by MG (22 ± 12%). Sperm in the other cryoprotectants (DMSO, DMA, and PROH) had low motilities (< 10%). When the cryoprotectants were increased to 30% all ($P = 0.69$) motility was eliminated within 10 min (Figure 3.2).

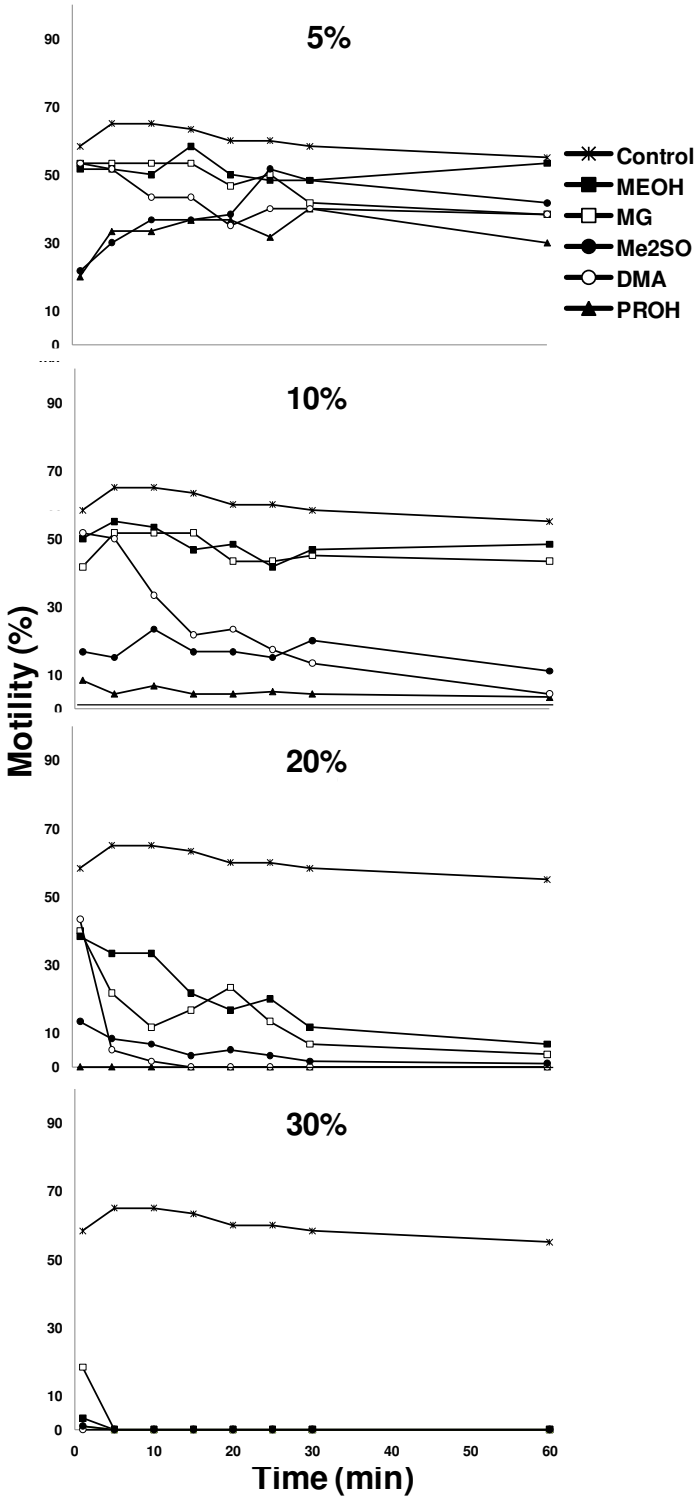

Figure 3.2. Acute toxicity to sperm of channel catfish of 5, 10, 20 and 30% of five cryoprotectants. Each point represents the mean of three replicates. The cryoprotectants used were, methanol (MeOH), methyl glycol (MG), dimethyl sulfoxide (DMSO), dimethyl acetamide (DMA), and propanediol (PROH).

Vitrification Device Configurations

From the pilot study, it was determined that each device had unique characteristics that influenced their suitability and practicality for use in vitrification. The volume held by the 20-µm cryoloop™ (< 1 µL) was too small to be practical for use in fertilization trials for channel catfish.

Expulsion of samples from the gel-loading tips and capillary tubes was difficult because of the high viscosity of the vitrification solutions. A device that proved to be advantageous was the cut standard straws, which offered the standard advantages of the straws (e.g. ease of labeling, handling, and storage) while decreasing the sample volume for faster cooling, and the cut edge provided easy loading and unloading of the small volumes. The nichrome and polystyrene inoculation loops also proved to be effective and easy to use. The polystyrene loops provided the additional advantage that they could be easily cut to lengths that would fit into goblets. Therefore, the vitrification apparatus chosen for further study were the cut standard straw, nichrome loop, and polystyrene loop.

Vitrification Procedures

Experiment 1. Evaluation of Cryoprotectant-free Vitrification:

Some twitching and vibration of sperm was observed after thawing, but no true progressive post-thaw motility was observed in any experiments. Mean neurulation (fertilization) for all experiments was low. None of the gynogenetic controls produced fertilization. Cryoprotectant-free vitrification in nichrome loops did not yield fertilization, and cryoprotectant-free vitrification in cut standard straws yielded low levels (<2%) of fertilization in 2 of 16 trials (Table 3.3).

Experiment 2. Evaluation of Two Apparatuses Using a Single Cryoprotectant:

Cut straws yielded more reproducibility (neurulation was observed in 16 of 16 trials) than polystyrene loops (neurulation in 13 of 16 trials) (Table 3.3). In addition, mean neurulation values were higher (~5%) for sperm vitrified in cut standard straws than for those in polystyrene loops, but were still low. This higher fertilization rate could have been related to the larger volume (20 µL vs. 10 µL) held by cut standard straws that translated into more sperm per egg (Table 3.3).

Table 3.3. Apparatus, cryoprotectants, sperm number per container, number of trials, and mean fertilization used for vitrification experiments in channel catfish performed from 2007 to 2010. Apparatus used were nichrome loops (15 µL), cut standard straws (20 µL), and polystyrene loops (10 µL). Fertilization was expressed as percentage of neurulated embryos in relation to the total number of eggs.

		Neurulation			
Apparatus	**Cryoprotectant**	**by # trials**	**Mean ± SD[a]**	**Sperm per container**	**Sperm-to-egg ratio**
Experiment 1 (4 males; 4 females)					
Nichrome loop	None	0 of 16	0	1.5×10^7	6.4×10^5
Cut standard straw[b]	None	2 of 16	2 ± 1	2.0×10^7	8.4×10^5
Control	–	32 of 32	56 ± 19	1.0×10^8	8.4×10^5
Experiment 2 (4 males; 4 females)					
Polystyrene loop	20% MG[c]	13 of 16	2 ± 1	6.0×10^6	2.0×10^5
Cut standard straw	20% MG	16 of 16	5 ± 2	1.2×10^7	5.1×10^5
Control	–	32 of 32	56 ± 19	1.0×10^8	8.4×10^5
Experiment 3 (4 males; 4 females for 10%MeOH and 2 females for 20%MeOH)					
Cut standard straw	10% MEOH[d]	11 of 15	3 ± 3	1.6×10^7	6.0×10^5
Cut standard straw	20% MeOH	5 of 8	9 ± 5	1.2×10^7	4.5×10^5
Control	–	8 of 8	65 ± 9	1.0×10^8	7.5×10^5
Experiment 4 (3 males; 1 female)					
Nichrome loop	5%MeOH + 10%MG + 20%PROH[e]	7 of 9	4 ± 3	1.9×10^6	6.1×10^4
Control	–	9 of 9	21 ± 7	1.0×10^8	6.4×10^5
Experiment 5 (3 males; 2 females)					
Polystyrene loop	10%MeOH+20%MG +1%X[f] + 1%Z[g]	8 of 12	3 ± 2	2.5×10^6	5.4×10^4
Control	–	18 of 18	21 ± 7	1.0×10^8	7.1×10^5
Cut standard straw	10%MeOH+20%MG +1%X + 1%Z	14 of 18	4 ± 3	5.0×10^6	1.6×10^5
Control	–	16 of 16	51 ± 9	1.0×10^8	6.6×10^5
Experiment 6 (3 males; 2 females)					
Polystyrene loop	MMP[h]	5 of 6	11 ± 11	5.0×10^6	9.1×10^4
Polystyrene loop	MEMG[i]	5 of 6	4 ± 3	5.0×10^6	9.1×10^4
Polystyrene loop	MEMGT[j]	4 of 6	4 ± 2	5.0×10^6	9.1×10^4
Control Female 1	–	9 of 9	83 ± 5	1.0×10^8	6.1×10^5
Polystyrene loop	MMP	2 of 6	3 ± 2	5.0×10^6	1.2×10^5
Polystyrene loop	MEMG	3 of 6	2 ± 1	5.0×10^6	1.2×10^5
Polystyrene loop	MEMGT	4 of 6	1 ± 1	5.0×10^6	1.2×10^5
Control Female 2	–	8 of 9	17 ± 4	1.0×10^8	7.8×10^5

[a]Mean and standard deviation from the samples that had neurulation; [b]Cut standard straws (0.25-mL); [c]Methyl glycol; [d]Methanol; [e]1,2-propanediol; [f]X-1000™; [g]Z-1000™;
[h]20%MeOH + 10%MG + 10% PROH; [i]20%MeOH + 20%MG;
[j]20%MeOH + 20%MG + 0.25 M Trehalose.

Experiment 3. Evaluation of Different Concentrations Using a Single Cryoprotectant:

Higher concentrations of cryoprotectants yielded higher mean neurulation values in all experiments. Comparison of fertilization with sperm vitrified in 10% and 20% MeOH in cut standard straws showed that 10% yielded reproducibility with neurulation observed in 11 of 15 trials (Table 3.3).

Experiment 4. Evaluation of Higher Cryoprotectant Concentrations by Combination of Cryoprotectants in a Three-step Addition:

Neurulation in the fresh sperm control for Experiment 4 was low (~21%) indicating poor egg quality. The vitrification solution (5% MeOH + 10% MG + 20% PROH) formed almost completely transparent glass (~80% by visual assessment). Although the glass was clearer when the solution was vitrified in a single step than after the stepwise additions, there was no benefit in adding the cryoprotectants in different steps, as the neurulation remained low (<10%) in all experiments (Table 3.3).

Experiment 5. Evaluation of Higher Cryoprotectant Concentrations by Combining Cryoprotectants in a Two-step Addition:

The egg quality assessed using fresh sperm for fertilization was low (< 50% neurulation). Glass formation was enhanced by the use of polymers (X-1000™ and Z-1000™) with the observation of an almost complete transparency (~90% by visual assessment). There was no benefit in adding the cryoprotectants in a two-step addition (Table 3.3), as neurulation remained low (<10%) for the two-step addition using either polystyrene loops or cut standard straws.

Experiment 6. Evaluation of Vitrification Solutions in One-step Addition:

Complete glass formation was observed in 20% MeOH + 10% MG + 10% PROH, 20% MeOH + 20% MG, and 20% MeOH + 20% MG + 0.25 M trehalose treatments vitrified in loops (Figure 3.3). The combination of cryoprotectants was difficult to evaluate due to poor egg quality, demonstrated by the low neurulation with fresh sperm. The egg quality had a direct relationship with the fertilization trials. For example, 20% MeOH + 10% MG + 10% PROH treatment for Female 1 yielded a mean neurulation of 11% while Female 2 yielded 3%. With fresh sperm, eggs from Female 1 had significantly higher neurulation (83%) than did Female 2

(17%) ($P < 0.001$). The highest neurulation rates (as % success) were observed using 20% MeOH + 10% MG + 10% PROH in polystyrene loops (as high as 25%), followed by 20% MeOH using cut standard straws (as high as 15%).

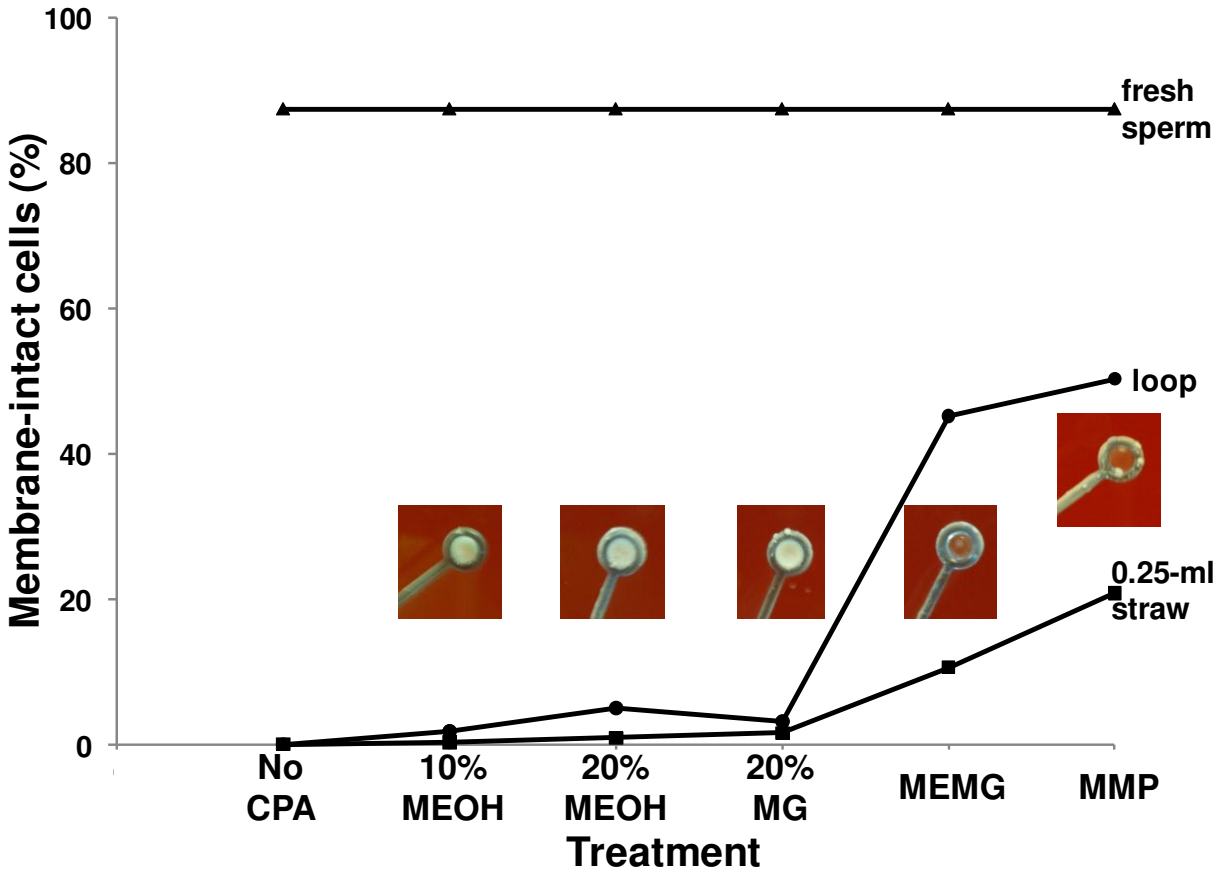

Figure 3.3 Membrane integrity of thawed sperm from three channel catfish as determined by flow cytometry. Sperm were cryopreserved in 10-µL polystyrene loops or 0.25-mL straws (250 µL sample volume). The treatments used were cryoprotectant-free (No CPA), 10% methanol (10% MeOH), 20% methanol (20% MeOH), 20% methyl glycol (20% MG), 20% methanol + 20% methyl glycol (MEMG), and 20% methanol + 10% methyl glycol + 10% propanediol (MMP). Assessment of ice crystals (milky color) or glass formation (clear) was evaluated visually for the loops with different treatments. Individual cryoprotectants with <20% concentration appeared milky indicating ice crystal formation, while the mixture of cryoprotectants (final concentration 40%) appeared transparent indicating glass formation.

Assessment of Membrane Integrity

There was a significant difference ($P < 0.001$) between loops and 0.25-mL straws in the percentage of membrane-intact sperm after vitrification. There was no significant difference ($P > 0.05$) among cryoprotectant-free vitrification and vitrification using 10% MeOH, 20% MeOH, and 20% MG. Membrane integrity using the vitrification solutions 20% MeOH + 20% MG and 20% MeOH + 10% MG + 10% PROH in a one-step addition was significantly higher ($P < 0.001$) than for the other treatments. The highest percentage of membrane-intact sperm was for those vitrify in 20% MeOH + 10% MG + 10% PROH in loops (50 ± 4%) followed by 20% MeOH + 20% MG in loops (45 ± 6%). In contrast, the percentage of intact sperm was lower for 20% MeOH + 10% MG + 10% PROH (21 ± 7%) and 20% MeOH + 20% MG in straws (11 ± 8%). There was no significant difference ($P = 0.8$) between the vitrification solutions 20% MeOH + 10% MG + 10% PROH in loops and 20% MeOH + 20% MG in loops. In general, as the cryoprotectant concentration increased, yielding glass-forming solutions, the membrane integrity also increased (Figure 3.3).

Discussion

The technique used in general to attain vitrification is rapid non-equilibrium cooling, which differs from traditional slow-cooling cryopreservation protocols in that dehydration and cryoprotectant permeation take place before cooling begins (Shaw and Jones 2003). This phenomenon can be enhanced by the use of high concentrations of cryoprotectants (40 to 60%) and an increase in cooling rate (>1,000°C/min) (Leibo 1989, Leibo and Songsasen 2002, Shaw and Jones 2003), although neither high cryoprotectant concentration nor increased cooling rates are essential for vitrification to occur. Partial or total intracellular vitrification can occur during conventional equilibrium cooling, and may be responsible for some degree of survival of cryopreserved samples (Vajta et al. 2009). Vitrification is now widely used to cryopreserve oocytes and embryos of several mammalian species (Vajta and Nagy 2006). In fishes, vitrification has been applied to cryopreservation of embryos, although results from some of these studies have been controversial (Edashige et al. 2006). Embryo survival has been reported but not successfully replicated in zebrafish (Chao et al. 1997), Japanese seaperch (*Lateolabrax japonicas*) (Tian et al. 2003), winter flounder (*Pseudopleuronectes americanus*) (Robles et al.

2005), and Japanese flounder (*Paralichthys olivaceus*) (Chen and Tian 2005). The limited success in fish embryo vitrification could be due to the large size (> 1 mm in diameter), complexity, and low permeability of the multicellular embryo, which could inhibit the entrance of cryoprotectants into the various compartments (Robles et al. 2009, Zhang 2004). With the exception of two studies in which the investigators observed vitrification in fish sperm by use of cryomicroscope (Andreev et al. 2009) or evaluated motility, membrane and mitochondrial integrity (Merino et al. 2011), to our knowledge there have been no studies that specifically addressed fish sperm vitrification or intended to develop streamlined protocols, although some cryopreservation studies have coincidentally produced vitrification (Table 3.1).

Acute toxicity of Cryoprotectants

Choosing the least toxic permeable cryoprotectant is one of the first steps in developing a cryopreservation protocol. Cryoprotectant toxicity and osmotic effects can be seen as limiting factors for cryopreservation by slow-cooling and vitrification (Fahy et al. 1987). The toxicity of a cryoprotectant is related to its concentration, the duration of exposure, and temperature. Most cryoprotectants tend to have toxic and hypertonic effects when used at concentrations that are effective for successful vitrification (Yavin and Arav 2007).

Cryopreservation studies in channel catfish began in the 1970s (Guest et al. 1976), and previous studies of acute cryoprotectant toxicity in channel catfish sperm evaluated low concentrations (< 15%), and long exposure times (> 10 min to days) (Christensen and Tiersch 1997, Tiersch et al. 1994). The high concentrations of cryoprotectant used in vitrification required a new evaluation of acute toxicity and shorter exposure times. Concentrations greater than 20% for six cryoprotectants were evaluated in this study and were found to be toxic to sperm. Methanol (5 M) and methyl glycol (2.6 M) at a concentration of 20% were the least toxic with an exposure time of less than 5 min. Methanol at 10% was the cryoprotectant of choice for channel catfish cryopreservation in previous studies (Christensen and Tiersch 2005). Methyl glycol has been used to cryopreserve sperm from some freshwater fishes (Maria et al. 2006, Viveiros and Godinho 2009), but this is the first study to evaluate methyl glycol as a cryoprotectant for channel catfish sperm. One possible reason that methanol and methyl glycol yielded higher survival at higher concentrations is because they produce less osmotic damage

than other cryoprotectants. The osmolality of methanol and methyl glycol at 20% in HBSS300 is close to 300 mOsmol/kg (the osmolality of HBSS300) (Tiersch et al. 1994), while the rest of the cryoprotectants tested in this study at 20% had osmolalities of greater than 1,500 mOsmol/kg.

In general, sperm from freshwater fishes become motile in response to a reduction of osmotic pressure (hypotonic to blood plasma) while sperm from marine fishes become active with an increase of osmotic pressure (hypertonic, sea water > 1,000 mOsmol/kg) (Tiersch et al. 2007). Activation of sperm motility in channel catfish occurs in the range of 35 – 270 mOsmol/kg, with complete activation occurring at 132 mOsmol/kg and below (Bates et al. 1996). Sperm from channel catfish are not adapted to deal with high osmotic pressures and this could explain the low survival observed at higher concentrations of some cryoprotectants.

Cryoprotectant-free Vitrification

Sperm cells are damaged primarily due to osmotic (solution) effects at slow cooling rates and intracellular ice formation at high cooling rates. These paired observations taken together are termed the "two-factor hypothesis" or "Oak Ridge curve" (Leibo and Mazur 1971). Recent publications have stated that there was no evidence of intracellular ice formation in human or horse sperm cooled at 3,000°C/min, and it was proposed that the cell damage observed was a result of an osmotic imbalance encountered during thawing (Morris 2006, Morris et al. 2007). Intracellular vitrification can be achieved relatively easily in cells such as spermatozoa because of their small size and high content of soluble macromolecules (such as proteins and sugars) that make the intracellular matrix highly viscous compared with oocytes and embryos (Isachenko et al. 2003, Isachenko et al. 2007a). Early attempts to vitrify mammalian spermatozoa resulted in low or no survival (Smith 1961). However, a breakthrough came in 2002, when human sperm were vitrified without conventional cryoprotectants by cryopreserving samples in thin films in copper cryoloops (Nawroth et al. 2002). The idea of using loops dated back to 1942, when human sperm were vitrified in films on wire loops by plunging into liquid nitrogen, resulting in sperm survival as high as 67%, but "negative results" were obtained with sperm from rat, mouse, guinea pig, rabbit, and bull (Hoagland and Pincus 1942). In another experiment, platinum loops were used to vitrify human sperm without cryoprotectants, but no motile sperm were observed after thawing (Parkes 1945). It has been suggested that survival of vitrified sperm without

cryoprotectants could be due to the presence of large amounts of osmotically inactive water bound to macromolecular structures, such as DNA and histones, or the presence of high molecular weight components in sperm that affect the viscosity and glass transition temperature of the intracellular cytosol (Isachenko et al. 2007a, Rama Raju et al. 2006).

Compared to those of mammals, fish sperm are small; for example, in most externally fertilizing teleost species the length of the sperm nucleus is < 5 µm, and the midpiece length is 2 to 4 µm (although the flagellum is 30 - 40 µm or longer) (Lahnsteiner and Patzner 2008). Attempts to cryopreserve fish sperm samples at slow cooling rates without cryoprotectants have yielded low survival (~1% post-thaw motility cooled at 40°C/min) (Christensen and Tiersch 1997) or no survival (Chao et al. 1975, Sneed and Clemens 1956, Wang et al. 2010, Yao et al. 2000), most likely due to the large sample volume (>0.25 mL) and slow rate of cooling which lead to injuries by long exposure to concentrated solutions (i.e. the solution effect). Attempts have been made to plunge samples into liquid nitrogen to increase the cooling rate, although none of these publications made reference to vitrification (Table 3.1). One previous study in which ampoules (0.2 – 0.5 mL) of undiluted sperm of Pacific oyster (*Crassostrea gigas*) were plunged into liquid nitrogen without use of cryoprotectants reported fertilization rates as high as 40% (Hwang and Chen 1973) although total vitrification was not a likely result given the large volume and use of ampoules.

In the present study, we evaluated a method reported for human sperm (Nawroth et al. 2002), using small volumes in loops plunged into liquid nitrogen without cryoprotectant. Fresh human sperm vitrified in this matter without addition of media yielded motilities of ~20% after warming (Nawroth et al. 2002). In the present study, two apparatuses were used for cryoprotectant-free sperm vitrification in channel catfish. Vitrification using loops did not yield neurulation, and low rates were also observed using cut standard straws. The loops that were used in this experiment (5 mm; 15 µL) were similar to the ones used for humans (5 mm; 20 µL) (Nawroth et al. 2002), and to our knowledge there are no previous publications that used cut standard straws to vitrify sperm. A similar method (open straw) was described previously to vitrify human sperm by adding 1 µL to the open end of a 0.25-mL straw, which was placed inside a 90-mm straw that was hermetically sealed (Isachenko et al. 2005). In the present study, cryoprotectant-free

vitrification using a 20-µL sample in cut standard straws yielded low fertilization (~2%) in two trials. Cut standard straws have been used to vitrify human blastocysts (Isachenko et al. 2007b), and they are easy to work with and can be used for different sample volumes. In addition, if there are concerns about cross-sample contamination, the cut standard straws can be inserted into a 0.5-mL straw that can be closed at both ends (Isachenko et al. 2007b). Recently sperm from rainbow trout (*Oncorhynchus mykiss*) were vitrified without cryoprotectants using the microdrop (20 µL) method (Merino et al. 2011). Motility and membrane integrity after vitrification ranged from 70 to 90%. Although no fertilization attempts were made, it was the first report of cryoprotectant-free vitrification in fish sperm.

Previous attempts in twenty studies to cryopreserve sperm from aquatic species by plunging them into liquid nitrogen have produced inconsistent results (Table 3.1). While one study using sperm from the Pacific herring (*Clupea pallasi*) yielded fertilization (assessed by neurulated embryos) as high as 95% (Pillai et al. 1994), the majority of the studies have been unsuccessful. Complete vitrification was not attained in the previous studies because of the low concentrations of cryoprotectant (<30%; most <15%), and large volumes (0.25 to 5 mL) used. For example, in the Pacific herring study, DMSO was used at 15% with 1.8-mL polypropylene microcentrifuge tubes. The minimum concentration of DMSO in 0.25-mL straws that will vitrify when cooled by plunging into liquid nitrogen is 39% (Ali and Shelton 2007). This indicates that that study did not result in complete vitrification. The high fertilization in the Pacific herring study was probably due to partial intracellular vitrification, because sperm from marine fishes have greater survival (as high as 80% vs. ~50% for freshwater fishes) during cryopreservation (Kopeika et al. 2007), and because of chemotaxis where the sperm are not active in seawater but become activated when coming in contact with the egg chorion (Morisawa 2008). Chemotaxis is an important factor in egg fertilization because herring eggs contain proteins that facilitate the union of the gametes. The high fertilization of the previous study could be explained by the unique fertilization strategy employing these proteins that guide the sperm into the micropyle (an opening were immersed in the egg chorion). The low success reported in most other studies in which samples were immersed into liquid nitrogen is likely due to a combination of insufficient cryoprotectant concentration, long pre-freeze exposure times, large sample volumes, and use of containers that inhibit heat transfer which translates into slow cooling rates (Table 3.1). Thus,

although using a potentially rapid cooling method (plunging in liquid nitrogen), none of these studies were designed to directly address vitrification. One study used a high cryoprotectant concentration (40% EG) that had the potential to achieve glass formation, but the exposure time was long (2 h), and the sperm likely experienced damage due to cryoprotectant toxicity before cooling began (Truscott et al. 1968). Recently, one study attained an ultra-rapid cooling rate (3,000 – 4,000°C/min) by using 10% DMSO and 10 µL samples of sperm from rainbow trout and Russian sturgeon (*Acipenser gueldenstaedtii*) in a 100-µm thick glass cell ("thin-layer freezing"). No ice crystals were observed by examination with a cryomicroscope. Sturgeon sperm cryopreserved by this method resulted in 90% egg fertilization (fresh sperm was defined as 100%) (Andreev et al. 2009). Although the focus of that study was to evaluate the formation of ice microparticles at different cooling rates with the use of different additives (i.e., egg yolk, sugars, and lipids), it nonetheless produced vitrification. That report focused on basic research rather than protocol development, and lacked practical details such as a description of the cooling method, post-thaw motility, fertilization assessment methods, percentage of fertilization from the control group, and number of females used.

In general in the present study, the use of low concentrations of cryoprotectants yielded low fertilization, while use of vitrification solutions containing high cryoprotectant concentrations increased fertilization. The concentration of cryoprotectants needed for vitrification of mammalian embryos is high (> 40%) and near the maximum tolerated by these cells (Mazur et al. 2008). There are a number of ways to reduce the concentration of individual cryoprotectants required for vitrification. For example, the application of high hydrostatic pressure, addition of non-permeating polymers or agents, combination of cryoprotectants, stepwise addition of cryoprotectants, and limiting of exposure time at high concentrations to a minimum (Fahy et al. 1984). In this study, the two most suitable cryoprotectants, based on acute toxicity (methanol and methyl glycol), were mixed to obtain additive effects of each agent. Methanol has a high rate of permeability and relatively low toxicity, but almost pure methanol (99.8%) exhibits little or no vitrification (Ali and Shelton 2007), while methyl glycol is considered a good glass former (it will vitrify at 40%) and may be useful in vitrification solutions (Robles et al. 2005). In addition to combining the cryoprotectants, addition of proprietary polymers such as X-1000™ and Z-1000™ has been used to inhibit ice formation (Wowk and Fahy 2002, Wowk 2005) and was able to enhance glass formation at lower concentrations of cryoprotectants in the present study.

Despite this, the neurulation rates of sperm vitrified using these polymers remained low in the present study. Similarly, trehalose has been used to cryopreserve sperm in fish species (Miyaki et al. 2005, Sean-in et al. 2009), but in this study did not improve the fertilization success of vitrified samples. Another way to reduce osmotic damage when using high concentrations is by stepwise addition of the cryoprotectants. In this study, three different addition methods were evaluated, but fertilization rates were low in all trials, suggesting that there was no advantage in adding the cryoprotectants in successive steps.

Overall, the highest neurulation obtained (25%) was from a mixture of three cryoprotectants (20% MeOH + 10% MG + 10% PROH) with a single-step addition. The reasons for this were most likely that the mixture reduced the aggregate toxicity of the cryoprotectants (although total osmolality was 1,700 mOsmol/kg), but the exposure time was held to a minimum (< 1 min). This was reflected in the flow cytometry data from which the highest membrane integrity using loops was for this cryoprotectant mixture (~50%). In addition, variation in the fertilization of eggs from two females with sperm vitrified using the same mixture of cryoprotectants emphasized the importance of egg quality, which when higher than 50% neurulation (in fresh sperm controls) showed fertilization for vitrified samples. Another variable in Experiment 6 was the use of a volume (5 mL) of activation solution that was lower than in the other experiments (10 mL). Lower volumes allowed more sperm contact with the egg, and a higher effective sperm-to-egg ratio.

Assessment of Membrane Integrity

Membrane integrity is a commonly used assay to estimate viability of cryopreserved sperm (Graham and Mocé 2005). Recognition of the benefits of evaluating plasma membrane functionality in fish sperm cryopreservation dates back to 1966 (Fribourgh 1966). Since then different protocols have been tested in aquatic organisms (Robles et al. 2008). The most common fluorophore combination used is SYBR-14 and PI (Martínez-Pastor et al. 2010), which provides simultaneous information on the proportions of membrane-intact and membrane-compromised cells. This method can be analyzed by fluorescence microscopy or by flow cytometry (Martínez-Pastor et al. 2010) that is a precise, sensitive, accurate, and rapid method of multiparameter, single-cell analysis (Mahfouz et al. 2009). Previous studies in human sperm vitrification have

evaluated DNA integrity, mitochondrial activity, and acrosomal status by use of flow cytometry. These studies concluded that sperm vitrification was similar to or better than standard cryopreservation (Isachenko et al. 2004a, Isachenko et al. 2008, Isachenko et al. 2004b).

In the present study, we evaluated membrane integrity as an additional means of assessing the effectiveness of vitrification. The first hypothesis that was tested was whether glass formation improved the survival of the sperm. The highest neurulation rate (25%) was observed using sperm cryopreserved with the vitrification solution containing 20% MeOH + 10% MG + 10% PROH, but some fertilization (≤ 15%) was observed using sperm frozen with a relatively low cryoprotectant concentration (20% MeOH). By assessing membrane integrity at different concentrations of cryoprotectants, we observed a direct relationship between viability and cryoprotectant concentration. In general, increasing the concentration of cryoprotectants led to more glass formation and increased sperm viability. This can be explained because vitrification can be partial or total. Total vitrification includes the glass formation of the whole sample including the extracellular and intracellular fractions, while partial vitrification includes the glass formation of intracellular water (Vajta and Nagy 2006, Vajta et al. 2009). In this study, total vitrification (e.g. complete transparency) yielded higher survival. A study of vitrification of primordial germ cells in zebrafish concluded that there was no marked relationship between the appearance of ice formation during cooling and the cell survival rate (Higaki et al. 2010b). But in a subsequent study, ice crystal formation lowered the survival of cells (to 1-26%) compared to glass formation (80%) (Higaki et al. 2010a).

The second hypothesis tested was whether there was any difference in vitrification using the same concentrations of cryoprotectants but different sample volumes. We compared the membrane integrity of sperm vitrified using loops (10 µL) and 0.25-mL straws (250 µL). When vitrification solutions were used, the membrane integrity of sperm in loops was more than twice that of sperm vitrified in straws. This is most likely because the smaller volumes had faster cooling and warming rates, and lower concentrations of cryoprotectants were required to achieve glass formation (Vajta and Nagy 2006). Increasing the concentration of cryoprotectants could improve vitrification in straws, but it is possible that the increased toxicity may not be tolerated by the sperm.

Conclusions

This report demonstrates the feasibility of using vitrification for fish sperm. Overall, fertilization (neurulation) values were low, and thus while the current technique could be used to reconstitute lines (especially in small aquarium fishes), it would require improvement and scaling up before being useful as a production method for large-bodied fishes such as catfish. Recently, vitrification has been used in to cryopreserve fish blastomeres (Cardona-Costa and Garcia-Ximenez 2007), testicular cells (i.e. spermatogonial stem cells) (Bono-Mestre et al. 2009), and primordial germ cells by whole- embryo freezing (Higaki et al. 2009, Higaki et al. 2010b). In addition, live fish have been produced by transplanting primordial germ cells recovered from vitrified embryos (Higaki et al. 2010a). This illustrates the importance and potential applications of vitrification as a feasible cryopreservation method. Vitrification is a simple, fast, and inexpensive method for preserving genetic resources that does not require equipment, and can be performed in the field. Because of the minute volumes needed to attain ultra-rapid cooling, vitrification is best suited for small fish or fishes that yield only small volumes of sperm.

Future work in sperm vitrification should focus on small (aquarium) fish, some of which (e.g. zebrafish) are extremely important and are widely used as biomedical models, and many of which are highly endangered and are typically overlooked in conservation programs. According to the International Union for Conservation of Nature and Natural Resources (IUCN) Red List, 65% of the ray-finned fish (Class Actinopterygii) listed as imperiled are less than 20 cm in length. Sperm vitrification could offer a new option for conservation biology in imperiled aquatic species. Samples can be preserved in remote locations such as on the riverbank or a boat, in a remote fish hatchery, or in developing countries, without the need for sophisticated procedures or equipment. In addition, vitrification could be a useful technique to preserve the genetic resources from aquatic model organisms. Laboratories around the world have produced tens of thousands of mutant, transgenic, and wild-type fish lines. Maintaining these valuable genotypes as live populations is expensive, risky, and beyond the capacity of even the largest stock centers (NCRR-NIH 2007). Currently more than 20,000 lines of zebrafish require preservation in

germplasm repositories (http://zfin.org) (Yang and Tiersch 2009). Vitrification could assist this process, and offers opportunities for gene banking of other materials such as oocytes, embryos, larvae, and stem cells not possible with conventional cryopreservation. This work provides a model for development of generalized protocols for sperm of aquatic species and that could be integrated into a standardized approach for vitrification of germplasm of aquatic species.

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Chapter 4*

Production of F_1 Offspring with Vitrified Sperm from a Live-bearing Fish, the Green Swordtail *Xiphophorus hellerii*

Aquarium fish models, such as zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), and *Xiphophorus*, have provided useful tools for the study of human diseases. Fishes are one of the few vertebrate model systems that can be used for high-throughput bioassays while at the same time providing physiologically relevant data derived from a whole organism (Lam and Gong 2010). For example, the use of *Xiphophorus* fishes in cancer research dates back to the 1920s when it was discovered that certain hybrids of platyfish (*X. maculatus*) and green swordtail (*X. hellerii*) develop melanomas spontaneously (Gordon 1927). Models of spontaneous and induced carcinogenesis for several tumor varieties can be produced by selective backcrossing among the 27 described *Xiphophorus* species. The *Xiphophorus* Genetic Stock Center (XGSC, www.xiphophorus.org) maintains more than 57 pedigreed lines. Several of these lines have been inbred in the XGSC for more than 50 years (Walter et al. 2006b). In addition to cancer research, *Xiphophorus* fishes are also used in other fields of study including evolution, behavioral ecology, sex determination, and for bio-geographical systematic molecular events leading to speciation (recently reviewed by Walter 2011). Furthermore, the genome (~830 MB) of *X. maculatus* (Jp 163 A strain), in its 109th generation of inbreeding, has recently been sequenced and a draft assembly produced (R. Walter, personal communication). This allows study of genetic regulation at the molecular level (Shen et al. 2011).

Because of the short generation time, high fecundity and easy maintenance of large numbers in a relatively small space, several thousand mutants and transgenic lines of oviparous aquarium fish models have been produced (Walter 2001, Hagedorn et al. 2009). Similar to the husbandry space limitations encountered in mouse breeding facilities, the large number of strains increases the cost of labor and maintenance of these facilities and is becoming overwhelming (Knight and Abbott 2002). Thus, there is a risk that many valuable strains could become lost and presents an immediate need to improve preservation of genetic resources from aquarium fish

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models (Yang and Tiersch 2009). Furthermore, many wild populations of these fishes have become imperiled. For example, human activities have negatively affected the natural habitats of *Xiphophorus* fishes leading to the decline of wild populations (Borowsky and Kallman 1991). Six species of this genus are imperiled, four of which are classified as severely endangered (*X. couchianus*, *X. gordoni*, *X. meyeri*, and *X. milleri*) (Jelks et al. 2008). Although population studies on these species in the wild are far from complete, conservation efforts need not be delayed while awaiting more thorough assessments. Cryopreservation is a technique that may be employed to address the need for preservation of these valuable research lines and for restoration or protection of imperiled species (Tiersch 2001).

Sperm cryopreservation efforts in *Xiphophorus* confront significant challenges. *Xiphophorus* are characterized by a small body size (2-4 cm), and fertilization is internal so artificial insemination is needed for fertility estimation of cryopreserved sperm. In addition, sperm sample availability is limited. In *X. hellerii* the maximum sperm volume available was calculated to be 9.2 µL (Huang et al. 2004b), while for *X. couchianus* the maximum volume was < 5 µL (Huang et al. 2004a). Small (µL) sample volume limits experimental replication and the numbers of treatments (Tiersch 2001). Despite these limitations, live young have been produced from cryopreserved sperm in *X. helleri* (Yang et al. 2007), *X. couchianus* (Yang et al. 2009), *X. maculatus* (Yang et al. 2011), and *X. variatus* (Yang et al. unpublished data) since the first research on sperm cryopreservation of *Xiphophorus* fishes in 2004 (Huang et al. 2004b).

Vitrification is a form of cryopreservation that utilizes rapid cooling rates (> 1,000°C/min compared with < 40°C/min for conventional cryopreservation) and high concentrations of cryoprotectants (40 - 60% compared with 5 - 15% for conventional cryopreservation) to form glass (non-crystalline ice). The ultra-rapid cooling is typically achieved by plunging samples directly into the liquid nitrogen (Vajta and Nagy 2006, Tucker and Liebermann 2007). In general, the smaller the sample volume, the higher the cooling rate, and the higher the probability of vitrification. Vitrification is therefore suited to cryopreservation of small volumes, and offers advantages for use in laboratory and field environments. Previous attempts have been made to cryopreserve fish sperm by direct plunging into liquid nitrogen, but inconsistent results were obtained (Chapter 3, Cuevas-Uribe et al. 2011). In one study that focused on standard

cryopreservation of sperm from *X. hellerii*, minimal motility (< 1%) was observed when 0.25-mL straws containing 80 µL of sperm in 14% glycerol were plunged into liquid nitrogen (Huang et al. 2004c), possibly because complete vitrification was not attained due to the low concentration of cryoprotectant and the relatively large sample volume used. Successful vitrification has previously been reported for sperm of mammals (human) (Nawroth et al. 2002) and channel catfish (*Ictalurus punctatus*) (Chapter 3, Cuevas-Uribe et al. 2011) by employment of loops designed to hold small sample volumes (20 µL). Very recently the success (post-thaw motility as high as 86%) of cryoprotectant-free vitrification in rainbow trout (*Oncorhynchus mykiss*) by use of the microdrop method (20 µL) has been published (Merino et al. 2011).

The present study evaluated the effectiveness of loops for vitrification of sperm from *X. hellerii.* The goal was to develop streamlined protocols that could be integrated into a standardized approach for vitrification of germplasm of aquatic species. The objectives were to: 1) estimate acute toxicity of cryoprotectants, alone and in combination, at concentrations ranging from 10 to 40%; 2) evaluate vitrification solutions; 3) compare different thawing methods; 4) evaluate membrane integrity of post-thaw sperm vitrified in different cryoprotectants, and 5) evaluate the fertility of vitrified sperm by artificial insemination. This is the first report of sperm vitrification in a live-bearing fish with production of offspring. Vitrification offers an alternative to conventional cryopreservation and it can be applied to small body-sized fishes such as ornamentals, endangered species, and biomedical models.

Materials and Methods

Animals

Male *X. hellerii* used in this study were obtained from EkkWill Waterlife Resources (Gibsonton, FL), Crystal River Aquarium (Crystal River, FL), Segrest Farms (Gibsonton, FL), and the XGSC (Texas State University, San Marcos, TX) for experiments performed between 2008 and 2009. Males used for the acute toxicity experiments were from EkkWill and Crystal River, and had an average (mean ± SD) body length of 4.3 ± 0.78 cm, and body weight of 1.6 ± 0.68 g. Males used for artificial insemination were from XGSC, and had a mean body length of 4.03 ± 0.65 cm, and body weight of 0.62 ± 0.20 g. Males used for the flow cytometry and acute

toxicity studies were from Segrest Farms, and had a mean body length of 4.23 ± 0.25 cm, and body weight of 1.55 ± 0.21 g.

All males were maintained at the Aquaculture Research Station of the Louisiana State University Agricultural Center in a recirculating aquaculture system using a bead filter at a density of 1 fish per 10 L. Fish were fed twice daily with commercial flakes (Tropical Mix, Aquatic Eco-Systems Inc., Apopka, FL) and live *Artemia* nauplii grown from cysts (INVE Aquaculture Inc.; Salt Lake City, UT). The bead filter was backwashed weekly and water quality was monitored weekly. The water quality standards were: alkalinity >100 mg/L, hardness >100 mg/L, and total ammonia nitrogen and nitrite <1 mg/L. Females used in 2008 for artificial insemination were *Xiphophorus maculatus* of the strain Jp Wild and *X. hellerii* of the albino strain, while females used in 2009 were *X. maculatus* and *X. hellerii* of the strain BxII (Walter et al. 2006a). Virgin females were selected by separation from mixed-sex broods prior to maturation (at around 6 weeks of age) (Walter et al. 2006b). Females were maintained at the XGSC and cultured following routine protocols (www.xiphophorus.org) which included feeding twice daily with *Artemia* and liver paste (Kazianis and Walter 2002). Guidelines from the Institutional Animal Care and Use Committees (IACUC) of Louisiana State University Agricultural Center and Texas State University were followed for animal care in this study. These IACUC animal protocols and inspections are current (IACUC no. 05-05F7651F62), as is the National Institutes of Health Protection from Research Risks approval.

Sperm Collection

Sperm were collected by crushing of dissected testis. Male fish were anesthetized on ice for 1 min, killed by decapitation, and blotted with a paper towel to dry the body. The testes (13.8 ± 10.3 mg, n = 101) were removed and separated from the surrounding lipid tissues while viewing with a dissection microscope (10-X magnification) and transferred to 1.5-mL centrifuge tubes for weighing. Sperm were released by crushing of the testis in Hanks' balanced salt solution at an osmolality of 500 mOsmol/kg (HBSS500) (Yang et al. 2009), and diluted to a final concentration of 1 x 10^8 cells/mL unless otherwise stated. Sperm concentration was estimated by use of a hemacytometer (Hausser Scientific, Horsham, PA), and osmolality was measured with a vapor pressure osmometer (Model 5520, Wescor Inc., Logan, UT).

Motility Estimation

Sperm motility was estimated using dark-field microscopy (Optiphot 2, Nikon, Inc., Garden City, NY) at 200-X magnification. The addition of 20 µL of HBSS at an osmolality of 300 mOsmol/kg (HBSS300) was used to activate 2 µL of sperm suspension placed on a glass slide; no coverslip was added to the sample. Motility was estimated subjectively based on observation of 3 to 5 different fields within 20 sec after activation, and expressed as the percentage of sperm swimming progressively forward within the sample; sperm that vibrated in place were not considered to be motile. For consistency, motility was evaluated by a single skilled operator in a blind protocol (the examiner did not know the treatment given).

Fluorescent Staining and Flow Cytometry

Sperm membrane integrity was evaluated with the fluorescent dyes SYBR-14 and propidium iodide (PI) (live/dead sperm viability kit, Molecular Probes, Eugene, OR). Duplicate aliquots of 250 µL of sperm sample at a concentration of 1 x 10^6 cells/mL were stained with 100 nm SYBR®-14 (membrane-permeant nucleic acid stain) and 12 µM PI for 10 min. Membrane integrity was assessed by analyzing 10 µL of sperm sample at a flow rate of 35 µL/min using an Accuri C6 flow cytometer, equipped with a 488-nm, 50-mW solid-state laser (Accuri Cytometers Inc., Ann Arbor, MI), and CFlow® software (version 1.0.202.1, Accuri Cytometers Inc.). Green fluorescence (SYBR 14) was detected with a 530 ± 15 nm bandpass filter (FL1, fluorescence detector 1), and red fluorescence (PI) was detected with a >670 nm longpass filter (FL3). Events were viewed on forward-scatter (FSC) vs. side-scatter (SSC) plots with gating to exclude non-sperm events, and gated events were viewed on a scatter plot showing FL1 vs. FL3 with fluorescence compensation to reduce spectral overlap. The proportion of intact sperm was expressed as a percentage of the fluorescent population (i.e. sperm stained with SYBR 14, PI, or both) to exclude non-sperm particles from calculations.

Vitrification of Sperm Samples

Sperm samples were prepared by crushing of dissected testes in HBSS500 at an initial volume of 5 times the testes weight, and the concentration was adjusted to 2 - 5 x 10^8 cells/mL. Cryoprotectant solutions were prepared at double-strength in HBSS500. To vitrify, sperm samples were mixed with double-strength cryoprotectants at room temperature, immediately

loaded (within 15 sec) into 10-µL polystyrene loops (Nunc™, Roskilde, Denmark) or 5-mm nichrome loops (~15 µL) (Cole-Parmer, Vernon Hills, IL) without equilibration, submerged in liquid nitrogen within 1 min (~50 sec after the mixing), and packed under liquid nitrogen into goblets for storage in a Dewar flask (Appendix A, SOP-2). After at least 24 h of storage in liquid nitrogen, the vitrified loops were thawed in 20 µL of HBSS300 at room temperature or other temperatures as noted, and the motility of thawed sperm was estimated within 30 sec.

For membrane integrity assessment of vitrified sperm after thawing, sperm samples were vitrified at 1 x 10^7 cells/mL in 10-µL polystyrene loops, and thawed by warming four loops directly in 300 µL of HBSS500 at 40°C to yield a sperm concentration of around 1 x 10^6 cells/mL. Duplicate aliquots (250 µL) of the thawed samples were stained with 100 nM SYBR14 and 12 µM PI, and analyzed by flow cytometry within 10 min of thawing as described above.

Artificial Insemination

The artificial insemination procedure used in this study was based on previously published protocols (see details in Yang et al. 2007 and Dong et al. 2009a). In brief, females were anesthetized in 0.01% MS-222 (w/v), and transferred to a Petri dish with the abdomen facing up. The tip end of the insemination device (injector) was filled with sperm sample and gently pushed into the genital duct (viewed at 10-X magnification), and the sperm sample (5 µL) from each male was injected into the genital duct. After insemination, the females were returned to fresh water for recovery and were maintained in aquaria (5 females in each tank) in the XGSC for harvest of live young. These tanks contained live plants (java moss, *Vesicularia dubyana*) to provide refuge for newborn fish, thereby reducing the chances of cannibalism. At 90 days after insemination or when live young were collected (whichever came first), the inseminated females were dissected for examination of the reproductive tract.

Experiment l: Acute Toxicity of Cryoprotectants

In the first trial, 49 males were dissected and testis samples from individual males or pooled samples from several males (2-8) were used depending on the volume collected. Three replicates were produced by pooling sperm from multiple males. Nine cryoprotectants, (1) 2-methyl-2,4-pentanediol (MPD; Acros Organics, Fair Lawn, NJ), (2) 1-methoxy-2-propanol (MP;

Acros Organics), (3) methyl glycol (2-methoxyethanol, MG; Sigma-Aldrich, St Louis, MO), (4) polyethylene glycol (PEG molecular weight 200; Sigma-Aldrich), (5) ethylene glycol (EG; Mallinckrodt Baker, Paris, KY), (6) 2,3-butanediol (BD; Acros Organics), (7) glycerol (Gly; Mallinckrodt Baker), (8) 1,2-propanediol (PROH; Sigma-Aldrich), and (9) dimethyl sulfoxide (DMSO; OmniSolv, France), were used at final concentrations of 5, 10, 15, 20, 25 and 30% (v/v) with two exposure temperatures (24°C room temperature and 4°C on ice). In the second trial, EG, Gly and PROH were evaluated at increased concentrations of 30, 35 and 40%. EG and Gly exposures were performed at room temperature (24°C) while PROH were held on ice (4°C). Three replicates were produced for each treatment with different fishes (n = 5). Cryoprotectant solutions were prepared in HBSS500 at double strength of the final concentrations, followed by mixing with sperm suspension at a ratio of 1:1 for toxicity estimation. Motility was estimated immediately (within 10 sec) and at 5, 10, 15, 20, 25, 30, and 60 min.

Experiment II. Acute Toxicity of Commercial Vitrification Solutions

Five commercial solutions, VitriFreeze™ Freezing Medium 1 and 2 (FertiPro N.V., Belgium), V_{EG}, VM3, X-1000™, and Z-1000™ (21st Century Medicine, Fontana, CA) were used at final concentrations of 10, 20, 30, 40 and 50% (v/v). Vitrification solutions were prepared in HBSS500 at double strength of the final concentrations, followed by mixing with the sperm suspension at a ratio of 1:1. Motility was estimated immediately (within 10 sec) and at 5-min intervals for 30 min, and finally at 60 min. Three replicates were produced for each treatment with different fishes (n = 4).

Experiment III. Acute Toxicity of Combined Cryoprotectants

Fifteen combinations from different cryoprotectants were tested: (1) 20% EG + 20% Gly, (2) 30% EG + 10% DMSO + 0.45 M trehalose dihydrate (Tre; Acros Organics), (3) 30% EG + 10% PROH, (4) 20% methanol (MeOH; Fisher Scientific, Fair Lawn, NJ) + 20% MG, (5) 40% Gly + 0.45 M Tre, (6) 30% EG + 10% BD, (7) 30% EG + 10% Gly, (8) 40% Gly, (9) 30% EG + 15% Gly, (10) 15% EG + 10% Gly + 15% DMSO + 1% X-1000™ + 1% Z-1000™, (11) 20% EG + 20% Gly + 0.45 M Tre, (12) 30% EG + 10% MeOH, (13) 20% DMSO + 10% PROH + 6% PEG + 15% acetamide (Sigma-Aldrich), (14) 40% EG + 0.45 M Tre, and (15) 40% EG. Double-strength cryoprotectant solutions were prepared in HBSS500 and mixed with sperm suspension

at a ratio of 1:1 at 24ºC (Appendix A, SOP-1). Motility was estimated immediately (within 10 sec) and at 5 and 10 min. Sperm from four males were used in this experiment, and three replicates were produced with two replicates from individual males and one from pooling of two males.

Experiment IV. Effect of Thawing Temperatures

Six vitrification solutions were tested: (1) 20% EG + 20% Gly, (2) 40% Gly, (3) 40% Gly + 0.45 M Tre, (4) 30% EG + 10% DMSO + 0.45 M Tre, (5) 15% EG + 10% Gly + 15% DMSO + 1% X-1000™ + 1% Z-1000™, and (6) 10% EG + 20% Gly + 5% DMSO + 1% X-1000™ + 1% Z-1000™. Double-strength cryoprotectant solutions were prepared in HBSS500 and diluted at 24 ºC with sperm suspension at a ratio of 1:1 (final sperm concentration 5 x 10^7 cells/mL). Samples were immediately loaded (within 15 sec) into 5-mm nichrome loops (~ 15 µL) (Cole-Parmer, Vernon Hills, IL) without equilibration, and submerged in liquid nitrogen within 1 min (~ 50 sec) after the addition of the vitrification solutions (Appendix A, SOP-2). Glass formation was assessed by observing the appearance of the vitrified sample (a milky appearance indicated ice crystal formation). Loops were thawed directly onto a microscope slide containing a 20 µL drop of HBSS300 at two temperatures (24 and 37°C) (Appendix A, SOP-3). The motility of each sample was estimated immediately after thawing. Sperm from five males were used in this experiment.

Experiment V. Effect of Cryoprotectant on Membrane Integrity by Flow Cytometry

Sperm samples from three males were used to evaluate the toxicity of three treatments: 1) 30% EG, 2) 35% Gly, and 3) 20% EG + 20%Gly. Sperm samples (2 x 10^6 cells/mL) were stained with 100 nM SYBR-14 in duplicate for 10 min, and mixed with the same volume (125 µL) of double-strength vitrification solution containing PI at a final concentration of 12 µM. The mixture was analyzed by flow cytometry as described above at 1 min and 5 min after the addition of the vitrification/PI solution. Post-thaw motility after vitrification for each treatment was evaluated.

Experiment VI. Artificial Insemination with Vitrified Sperm

In July 2008, testes from 10 *X. hellerii* males were vitrified at a final concentration of 5 x 10^8 cells/mL with Gly (final concentration 40% v/v) in 5-mm nichrome loops. For artificial insemination, the vitrified loops were thawed in 20 µL of HBSS300 at 24°C after 24 h of storage in liquid nitrogen. Females of *X. maculatus* (n = 20) and albino females of *X. hellerii* (n = 5) were used for artificial insemination. Each loop was thawed in a 5 µL drop of HBSS300 at 24°C, and 5 µL of the thawed sperm sample (~ 7 x 10^5 cells) were injected into the female within 3 min after thawing. To evaluate artificial insemination success, fresh sperm samples were collected from males of *X. maculatus* (n = 15) and used to inseminate females of *X. hellerii* (n = 15) and *X. maculatus* (n =15).

In July 2009, testes from 20 *X. hellerii* males were vitrified at final concentrations of 5 x 10^8 cells/mL (5 males) or 2 x 10^8 cells/mL (15 males) with 20% EG + 20% Gly in 10-µL polystyrene loops. Artificial insemination was performed in four groups. For the first group, each loop from individual males (n = 10) were thawed in a 5-µL drop of HBSS300 at 24°C, and 5 µL of the thawed sperm sample (~ 1 x 10^5 cells) were injected into females of *X. hellerii* (n = 10) and *X. maculatus* (n = 10) within 3 min after thawing. For Group two, three loops of vitrified samples from each individual male (n = 10) were thawed into 1.5-mL microcentrifuge tubes containing 500 µL of HBSS500 at 24°C (Appendix A, SOP-4). For concentrating and washing, the thawed sperm suspensions were centrifuged (1000 x *g*) for 5 min at 4°C, the supernatant was decanted, and the sperm pellet was re-suspended by adding 100 µL of fresh HBSS500. Centrifugation was repeated (1000 x *g* for 5 min at 4°C), and the supernatant decanted again. For artificial insemination, the sperm pellet (1.5 x 10^6 to 2.5 x 10^6 cells) was suspended into a total volume of 5 µL of fresh HBSS500 prior to injection. Females of *X. hellerii* (n = 5) and *X. maculatus* (n = 5) were inseminated with samples from 10 males. For Group three (designated the "chemical control"), fresh sperm from *X. hellerii* (n = 5) were exposed to 20% EG + 20% Gly and used for artificial insemination of females of *X. hellerii* (n = 5) and *X. maculatus* (n = 5). For each female, 5 µL of the mixture sample (2.5 x 10^6 cells) were injected within 3 min after addition of cryoprotectant. For Group four, female *X. hellerii* (n = 15) from the same batch as the females used in the other three groups were used for artificial insemination with fresh sperm

from *X. variatus* (concentration range 2 x 10^8 to 1 x 10^9 cells/mL). This group was used as a control to evaluate the success of the artificial insemination procedure.

Data Analysis

Data were analyzed as a factorial randomized block design. Analysis was conducted using a mixed ANOVA procedure for all interactions. For acute toxicity experiments, the fixed treatment variables were: cryoprotectant, concentration, temperature, and incubation time. The green swordtail males were grouped in a block to remove variation among individual motility from the error term. The dependant variable was sperm motility (percent). The control (fresh sperm) was excluded from the model, but was used as a reference to ensure sperm viability. For the thawing experiment, the fixed treatments were temperature and vitrification solution, and the dependant variable was post-thaw motility (percent). Membrane integrity data were analyzed using a mixed ANOVA procedure with cryoprotectants as fixed treatments and membrane intact (percent) as a dependent variable. The control (fresh sperm) was included in the model. Statistical differences were determined at an $\alpha = 0.05$ level using Tukey's adjustment. Statistical analyses were performed using SAS software (Statistical Analysis System Inc., version 9.1; SAS institute, Cary, NC).

Results

Experiment I: Acute Toxicity of Single Cryoprotectants

The motility of fresh sperm before incubation with cryoprotectants was 60 ± 12% (mean ± SD). In the first trial, the addition of cryoprotectants at two temperatures (24 and 4°C) was significantly different for the cryoprotectants EG ($P = 0.004$), BD ($P = 0.014$), Gly ($P < 0.001$), and DMSO ($P = 0.004$), where toxicity was less evident at room temperature except for BD. The least toxic cryoprotectant was Gly, in which sperm showed no significant differences in motility among concentrations as high as 25% in all time intervals ($P = 0.112$) (Figure 4.1). EG showed no significant differences among concentrations as high at 25% and for as long as 30 min ($P = 0.466$). The highest concentrations without significant differences for the rest of the cryoprotectants were 20% DMSO ($P = 0.773$), 15% PROH ($P = 0.530$), 15% BD ($P = 0.490$), 10% MG ($P = 0.109$), and 10% MP ($P = 0.107$) (Figure 4.1). The highest concentration that

could be used for MPD and PEG was 5%. The longest time without significant motility reduction for these cryoprotectants at 5% was 60 min for PEG ($P = 0.174$), and 30 min for MPD ($P = 0.055$).

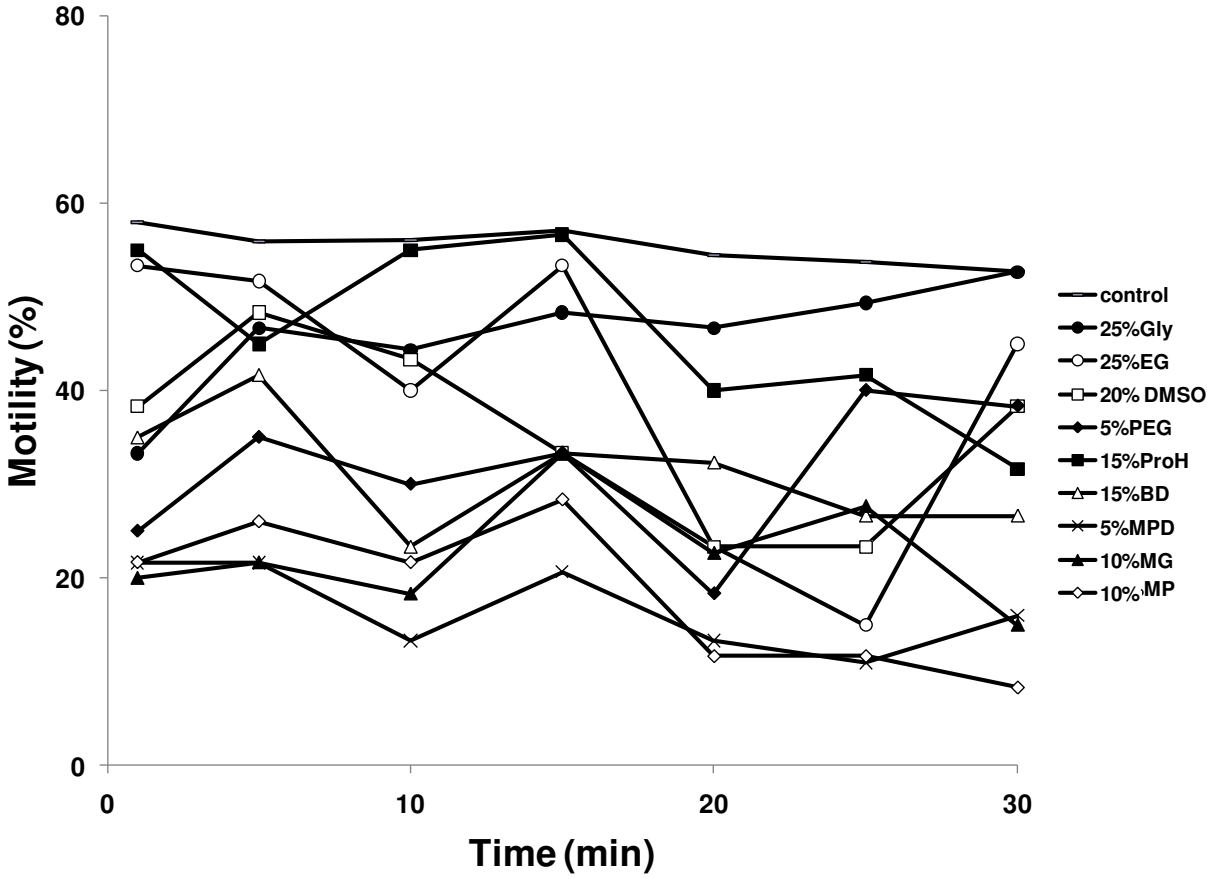

Figure 4.1. Percent sperm motility of green swordtail *Xiphophorus hellerii* in samples incubated with different cryoprotectants. The cryoprotectants used were, glycerol (Gly), ethylene glycol (EG), dimethyl sulfoxide (DMSO), polyethylene glycol (PEG), propanediol (PROH), butanediol (BD), methyl pentanediol (MPD), methyl glycol (MG), and methoxy propanol (MP). Each point represents the mean of three replicates; error bars were omitted for clarity.

In the second trial with higher concentrations of cryoprotectants (30 – 40%), EG was not significantly different from Gly ($P = 0.837$), but it was significantly different from PROH ($P = 0.037$). Gly and PROH were significantly different from each other ($P = 0.025$). EG at concentrations of 30% and 35% were not significantly different ($P = 0.083$). Glass formation was observed for concentrations of 35% PROH, 35% Gly, and 45% EG (Figure 4.2; Table 4.1), although only partial glass formation could be observed at 40% EG.

Table 4.1. Sperm motility of green swordtail *Xiphophorus hellerii* (n = 3) after exposure to vitrifying solutions.

Time (min)	Control	40% EG	35% Gly	40% Gly	20% EG + 20% Gly	35% PROH	40% VM3
< 1	61 ± 9	18 ± 10	37 ± 6	18 ± 11	20 ± 21	18 ± 13	12 ± 8
5	58 ± 10	11 ± 15	18 ± 18	13 ± 8	4 ± 1	5 ± 9	0
10	61 ± 9	4 ± 8	13 ± 15	24 ± 12	0	1 ± 2	0

EG = ethylene glycol; Gly = glycerol; PROH = 1,2 propanediol; VM3 vitrification solution.

Experiment II. Acute Toxicity of Commercial Vitrification Solutions

The motility of fresh sperm before incubation with vitrification solutions was 50 ± 0%. The least toxic commercial vitrification solution was VitriFreeze™ Freezing Medium 1, which showed no significant effect on motility among all the concentrations tested (as high as 50%). For VitriFreeze™ Freezing Medium 2, there was no difference within the first 5 min of exposure to concentrations as high as 30%. When VitriFreeze™ Freezing Medium 2 was used for less than 1 min, there was no difference at concentrations as high as 50%. When samples were exposed to VM3 for less than 1 min, there was no difference in motility at concentrations as high as 30% ($P = 0.572$). For the polymer X-1000™ there was no significant difference in motility at concentrations as high as 30% and at all time intervals ($P = 0.347$). There was no difference between V_{EG} and Z-1000™ ($P = 0.803$). The highest concentration that could be used for V_{EG} and Z-1000™ was 10% for less than 1 min. Glass formation was observed only in 40% VM3 (Table 4.1).

Experiment III. Acute Toxicity of Combined Cryoprotectants

The motility of fresh sperm before incubation with cryoprotectants was 65 ± 10%. Time was an important factor in the toxicity of combined cryoprotectants, which could be separated into three groups at <1 min. In the first group (20% MeOH + 20% MG, 30% EG + 10% BD, 30% EG + 15% Gly, 20% EG + 20% Gly + Tre, 30% EG + 10% MeOH, and 20% DMSO + 10% PROH + 6% PG + Ace), motility estimates were close to zero at <1 min and remained the same afterward. In the second group there were no significant differences in motility at any time interval. The least toxic cryoprotectant from this group and from this experiment was 40% Gly, for which the average motility at <1 min was 18% and was not significantly different through

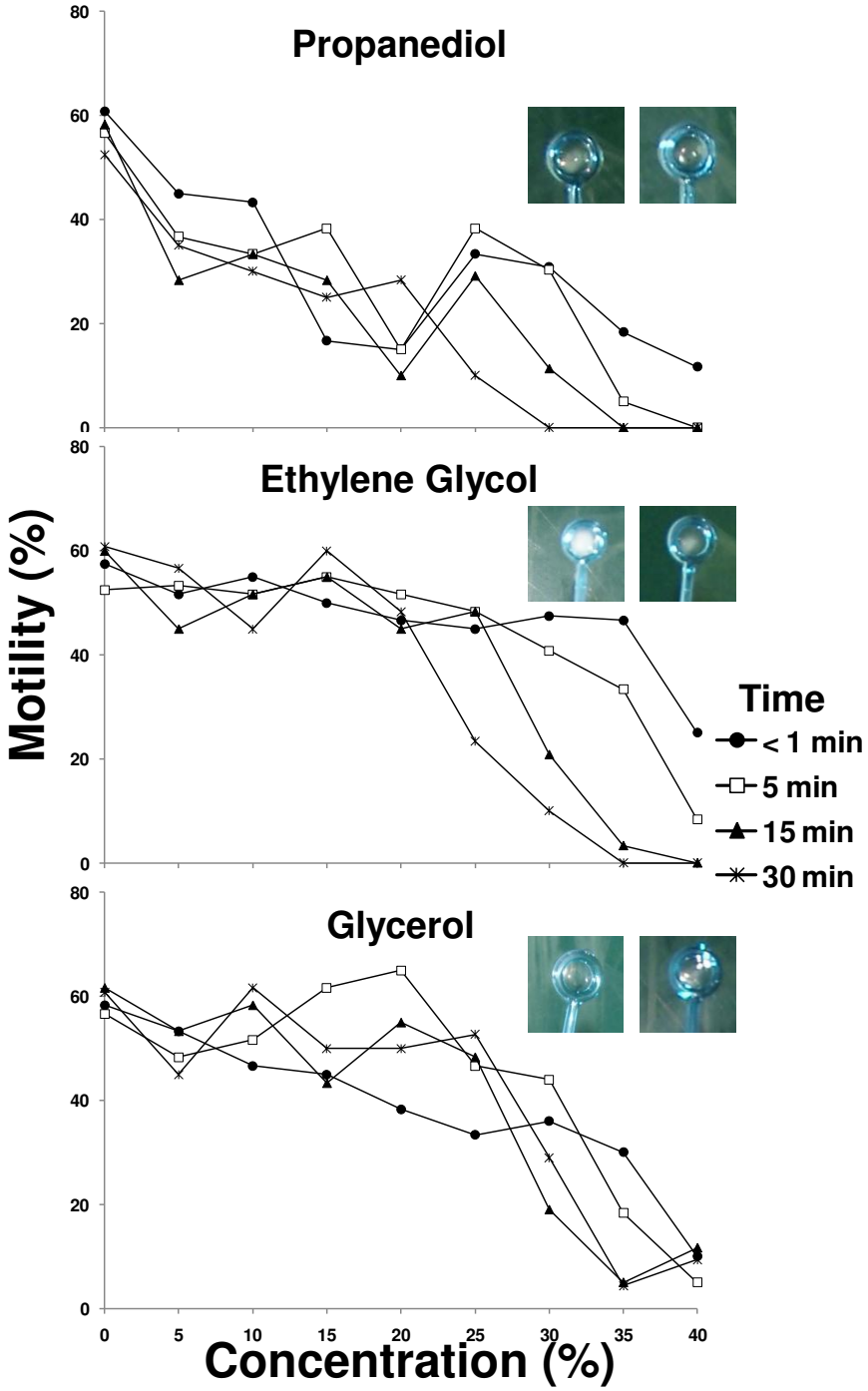

Figure 4.2. Sperm motility of green swordtail *Xiphophorus hellerii* when suspended at different concentrations of cryoprotectants at 1 min, 5 min, 15 min, and 30 min. As concentration increased, exposure time played a major role in toxicity with the highest motility observed just after the addition (< 1 min) of cryoprotectants. Some glass formation appeared in the polystyrene loops (inset pictures) at 35% and 40% for each cryoprotectant (identified by a transparent state, crystallization appeared milky). Each point represents the mean of three replicates.

time (P = 0.454). The other combined cryoprotectants from the second group were 30% EG + 10% PROH (15% motility at <1 min; no difference through time P = 0.137), 30% EG + 10% Gly (12% motility at <1 min; no difference through time P = 0.708), and 40%EG (12% motility at <1 min; no difference through time P = 0.851). In the third group, motility was highest at <1 min but declined at subsequent time intervals. Combined cryoprotectants from this group were 20% EG + 20% Gly, 30% EG + 10% DMSO + Tre, 40% Gly + Tre, 15% EG + 10% Gly + 15% DMSO + XZ (Figure 4.3), and 40% EG + Tre (motility at <1 min was 37%, and at 5 min was 7%).

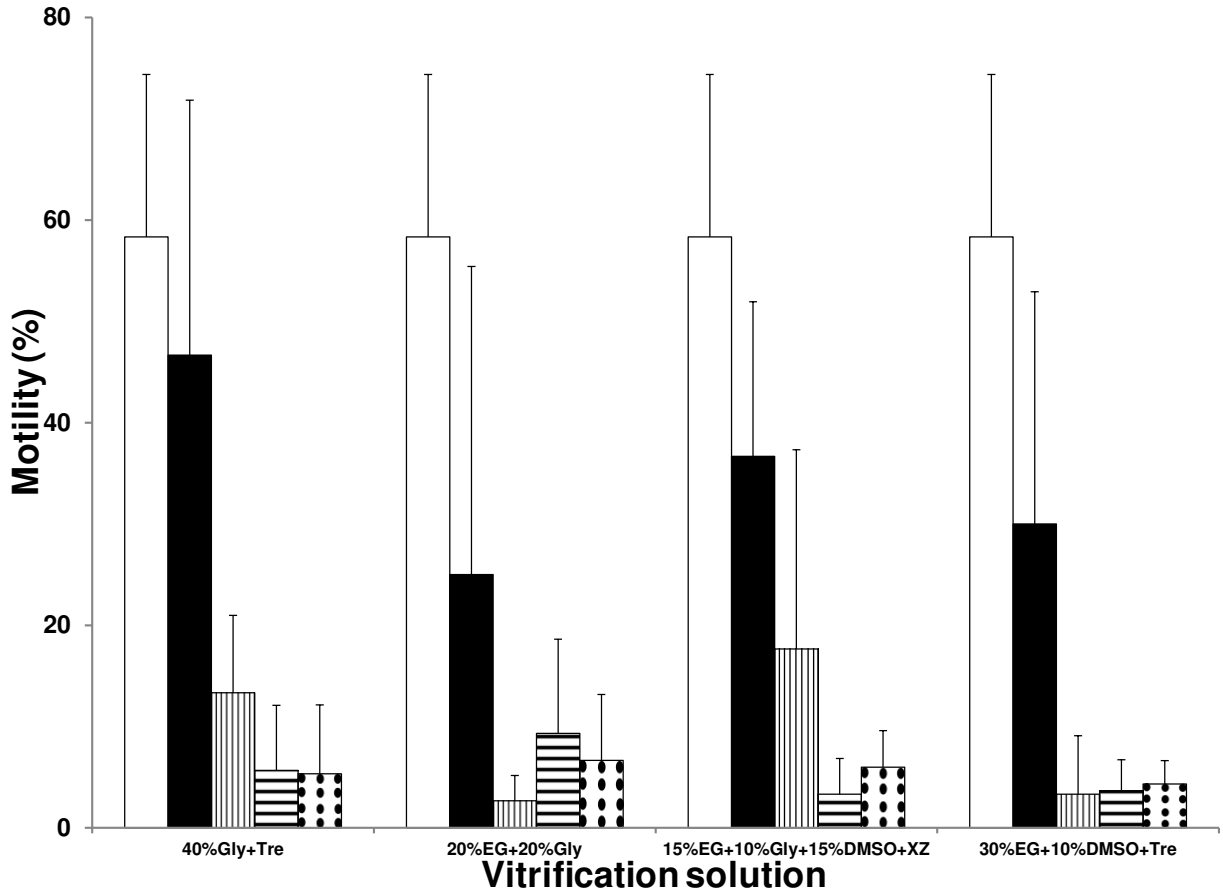

Figure 4.3. Sperm motility (mean ± SD) of green swordtail *Xiphophorus hellerii* (n = 3 males) in relation to exposure to vitrification solutions for 1 min (black bars) and 5 min (vertical stripes), and thawed at 24°C (horizontal stripes) or at 37°C (dotted bars). Motility of control samples was assessed before the addition of cryoprotectants (white bars). There was a significant difference in sperm motility between exposure at 1 min and 5 min. There was no significant difference in post-thaw sperm motility due to thawing at 24°C or at 37°C in all treatments.

Experiment IV. Effect of Thawing Temperatures

The motility of fresh sperm before vitrification was 64 ± 13%. There were no significant differences ($P = 0.945$) in post-thaw motility of sperm thawed at 24°C or at 37°C in all the treatments (Figure 4.3). In all treatments, the only significant differences found were between 20% EG + 20% Gly and 30% EG + 10% DMSO + Tre ($P = 0.033$). The highest post-thaw motility was for samples vitrified in 20% EG + 20% Gly (ranging from 3 to 20%) followed by 40% Gly + Tre (ranging from 1 to 15%). In general, the sperm in most vitrification solutions had a tendency towards vibration rather than what we considered to be progressive motility. This could be due to low inherent motility (perhaps due to cellular damage), or could be in part due to the high viscosity of the solutions which impeded sperm movement. The highest post-thaw sperm vibration was for 20% EG + 20% Gly (vibration 54 ± 14%) followed by 40% Gly (vibration 53 ± 12%).

Experiment V. Effect of Cryoprotectant on Sperm Membrane Integrity

The fresh sperm motility was 47 ± 6%, the percentage of membrane-intact sperm cells before vitrification was 87 ± 2%. For the cryoprotectant toxicity, the percentage membrane-intact sperm in the 30% EG treatment did not differ significantly from the control ($P = 0.490$), but membrane integrity in the 35% Gly ($P = 0.002$) and 20% EG + 20% Gly ($P < 0.001$) treatments was significantly lower than in the control. There was a significant difference in sperm membrane integrity between < 1 min and at 5 min for the treatments 30% Gly ($P = 0.048$), 35% Gly ($P = 0.022$), and 20% EG + 20% Gly ($P < 0.001$) but not for the control ($P = 0.961$). After vitrification, the percentage of membrane-intact post-thaw sperm was low (< 12%) which corresponded with the post-thaw motility (< 7%) (Figure 4.4). There was no significant difference in the percentage of post-thaw membrane-intact sperm between 35% Gly and 20% EG + 20% Gly ($P = 0.590$).

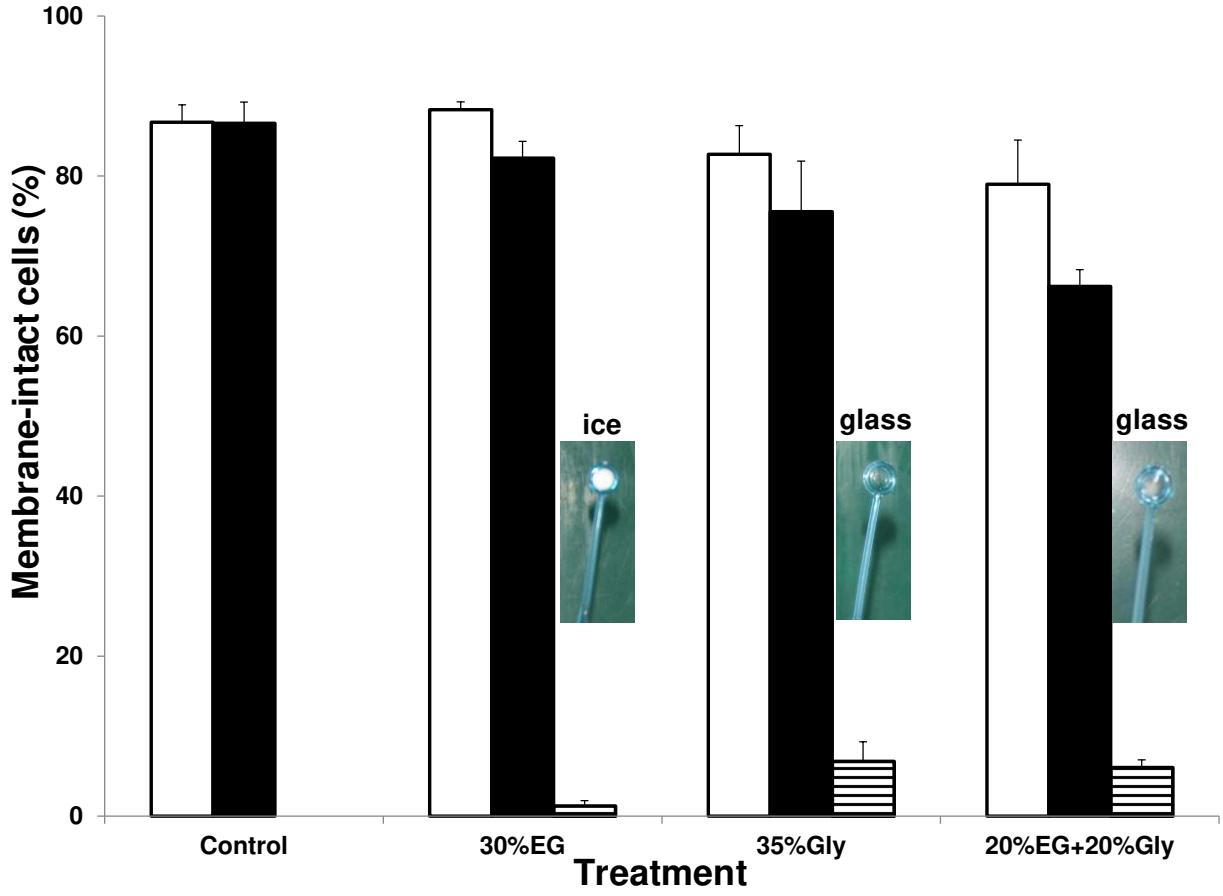

Figure 4.4. Sperm membrane integrity (mean ± SD) of green swordtail *Xiphophorus hellerii* (n = 3 males) in relation to exposure to cryoprotectants for 1 min (white bars) and 5 min (black bars), and post-thaw (horizontal stripes). The control values (fresh sperm) were not significantly different from 30% EG but were significantly different from 35% Gly and 20% EG + 20% Gly. There were fewer than 10% membrane-intact cells after vitrification. Polystyrene loops were evaluated for glass formation for each treatment. Vitrification produced a clear, transparent glass rather than an opaque, milky solid, caused by the appearance of ice crystals.

Experiment VI. Artificial Insemination with Vitrified Sperm

The motility of fresh sperm used for the artificial insemination was 71 ± 11%. For the first insemination trial in 2008 using sperm from one loop of vitrified samples in 40% Gly, none of the 25 females inseminated yielded offspring. In the control group, which consisted of 30 females that were inseminated with fresh sperm, 14 females contained embryos or oocytes after dissection. Of the 15 *X. hellerii* females from the control, 14 live offspring were observed and 3 females contained secondary growth oocytes at advanced vitellogenesis (abundant oil droplets and yolk globules) (~ 6 oocytes per female) after dissection, and of the 15 *X. maculatus* females,

2 live offspring were observed and 11 females contained secondary growth oocytes (~ 7 oocytes per female) after dissection. In the second insemination trial in 2009, none of the 20 females in Group 1 that were inseminated with vitrified sperm from a single loop yielded offspring. From Group 2 that consisted of 10 females inseminated with concentrated and washed sperm pooled from three loops (pellet 1.5 x 10^6 cells), five *X. hellerii* females contained secondary growth oocytes. Four live offspring (3 females and 1 male) and 31 oocytes were collected from these five females. Each female contained an average of six oocytes after dissection at 130 days post-insemination. The other five *X. maculatus* females from Group 2 that were inseminated with the concentrated and washed sperm from three loops (pellet containing 2.5 x 10^6 cells) did not yield offspring. In Group 3 (chemical control), which consisted of 10 females that were inseminated with fresh sperm with 20% EG + 20% Gly, none of the females yielded offspring. In Group 4 (control), which consisted of 15 females that were inseminated with fresh sperm, 3 live offspring were observed and none of the females contained embryos or oocytes after dissection.

Discussion

Aquarium fish such as zebrafish, medaka, and *Xiphophorus* have proven to be valuable as models of disease for molecular genetic studies. As vertebrates, fish and humans share most developmental processes, physiological mechanisms, and organ systems. By producing fish mutants, human diseases can be modeled and can provide experimental systems to aid pathological investigations or for use in screening of therapeutics (Lam and Gong 2010). Certain fish species possess novel attributes making them valuable models for specific diseases; for example, *Xiphophorus* provides a long-standing model for melanoma. Genetic control of tumor susceptibility in *Xiphophorus* has been investigated in pure strains and in interspecific hybrids for a variety of spontaneous and induced neoplasias (Hawkins et al. 2001). Inbred lines have been available for research since 1939 from the *Xiphophorus* Genetic Stock Center (XGSC) (Walter et al. 2006b). Despite the significant costs to maintain and generate these pedigreed fish lines, few alternatives currently exist to safely preserve important individuals, strains, and lines that may include endangered species. Of the 27 species of *Xiphophorus*, conventional cryopreservation of germplasm yielding offspring has been reported in only 3 species (*X. helleri* , Yang et al. 2007; *X. couchianus,* Yang et al. 2009; and *X. maculatus*, Yang et al. 2011).

Cryopreservation and artificial insemination efforts in *Xiphophorus* are faced with four major challenges: 1) limited volume of sperm samples (< 10 µL); 2) a low percentage (20 - 30%) of females producing offspring by artificial insemination for control (fresh) sperm; 3) the need for virgin females because sperm can be stored in the female reproductive tract, and 4) low control over the fertilization process due to difficulties in selecting spawning-capable females (Yang et al. 2009).

Conventional cryopreservation has been applied for the protection of genetic resources in fish and shellfish, but is not always practical for use in remote locations or in developing countries (often the places where preservation is most necessary). Vitrification is considered to provide an alternative to standard cryopreservation and has been used successfully for cryopreservation of spermatozoa, embryos, oocytes, stem cells, and organs from several mammalian species (Tucker and Liebermann 2007, Saragusty and Arav 2011). The advantages of vitrification are that it does not require expensive equipment, it is simple, fast, and can be used to preserve samples in the field especially in remote, inaccessible areas. In addition, it offers perhaps the greatest potential for success in overcoming the challenges for preservation of fish embryos. At present, there are three studies that have reported vitrification of fish sperm. In one study, the investigators observed sperm vitrification in rainbow trout (listed in the report as *O. mikiss*) and Russian sturgeon *Acipenser gueldenstaedtii* (listed in the report as *A. guldenshtadti*) by use of cryomicroscopy (Andreev et al. 2009). The second study specifically addressed fish sperm vitrification and development of a streamlined protocol for sperm vitrification in channel catfish (Chapter 3, Cuevas-Uribe et al. 2011), and a recent study reported sperm vitrification in rainbow trout without the use of cryoprotectants, but no production of offspring was reported (Merino et al. 2011). These are all large-bodied (e.g. > 2.5 kg) externally fertilizing species. In the present study, we report the first successful production of offspring from vitrified sperm in a live-bearing fish.

Identifying Suitable Cryoprotectants

One of the first steps in protocol development is the measurement of acute toxicity and selection of the least toxic vitrification solutions. Sperm vitrification is new in fish and there is little knowledge of cryoprotectants that vitrify at non-toxic concentrations. There have been no

published studies that specifically address acute toxicity of cryoprotectants in *X. hellerii.* A previous study in *X. hellerii* estimated the toxicity of cryoprotectants after thawing (Huang et al. 2004c). The cryoprotectants used in that study were DMSO, dimethyl formamide, dimethyl acetamide, PROH, MeOH, and sucrose at concentrations of 6 and 10%, and with an equilibration time of 10 min. Unfortunately, that study did not report sperm motility after the equilibration time and prior to freezing, so it is difficult to differentiate the effect of the cryoprotectants from the effects of freezing and thawing (Tiersch 2011). In addition, sperm concentration was estimated and no attempts were made to adjust the concentration to an established value (Huang et al. 2004c).

In the present study, we tested the effect of nine cryoprotectants at six concentrations (5 – 30%) and at two exposure temperatures on sperm at one standardized concentration (1 x 10^8 cells/mL). We evaluated motility immediately (within 10 sec) after the addition of the cryoprotectant, and at seven time intervals (as long as 60 min). This first screening identified three potential cryoprotectants (EG, Gly, and PROH) that appeared to be the least toxic at concentrations suitable for vitrification. Because vitrification requires high concentrations of cryoprotectants, we evaluated concentrations as high as 40% of these cryoprotectants. Temperature influenced the toxicity of some cryoprotectants. For example, Gly was least toxic at room temperature (24°C) compared to cold (4°C), which is different from human sperm where lower temperatures reduced the toxicity of Gly (Clarke et al. 2004). Temperature plays an important role in the addition of cryoprotectants because it interacts with factors such as chilling sensitivity of the gametes, toxic properties of the cryoprotectant, and permeation rate (lower temperatures require a longer period of equilibration) (Lawson et al. 2011, Fuller 2004). Because cryoprotectant exposure time is kept to a minimum in vitrification, high concentrations of cryoprotectants are usually added at room temperature (Kuleshova et al. 2007, Jain and Paulson 2006). One aim in vitrification (non-equilibrium cooling) protocols is to dehydrate the cells before cooling begins by using high concentrations of cryoprotectants (Chapter 2, Cuevas-Uribe and Tiersch 2011). Even so, it is possible to attain vitrification in the absence of cryoprotectants as was discovered for human sperm (Isachenko et al. 2005, Isachenko et al. 2003) and recently in rainbow trout (Merino et al. 2011).

Evaluating Vitrification Solutions

Most cryoprotectants tend to have toxic and hypertonic effects when used at concentrations that are effective for vitrification (Yavin et al. 2009). There are a number of ways of varying practicality that can be used to reduce the concentration of individual cryoprotectants required for vitrification, including applying high hydrostatic pressure, step-wise addition of cryoprotectants, combination of cryoprotectants, addition of non-permeating polymers or sugars, and reducing exposure time at high concentrations to a minimum (Fahy et al. 1984). In addition, the toxicity of cryoprotectants can be counteracted by the use of "toxicity neutralizers" such as formamide or urea (Fahy 2010).

Commercial vitrification solutions are not widely used in fish cryopreservation. This could be due to the cost, because most of the commercial solutions are designed for use with mammals. A previous study in fish embryo vitrification used the polymer X-1000™ to inhibit ice formation (Cabrita et al. 2006). In this study, the commercial polymers (X-1000™ and Z-1000™) were not toxic at the recommended concentration (1%) (Wowk and Fahy 2002). Another non-permeating cryoprotectant that has been used to enhance glass formation and cell biostabilization is trehalose. This disaccharide has been used to cryopreserve sperm from fishes such as rainbow trout (Maisse 1994), longtooth grouper (*Epinephelus bruneus*, formerly *E. moara*) (Miyaki et al. 2005), humpback grouper (*Cromileptes altivelis*) (Sean-in et al. 2009), and orange-spotted grouper (*Epinephelus coioides*) (Peatpisut and Bart 2010). In the present study, we decided to mix cryoprotectants to combine the additive actions of each, such as permeability and glass formation. The combination of cryoprotectants has been reported to reduce toxicity compared to high concentrations of individual cryoprotectants (Weiss et al. 2010). In the present study, the motility of sperm exposed to the combination of Gly and trehalose immediately after addition (< 1 min) was higher than for sperm exposed to Gly alone, but Gly alone yielded higher motilities over time. This is probably because at the time of cryoprotectant addition there was a rapid osmotic shock. This resulted in sperm activation and motility, which declined during the equilibration period. A similar reduction of motility was observed in *X. hellerii* when the thawed sperm were immediately diluted in HBSS creating a rapid change in osmolality (from as high as 2608 to as low as 599 mOsmol/kg) which caused sperm volume to change (Yang et al. 2006).

In this study, the combination of acetamide and DMSO (Fahy 2010) did not reduce damaging effects. Overall the combinations of cryoprotectants (vitrification solutions) were less toxic than individual cryoprotectants. This is in agreement with the standard mammalian procedures for vitrification in which a combination is often used to increase viscosity, increase the glass transition temperature, and reduce the level of toxicity (Saragusty and Arav 2011). In the present study, exposure time played a major role in toxicity with the highest motility observed just after the addition (< 1 min) of cryoprotectants. We decided to use vitrification solutions containing Gly because it was the cryoprotectant of choice for conventional cryopreservation for *Xiphophorus*, and because it was the least toxic in the present study at high concentrations (i.e. 25%). It has been suggested that as the concentration of Gly increases, the post-thaw motility of green swordtail sperm increases (Huang et al. 2004c). In addition to Gly, we decided to use DMSO because in previous studies it was used for conventional cryopreservation in green swordtail (Huang et al. 2004b, Huang et al. 2004c). Post-thaw motilities in a previous study of conventional cryopreservation using 20% DMSO were around 15%, while they were 50% for 20% Gly (Huang et al. 2004c).

Effect of Thawing Temperatures

Warming has been a topic of interest in recent studies in vitrification (Seki and Mazur 2009, Mazur and Seki 2011). Previous studies have focused on achieving the highest possible cooling rates (for example "ultravitrification" 250,000°C/min; Criado et al. 2011). This is because there is an inverse relationship between the rates of cooling and warming, and the concentration of cryoprotectant (i.e. the faster the cooling and warming, the lower the concentration of cryoprotectant needed and *vice versa* (Mazur et al. 2008)). Overall, the high concentrations of cryoprotectants required are near the maximum tolerable limits of cells. This is one of the reasons that previous studies focused on minimum volume methods to attain high cooling rates and prevent ice formation (Vajta and Nagy 2006). Even so, neither high cryoprotectant concentration nor increased cooling rates are essential for vitrification to occur. Partial (usually) or total intracellular vitrification can occur incidentally during conventional cryopreservation, and may be responsible for some degree of survival of cryopreserved samples (Vajta et al. 2009). A recent publication found that a wide range of cooling rates (160 –

250°C/min) could produce vitrification of human sperm (Isachenko et al. 2004). In addition, cryopreservation of rhesus monkey (*Macaca mulatta*) sperm was attained at cooling rates of 220°C/min with the absence of permeable cryoprotectants (Dong et al. 2009b).

Previous studies in *X. hellerii* achieved cooling rates of 200°C/min by the use of a differential scanning calorimeter (DSC) but caused the sperm cells to lyse and become osmotically inactive (Thirumala et al. 2005). In the same study, the optimal cooling rate predicted for samples without cryoprotectant was 90°C/min. In a different study, minimal post-thaw motility (<1%) was reported when 0.25-mL straws containing 80 µL samples of *X. hellerii* sperm with 14% Gly were plunged into liquid nitrogen (Huang et al. 2004c). The cooling rate for a 0.25-mL straw was estimated to range from 1,700°C/min (Yavin et al. 2009) to 2,500°C/min (Rall and Fahy 1985). In the present study, we used loops (5 mm; 15 µL) similar to those used previously for human sperm vitrification (5 mm; 20 µL) (Nawroth et al. 2002). The cooling rate estimated for the loops could be as fast as 720,000°C/min (Isachenko et al. 2003), while the warming rate at 37°C could be as fast as 200,000°C/min (Katkov et al. 2003). In the present study, there was no significant difference in motility between the two thawing temperatures (24 and 37°C) tested. This means that the combination of the small volumes used and the two temperatures yielded warming rates fast enough to avoid ice crystal formation (devitrification) or recrystallization of small intracellular ice crystals produced during cooling.

This result is in agreement with a recent publication that found that high warming rates (118,000°C/min) yielded high survival of mouse oocytes (*Mus musculus*) (70 – 85%) regardless of the cooling rate used (95 to 69,250°C/min) (Mazur and Seki 2011). This fast warming rate could explain results from a previous report in *X. hellerii* in which samples were cooled at a relatively fast rate (200°C/min) but the sperm were destroyed due to the slow warming rate used (20°C/min) (Thirumala et al. 2005). In addition, *X. hellerii* sperm in 0.25-mL straws that were plunged into liquid nitrogen (cooling of 1,700 to 2,500°C/min) and thawed for 7 sec in a 40°C water bath (thawing of around 1,300 to 2,500°C/min) yielded minimal motility (<1%) (Huang et al. 2004c). The reason for this could be the use of a slow warming rate compared with the rate suggested recently (118,000°C/min; Mazur and Seki 2011). Another reason could be the use of low cryoprotectant concentrations (14% Gly), which means that the glass transition temperature was low (below -120°C).

However, the big question that remains is: What is the source of sperm damage during vitrification? There is controversy over whether intracellular ice is formed in the sperm during rapid rates of cooling. While some studies suggest that at cooling rates as high as 3,000°C/min there is no formation of intracellular ice (Morris 2006, Morris et al. 2007), other studies suggest that intracellular ice starts forming at a cooling rate of 2,000°C/min but not in cells that are cooled at 250-1,000°C/min (Mazur and Koshimoto 2002). The intracellular nucleation temperature inferred by DSC for *X. hellerii* was -30°C (Thirumala et al. 2005). In a similar live-bearing fish *Poecilia reticulata*, the nucleation temperature was calculated to be in the range of -25 to -32°C at cooling rates from 5 to 100°C/min (Wang et al. 2010). It has been suggested that survival of vitrified sperm could be due to the presence of large amounts of osmotically inactive water bound to macromolecular structures, such as DNA and histones, or the presence of high molecular weight components in sperm that affect the viscosity and glass transition temperature of the intracellular cytosol (Isachenko et al. 2004, Rama Raju et al. 2006). To help identify if sperm were damaged by cryoprotectant toxicity, osmotic shock, or intracellular ice formation, in the present study we evaluated membrane integrity before and after the addition of the cryoprotectants, and after vitrification.

Effect of Cryoprotectant on Membrane Integrity

Flow cytometry is a useful technique to evaluate sperm quality parameters such as membrane integrity, mitochondrial status, and DNA damage (Graham 2001, Martínez-Pastor et al. 2010). A previous study in *Xiphophorus* used flow cytometry to measure nuclear DNA content in nine species, and was able to differentiate sex based on sex chromosomes heteromorphism in four species (Tiersch et al. 1989). In the present study we used flow cytometry to evaluate membrane integrity in response to cryoprotectant toxicity and after vitrification. Because vitrification uses high cryoprotectant concentrations, we evaluated membrane integrity at two time intervals, just after the addition of cryoprotectant solution (1 min) and after 5 min of exposure. We decided to use three treatments, based on our toxicity study and on the glass formation characteristics of these solutions. Although 30% EG (5.4 mol/L) did not form a glass, we tested it because previous work in mammals had success with a similar vitrification solution (VS14 = 5.5 mol/L EG + 1 mol/L sucrose (Ali and Shelton 2007)).

Gly at 35% was the minimum concentration needed to form glass in loops in the present study. The mixture of EG and Gly is a common combination used in the development of vitrification solutions (Ali and Shelton 2007). We used 20% EG (3.6 mol/L) + 20% Gly (2.7 mol/L) which corresponded to 80% of the vitrification solution VS5 (100% VS5 contains 4.5 mol/L EG + 3.5 mol/L Gly (Ali and Shelton 2007)). After 5 min of the addition of these solutions, the percentage of sperm with intact membranes remained relatively high (> 66%). But after vitrification, damage to the membrane was significant (highest value for membrane-intact sperm was 12% for 35% Gly). The lowest percentage of membrane-intact sperm (1%) was for 30% EG, which was the solution that did not form glass. The highest value for membrane-intact sperm was for 35% Gly (7%) and 20% EG + 20% Gly (6%) which corresponded with post-thaw motility (< 8%). Although we used high cooling and warming rates, glass formation needs to be attained for the sperm to survive, as low cryoprotectant concentrations that did not form glass had minimum motility (< 1%) after thawing.

This finding is similar to a previous study in channel catfish where higher concentrations of cryoprotectants that led to glass formation had higher sperm viability (Chapter 3, Cuevas-Uribe et al. 2011). But this finding is different from previous studies where human sperm was vitrified without cryoprotectant (Nawroth et al. 2002). Human sperm contains large amounts of proteins, sugars, and other components that make the cytosol highly viscous and this may provide a degree of natural protection (Isachenko et al. 2007). Fish sperm, in contrast, are characterized by low protein concentrations, containing mainly mineral compounds and low concentrations of other organic substances such as sugars (Ciereszko 2008). *Xiphophorus* sperm are similar in size to human sperm (Thirumala et al. 2005, Mortimer and Menkveld 2001), with a head length of 3.6 µm (human 4 - 5 µm), midpiece length of 6.8 µm (human 7 - 8 µm), and flagellum length of 43 µm (human 45 µm). However, *Xiphophorus* sperm have a higher surface area-to-volume ratio than human sperm (6.81 for *Xiphophorus* and 4.8 for humans) and a higher osmotically inactive cell volume than humans (0.6 for *Xiphophorus* and 0.5 for humans) (Thirumala et al. 2005, Mortimer and Menkveld 2001). These characteristics should allow the sperm from *Xiphophorus* to have higher hydraulic conductivity (water loss), higher cryoprotectant permeability, and in theory better cryopreservation success. Based on the membrane integrity results in the present study, we suggest that the plasma membrane is damaged either by intracellular ice formation (Thirumala et al. 2005) or by changes in the

physical properties of the extracellular environment (Morris 2006). In contrast, a recent publication reported post-thaw motilities as high as 86% and membrane integrity of 90% of sperm from rainbow trout that was vitrified without the use of cryoprotectants (Merino et al. 2011). Because of the numerous biological differences in gametes between these species and technical differences between these studies, more research is required to make conclusive comparisons.

Artificial Insemination of Vitrified Sperm

One of the most important sperm quality tests for cryopreserved sperm is the ability to fertilize eggs and produce offspring. Artificial insemination of live-bearing fishes is required to achieve fertilization. Studies on artificial insemination date back to 1914 when hybrids were produced from *X. hellerii* and *X. maculatus* (Koßwig 1927) and since then several artificial insemination protocols have been published using fresh sperm (Clark 1950, Kazianis et al. 2002, McGovern-Hopkins et al. 2003) and cryopreserved sperm (Yang et al. 2007, Dong et al. 2009a). Artificial insemination involves the injection of sperm (2 - 5 µL) into the female reproductive tract and pregnancy monitoring for as long as 90 days because females can store sperm and can delay fertilization (Kazianis and Walter 2002, Uribe et al. 2009). Success rates for artificial insemination using fresh sperm are usually 20 - 30% (Yang et al. 2009), although one study reported 50% success by insemination of at least 40 sperm bundles (spermatozeugmata) into the female (a single male can provide as many as 3000 bundles per stripping (Greven 2005)) (Gasparini et al. 2010). Previously reported success rates for artificial insemination using cryopreserved sperm ranged from 10 to 20% (Yang et al. 2007, Yang et al. 2009), although one study reported a rate as high as 40% fertilization (Yang et al. 2011). Thawed sperm requires washing by centrifugation and resuspension of the pellet to remove the cryoprotectant solution and to concentrate the sperm into a small volume.

One of the constraints of artificial insemination, in addition to the need to use virgin females, is the uncontrolled variable of selection of spawning-capable females (Yang et al. 2011). In the present study, we decided in the first trial not to remove the cryoprotectant because the volume held by the loop (15 µL) was small enough for artificial insemination, but of the 25 artificial inseminations with unwashed vitrified sperm none yielded offspring. To determine

whether cryoprotectants played a major role in the lack of fertilization, in the second we decided trial to add a chemical control that included fresh sperm with the cryoprotectant. In addition, in the second trial we inseminated small volumes with higher sperm concentration by centrifugation of vitrified sperm from three loops. From the 20 females inseminated with vitrified sperm without centrifugation, none yielded offspring, and neither did the 10 females inseminated with the chemical control. Five of ten *X. hellerii* females yielded offspring from the concentrated and washed sperm after centrifugation, while the five *X. maculatus* females did not yield offspring. Four live young were collected from the aquarium, and 31 oocytes were collected from these five *X. hellerii* females after dissection.

Previous studies demonstrated the production of offspring from cryopreserved sperm, and in this study we demonstrated fertilization and production of live young from vitrified sperm. Because there was not an evaluation of the fecundity of the females before insemination and there was no control for the selection of spawning-capable females, it is difficult to make a strong conclusion of why only females from the same species as the males were the ones that yielded offspring. While some studies support the concept of sperm competition (Gasparini et al. 2010, Smith and Ryan 2010), in this study we standardized the sperm concentration, and the characteristics of the males such as body size and sword length were similar. This is not the only report of fertilization after artificial insemination in *X. hellerii* females. In a previous study that used cryopreserved sperm from *X. maculatus*, none of the 20 *X. maculatus* females yielded offspring, but live young were produced from *X. hellerii* females (Yang et al. 2011). Highly inbred *X. maculatus* lines such as Coatzacoalcos were reported to have a high percentage of infertile females due to an ovarian regression syndrome (Burns and Kallman 1985). But previous studies that used *X. maculatus* females from other lines (e.g., Jp) produced offspring from cryopreserved sperm from *X. hellerii* (Yang et al. 2007) and *X. couchianus* (Yang et al. 2009). Further research is needed to study the fecundity and ovarian maturation of female *Xiphophorus* as recently suggested (Yang et al. 2011).

Of the 95 described species of live-bearing fishes (family Poeciliidae) in North America, 33% are imperiled (Jelks et al. 2008). A generalized protocol using conventional cryopreservation for live-bearing fishes such as mollies, guppies and *Xiphophorus* has been

described (Huang et al. 2009). In this study, we demonstrated a different technique that could be used to cryopreserve sperm samples from live-bearing *Xiphophorus* fishes (Table 4.2).

Table 4.2. Comparison of protocols for sperm cryopreservation of green swordtail *Xiphophorus hellerii*.

Parameter	Conventional cryopreservation[a]	Vitrification[b]
% Cryoprotectant	14% Gly	20% EG + 20% Gly
Exposure time	10 – 120 min	< 1 min
Apparatus	0.25-mL straw	5-mm loops
Sample volume	80 – 220 µL	~ 10 µL
Cooling rate	20 – 30°C/min	50,000 – 720,000°C/min[c]
Thawing rate	1,300 – 2,500°C/min[d]	200,000°C/min

Gly = glycerol; EG = ethylene glycol; [a]Yang and Tiersch 2009; [b]This study; [c]Isachenko et al. 2003; [d]Rall and Fahy 1985.

Vitrification is a simple technique that is well suited for use with small-bodied species, does not require specialized equipment, and offers advantages for use in the field. Furthermore, vitrification can be used to reconstitute lines from valuable biomedical models, conserve mutants for development of novel lines for ornamental aquaculture, and transport frozen sperm from the field to the laboratory to expand genetic resources. Further research is needed to evaluate whether vitrification can be applied with other species such as marine fishes.

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Chapter 5

Vitrification of Sperm from Marine Fishes: Effect on Motility and Membrane Integrity

Cryopreservation has proven to be a useful tool for improvement, maintenance, and distribution of genetic resources in aquatic species (Tiersch 2011a). There are two general procedures to attain cryopreservation: (1) slow equilibrium freezing, commonly referred to as "slow freezing", and (2) rapid non-equilibrium vitrification, often referred to as "ultra-rapid cooling". The main differences between these two procedures are the concentration of cryoprotectants, and the cooling and warming rates. The main purpose of these procedures is to avoid osmotic damage and intracellular ice formation, which can have negative effects on gamete survival. While in equilibrium cooling this is attained by cellular dehydration (maintaining an osmotic equilibrium between intracellular and extracellular compartments), in vitrification ice crystal formation is avoided by converting the solution directly into a viscous glass (Mazur et al. 2008). Glass is formed when solutions reach the glass transition temperature (-130°C for water). This temperature can be raised by the addition of cryoprotectants, making it easier to attain. However, the high concentrations of cryoprotectants required for vitrification are near the maximum tolerated by cells (Fahy et al. 1984). As such, the typical approach to vitrification is to reach the glass transition temperature as fast as possible through rapid cooling, and by increasing the concentration of cryoprotectants, bypassing or rapidly passing through the critical temperature range of ice formation (between -5 and -40°C) (Shaw and Jones 2003).

Vitrification has been applied for the cryopreservation of fish eggs and embryos, although results from some of these studies have been controversial (Chapter 2, Cuevas-Uribe and Tiersch 2011; Edashige et al. 2006; Hagedorn and Kleinhans 2011). Recently, vitrification has been applied successfully in freshwater fishes, and offspring were produced from vitrified sperm samples of Russian sturgeon (*Acipenser gueldenstaedtii*) (Andreev et al. 2009), channel catfish (*Ictalurus punctatus*) (Chapter 3, Cuevas-Uribe et al. 2011a), and green swordtail (*Xiphophorus hellerii*) (Chapter 4, Cuevas-Uribe et al. 2011b). Despite this, sperm vitrification remains unexplored in marine fishes. It is generally accepted that sperm from marine fishes have better resistance to cryopreservation when compared with freshwater species (Drokin et al. 1998; Suquet et al. 2000), and this may be due to adaptations of marine fish sperm to deal with osmotic changes between body tissues and the external environment, or differences in sperm membrane

composition (Kopeika and Kopeika 2008). Based on the previous use of sperm vitrification in freshwater species, we decided to evaluate the utility of sperm vitrification when applied to marine fishes.

Global marine fisheries are in crisis and about 32% of fish stocks are either overexploited (28%), depleted (3%) or recovering (1%) (FAO 2010). The decline of marine fisheries and the increasing consumer demand for seafood have precipitated an increase in intensive fish farming (Tal et al. 2009). As marine fish farming expands, there is an increasing need to apply sperm cryopreservation for repository development. Sperm cryopreservation can be used to preserve genetic resources from stocks of fishes that are endangered and to aid in replenishing fisheries (Wayman et al. 1996). The spotted seatrout (*Cynoscion nebulosus*), red drum (*Sciaenops ocellatus*), and red snapper (*Lutjanus campechanus*) are popular sport fishes in the Gulf of Mexico, but habitat destruction, climate change, and chronic overfishing have resulted in significant population declines in these species (Rummer 2007). Although sperm cryopreservation protocols have been developed for these species (Riley et al. 2004; Wayman et al. 1996; Wayman et al. 1998), vitrification has a great advantage over conventional cryopreservation due to its simplicity and speed. Further, it does not require specialized equipment, making it more user friendly especially for on-farm procedures and field work in remote sites such as coastal areas (Saragusty and Arav 2011). Although slow freezing has been proven effective, it requires more time and typically the use of expensive equipment (Moore and Bonilla 2006).

Based on previous cryopreservation studies done in spotted seatrout, red drum, and red snapper, and on the availability of gametes from recreational fisheries, we decided to use these fishes to evaluate if sperm vitrification could be applied to marine fishes. The overall goal of this project was to develop a standardized approach for vitrification of aquatic species germplasm. The specific objectives in this study of marine fishes were to: 1) estimate acute toxicity of cryoprotectants at varied concentrations; 2) evaluate vitrification solutions; 3) evaluate different thawing solutions, and 4) evaluate sperm quality after thawing by examination of motility and membrane integrity. We report the first successful sperm vitrification in a marine fish. Vitrification offers an alternative to conventional cryopreservation and it is ideally suited to work

with small volumes, offering a practical and simple approach to rapid cryopreservation of gametes.

Materials and Methods

Fish and Sperm Collection

Fish were collected by recreational anglers on charter boats off coastal Louisiana during June (for spotted seatrout and red snapper) and October (for red drum) of 2009. After capture, fish were placed in insulated coolers with ice and transported to either Coco Marina (Cocodrie, LA) or Sportman's Paradise (Chauvin, LA). Testes were removed within 4 – 8 h of capture, before the fish were cleaned and filleted for customers. Testes were placed in 4-L Ziploc® freezer bags (S.C. Johnson and Son, Inc., Racine, WI) with calcium-free Hanks' balanced salt solution (C-F HBSS) (Riley et al. 2008). The C-F HBSS was composed of 5.26 g/L NaCl, 0.26 g/L KCl, 0.13 g/L $MgSO_4$ x $7H_2O$, 0.04 g/L Na_2HPO_4, 0.04 g/L KH_2PO_4, 0.23 g/L $NaHCO_3$, 0.66 g/L $C_6H_{12}O_6$ adjusted to 200 mOsmol/kg with ultrapure water. The osmolality was measured with a vapor pressure osmometer (Model 5520, Wescor Inc., Logan, UT). The samples were placed on ice and transported to the Louisiana Universities Marine Consortium in Cocodrie (10 min) where the testes were removed from the bags, blotted dry with paper towel, and weighed. For spotted seatrout, sperm were released by crushing of testes in a quart Ziploc® freezer bag after addition of 1 mL (volume) of C-F HBSS per 1 g (weight) of testes. The sperm suspensions were filtered through a mesh series consisting of a 7.62-cm round mesh strainer (1-mm mesh), a 15.24-cm round mesh strainer (0.5-mm mesh), and a 200-µm mesh filter. Testes from red drum and red snapper were sliced to release sperm. Sperm were collected in 50-mL plastic centrifuge tubes (Corning, NY) and diluted 1:3 (v:v) with C-F HBSS. Sperm concentration was estimated by use of a hemacytometer (Hausser Scientific, Horsham, PA) and diluted to a final concentration of 2 x 10^9 sperm/mL with C-F HBSS. The sperm solutions were refrigerated at 4°C until use in cryopreservation experiments.

Estimation of Sperm Motility

The percent motility in each sperm sample was estimated using darkfield microscopy at 200-x magnification. Motility was determined as the percentage of sperm that were actively moving forward. Sperm that vibrated in place without forward movement were not considered to be motile. Activation of sperm from all three species was initiated by placing 2 µL of sperm onto a microscope slide and diluting it with 20 µL of filtered seawater collected from the Gulf of Mexico, at 970 mOsmol/kg (~30 ppt). Estimates of motility were made within 10 sec of adding the seawater (Appendix A, SOP-3).

Acute Toxicity of Individual Cryoprotectants

Sperm from three spotted seatrout and three red snapper males were collected and diluted to a final concentration of 2 x 10^9 sperm/mL with C-F HBSS. Eight cryoprotectants at different concentrations were evaluated for their effects on sperm motility over 15 min at 4°C: (1) ethylene glycol (EG; Mallinckrodt Baker, Paris, KY) was used at 10%, 15%, 20%, 25% and 30%; (2) dimethyl sulfoxide (DMSO; OmniSolv, France) was used at 15%, 20%, 25% and 30%; (3) 1,2-propanediol (PROH; Sigma-Aldrich, St Louis, MO) and (4) glycerol (Gly; Mallinckrodt Baker) were used at 10%, 15% and 20%; (5) methanol (MeOH; Fisher Scientific, Fair Lawn, NJ) and (6) methyl glycol (2-methoxyethanol, MG; Sigma-Aldrich) were used at 15% and 20%; (7) polyethylene glycol (PEG MW 200; Sigma-Aldrich) and (8) 2,3-butanediol (BD; Acros Ogranics, Fair Lawn, NJ) were used at 10%. Cryoprotectant solutions were prepared in C-F HBSS at double the final concentration and kept cold (4°C) before being added at that temperature to the sperm suspension at a ratio of 1:1 (100 µL of cryoprotectant solution : 100 µL of sperm suspension). Motility was estimated immediately (within 10 sec) and at 5, 10, and 15 min. Three replicates were produced for each treatment with the different fishes.

Acute Toxicity of Combined Cryoprotectants

Sperm from three spotted seatrout and three red snapper males were collected and diluted to a final concentration of 2 x 10^9 sperm/mL with C-F HBSS. Twenty-nine different cryoprotectant combinations were tested (Table 5.1). For each, double-strength cryoprotectant solutions were prepared in C-F HBSS and mixed with sperm suspensions at a ratio of 1:1 at 4°C.

Motility was estimated immediately (within 10 sec) and at 5 and 10 min. Three replicates were produced for each treatment with different fishes.

Table 5.1. Combined cryoprotectants used for the acute toxicity experiment. Glass formation was assessed by plunging samples into liquid nitrogen. Appearance of glass (clear, transparent) or ice crystals (opaque, milky) was observed. The cryoprotectant were: ethylene glycol (EG), acetamide (Ace), dimethyl sulfoxide (DMSO), trehalose (Tre), propanediol (PROH), polyvinyl alcohol (PVA), methyl glycol (MG), glycerol (Gly), methanol (MeOH), butanediol (BD), polyethylene glycol (PEG), X-1000™ (X), and Z-1000™ (Z).

Combined cryoprotectants	**Abbreviation**	**Glass (%)**
20% EG + 15% Ace	20EA	0
25% DMSO + 0.25 M Tre	25DT	0
30% DMSO + 0.25 M Tre	30DT	0
30% EG + 0.25 M Tre	30ET	10
35% DMSO + 0.25 M Tre	35DT	10
35% EG + 0.25 M Tre	35ET	60
35% PROH + 0.25 M Tre	35PT	80
35% PROH + 3% PVA	35PP	70
40% DMSO + 0.25 M Tre	40DT	100
40% EG + 0.25M Tre	40ET	100
20% DMSO + 20% EG	20D20E	90
20% DMSO + 20% MG	20D20M	90
20% DMSO + 20% PROH	20D20P	100
20% DMSO + 20%Gly	20D20G	95
20% EG + 20% Gly	20E20G	50
20% EG + 20% MG	20E20M	90
20% MeOH + 20% MG	20Me20M	100
25% DMSO + 15% EG	25D15E	90
30% DMSO + 10% BD	30D10B	100
30% EG + 10% BD	30E10B	100
30% EG + 10% MeOH	30E10Me	100
10% DMSO + 30% EG + 0.25 M Tre	10D30ET	95
15% DMSO + 15% EG + 10% Gly	15D15E10G	50
20% DMSO + 15% EG + 0.25 M Tre	20D15ET	0
20% DMSO + 10% EG + 10% PROH	20D10E10P	100
20% DMSO + 10% MG + 10% PROH	20D10M10P	100
20% DMSO + 10% PROH + 6% PEG + 15% Ace	DPPA	30
25% DMSO + 10% BD + 15% Ace	25D10BA	100
25% DMSO + 15% EG + 15% Ace	25D15EA	95
35% EG + 1% X + 1% Z[a]	35EXZ	80
15% DMSO + 15% EG + 10% Gly + 1% X + 1% Z	DEGXZ	100

[a]not used in cryoprotectant toxicity experiment.

Vitrification of Sperm and Glass Formation Assessment

The general procedure for vitrification was as follows. Sperm were diluted to a concentration of 2 x 10^9 sperm/mL with C-F HBSS. Double-strength cryoprotectant solutions were prepared in C-F HBSS, and mixed with sperm suspensions at a ratio of 1:1 (Appendix A, SOP-1). Samples were loaded within 15 sec into 10-µL polystyrene loops (Nunc™, Roskilde, Denmark) without equilibration, and individually submerged in liquid nitrogen within 1 min (~50 sec) of the addition of the cryoprotectant solution (Appendix A, SOP-2). Glass formation was assessed by observing the appearance of the vitrified sample. A milky appearance indicated ice crystal formation, and a clear transparent appearance indicated glass formation (Ali and Shelton 1993). Loops were stored in goblets (three per goblet) in liquid nitrogen. After at least 12 h of storage in liquid nitrogen, the vitrified loop samples were thawed directly onto a microscope slide containing a 30-µL drop of filter seawater (~1,000 mOsmol/kg) at room temperature (24°C), and the motility of thawed sperm was estimated within 10 sec.

Evaluation of Thawing Solutions

Based on the toxicity of combined cryoprotectants and on the vitrification characteristics, eight vitrification solutions were selected to vitrify sperm of red snapper from three males: 35ET, 40ET, 20E20G, 30E10B, 10D30ET, 40% EG, 35EXZ, and DEGXZ (Table 1). In addition to these vitrification solutions, sperm were also vitrified without the use of cryoprotectants (cryoprotectant-free vitrification). The vitrification solutions tested for sperm from three spotted seatrout were: 40ET, 10D30ET, and DEGXZ (Table 5.1). The vitrification procedure was performed as described above. Loops were thawed directly onto a microscope slide containing 30 µL of two solutions: filtered seawater (~1,000 mOsmol/kg) or 10% DMSO in seawater (~2,000 mOsmol/kg) at room temperature (24°C). The motility of each sample was estimated immediately after thawing. All trials were replicated a minimum of two times for each individual male.

Membrane Integrity Assessment

Sperm samples from three spotted seatrout, three red snapper, and four red drum were used in this experiment. Sperm samples were vitrified as described in Section 2.5 using three treatments (10D30ET, DEGXZ, and 40ET; Table 5.1) for spotted seatrout and red snapper, and

two treatments (10D30ET and DEGXZ) for red drum. Sperm membrane integrity was evaluated with the fluorescent dyes SYBR-14 and propidium iodide (PI) (live/dead sperm viability kit, Molecular Probes, Eugene, OR). Each loop was thawed directly in 495 µL of C-F HBSS at room temperature (24°C), and duplicate aliquots of 250 µL of sperm sample at a concentration of ~5 x 10^6 cells/mL were stained with 100 nm SYBR-14 and 12 µM PI for 10 min. Membrane integrity was assessed by analyzing 10 µL of sperm sample at a flow rate of 35 µL/min using an Accuri C6 flow cytometer, equipped with a 488-nm, 50-mW solid-state laser (Accuri Cytometers Inc., Ann Arbor, MI), and CFlow® software (version 1.0.202.1, Accuri Cytometers Inc.). Green fluorescence (SYBR 14) was detected with a 530 ± 15 nm bandpass filter (FL1), and red fluorescence (PI) was detected with a >670 nm longpass filter (FL3). Events were viewed on forward-scatter (FSC) vs. side-scatter (SSC) plots with gating to exclude non-sperm events. Gated events were viewed on a scatter plot showing FL1 vs. FL3 with fluorescence compensation to reduce spectral overlap. The proportion of intact sperm was expressed as a percentage of the fluorescent population (i.e. sperm stained with SYBR 14, PI, or both) to exclude non-sperm particles from calculations.

Statistical Analysis

Data were analyzed as a factorial randomized block design. Analysis was conducted using a mixed ANOVA procedure for all interactions. For acute toxicity experiments, the fixed treatment variables were: cryoprotectant, concentration, and incubation time. The males were grouped in a block to remove variation among individual motility from the error term. The dependent variable was sperm motility (percent). The control (fresh sperm) was excluded from the model, but was used as a reference to ensure sperm viability. For the thawing experiment, the fixed treatments were vitrification solution and thawing solution, and the dependent variable was post-thaw motility (percent). For membrane integrity experiments, the fixed treatments were cryoprotectants, and the dependent variable was membrane intact (percent). Correlation between membrane-intact and post-thaw motility was estimated. Statistical differences were determined at an $\alpha = 0.05$ level using Tukey's adjustment. Statistical analyses were performed using SAS software (Statistical Analysis System Inc., version 9.1; SAS institute, Cary, NC).

Results

Collection of Fish, Testes, and Sperm

In total, 80 spotted seatrout, 29 red snapper, and six red drum males were collected from the recreational fishery. The total body length (TL) (mean ± SD) was 40.6 ± 5.9 cm for spotted seatrout, 63.0 ± 6.6 cm for red snapper, and 90.4 ± 13.4 cm for red drum. Testes weight (mean ± SD) was 9.6 ± 5.0 g for spotted seatrout, 35.5 ± 27.4 g for red snapper, and 95.8 ± 79.5 for red drum. Testes size and development was variable among similar-sized fish.

Acute Toxicity of Individual Cryoprotectants

Spotted seatrout: The motility of fresh sperm before incubation with cryoprotectant was 70 ± 17% (mean ± SD). The highest concentration without significantly different motility was 20% for EG, DMSO, and MG and 15% for PROH (Table 5.2). DMSO at 20% was not significantly different in motility from EG at 20% ($P = 0.066$), or from MG at 20% ($P = 0.665$).

Red snapper: The motility of fresh sperm before incubation with cryoprotectant was 77 ± 6% (mean ± SD). The highest concentration that did not significantly affect motility was 25% for EG, 20% for DMSO, PROH, MeOH, and MG (Table 5.3). DMSO at 20% was not significantly different from EG at 25% ($P = 0.137$), or from MG at 20% ($P = 0.201$), or PROH at 20% ($P = 0.667$).

Acute toxicity of Combined Cryoprotectants

Spotted seatrout: The motility of fresh sperm before incubation with combined cryoprotectant was 73 ± 21%. Time was a critical factor in the toxicity of combined cryoprotectants, which could be separated into two groups at <1 min. In the first group motility estimates were close to zero at time one and remained the same afterward (Table 5.4). In the second group there was a significant difference in motility between time one (< 1 min) and at 5 min. From the 29 combined cryoprotectants solutions, 18 solutions vitrified and formed a transparent glass (> 70%) (Table 5.1).

Table 5.2. Acute toxicity of various cryoprotectants to sperm of spotted seatrout. Values presented are mean ± SD (n = 3).

Cryoprotectant	Concentration	Incubation time (min)			
		< 1	5	10	15
None (Control)		61 ± 3	64 ± 2	64 ± 2	63 ± 3
Ethylene glycol	10%	53 ± 15	67 ± 15	63 ± 15	63 ± 15
	15%	63 ± 15	55 ± 22	57 ± 20	57 ± 20
	20%	58 ± 19	57 ± 15	58 ± 12	53 ± 15
	25%	53 ± 15	45 ± 9	45 ± 13	37 ± 28
	30%	47 ± 30	38 ± 30	18 ± 18	7 ± 3
Dimethyl sulfoxide	15%	63 ± 15	67 ± 15	67 ± 11	52 ± 3
	20%	60 ± 10	60 ± 17	63 ± 20	48 ± 22
	25%	67 ± 15	53 ± 28	38 ± 29	18 ± 13
	30%	33 ± 17	2 ± 1	0 ± 0	0 ± 0
Propanediol	10%	60 ± 20	63 ± 11	50 ± 0	48 ± 19
	15%	67 ± 15	57 ± 11	37 ± 27	10 ± 8
	20%	63 ± 21	50 ± 30	22 ± 24	1 ± 1
Glycerol	10%	57 ± 20	38 ± 20	12 ± 12	9 ± 13
	15%	50 ± 20	32 ± 27	12 ± 16	13 ± 16
	20%	63 ± 12	10 ± 17	2 ± 2	2 ± 2
Methanol	15%	67 ± 15	53 ± 15	40 ± 10	37 ± 15
	20%	67 ± 15	47 ± 11	12 ± 11	1 ± 1
Methyl glycol	15%	70 ± 10	63 ± 15	67 ± 11	57 ± 20
	20%	67 ± 15	60 ± 20	57 ± 25	37 ± 25
Polyethylene glycol	10%	60 ± 17	35 ± 27	2 ± 2	0 ± 0
Butanediol	10%	63 ± 15	60 ± 10	53 ± 15	57 ± 15

Table 5.3. Acute toxicity of various cryoprotectants to sperm of red snapper. Values presented are mean ± SD (n = 3).

		Incubation time (min)			
Cryoprotectant	**Concentration**	**< 1**	**5**	**10**	**15**
None (Control)		77 ± 3	77 ± 1	75 ± 3	74 ± 3
Ethylene glycol	10%	73 ± 6	77 ± 6	80 ± 0	77 ± 6
	15%	80 ± 0	77 ± 6	72 ± 3	77 ± 6
	20%	73 ± 11	73 ± 6	73 ± 6	73 ± 6
	25%	77 ± 6	77 ± 6	70 ± 0	70 ± 10
	30%	73 ± 6	57 ± 11	57 ± 11	55 ± 13
Dimethyl sulfoxide	15%	73 ± 6	77 ± 6	75 ± 9	73 ± 6
	20%	80 ± 0	80 ± 0	80 ± 0	77 ± 6
	25%	80 ± 0	63 ± 11	60 ± 8	30 ± 21
	30%	73 ± 6	32 ± 14	3 ± 2	0 ± 0
Propanediol	10%	77 ± 6	72 ± 8	73 ± 6	70 ± 5
	15%	80 ± 0	65 ± 21	77 ± 6	73 ± 11
	20%	80 ± 0	80 ± 0	80 ± 0	70 ± 0
Glycerol	10%	80 ± 0	3 ± 3	3 ± 2	2 ± 2
	15%	77 ± 6	2 ± 2	1 ± 1	1 ± 1
	20%	67 ± 15	0 ± 0	0 ± 0	0 ± 0
Methanol	15%	82 ± 3	77 ± 6	77 ± 6	72 ± 3
	20%	75 ± 5	68 ± 7	65 ± 5	65 ± 5
Methyl glycol	15%	78 ± 3	80 ± 0	70 ± 17	63 ± 20
	20%	77 ± 6	77 ± 6	77 ± 6	67 ± 15
Polyethylene glycol	10%	80 ± 0	77 ± 6	63 ± 11	63 ± 11
Butanediol	10%	72 ± 3	63 ± 6	67 ± 6	65 ± 5

Table 5.4. Sperm motility (mean ± SD) of spotted seatrout (*Cynoscion nebulosus*) in relation to combined cryoprotectant solutions were compared at two exposure times. Treatments with a significant difference in motility between the two times were grouped on the left side while treatments without difference in motility were grouped on the right side.

	Motility				Motility		
Treatment	**< 1 min**	**5 min**	***P* - value**	**Treatment**	**< 1 min**	**5 min**	***P* - value**
30DT	63 ± 15	3 ± 2	<0.001	20Me20M	0 ± 0	0 ± 0	1.000
15D15E10G	63 ± 15	1 ± 1	<0.001	20E20M	0 ± 0	0 ± 0	1.000
20D15ET	62 ± 28	37 ± 27	<0.001	30D10B	0 ± 0	0 ± 0	1.000
25DT	60 ± 26	28 ± 36	<0.001	40DT	0 ± 0	0 ± 0	1.000
20E20G	53 ± 23	0 ± 0	<0.001	20D10M10P	1 ± 1	0 ± 0	1.000
35PP	53 ± 23	3 ± 2	<0.001	20D20M	2 ± 2	0 ± 0	0.833
20EA	52 ± 8	17 ± 20	<0.001	30E10B	5 ± 4	2 ± 3	0.527
35PT	52 ± 10	0 ± 0	<0.001	25D15E	7 ± 6	0 ± 0	0.423
35ET	50 ± 26	22 ± 24	0.001	25D10BA	7 ± 11	0 ± 0	0.399
35DT	42 ± 35	0 ± 0	<0.001	25D15EA	10 ± 17	0 ± 0	0.207
10D30ET	38 ± 10	7 ± 7	<0.001	20D20P	12 ± 16	0 ± 0	0.178
40ET	33 ± 32	4 ± 1	<0.001	20D20G	15 ± 9	0 ± 0	0.065
DPPA	25 ± 32	0 ± 0	0.002	20D10E10P	15 ± 13	0 ± 0	0.059
30E10Me	18 ± 27	0 ± 0	0.022	30ET	63 ± 25	57 ± 25	0.399
20D20E	17 ± 28	0 ± 0	0.033				

Red snapper: The motility of fresh sperm before incubation with combined cryoprotectant was 77 ± 6%. Time was a critical factor in the toxicity of combined cryoprotectants, which could be separated into three groups at <1 min. In the first group motility estimates were close to zero at time one and remained the same afterward (Table 5.5). In the second group there was no significant difference in motility between time one and at 5 min. In the third group there was a significant difference in motility between time one (< 1 min) and at 5 min.

Effect of Thawing Solutions

Spotted seatrout: The motility of fresh sperm before vitrification was 77 ± 11%. There was no significant difference in motility between thawing in seawater or in seawater containing 10% DMSO ($P = 0.709$). The highest post-thaw motility was 70% for DEGXZ, followed by 60% for 10D30ET (Table 5.6). There was a significant difference in motility among treatments ($P < 0.001$).

Table 5.5. Sperm motility (mean ± SD) of red snapper (*Lutjanus campechanus*) in relation to combined cryoprotectant solutions were compared at two exposure times. Treatments with a significant difference in motility between the two times were grouped on the left side while treatments without differences in motility were grouped on the right side.

	Motility				Motility		
Treatment	**< 1 min**	**5 min**	***P* - value**	**Treatment**	**< 1 min**	**5 min**	***P* - value**
35PT	77 ± 6	2 ± 1	<0.001				
20D15ET	77 ± 6	20 ± 13	<0.001	No difference time 1 and 10 min			
40ET	77 ± 6	53 ± 25	0.004				
35PP	75 ± 5	22 ± 32	<0.001	20D20P	12 ± 16	0 ± 0	0.153
10D30ET	75 ± 5	47 ± 15	0.001	20D10M10P	7 ± 11	1 ± 1	0.474
25DT	73 ± 6	38 ± 29	<0.001	20E20M	5 ± 5	0 ± 0	0.527
15D15E10G	73 ± 12	10 ± 8	<0.001	40DT	3 ± 2	0 ± 0	0.736
30DT	70 ± 10	14 ± 22	<0.001	25D15EA	2 ± 2	0 ± 0	0.800
25D15E	63 ± 6	0 ± 0	<0.001	30D10B	0 ± 0	0 ± 0	1.000
20D20E	63 ± 12	5 ± 8	<0.001	25D10BA	0 ± 0	0 ± 0	1.000
20D10E10P	57 ± 6	0 ± 0	<0.001	20D20M	0 ± 0	0 ± 0	1.000
20E20G	57 ± 21	0 ± 0	<0.001	20Me20M	0 ± 0	0 ± 0	1.000
30E10B	47 ± 32	27 ± 10	0.013				
30E10Me	43 ± 6	0 ± 0	<0.001	No difference time 1 and 5 min			
35DT	35 ± 5	0 ± 0	<0.001	35ET	78 ± 3	63 ± 15	0.060
DPPA	27 ± 23	0 ± 0	0.001	30ET	77 ± 6	70 ± 10	0.400
20D20G	17 ± 20	0 ± 0	0.037	20EA	75 ± 5	63 ± 11	0.142

Red snapper: The motility of fresh sperm before vitrification was 77 ± 6%. There was no significant difference in motility between thawing in seawater or in seawater containing 10% DMSO ($P = 0.708$). The highest post-thaw motility was 60% for 10D30ET but this was not different from DEGXZ and 40ET (Table 5.6). Motility in the 35ET treatment was not different from 40% EG ($P = 0.051$) and 20E20G ($P = 0.284$). Post-thaw motility was not observed with cryoprotectant-free vitrification. Motility in the 30E10B treatment was not significantly different from motility in the 35EXZ treatment ($P = 0.489$).

Effect of Vitrification on Sperm Membrane Integrity

Spotted seatrout: There was a weak correlation between post-thaw motility and membrane integrity (r = 0.241). The treatment with the highest percentage of membrane-intact sperm did not correlate with the treatment with the highest motility (Table 5.6). There was no significant difference in the percentage of membrane-intact sperm among all the treatments ($P > 0.279$).

Table 5.6. Sperm motility and membrane integrity (mean ± SD) after thawing for different vitrification solutions from three males of spotted seatrout and red snapper (fresh motility ~77%). Two thawing solutions were compared: seawater (1000 mOsmol/kg) and 10% DMSO in seawater (2000 mOsmol/kg). There was no difference in motility between the thawing solutions.

		Post-thaw motility (%)		Membrane-intact
Species	**Treatment**	**Seawater**	**10% DMSO**	**(%)**
Spotted seatrout	DEGXZ	58 ± 9	62 ± 8	19 ± 3
	10D30ET	44 ± 12	42 ± 16	22 ± 2
	40ET	13 ± 9	15 ± 9	23 ± 3
Red snapper	DEGXZ	38 ± 10	37 ± 9	9 ± 2
	10D30ET	43 ± 14	38 ± 13	22 ± 6
	40ET	40 ± 7	33 ± 5	12 ± 4
	20E20G	23 ± 4	22 ± 7	—
	35ET	23 ± 11	21 ± 10	—
	40% EG	10 ± 8	21 ± 11	—
	35EXZ	7 ± 3	8 ± 3	—
	30E10B	2 ± 2	8 ± 4	—
	No CPA[a]	0 ± 0	0 ± 0	—

[a]Cryoprotectant-free vitrification

Red snapper: There was a low correlation between post-thaw motility and membrane integrity (r = 0.411). The highest percentage of membrane-intact sperm was 31% for 10D30ET, but there was no difference in membrane integrity among treatments ($P > 0.061$) (Table 5.6).

Red drum: Fresh sperm motility (79 ± 6%) was positively correlated (r = 0.83) with membrane-intact cells (92 ± 6%), but there was a low correlation between motility and membrane integrity of thawed sperm (r = 0.322). Although the post-thaw motility was significantly different between DEGXZ (as high as 40%) and 10D30ET (as high as 25%) ($P = 0.027$), the percentage of membrane-intact sperm was not different between these two treatments ($P = 0.833$) (Figure 5.1).

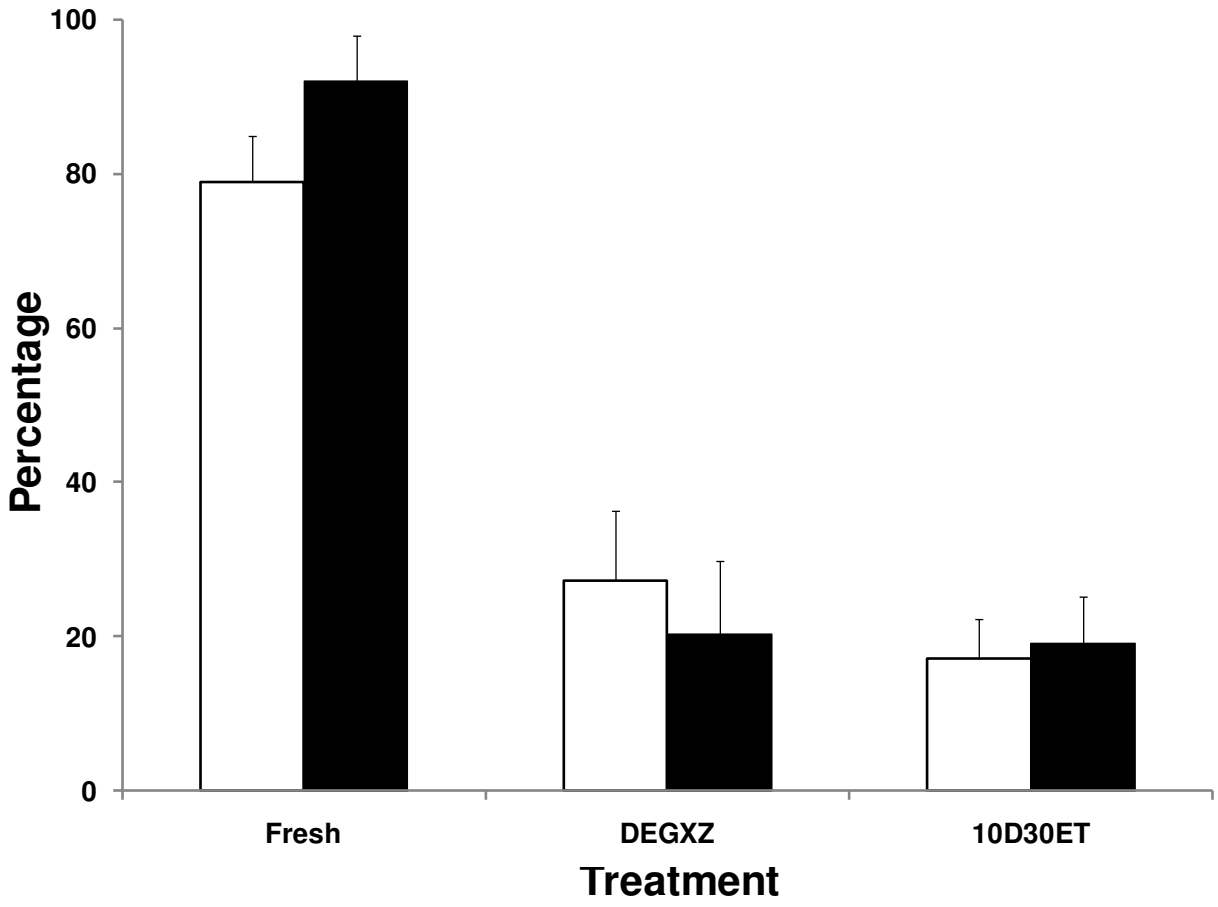

Figure 5.1. Sperm motility (white bars) and membrane integrity (black bars) of red drum (*Sciaenops ocellatus*) (mean ± SD). Sperm were vitrified with 15% DMSO + 15% EG + 10% Gly + 1% X-1000™ + 1% Z-1000™ (DEGXZ), and 10% DMSO + 30% EG + 0.25 M Trehalose (10D30ET). Post-thaw motility was significantly higher for the DEGXZ treatment than for the 10D30ET treatment. The percentage of membrane-intact sperm cells was not significantly different between treatments ($P = 0.833$).

Discussion

Changes in marine biodiversity are caused directly by anthropogenic disturbances such as exploitation, pollution, and habitat destruction, or indirectly through climate change and related perturbations of ocean biogeochemistry (Worm et al. 2006). Our current knowledge of marine biodiversity is not evenly distributed; for example, we know much more about large-bodied species than smaller species. The upper 200 m of the pelagic oceanic environments are well studied, whereas the deep ocean is relatively unknown (Di Poi et al. 2011). It is estimated that as

many as 63% of fish stocks worldwide require rebuilding (Worm et al. 2009), and there is an urgent need for immediate and decisive conservation action (Jackson 2010).

Cryopreservation can contribute to conservation programs, but conventional techniques often require specialized equipment that is unsuitable for use in field environments. New approaches that can be easily used in the field to cryopreserve samples and be applied to assist in conservation programs are urgently needed. Vitrification is well suited for use in the field, does not require expensive equipment and is simple, fast, inexpensive, and offers a new option for conservation biology (Saragusty and Arav 2011). Although vitrification has proven to be a useful tool for the cryopreservation of embryos in mammals, it has yielded controversial results in fish embryos (Hagedorn and Kleinhans 2011). Sperm vitrification in freshwater fishes has had limited success, largely because vitrification requires high concentrations of cryoprotectants (40 - 60%) which translates into high osmotic pressures (>2,000 mOsmol/kg). Exposure to these conditions can damage sperm by chemical toxicity and osmotic effects including changes in plasma membrane integrity (Dzuba and Kopeika 2002). Sperm from freshwater fishes are not adapted to deal with high osmotic pressure, as they generally become active in response to reduced osmotic pressure. Sperm of marine species respond in the opposite manner, with motility activated by increased osmotic pressure (>1,000 mOsmol/kg) (Morisawa 2008). The goal of the present project in marine fishes was to evaluate the potential to develop a standardized approach for vitrification of aquatic species germplasm.

Acute Toxicity of Individual Cryoprotectants

Successful cryopreservation protocols are dependent upon optimizing cooling and warming rates in conjunction with cryoprotective strategies. The key to successful vitrification is to achieve high cooling and warming rates and a high but tolerable subtoxic concentration of cryoprotectants. The major factors to be considered in assessing the toxicity of cryoprotectants are their concentration, the exposure temperature, and the time of incubation or exposure in solution (Chian 2010). Previous cryopreservation studies in spotted seatrout and red drum did not evaluate the acute toxicity of cryoprotectants (Wayman et al. 1996; Wayman et al. 1998). In the case of spotted seatrout one equilibration time of 15 min was evaluated with 4 cryoprotectants (MeOH, Gly, DMSO, and n,n-dimethyl acetamide: DMA) at 2 concentrations (5 and 10%).

Equilibration motility at 15 min in this previous study was high for all treatments (>50% vs. control 70%), except when Gly was used (1%) (Wayman et al. 1996). In the case of red snapper, acute toxicity of 4 cryoprotectants (DMSO, Gly, MeOH, and DMA) at 5 concentrations (as high as 25%) was evaluated in a previous study (Riley et al. 2004). Motility was estimated after the addition of cryoprotectant and every 15 min for as long as 60 min. The results of this study indicated that the sperm from red snapper can tolerate high concentrations of cryoprotectants. Motilities after 15 min exposure to cryoprotectants were 50% for 25% DMSO, 40% for 25% MeOH, and 40% for 25% DMA (control 95%). Although glycerol was toxic at higher concentrations, at 10% the motility was 40% immediately after addition but dropped to 20% at 15 min. To minimize the toxicity of cryoprotectants at higher concentrations, the exposure time can be shortened (Tiersch 2011b).

The present study evaluated relatively high concentrations of cryoprotectants (as high as 30%) at regular exposure times (every 5 min). The acute toxicity experiment was done in June and the only gametes available were from spotted seatrout and red snapper. No further attempts were made to estimate acute toxicity in red drum, which spawn in September to October in the Gulf of Mexico. The least toxic cryoprotectant at higher concentrations was EG, followed by DMSO. Comparing these results with previous studies, DMSO was the least toxic and Gly was one of the most toxic. DMSO is the most common cryoprotectant used for sperm cryopreservation of marine fishes (Gwo 2011). The acute toxicity experiment indicated which of the cryoprotectants were relatively non-toxic at different concentrations and times, and those meeting these criteria were selected for the combined cryoprotectant study.

Acute Toxicity of Combined Cryoprotectants

Upon cooling, vitrification solutions can form a clear transparent solid (glass) rather than an opaque, milky solid (indicating the formation of ice crystals) (Ali and Shelton 1993). Most cryoprotectants tend to have toxic and hypertonic effects when used at concentrations that are effective for successful vitrification (Yavin and Arav 2007). Two common approaches to decrease this toxicity are to combine cryoprotectants, and to limit the exposure time to a minimum. Generally a mixture of cryoprotectants has a lower aggregate toxicity to cells because it vitrifies at lower concentrations, and it combines the additive properties (such as permeability

and glass formation) of each cryoprotectant (Ali and Shelton 2007). In addition, the concentration needed to vitrify can be reduced by adding small quantities of ice-blockers, such as polymers that directly inhibit ice nucleation and growth (Wowk 2010). Furthermore, the addition of sugars, especially disaccharides such as trehalose, can enhance glass formation and reduce the concentrations of cryoprotectants required for vitrification (Fuller 2004). Another approach to reduce the toxicity of cryoprotectants is to use "toxicity neutralizers", which counteract the toxicity of cryoprotectants. Acetamide, for example, has been recommended to block the damaging effect of DMSO (Fahy 1984). However a recent study demonstrated that the benefits of adding acetamide were limited, and that other amides such as formamide work better (Fahy 2010).

In the present study, 29 combinations of cryoprotectants were evaluated for toxicity and glass formation. It should be noted that what appears to be a transparent glass can contain ice nuclei and ice crystals, because the crystals only become optically detectable once they become larger than the wavelength of light (Shaw and Jones 2003). In the present study, the combination of cryoprotectants were highly toxic, especially the vitrifying solutions (those that formed glass). Acetamide did not decrease the toxicity of cryoprotectants, which is in agreement with the limited neutralization reported previously (Fahy, 2010). The addition of trehalose improved sperm survival in spotted seatrout. For example, 30% EG at 10 min yielded a motility of 18%, while 30% EG plus trehalose yielded a motility at 10 min of 45%. Solutions that vitrified and proved to be of limited toxicity to sperm were tested further for their effect on sperm survival after vitrification.

Effect of Thawing Solutions

Exposure to high cryoprotectant concentrations can be toxic to cells, and can result in osmotic damage. Cells exposed to permeating cryoprotectants undergo extensive initial dehydration followed by rehydration, and swelling when the cryoprotectants are removed. A strategy to decrease the osmotic effects of cryoprotectants is the stepwise addition or removal of cryoprotectants (multistep dilution procedure) (Gao et al. 1997). For fish sperm cryopreservation, it has been recommended to add cryoprotectant slowly and gradually (Kopeika et al. 2007), but as long as the cryoprotectant is diluted previously with an extender solution, it can be added to

the sperm (e.g. 1:1) without further stepwise addition (Wayman and Tiersch 2011). In the present study, no attempt was made to load the cryoprotectant using a multistep procedure, but because there were no previous publications on sperm vitrification in marine fishes, the dilution of the thawed sample was compared using two warming solutions. Because sperm were vitrified at high osmotic pressures (>2,000 mOsmol/kg), one warming solution had higher osmotic pressure (10% DMSO in seawater; 2,000 mOsmol/kg) than seawater (1,000 mOsmol/kg). Thawing in a higher osmotic pressure could reduce osmotic changes in cell volume, but in the present study there was no significant difference between thawing directly in seawater or at higher osmotic pressure.

The other important factor that plays a key role in thawing is the temperature. A previous study of sperm vitrification using loops compared two warming temperatures (24 and 37°C) in the green swordtail, and concluded that there was no significant difference in post-thaw motility between temperatures (Chapter 4, Cuevas-Uribe et al. 2011b). Using either of these temperatures yielded warming rates that were fast enough to avoid ice formation during warming (devitrification). Based on this, in the present study a single warming temperature was used.

In the present study, eight vitrification solutions were used with sperm of red snapper. The addition of trehalose, as previously observed in the toxicity experiment, improved the post-thaw motility. For example, 40% EG alone yielded a post-thaw motility of 10%, while 40% EG plus trehalose yielded 40% motility. Trehalose has been used in previous cryopreservation studies, and high fertilization rates (95%) were obtained with thawed sperm of longtooth grouper (*Epinephelus bruneus* formerly *E. moara*) using trehalose as the sole cryoprotectant (Miyaki et al. 2005). In a similar study in orange-spotted grouper (*E. coloides*) trehalose was used as a cryoprotectant, and high fertilization rates (82%) were obtained with thawed sperm (Peatpisut and Bart 2010). Although in the present study trehalose was not used alone, trehalose was used to enhance glass formation in combination with other cryoprotectants. Other vitrification enhancers include synthetic ice- blocking agents such as the polymers X-1000™ and Z-1000™ (Wowk and Fahy 2002). In the present study, combination of 15% DMSO + 15% EG + 10% Gly did not form a complete glass (~50% glass), but when the "ice blocker" polymers were added, 100% glass formation was observed. When this combination (DEGXZ) was used to vitrify sperm, average post-thaw motility was 58% for spotted seatrout, 38% for red snapper, and 30%

for red drum. The combination of DMSO and EG is one of the most popular combinations used in vitrification for mammals (Quinn, 2010). DMSO is a good glass former while EG is a weak glass former (Quinn 2010), but EG is less toxic and permeates faster than DMSO (Gilmore et al. 1995). The DMSO and EG combination decreases the total cryoprotectant concentration necessary to form glass and can improve viability. In the present study trehalose was added to the combination of DMSO and EG (10D30ET), and average post-thaw motility was 44% for spotted seatrout, 43% for red snapper, and 20% for red drum. It should be noted that the lower the concentration of cryoprotectant, the faster the cooling rate that was needed to avoid ice formation. The cooling rate calculated for loops similar to those used in the present study was as fast as 720,000°C/min (Isachenko et al. 2003). This high cooling rate was due to the minimal volume used. The disadvantage of using small-volume samples is the susceptibility to accidental warming (Vajta et al. 2009). Devitrification (ice formation during warming) happens faster than during cooling because ice nucleation occurs at lower temperatures than ice growth (Wowk 2010). That is one of the reasons for applying high warming rates to obtain high survival of vitrified oocytes and embryos (Kuwayama and Leibo 2010).

By use of ultra-rapid cooling and warming rates, and small sample volumes (20-µL in cryoloops), human sperm was vitrified without the use of cryoprotectants (Nawroth et al. 2002). A similar technique was used to vitrify sperm of channel catfish without cryoprotectants, and yielded fertilization (<2%) in 2 of 16 trials (Chapter 3, Cuevas-Uribe et al. 2011a). In a study using the microdrop method (20 µL dropped directly into liquid nitrogen), sperm from rainbow trout (*Oncorhynchus mykiss*) were vitrified without addition of cryoprotectant, and yielded post-thaw motilities as high as 86% (Merino et al. 2011). In the present study, sperm from red snapper was vitrified without cryoprotectants using 10-µL polystyrene loops but no post-thaw motility was observed. There are several differences between these studies, including sperm characteristics (such as cell size, and properties and composition of the cell membrane), and the methodology used (such as extender type, sperm centrifugation, apparatus used, and volume). Due to these differences it is problematic to directly compare these studies without further research.

Effect of Vitrification on Sperm Membrane Integrity

Cryopreservation can alter the cellular structures and physiology of sperm. There are no routinely accepted criteria for estimating sperm quality of aquatic species, especially in comparison to that available for humans (WHO 2010) and livestock (Chenoweth et al. 1992). The quality of cryopreserved aquatic sperm is often estimated by post-thaw motility but this is not always correlated with fertilization (He and Woods 2004; Warnecke and Pluta 2003). Damage induced by cryopreservation can occur to specific structures that would not be detectable by studying a single assay (such as motility) (Tiersch 2011b). The spermatozoal plasma membrane is one of the main structures affected by cryopreservation, and it is an important component in the maintenance of sperm viability (Jenkins 2011). One of the most common tests for plasma membrane integrity in aquatic species is the SYBR 14/ propidium iodide (PI) assay, often referred to as a "sperm viability" assay (Daly and Tiersch 2011). This assay was used in previous cryopreservation study of red snapper (Riley 2002). As in the present study, no correlation was found between post-thaw sperm motility and membrane integrity, and motility estimates were higher than the estimated percentage of membrane-intact sperm. Also, in the present study there was no difference in the percentage of membrane-intact sperm among the treatments used. Estimation of membrane integrity was done after 10 min of thawing and staining the cells with the fluorescent dyes. More research needs to be done to evaluate if the integrity of the membrane from vitrified sperm changes over time, especially right after thawing.

The ultimate measure of sperm quality is fertilizing capacity. Attempts were made to fertilize eggs collected from wild-caught females using vitrified sperm but poor quality eggs (<10% fertilization using fresh sperm) were collected that year (unpublished results). No further fertilization attempts were made in the following breeding season due to the Deepwater Horizon oil spill that took place close to the research location. Future studies need to be conducted to evaluate the fertilization capacity of vitrified sperm.

In summary, the present study demonstrated the feasibility of using vitrification to cryopreserve sperm from marine fishes. Based on the acute toxicity of cryoprotectants, vitrification solutions were developed and successfully applied for sperm from spotted seatrout, red snapper, and red drum. Compared to previous vitrification studies in freshwater species,

marine fish sperm had higher survival after vitrification, perhaps because they are adapted to deal with higher osmotic pressures. One important component of some of the vitrification solutions used in the present study was the disaccharide sugar trehalose, which has previously been used to cryopreserve sperm of marine fishes. Future studies should systematically evaluate trehalose in relation to the different functions that it could provide in the cryoprotectant solution (e.g., as an energy substrate or in biostabilization of the cell).

This is the first report on sperm vitrification in marine fishes. This procedure should offer an alternative approach to conventional cryopreservation for conservation of valuable genetic lineages, such as endangered species, "model" strains used in research, and improved farmed strains. Furthermore, sperm vitrification can be used to transport cryopreserved sperm from the field to the laboratory to expand the genetic resources available for germplasm repositories. Due to the small volumes used, this technique could be utilized to reconstitute lines, but it would require improvement before being useful as a production method. Additional fertilization trials are needed to further evaluate the efficiency of the technique, and because vitrification can be performed in the field, this work could be extended to management programs of endangered species.

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Chapter 6

Vitrification as an Alternative Approach for Sperm Cryopreservation in Marine Fishes

Cryopreservation technology in aquatic species has enhanced hatchery and aquaculture operations by providing flexibility in spawning of females, greater control in breeding programs, and the ability to store favorable genes for extended periods. In addition, concern for native fish populations has resulted in examining sperm cryopreservation as a means to preserve genetic material and transfer genes between wild and hatchery populations (Tiersch 2011a). Problems typically associated with sperm cryopreservation for threatened and endangered fishes are the availability of captive broodstock and the lack of cryopreservation expertise (Tiersch et al. 2004). New and easy approaches to cryopreserve samples in the field are needed (Tiersch et al. 2004). Vitrification is an alternative approach to cryopreservation in which glass (non-crystalline ice) is formed by ultra-rapid cooling (>1,000°C/min) and the use of very high concentrations of cryoprotectants (40 to 50%) (Fahy and Rall 2007).Vitrification has a great advantage over conventional cryopreservation due to its simplicity, speed and utility for field and on-farm applications with no additional equipment required (Moore and Bonilla 2006; Saragusty and Arav 2011).

Vitrification is not practical as an aquaculture production method due to the small volumes (~20 µL) used, however offers a new approach to preserve genetic resources and to reconstitute strains or lines. Vitrification is best suited for use in three areas: biomedical research fish models, genetically improved lines, and imperiled species. There are fish species that produce small sperm volumes, such as in zebrafish (*Danio rerio*) (<5 µL) (Jing et al. 2009) and *Xiphophorus* (<9 µL) (Huang et al. 2004). In other larger species it is difficult to collect large sperm volumes, such as in southern flounder (*Paralichthys lethostigma*) (<500 µL) (Daniels 2000) and the endangered Apache trout (*Oncorhynchus apache*) (<500 µL) (David et al. 2011). Furthermore genetic improvement of fish has been associated with diminished reproductive performance in some species. For example, very small sperm volumes (<100 µL) were produced by Atlantic salmon (*Salmo salar*) broodstock from a selection program for fast growth and late maturation (Zohar 1996) compared with wild fish (>10 mL) (Kazakov 1981), and sex-reversed males from dusky grouper (*Epinephelus marginatus*) produced small sperm volumes (<400 µL)

(Cabrita et al. 2009). Vitrification fits perfectly with the need to conserve germplasm from these important species.

Sperm vitrification has been applied in freshwater fishes and offspring were produced from vitrified sperm samples of Russian sturgeon (*Acipenser gueldenstaedtii*) (Andreev et al. 2009), channel catfish (*Ictalurus punctatus*) (Chapter 3, Cuevas-Uribe et al. 2011a), and the live-bearing fish green swordtail (*Xiphophorus helleri*) (Chapter 4, Cuevas-Uribe et al. 2011b). Our recent studies evaluated sperm vitrification in three marine species: spotted seatrout (*Cynoscion nebulosus*), red drum (*Sciaenops ocellatus*), and red snapper (*Lutjanus campechanus*), with sperm motility and membrane integrity as general indicators of gamete quality (Chapter 5, Cuevas-Uribe 2011). However, these studies did not attempt fertilization or offspring production. The purpose of the present study was to use the information from previous studies and evaluate the fertilization ability of vitrified sperm in marine fish. Southern flounder were chosen because they are difficult to strip sperm, and produce very small quantities of sperm (Watanabe and Daniels 2010). In addition, these fish are used in breeding programs to produce all-female fingerlings (Daniels et al. 2010; Luckenbach et al. 2002). The production of sex-reversed males (genotypic XX) is tedious and labor intensive (Morgan et al. 2006). Vitrification could offer a new way to protect this investment and reconstitute these valuable sex-reversed males.

The goal of this project was to develop a standardized approach for vitrification of aquatic species germplasm. The specific objectives in the present study were to: 1) evaluate thawing methods and vitrification solutions; 2) evaluate the post-thaw membrane integrity of sperm vitrified in different cryoprotectant solutions; 3) examine the relationship between membrane integrity and motility, and 4) evaluate the ability of vitrified sperm to fertilize eggs. Here we report the first successful fertilization by vitrified sperm in a marine fish. Vitrification is well suited for use in the field and offers a new option for conservation biology.

Materials and Methods

Collection of Sperm

Adult southern flounder broodstock held at the North Carolina State University (NCSU) Lake Wheeler Facilities (Raleigh, NC) were induced to spawn by manipulation of photoperiod and temperature (Daniels and Watanabe 2002; Watanabe et al. 2006) during April, 2009, and March-April and August, 2010. The fish were cultured in an artificial seawater (33 ppt) (Crystal Sea Marinemix, Marine Enterprises International Inc., Baltimore, MD) system with a photoperiod of 9 h light:15 h dark at 16 ºC, and they were fed every other day to satiation with BioBrood pellets (Bio-Oregon®, Longview, WA). The fish used were 3-year- old F_3 sex-reversed males (XX neomales), which weighed 0.41 ± 0.23 kg (average ± SD). Males were anesthetized with tricaine methanesulfonate (40 mg/L) (MS-222; Argent Chemical Laboratories, Redmond, WA) and checked for spermiation by applying pressure to the gonadal area. Spermiating males were dried with towels, and sperm was aspirated into 1-mL pipette tips by applying slight pressure to the abdomen. Care was taken to avoid contamination of the samples with urine, feces, or water. Feed was withheld for 2 days before sperm collection to avoid the release of feces during handling. The sperm samples and a sample of the seawater from the system were secured in ZipLoc® bags and shipped overnight to the Aquaculture Research Station (ARS) of the Louisiana State University Agricultural Center in a styrofoam box with two foam refrigerant blocks frozen to -20ºC. A cardboard divider was placed on top of the refrigerant blocks to avoid direct contact with the samples (Tiersch 2011c).

Motility Estimation and Preparation of Sperm Samples

Upon arrival at the ARS, samples were inverted to mix, and motility was estimated using darkfield microscopy (Optiphot-2, Nikon Inc., Garden City, NY) at 200-x magnification. Sperm suspension (1 µL) was placed on a glass slide, and was activated by mixing with 20 µL of seawater (995 mOsmol/kg). Motility estimation was based on an observation of 3-5 different fields within 20 sec after activation, and expressed as the percentage of sperm swimming progressively forward within the sample. Sperm cells that vibrated in place were not considered to be motile.

Sperm concentration was estimated by measuring absorbance of 2-µL aliquots at 601 nm wavelength using a microspectrophotometer (Nanodrop 1000, Thermo Scientific, Wilmington, DE), and by using these absorbance values in the following calculation:

Sperm concentration (cells/mL) = absorbance x $9.77 \times 10^8 - 7.68 \times 10^7$

This equation was generated from a standard curve between absorbance readings of serially diluted sperm suspensions and the sperm concentration ($r^2 = 0.987$) as determined by the use of a hemacytometer (Appendix B, Cuevas-Uribe and Tiersch 2011a).

Samples were diluted to a final concentration of 2×10^9 cells/mL using sperm motility inhibiting saline solution (SMIS) (Lahnsteiner 2000). The SMIS was composed of 600 mg NaCl, 315 mg KCl, 15 mg $CaCl_2 \cdot 2H_2O$, 20 mg $MgSO_4 \cdot 7H_2O$, 470 mg HEPES in 100 Ml of ultrapure water [pH 7.8], with 1.5 g bovine serum albumin, and 0.5 g sucrose at 324 mOsmol/kg. The osmolality was measured with a vapor pressure osmometer (Model 5520, Wescor Inc., Logan, UT). The samples were placed on ice until use in vitrification experiments.

Sperm Vitrification

Sperm were diluted to a concentration of 2×10^9 cells/mL with SMIS. Cryoprotectant solutions were prepared at double-strength in SMIS. For vitrification sperm samples were mixed with double-strength cryoprotectants at a ratio of 1:1 (v/v). Samples were immediately loaded (within 15 sec) into 10-µL polystyrene loops (Nunc™, Roskilde, Denmark) without equilibration, and individually submerged in liquid nitrogen within 1 min (~50 sec) of the addition of the cryoprotectant solution (Appendix A, SOP-2). Loops were stored in goblets (three per goblet) in liquid nitrogen. After at least 12 h of storage in liquid nitrogen, the vitrified loop samples were thawed directly onto a microscope slide containing a 30 µL drop of seawater (~1,000 mOsmol/kg) at room temperature (24°C) or other temperatures as noted, and the motility of thawed sperm was estimated within 30 sec.

Experiment 1: Effect of Thawing Temperatures

The cryoprotectants used were: dimethyl sulfoxide (DMSO; OmniSolv, France), ethylene glycol (EG; Mallinckrodt Baker, Paris, KY), 1,2-propanediol (PROH; Sigma-Aldrich, St Louis, MO), and glycerol (Gly; Mallinckrodt Baker). Six vitrification solutions were tested: (1) 20% DMSO + 20% EG, (2) 20% DMSO + 20% PROH, (3) 20% DMSO + 20% Gly, (4) 20% EG + 20% PROH, (5) 20% EG + 20% Gly, and (6) 20% PROH + 20% Gly. Double-strength cryoprotectant solutions were prepared in SMIS and diluted at 4ºC with sperm suspension at a ratio of 1:1 (final sperm concentration 1 x 10^9 cells/mL) (Appendix A, SOP-1). Samples were immediately loaded (within 15 sec) into 10-µL polystyrene loops without equilibration, and submerged in liquid nitrogen within 1 min after the addition of the vitrification solutions.

Glass formation was assessed by observing the appearance of the vitrified sample (a milky appearance indicated ice crystal formation) (Ali and Shelton 1993). Loops were thawed directly onto a microscope slide containing a 30 µL drop of seawater at two temperatures (21 and 37°C). The motility of each sample was estimated immediately after thawing (Appendix A, SOP-3). Sperm from three males were used in this experiment.

Experiment 2: Evaluation of Vitrification Solutions

Sperm samples from three males were used to evaluate three vitrification solutions: (1) 20% EG + 20% Gly, (2) 10% DMSO + 30% EG + 0.25 M trehalose dehydrate (Tre; Acros Organics, Fair Lawn, NJ), and (3) 15% DMSO + 15% EG + 10% Gly + 1% X-1000™ (21^{st} Century Medicine, Fontana, CA) + 1% Z-1000™ (21^{st} Century Medicine) (DEGXZ). The general vitrification procedure was performed. Loops were thawed directly onto a microscope slide containing 30 µL of seawater at room temperature (24°C). The motility of each sample was estimated immediately after thawing. All trials were replicated a minimum of two times for each individual male.

Experiment 3: Effect of Vitrification Solutions on Membrane Integrity

Sperm samples from three males were used in this experiment. Sperm samples were vitrified using three vitrification solutions: (1) 20% DMSO + 20% EG, (2) 20% DMSO + 20% Gly, and (3) 20% EG + 20% Gly. The samples were stored in liquid nitrogen for 13 days before flow cytometry analysis. To thaw the sperm, each loop was warmed directly in 495 µL of SMIS

at room temperature (24 ºC) to yield a sperm concentration of around 5 x 10^6 cells/mL. To evaluate membrane integrity, fresh and thawed sperm were filtered through 35-µm nylon mesh and duplicate aliquots of 250 µL were stained with the fluorescent dyes SYBR-14 and propidium iodide (PI) (live/dead sperm viability kit, Molecular Probes, Eugene, OR). Final concentrations of the fluorescent dyes were 100 nM SYBR-14 and 12 µM PI, and samples were incubated in the dark for 10 min at room temperature prior to analysis. Flow cytometry was performed using an instrument (C6 Accuri Cytometers Inc., Ann Arbor, MI) equipped with a 488-nm, 50-mW solid-state laser. Flow cytometer performance was assessed using fluorescent validation beads (Spherotech, Accuri Cytometers Inc.) to ensure that coefficient of variation values were < 3.0% (calculated based on full peak height) for the fluorescence detectors (FL1, FL2, FL3, and FL4). Each microcentrifuge tube was flicked gently 3 times with a finger prior to analysis to ensure suspension of the cells, and 10 µL of sample were analysed at a flow rate of 35 µL/min using Cflow® software (version 1.0.202.1, Accuri Cytometers Inc.). Green fluorescence (SYBR 14) was detected with a 530 ± 15 nm bandpass filter (FL1), and red fluorescence (PI) was detected with a >670 nm longpass filter (FL3). Events were viewed on forward-scatter (FSC) vs. side-scatter (SSC) plots, and a gate (used to define target cells within the total event population) was drawn around the sperm population to exclude non-sperm events. Gated events were viewed on a scatter plot showing FL1 vs. FL3 with fluorescence compensation based on the computed median fluorescence values of the different populations to reduce spectral overlap. Sperm that stained with SYBR 14 alone were considered to have an intact membrane, and those that stained with both SYBR 14 and PI or PI alone were considered to be membrane-compromised.

Experiment 4: Fertilization Trials

Females (body weight 1.09 ± 0.28 kg) were injected intramuscular with 0.5 mL/kg of Ovaprim® yielding 10µg/kg salmon gonadotropin releasing hormone analogue + 10 µg/kg of Domperidone (Syndel International Inc., Vancouver, British Columbia) at the North Carolina State University Lake Wheeler Facilities. The hormones were administered in two injections. The first injection was 10% of the total dosage; the second injection, given 24 h later, was the remaining 90%. Eggs were collected approximately 48 h after the second injection. Aliquots of 0.1 mL of eggs (129 ± 35 eggs) were placed into 60-mL plastic cups, and held (< 1 h) for the fertilization trials.

Based on the results of the previous experiments in April, 2009, sperm samples from three males were vitrified at a final concentration of 5 x 10^8 cell/mL using 20% EG + 20% Gly in 10-µL polystyrene loops at the ARS and shipped overnight to NCSU for the fertilization trials. In addition to the vitrification solution, sperm samples from these three males were vitrified without the use of cryoprotectants (cryoprotectant-free vitrification) at a final concentration of 1 x 10^9 cells/mL. For artificial fertilization, three loops of vitrified samples from each individual male were thawed into 15-mL conical tubes (Corning, NY) containing 5 mL of seawater at ~20 ºC (Appendix A, SOP-4). Loops contained in the tube were gently agitated for <10 sec and the suspensions were mixed with egg aliquots. Other aliquots of eggs were mixed with a pool of fresh sperm from at least two males and were used as a control to evaluate the egg quality. Eggs from three females were used for the fertilization trials during this year. The eggs were incubated at ~20 ºC. Fertilization rate was estimated by assessing embryo development to 64-128 cell division stage (3-5 h after fertilization) by use of a dissecting microscope.

In March, 2011, sperm samples from three males were vitrified at a final concentration of 1 x 10^9 cell/mL using 20% EG + 20% Gly in 10-µL polystyrene loops at the ARS and shipped overnight to NCSU for fertilization trials done in February-March , 2011, as described above. Eggs from eight females were used for the fertilization trials during that year.

Statistical Analysis

Data were analyzed using SAS software (Statistical Analysis System Inc., version 9.1; SAS institute, Cary, NC). Analyses were conducted using a mixed ANOVA procedure for all interactions. For the thawing experiment, the fixed treatments were temperature and vitrification solution, and the dependent variable was post-thaw motility. Membrane integrity data were analyzed using a mixed ANOVA procedure with cryoprotectants as fixed treatments and membrane intact as a dependent variable. Percentage data were arcsine-square-root transformed for normalization before analysis, and post hoc Tukey's test was used to locate differences. The significance level was set at $P < 0.05$.

Results

Experiment 1: Thawing Temperatures

The motility of fresh sperm before vitrification was 50 ± 10%. There were no significant differences ($P = 0.697$) in post-thaw motility of sperm thawed at 21°C or at 37°C in all the treatments (Table 6.1). The highest post-thaw motility was 35% for 20% EG + 20% Gly and 20% DMSO + 20% Gly and these were not significantly different ($P = 0.606$). The motility (14 ± 10%) in the vitrification solution of 20% PROH + 20% Gly was not significantly different from motility (21 ± 9%) in 20% DMSO + 20% Gly ($P = 0.059$), but it was significantly different from motility (22 ± 7%) in 20% EG + 20% Gly ($P = 0.018$). The motility in 20% DMSO + 20% EG (2 ± 2%), 20% DMSO + 20% PROH (2 ± 1%), and 20% EG + 20% PROH (3 ± 3%) were not significantly different ($P > 0.446$). Total glass formation was observed in all vitrification solutions; except for 20% DMSO + 20% Gly that formed around 80-90% glass (<20% had a milky appearance).

Table 6.1. Sperm motility (mean ± SD) after thawing and molarity and osmolality for different vitrification solutions: DMSO, dimethyl sulfoxide; EG, ethylene glycol; PROH, propanediol; Gly, glycerol; Tre, trehalose; X, X-1000™; Z, Z-1000™.

	Thawing			Osmolality
Vitrification solution	**21 ºC**	**24 ºC**	**37 ºC**	**mOsmol/kg**
20% DMSO + 20% EG	2 ± 2	—	2 ± 2	5988
20% DMSO + 20% PROH	2 ± 2	—	1 ± 1	4906
20% DMSO + 20% Gly	20 ± 12	—	21 ± 6	5542
20% EG + 20% PROH	3 ± 4	—	3 ± 3	5150
20% PROH + 20% Gly	15 ± 12	—	12 ± 8	4680
20% EG + 20% Gly	19 ± 5	28 ± 9	26 ± 8	7594
10% DMSO + 30% EG + 0.25 M Tre	—	7 ± 3	—	6104
15% DMSO+15%EG+10%Gly+1%X+1%Z	—	14 ± 10	—	7510

Experiment 2: Vitrification Solutions

The motility of fresh sperm before vitrification was 60 ± 10%. The highest post-thaw motility was 40% for 20% EG + 20% Gly, followed by 30% for 15% DMSO + 15% EG + 10% Gly + 1% X-1000™ + 1% Z-1000™ (DEGXZ). These two treatments were significantly different in motility ($P = 0.039$) (Table 6.1). Motility (7 ± 3%) in 10% DMSO + 30% EG + 0.25 M Tre was not significantly different from motility (14 ± 10%) in DEGXZ ($P = 0.114$) but it was significantly different from 20% EG + 20% Gly (28 ± 9%) ($P = 0.008$).

Experiment 3: Membrane Integrity

Fresh sperm motility (57 ± 9%) was positively correlated (r = 0.80) with membrane-intact cells (89 ± 1%) but there was a significant difference between them (P <0.001) (Table 6.2). There was no correlation between motility of thawed sperm and membrane integrity in all treatments (Table 6.2). The highest percentage of membrane-intact sperm after vitrification (17%) was for 20% EG + 20% Gly, and there was no significant difference in motility (P = 0.252). The vitrification solution 20% EG + 20% Gly was not significantly different in motility (P = 0.076) from 20% DMSO + 20% Gly, but it was significantly different in membrane integrity (P = 0.037). The vitrification solution 20% DMSO + 20% EG was significantly different in membrane integrity from 20% EG + 20% Gly (P = 0.004) and from 20% DMSO + 20% Gly (P = 0.045).

Table 6.2. Percentage motility and membrane integrity (mean ± SD) of sperm of southern flounder prior to and following vitrification using different treatments.

Treatment	**% Motility**	**% Intact**	***P* -value**	**Correlation (r)**
Fresh	57 ± 9	89 ± 1	<0.01	0.80
20% DMSO + 20% EG	0 ± 1	2 ± 1	<0.01	-0.26
20% DMSO + 20% Gly	7 ± 6	6 ± 4	0.42	-0.33
20% EG + 20% Gly	13 ± 6	11 ± 4	0.25	0.18

Experiment 4: Fertilization Trials

From the three females used in 2009, only one female yielded usable eggs (>20% control fertilization). Fertilization from vitrified sperm of one male yielded the same fertilization as the fresh sperm control (Table 6.3). But the vitrified sperm from the other two males yielded low fertilization (<5%). Cryoprotectant-free vitrification did not yield fertilization.

From the eight females used in 2011, data from four females with control fertilization of >9% were used. The same males were used for females 1, 2 and 3 (Table 6.3). Due to logistic problems no fertilization was attempted for males 1 and 3 with female 3 (Table 6.3). There was large male-to-male variation in the fertilization trials. For example, male 2 yielded 23% fertilization with female 1, while male 3 yielded 6% with the same female. In female 2, the fertilization from vitrified sperm yielded the same fertilization as the fresh control. Fertilization for female 4 yielded low levels of fertilization (<10% vs 20% control) (Table 6.3).

Table 6.3. Fertilization percentage (mean ± SD) assessed at the 64-128 cell division stage. The same males were used for females 1, 2 and 3 in 2011.

		Male				
Female	**Year**	**1**	**2**	**3**	**Average**	**Control**
1[a]	2009	50 ± 20	3 ± 2	3 ± 1	19 ± 25	50 ± 0
1[b]	2011	12	23	6	14 ± 9	54 ± 5
2[b]	2011	8	7	8	8 ± 1	9 ± 2
3[b]	2011	—	13	—	13 ± 0	27 ± 6
4[c]	2011	1 ± 0	0 ± 0	9 ± 2	3 ± 4	20 ± 4

[a]Mean ± SD of three replicates of egg batches
[b]No replicates for individual males
[c]Mean ± SD of two replicates of egg batches

Discussion

The production of sex-reversed males by chromosome set and sex manipulation can produce disruptions in gonadal development (Devlin and Nagahama 2002). For example, some sex-reversed males of rainbow trout (*Oncorhynchus mykiss*) lack sperm ducts, and therefore sperm cannot be stripped (Bye and Lincoln 1986). These sex-reversed males need to be sacrificed to collect the sperm. The creation of an all-female offspring system through the indirect method (combining sex reversal and breeding) or by combining gynogenesis and sex reversal is tedious and time-consuming (Piferrer 2001). This is due to the time required for progeny testing and the low survival of the gynogens. For example, the survival as percentage hatch of southern flounder after the induction of meiotic gynogenesis was <2% (Morgan et al. 2006). After the establishment of an all-female system, it is relatively easy to produce sex-reversed males by masculizing a small portion of the offspring. But if for any reason the sex-reversed males are lost, the entire process needs to be repeated.

Although standard cryopreservation is a proven method for long-term storage of genetic material, vitrification is an attractive alternative which has been used in mammals for the cryopreservation of spermatozoa, embryos, oocytes, stem cells, and organs (Tucker and Liebermann 2007). It offers a new approach to expand genetic resources, to protect the stocks, to reconstitute lines, and to transport frozen sperm from the field to the laboratory. Studies in fish vitrification can be traced back to 1938 when Basile Luyet attempted to vitrify juvenile goldfish

(*Carassius auratus*) (40 mm, standard length) by plunging the fish into liquid air (-194 ºC) (Luyet 1938). Several attempts have been made to vitrify fish embryos with limited or no success (reviewed in Cuevas-Uribe and Tiersch 2011b, Chapter 2). Recently vitrification has been applied for the cryopreservation of fish sperm. Sperm vitrification protocols have been developed in marine species (Chapter 5, Cuevas-Uribe 2011), and these protocols yielded motility as high as 60%, and membrane integrity as high as 23% in spotted seatrout, red snapper, and red drum. However, these studies did not attempt fertilization or offspring production. One of the most important assessment methods for sperm quality of cryopreserved sperm is the ability to fertilize eggs and produce offspring. The purpose of the present study was to evaluate the fertilization ability of vitrified sperm.

The greatest challenge in applying the vitrification approach is to formulate an appropriate vitrification solution and develop equilibration and dilution procedures that minimize osmotic and toxic injury (Rall 1991). In the present study, we used vitrification solutions developed from previous studies with marine fish (Chapter 5, Cuevas-Uribe 2011), and the green swordtail (Chapter 4, Cuevas-Uribe et al. 2011b). There are no previous studies in cryopreservation in southern flounder. Cryoprotectants were selected to form the vitrification solutions based on previous cryopreservation studies from other flatfishes. For example, DMSO was used to cryopreserve sperm from turbot (*Scophthalmus maximus*) (Chereguini et al. 1997) and spotted halibut (*Verasper variegatus*) (Tian et al. 2008); PROH was used to cryopreserve sperm from yellowtail flounder (*Limanda ferruginea*) (Richardson et al. 1999) and winter flounder (*Pseudopleuronectes americanus*) (Rideout et al. 2003), and Gly was used to cryopreserve sperm from olive flounder (*Paralichthys olivaceus*) (Zhang et al. 2003) and east coast sole (*Austroglossus pectoralis*) (Markovina and Kaiser 2009). All of these previous studies used comparatively low concentrations of cryoprotectants (<15%). The high concentrations of cryoprotectants required for vitrification (>40%) are near to the maximum tolerated by cells (Mazur et al. 2008). To decrease the toxicity of individual cryoprotectants, a mixture is often used because it can combine the cumulative properties of each cryoprotectant such as permeability and glass formation (Weiss et al. 2010).

In Experiment 1 of the present study, four cryoprotectants were chosen, and eight vitrification solutions were tested. The solutions that contained Gly yielded the highest post-thaw

motilities. There was not a relationship between osmolality of the vitrification solutions and the post-thaw motility. This was different from previous studies in mammals which suggested lowering the molarity to reduce the toxicity of the vitrification solutions (Ali and Shelton 2007). The highest post-thaw motility was for the combination of Gly and EG. Previous studies in turbot and spotted halibut that used EG for standard cryopreservation at 15% reported low post-thaw motilities (< 5%) (Dreanno et al. 1997; Tian et al. 2008). In a vitrification study that used 20% EG + 20% Gly to vitrify sperm from red snapper, the average post-thaw motility was 23% (Chapter 5, Cuevas-Uribe 2011) which was similar to the motility yielded in this study (26%). In a vitrification study with the freshwater green swordtail, the same vitrification solutions yielded post-thaw motilities of ~10% (Chapter 4, Cuevas-Uribe et al. 2011b).

In the present study the two temperatures (21 and 37 ºC) used to thaw the samples were not significantly different in motility. This corresponded with a previous study with the green swordtail in which no difference in motility was found between thawing at 24 or 37 ºC (Chapter 4, Cuevas-Uribe et al. 2011b). Due to the small volumes each of these temperatures yielded warming rates that were fast enough to avoid ice formation (devitrification). The warming rate estimated for a similar loop at 37 ºC could be as fast as 200,000 ºC/min (Katkov et al. 2003). This result is in agreement with a recent report that high warming rate (118,000 ºC/min) was more critical than cooling rates (95 to 69,250 ºC/min) in the survival of vitrified oocytes and embryos in mammals (Mazur and Seki 2011).

In Experiment 2, two vitrification solutions developed for use with the marine fishes (red snapper, spotted seatrout, and red drum) were evaluated (Chapter 5, Cuevas-Uribe 2011). To enhance glass formation, trehalose or the polymers X-1000™ and Z-1000™ were added to cryoprotectant mixtures. A previous study with marine fishes that used the vitrification solution 10% DMSO + 30% EG + 0.25 M Trehalose yielded high post-thaw motility in red snapper (~40%), spotted seatrout (~40%), and red drum (~20%) (Chapter 5, Cuevas-Uribe 2011). In the present study this vitrification solution yielded low post-thaw motility (~7%). The addition of trehalose did not increase the survival of southern flounder sperm after vitrification. This result is similar to a previous study that used the same vitrification solution in the green swordtail and resulted in low post-thaw motility (~4%) (Chapter 4, Cuevas-Uribe et al. 2011b). Trehalose has been shown to have a protective benefit in standard cryopreservation for marine fishes such as in sex-reversed orange-spotted grouper (*Epinephelus coioides*) (Peatpisut and Bart 2010), and

longtooth grouper (*Epinephelus bruneus*) (Miyaki et al. 2005). It seems that the benefits of trehalose is species dependent.

The other macromolecules used in the present study were the commercial polymers X-1000™ and Z-1000™. In addition to reducing the cryoprotectant concentration needed to vitrify, these polymers can act as "ice blockers" which inhibit ice nucleation and growth (Wowk and Fahy 2002). The vitrification solution DEGXZ was used with sperm from marine fishes, yielding high post-thaw motilities in red snapper (~40%), spotted seatrout (~60%), and red drum (~30%) (Chapter 5, Cuevas-Uribe 2011). In the present study this vitrification solution yielded post-thaw motility of ~14%.

The highest post-thaw motility in Experiments 1 and 2 was using 20% EG + 20% Gly. These cryoprotectants have low toxicity (Shaw and Jones 2003) but Gly is a poor glass former (forms glass at 46% concentration) compared to EG (forms glass at 40% concentration), and that EG permeates faster than Gly (Shaw and Jones 2003). The combination of Gly and EG is one the most common combinations used in vitrification (Ali and Shelton 2007). This mixture between a poor glass former and a fast permeable cryoprotectant was advantageous in the present study for southern flounder (post-thaw motility 20-30%). This solution was used in red snapper (~20%) and the green swordtail (~10%) (Chapter 5, Cuevas-Uribe 2011). Because this vitrification solution yielded the highest motilities, it was further evaluated for membrane integrity.

Sperm motility alone is not necessarily a reliable predictor of fertilization (Kopeika and Kopeika 2008). The high concentrations of cryoprotectants (>40%), and the high osmotic pressures (>4500 mOsmol/kg) from the vitrification solutions could damage sperm by chemical toxicity and osmotic effects including changes in plasma membrane integrity. If the plasma membrane is not functionally intact the sperm is compromised in viability and the capability to fertilize (Silva and Gadella 2006). One of the most common assays to assess plasma membrane integrity in fresh and post-thaw sperm is the SYBR 14/ propidium iodide stain combination, often referred to as a "Live/Dead" or "sperm viability" assay. For this analysis, "viable sperm" are defined as cells that possess an intact plasma membrane. In a previous study with sperm vitrification in channel catfish, the percentage of membrane-intact cells increased as the cryoprotectants led to more glass formation (Chapter 3, Cuevas-Uribe et al. 2011a). In another study with green swordtail sperm, membrane integrity was evaluated after the addition of the

vitrification solutions just before vitrification, and after vitrification. Before vitrification membrane-intact cells were ~70%, and around 10% viable sperm were recorded after vitrification (Chapter 4, Cuevas-Uribe et al. 2011b). In the present study the highest percentage of membrane-intact cells was for the vitrification solution 20% EG + 20% Gly, which corresponded with the treatment with the highest post-thaw motility. Coincidentally this vitrification solution did not contain DMSO. The post-thaw motilities in this experiment (Experiment 3) were lower than in the previous experiments. For example, the post-thaw motility in this experiment for the treatment 20% DMSO + 20% Gly (~7%) was different from Experiment 1 (~20%). In a similar manner the post-thaw motility for 20% EG + 20% Gly in this experiment (~13%) was different from Experiment 1 (19-26%), and Experiment 2 (~28%). These differences could be due to male-to-male variation or because of incomplete recrudescence. The males from Experiment 2 were stripped in March while the males used for the flow cytometry experiment were stripped 5 months later. Although these males were held in environmentally-controlled recirculating systems, a minimum of 5 months is required for recrudescence to regain the requisite energy and storage depots such as lipids (Watanabe et al. 2006).

The low correlation between post-thaw motility and membrane integrity could be due to male-to-male variation. It is important to mention that one loop was used for motility while another loop was used for membrane integrity. Although the loops came from the same male, there was loop-to-loop variation. For example, post-thaw motility from one loop using the treatment 20% DMSO + 20% Gly was 20% while another loop from the same male and treatment was 5%. This treatment also yielded post-thaw motility of 5% in two loops in a different male. One possible reason for loop-to-loop variation could be due to the small volumes involved which increased the susceptibility to accidental warming during handling and storage. However, more research needs to be done to determine the sources of this variation.

The highest post-thaw motility and membrane integrity was observed for 20% EG + 20% Gly. This vitrification solution was subsequently used for the fertilization trials. The criterion for fertilization was examination of the incubated eggs for early stages of embryonic cleavage. Fertilization rate was determined at 3 to 6 h post-fertilization. At this time the embryos are in a

multicellular stage (64-128 cell division stage) that is easily identified (Daniels 2000). This fertilization criterion had been used in previous reproductive studies in southern flounder (Berlinsky et al. 1996). In the present study, fertilization percentage varied among females and males. Vitrified sperm yielded fertilization as high as 70% (fresh sperm control 50%). On average, fertilization with vitrified sperm ranged from 10 to 20%. There were times that fertilization percent was low; this could be due to the loop-to-loop variation or to the difference among egg batches from the same female. A previous study noted that fertilization rates varied among egg batches from the same female (Berlinsky et al. 1996). This could be due to the reproductive characteristics of southern flounder, which is a serial spawner (i.e. multiple clutch, group synchronous), producing multiple batches of eggs during the spawning season in intervals of 3-4 days (Watanabe and Daniels 2010).

The sperm samples vitrified without cryoprotectants (cryoprotectant-free vitrification) did not yield fertilization. This is in contrast to results in a recent study of rainbow trout that reported motility of ~80% after cryoprotectant-free vitrification (Merino et al. 2011). No fertilization trials were done in the rainbow trout study, and there was no clear description of motility assessment. In another attempt of cryoprotectant-free vitrification, done in red drum, no motility was observed (Chapter 5, Cuevas-Uribe 2011). For estimation of percent motility, only sperm that are actively swimming in a forward motion should be included (Tiersch 2011b). In addition, the rainbow trout study reported mitochondrial membrane potentials of ~50% when bovine serum albumin was used. In the present study, the extender used (SMIS) contained bovine serum albumin and no motility was observed. Cryoprotectant-free vitrification has proved to be a useful tool in mammals. For example, in humans cryoprotectant-free vitrification yielded fertilization equal to slow cooling (Isachenko et al. 2004). In fish, cryoprotectant-free vitrification in channel catfish yielded limited success (<2% fertilization in 2 of 16 trials) (Chapter 3, Cuevas-Uribe et al. 2011a). More research needs to be done to evaluate the fertilization ability of cryoprotectant-free sperm vitrification in other fishes.

It should be noted that one of the disadvantages of vitrification is potential microbial and viral contamination especially in open systems such as the loops used in this study. There are various approaches to prevent contamination such the use of heat-sealed methods of an extra container and use of sterile liquid nitrogen (Bielanski and Vajta 2009). More research needs to be

done to evaluate the levels of contamination when samples are directly plunged into liquid nitrogen.

As more marine fish become imperiled directly from anthropogenic disturbances such as exploitation, and habitat loss and degradation, or indirectly through climate change, there is an urgent need for immediate and decisive conservation action. As much as 65% of fish stocks worldwide require rebuilding (Worm et al. 2009), and marine fisheries has been projected to collapse (losses in catch of 90% below the historic maximum) by 2048 (Worm et al. 2006). As an example, inshore trawling by the shrimp fishery in North Carolina may result in bycatch as high as 18 kg of fish per kg of shrimp (Miller et al. 2010). This bycatch is composed of 67% flatfishes, of which 73% were juvenile southern flounder and 26% juvenile summer flounder (*Paralichthys dentatus*). Conservation efforts need not be delayed while awaiting more species to become endangered. Cryopreservation can contribute to conservation programs, but conventional techniques require specialized equipment unsuitable for use in field environments. New approaches that can be easily used in the field to cryopreserve samples and be applied to assist in conservation programs are urgently needed. In the present study we offer a technique that could be used in the field and yields relatively good fertilization considering that the control fertilization was 50%, and the vitrified sperm fertilization was 10-20%, similar to the 20-30% of conventional cryopreservation (Hu et al. unpublished data). The urgent situation of some species demand attempts for store germplasm even in face of low expectation of success (Holt et al. 2003). Protection of genetic diversity by sperm vitrification offers a new approach for conservation biology.

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Chapter 7
Summary and Conclusions

Fishes are the largest and most diverse group of vertebrates with 32,000 described species (IUCN Red List 2011). Fishes are of immense value to humans as food, sport and ornamental resources. However fish species conservation, with few exceptions, has largely been neglected (Paxton and Eschmeyer 2003). One of the reasons for this is that the status of populations in the wild is difficult to assess because fishes are largely invisible compared to terrestrial species (Barton 2007). Currently only 29% of the total number of described species have been evaluated in the wild (IUCN Red List 2011), and more than 2,011of the 9,352 species evaluated in the wild are currently known to be threatened. Nearly 50% of freshwater fishes in Europe (Anon. 2010) and 40% in North America are considered to be threatened (Jelks et al. 2008). Extinction rates for freshwater species are four to six times higher than those for terrestrial or marine species (Vincent and Hall 1996; Mooney et al. 2009). In addition, it is not easy to assess the status of marine fishes because widespread species, such as migratory tuna, have separate populations under varying degrees of threats (Paxton and Eschmeyer 2003).

In the past, it was believed that marine species were safe because of the size of the oceans, huge population sizes, long-distance dispersal, astounding fecundity, and the pelagic nature of their eggs and larvae (Myers and Ottensmeyer 2005). However, these assumptions are proving to be false, yet persistence of the "extinction-proof" myth leads to denial that extinction is occurring on a massive scale and that humans are largely responsible (Myers and Ottensmeyer 2005). At least 396 marine fish species are known to be globally threatened (IUCN Red List 2011). Around 40% of coral reefs have been lost over the past 40 years, and losses continue at the rate of 1 to 2% per year (Bruno et al. 2007). Although coral reefs make up only 0.2% in area of the marine environment, they harbor around one third of all described marine species (Veron et al. 2009) and over 4,000 species of teleost fishes are associated with coral reefs (Munday et al. 2008). Without prompt action, coral reefs and many of their associated animals may cease to exist within the next 40 years, causing the first global extinction of a worldwide ecosystem within history (Veron et al. 2009).

As the world population increases and the average consumption of fish per person is 17 kg per year (FAO 2010), there is a severe decline in wild fish stocks or populations, and fisheries are already at or above sustainable levels. It is estimated that as many as 63% of fish stocks worldwide require rebuilding (Worm et al. 2009). Irreversible genetic losses can occur, compromising species integrity and fisheries resources. Aquaculture, sometimes associated with restocking, is not only important for increasing food production but is potentially a key means of promoting the recovery of overfished stocks by reducing fishing pressure and enhancing wild stocks (Liu and De Mitcheson 2008). Yet beyond preservation of genetic resources, there is additional potential for cryopreservation to provide commercial aquaculture with avenues for genetic progress in domestication and agronomic performance (Silverstein 2011). Just as the dairy industry, cryopreservation has become essential for the production of genetic improvement by acceleration of selective breeding efforts, maintenance of improved lines, and distribution of superior genetic material (Wolters and Tiersch 2004). Aquaculture accounts for half of total food fish supply (FAO 2010). But future growth in aquaculture is constrained by losses in genetic resources, which compromise genetic progress, and the availability of wild marine fish for aquaculture feed. Aquaculture uses 70% of the global fishmeal and 90% of the fish oil (Tacon and Metian 2008). Loss in biodiversity ultimately results in threats to food security. Successful aquaculture must be sustainable (i.e. aquaculture should be aiding the ocean not depleting it). A sustainable industry reduces waste discharges; it uses resources efficiently; it is responsible to consumer and communities; and it has a high regard and appreciation for maintaining the quality of the surrounding ecosystems (O'Bryen and Lee 2007).

Although the traditional goal of conservation programs is to preserve genetic variation within targeted populations of a species (Rall 1993), the goal in fishes is to also conserve the resource and its sustainable use. Research on the conservation of fishes has followed two routes: concerns with sustainability of commercially caught species, and the concern with maintenance and conservation of particular species and biodiversity (Anon. 2010). Fisheries managers protect or conserve fishes for human consumption or use, but not always sustainably (Reid and Hall 2003). The goals of fisheries managers should approximate those of conservation biologists in seeking long-term viability of fish populations (Vincent and Hall 1996). A change of paradigm is

required involving integrated research and management (i.e. conservation and sustainable use) (Anon. 2010).

Controversy associated with the field of conservation biology is associated with identifying the best method to maintain "genetic fitness" for future generations (Rall 1993). Conservation efforts are often classified into: (1) *in situ* programs that protect and manage animal populations within their natural, native habitat; and (2) *ex situ* programs that remove individuals, gametes or embryos from wild populations for controlled breeding and management in captivity (Rall 1993). *Ex situ* conservation can be *in vivo* as individuals or populations held in research establishments and aquaria, or *in vitro* as cryopreserved sperm, or more rarely as embryos and tissues containing DNA (Pullin 2008) (Figure 7.1). In an ideal world, habitat preservation would always be the highest priority, helping to protect entire ecosystems and species assemblages simultaneously (Pukazhenthi et al. 2006). However *in situ* conservation alone cannot be relied upon to ensure the long-term viability of species at risk. This is especially true when habitats have undergone extensive change and are under constant threat from environmental changes (or global environmental impacts) and anthropogenic disturbances (Figure 7.1). Although *ex situ* approaches are never preferred over sustaining truly natural systems, gene banking provides long-term security for maintaining biodiversity. Currently *ex situ* management offers the only hope for some populations until the immediate crises can be resolved and sufficient genetic diversity can be preserved for future reintroduction into restored habitats (Rall 1993). However, *ex situ* approaches should be complementary rather than competitive with other conservation approaches (Pukazhenthi et al. 2006). *Ex situ* collections should be built with several potential uses in mind, including: population regeneration (in worst-case scenarios), maintenance of genetic diversity in populations of interest, providing a source of diversity that industry can use when market conditions change (by protecting farmable aquatic species to ensure their future availability for use in aquaculture), providing a source of germplasm for the development of new research or industry lines, and providing a source of DNA for research purposes (Blackburn 2011). *Ex situ* conservation becomes the main or only approach to ensure long-term conservation and availability (Pullin 2008).

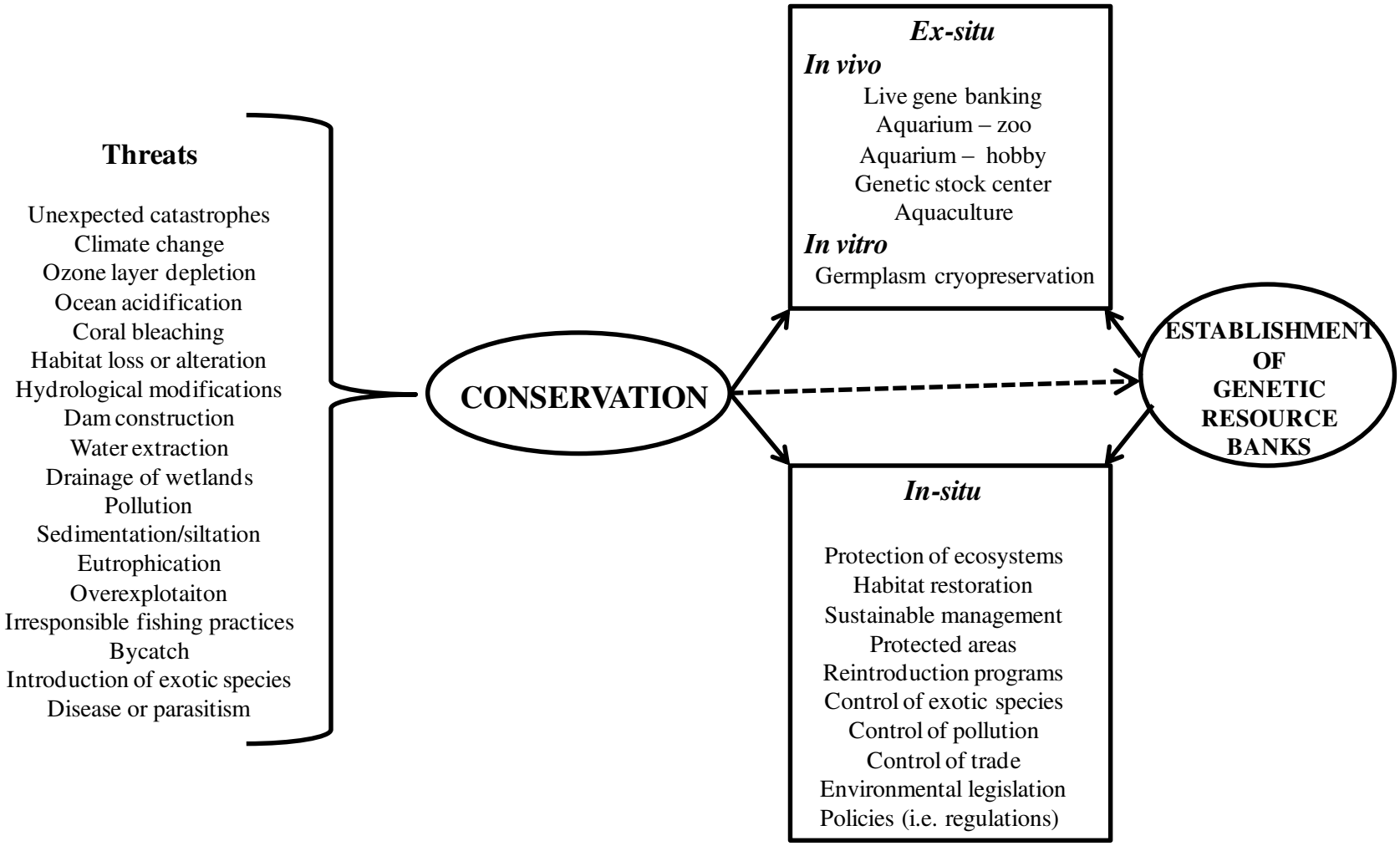

Figure 7.1. Threats that affect fish species and the approaches that can be used for conservation. *In situ* conservation focuses mainly on habitat recovery, so as to prevent degradation or loss. *In situ* conservation alone may not be enough to ensure fish survival because the threats that cause population decline interact, multiply and have additive and synergistic effects. Genetic resource banks can provide a warehouse for conservation approaches to withdraw and distribute genetic material. This material can be used to infuse populations with genetic variation. Genetic resource banks provide insurance against complete loss of highly endangered species.

This dissertation addressed an alternative approach from conventional sperm cryopreservation that can serve in basic and applied research for *in situ* and *ex situ* conservation efforts. The overall goal of this project was to develop streamlined protocols that could be integrated into a standardized approach for vitrification of aquatic species germplasm. The specific objectives were to: 1) identify suitable vitrification solutions by measuring acute toxicity of cryoprotectants at various concentrations; 2) determine appropriate cooling rates by evaluating apparatus configurations and volumes of samples; 3) determine effective warming methods; 4) evaluate the quality of thawed sperm; 5) evaluate the ability to fertilize eggs, and 6) develop approaches to integrate vitrification protocols into existing working repository systems for cryopreserved sperm.

Because sperm vitrification was a new approach in fish, channel catfish (*Ictalurus punctatus*) was first used as a model species to evaluate the feasibility of the method (Chapter 3). Sperm vitrification was successfully applied and offspring were produced (Table 7.1). In general, fertilization values were low (< 10%) for the experiments in which low concentrations of cryoprotectants were used, while fertilization values were higher (around 25%) for experiments in which vitrification solutions (high cryoprotectant concentrations that form glass) were used. This was reflected in flow cytometry data that revealed highest membrane integrity for the use of vitrification solutions. The use of small volumes in loops (10 µL) yielded a higher percentage of membrane-intact cells than 0.25-mL straws. Vitrification could be used to reconstitute lines (especially in small aquarium fishes), but it will require improvement and scaling up before being useful as a production method.

Fishes have become increasingly important as laboratory and assay organisms. Biomedical research using fish as model organisms has produced thousands of mutants and transgenic animals – a genetic resource that represents enormous scientific and informational investment and value (Varga and Tiersch 2011). The utility value of these resources is now increasingly limited by the constraints of maintaining the fish strains alive, and there is potential danger of loss for them all (Varga and Tiersch 2011). Vitrification fits perfectly for work with the limited sperm volumes available from these biomedical fishes to safeguard and ensure the continued availability of unique genotypes and mutants. Vitrification is a simple technique that is

Table 7.1. Summary of the experiments used to validate final procedures developed for sperm vitrification in research Chapters 3 through 6 (mean ± SD). The cryoprotectants used were: methanol (MeOH), methyl glycol (MG), propanediol (PROH), glycerol (Gly), ethylene glycol (EG), dimethyl sulfoxide (DMSO), Trehalose (Tre), X-1000™ (X), and Z-1000™ (Z).

Species	N	Vitrification solution	Fresh		Post-thaw		Fertilization	
			Motility (%)	Membrane -intact (%)	Motility (%)	Membrane -intact (%)	Mean (%)	Highest (%)
Channel catfish	6	20%MeOH+10%MG+10%PROH	64 ± 13	88 ± 1	<1	50 ± 4	11 ± 11	25
	6	20%MeOH+20%MG	64 ± 13	88 ± 1	<1	45 ± 6	4 ± 3	9
Green swordtail	10	20%Gly+20%EG	58 ± 14	87 ± 0.3	8 ± 7	6 ± 1	5 of 10[a]	4 fish
	3	10%DMSO+30%EG+0.25M Tre	64 ± 13	—	4 ± 3	—	—	—
	3	15%DMSO+15%EG+10%Gly+XZ[b]	77 ± 11	—	58 ± 9	19 ± 3	—	—
Red snapper	3	20%Gly+20%EG	77 ± 6	—	23 ± 4	—	—	—
	3	40%EG+0.25M Tre	77 ± 6	—	40 ± 7	12 ± 4	—	—
	3	10%DMSO+30%EG+0.25M Tre	77 ± 6	—	43 ± 14	22 ± 6	—	—
	3	15%DMSO+15%EG+10%Gly+XZ[b]	77 ± 6	—	38 ± 10	9 ± 2	—	—
Red drum	3	10%DMSO+30%EG+0.25M Tre	79 ± 6	92 ± 6	17 ± 5	19 ± 6	—	—
	3	15%DMSO+15%EG+10%Gly+XZ[b]	79 ± 6	92 ± 6	27 ± 9	20 ± 10	—	—
Southern flounder	22	20%Gly+20%EG	57 ± 9	89 ± 1	13 ± 6	11 ± 4	12 ± 17	71
	3	10%DMSO+30%EG+0.25M Tre	60 ± 10	—	7 ± 3	—	—	—
	3	15%DMSO+15%EG+10%Gly+XZ[b]	60 ± 10	—	14 ± 10	—	—	—

[a]Ten females were inseminated; five produced oocytes
[b]1% X-1000™ + 1% Z-1000™

well suited for use with small-bodied species, does not require specialized equipment, and offers advantages for use in the field. Vitrification was applied in this project to the biomedical model *Xiphophorus hellerii* (Chapter 4). Offspring were produced from this live-bearing fish after artificial insemination with vitrified sperm (Table 7.1). Motility of sperm in the acute toxicity experiment decreased as the cryoprotectant concentration increased. Accordingly, the least toxic cryoprotectants were combined to form vitrification solutions. The use of two warming temperatures (24 or 37ºC) yielded no difference in motility. There was no detectable damage to the sperm cell membrane immediately after the addition of vitrification solutions and before vitrification. As such, the damage occurred during cooling and warming. Offspring were produced for the first time with vitrified sperm in live-bearing fishes from virgin females inseminated with sperm frozen in loops with 20% glycerol + 20% ethylene glycol (Table 7.1).

Conventional cryopreservation techniques require specialized equipment that is unsuitable for use in field environments. The simplicity of vitrification makes it well suited for use in the field without the need for expensive equipment. In this project, sperm were collected from marine fishes captured from the recreational fishery. Sperm vitrification from marine fishes proved to be reliable in salvaging genetic material in the field from dead fish (Chapter 5). Post-thaw motility in marine fishes was higher than in freshwater fishes (Table 7.1). One possible reason for this difference is that sperm from marine fishes are adapted to deal with high osmotic pressures (sea water > 1,000 mOsmol/kg) when compared with freshwater fish (20 mOsmol/kg). Based on these data and previous research in freshwater species, adaptations by marine fishes could explain the survival in the high cryoprotectant concentrations (40- 60%) required for vitrification which translate to high osmotic pressures during equilibration (> 2,000 mOsmol/kg). There were differences however in post-thaw motility among marine fishes using the same vitrification solution. This could be due to the differences in sperm characteristics such as properties and composition of the cell membrane.

To translate the research findings into applied benefits, such as species conservation and preservation of genetic diversity, sperm from sex-reversed southern flounder (*Paralichthys lethostigma*) was vitrified (Chapter 6). On average, fertilization with vitrified sperm ranged from 10 to 20% (Table 7.1). Vitrification offers the means to safeguard the results of aquaculture

breeding programs by conserving improved germplasm from genetically important individuals, and making this material available over time and distance. Vitrification offers a new approach to expand genetic resources and offers new options for aquaculture and conservation biology.

The simplicity, speed, and utility for field and on-farm application with no additional equipment makes vitrification an attractive alternative for germplasm cryopreservation. The application of vitrification to conservation programs could be directly integrated with the existing of planned germplasm cryopreservation for long-term storage in genetic resource banks. Because fish in the wild are difficult to see and evaluate, inventories of fishes are incomplete and rates of species loss may be higher than currently estimated (Lévêque et al. 2008). As such, it is possible that many fish species may never be described before their extinction (Anon. 2010). Germplasm storage from extremely threatened species should be undertaken to prevent complete loss of the genetic information (Thorpe et al. 1995) because this could be the only chance of survival for some species (Rall 1993). The urgent situation of some species demands attempts to store germplasm even with low expectations of success (Holt et al. 2003). Genetic diversity in threatened populations can be protected from unforeseen dangers or predicted changes by cryopreserving sperm from adequate number of males (Pukazhenthi and Wildt 2004). Genetic diversity can be thus maintained as frozen sperm in liquid nitrogen, and cryopreserved samples can be regarded as being genetically equivalent to living animals (Holt et al. 2003). Field-friendly methods are needed in remote geographic areas, where the majority of critically endangered species occur.

Sperm vitrification has enormous potential because it can be applied in most field conditions, without removing animals from the wild. The results of this dissertation demonstrate that vitrification can be applied to freshwater, viviparous, and marine fishes (a species panel chosen to address fish diversity). Offspring were produced from vitrified sperm from each of these categories; and higher fertilization resulted when using vitrifying (i.e. glass-forming) solutions. Sperm from fishes can withstand the high concentrations of cryoprotectants needed for vitrification, especially marine fishes. However, because the sample volumes used were small (~20 µL), vitrification is currently best suited for use with small fishes in the areas of biomedical research models, genetic improvement of lines, and germplasm and gene-banking for imperiled

species. The work of this dissertation confirms that sperm vitrification should be considered as an alternative approach to conventional cryopreservation.

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Appendix A
Standard Operating Procedures

SOP-1. Preparation of Vitrification Solutions

Materials needed:

Latex gloves
Permanent marking pen
Scissors
Micropipette and tips (100 and 1000 µL)
Vortex mixer
1.5-mL microcentrifuge tubes
Rack for microcentrifuge tubes
Balance
Weigh boats
Laboratory notebook

For catfish:
1 L of Hanks' balanced salt solution prepared at 300 mOsmol/kg (HBSS300) (Tiersch et al. 1994)
Methanol (reagent grade)
Mehtyl glycol (2-methoxyethanol)
1,2-Proanediol

For *Xiphophorus*:
1 L of Hanks' balanced salt solution prepared at 500 mOsmol/kg (HBSS500) (Yang et al. 2009)
Ethylene glycol
Glycerol

For spotted seatrout, red drum, and red snapper:
1 L of calcium-free Hanks' balanced salt solution prepared at 200 mOsmol/kg (C-F HBSS) (Riley et al. 2008)
Dimethyl sulfoxide (DMSO)
Ethylene glycol
Trehalose

For southern flounder:
1 L of sperm motility inhibiting saline solution prepared at ~320 mOsmol/kg (SMIS) (Lahnsteiner 2000)
Ethylene glycol
Glycerol

Procedure:

For catfish to prepare 1 mL of 40% methanol + 20% methyl glycol + 20% propanediol:
1. In one microcentrifuge tube add 400 µL methanol, 200 µL methyl glycol, 200 µL of propanediol, and 200 µL HBSS300.
2. Vortex and keep under refrigeration until further use.
3. This vitrification solution is at double the concentration, and is to be used at a ratio of 1:1 with sperm suspension.
Note: some cryoprotectants are alcohol based and will evaporate if left open for a long time.

For *Xiphophorus* to prepare 1 mL of 40% ethylene glycol + 40% glycerol:
1. Glycerol is viscous and difficult to pipet. Cut ~0.5 cm off the end of a standard 1000-µL tip. Add 400 µL of glycerol in a microcentrifuge tube.
2. In the same microcentrifuge tube add 400 µL of ethylene glycol and 200 µL of HBSS500.
3. Vortex and keep under refrigeration until further use.
4. This vitrification solution is at double the concentration, and is to be used at a ratio of 1:1 with sperm suspension.
Note: Be sure that the vitrification sample is well mixed before use.

For spotted seatrout, red drum, and red snapper to prepare 1 mL of 20% DMSO + 60% ethylene glycol + 0.5 M trehalose:
1. The molecular weight of trehalose is 378.33. To prepare a 0.5 M solution in 1 mL, add 189.2 mg in a microcentrifuge tube.
2. In the same microcentrifuge tube add 200 µL of DMSO, 600 µL ethylene glycol, and 200 µL of C-F HBSS.
3. Vortex and keep under refrigeration until further use.
4. This vitrification solution is at double the concentration, and is to be used at a ratio of 1:1 with sperm suspension.
Note: Trehalose is difficult to dissolve, be sure to vortex after the addition of each cryoprotectant.

For southern flounder to prepare 1 mL of 40% ethylene glycol + 40% glycerol:
1. Glycerol is viscous and difficult to pipet. Cut ~0.5 cm off the end of a standard 1000-µL tip. Add 400 µL of glycerol in a microcentrifuge tube.
2. In the same microcentrifuge tube add 400 µL of ethylene glycol and 200 µL of SMIS.
3. Vortex and keep under refrigeration until further use.
4. This vitrification solution is at double the concentration, and is to be used at a ratio of 1:1 with sperm suspension.
Note: Be sure that the vitrification sample is well mixed before use.

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SOP-2. Sperm Vitrification Procedure

Materials needed:

Latex gloves
Disposable inoculating 10-µL loops
Petri dish (100 x 15 mm)
Micropipette and tips (100 µL)
Canes and goblets
Dewar flask
Liquid nitrogen
1.5-mL microcentrifuge tubes
Rack for microcentrifuge tubes
Styrofoam box
Vitrification solutions
Permanent marking pen
Laboratory notebook
Long tweezers

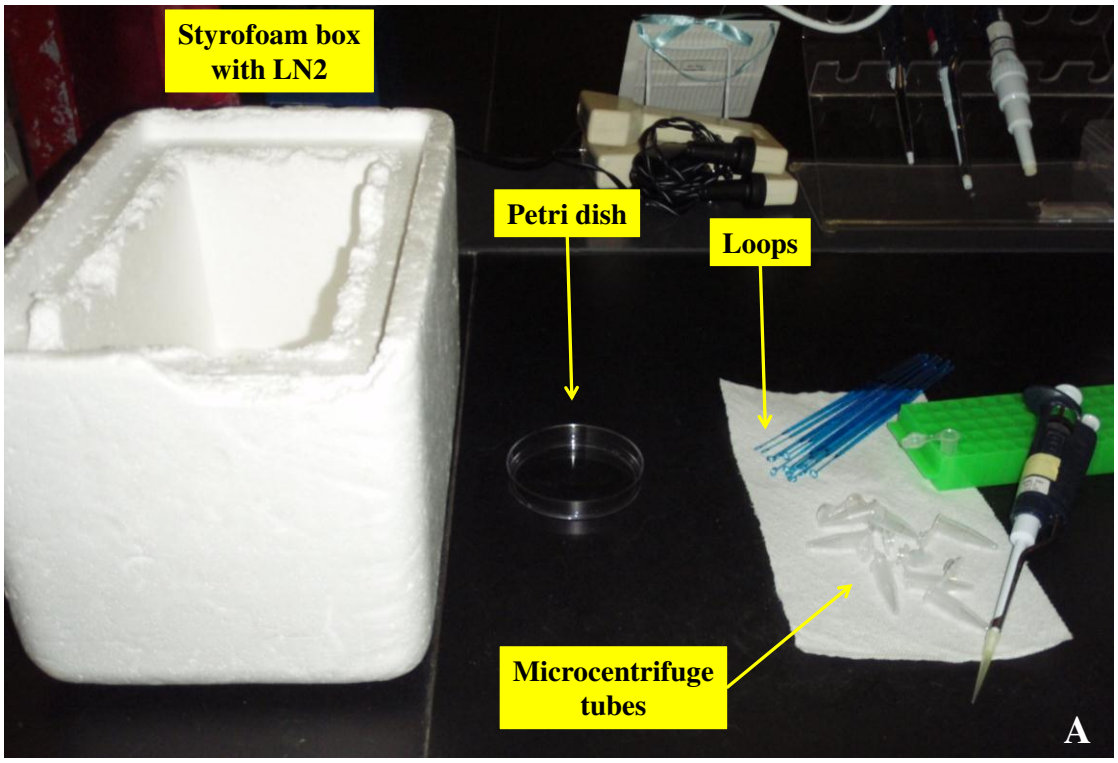
Procedure:

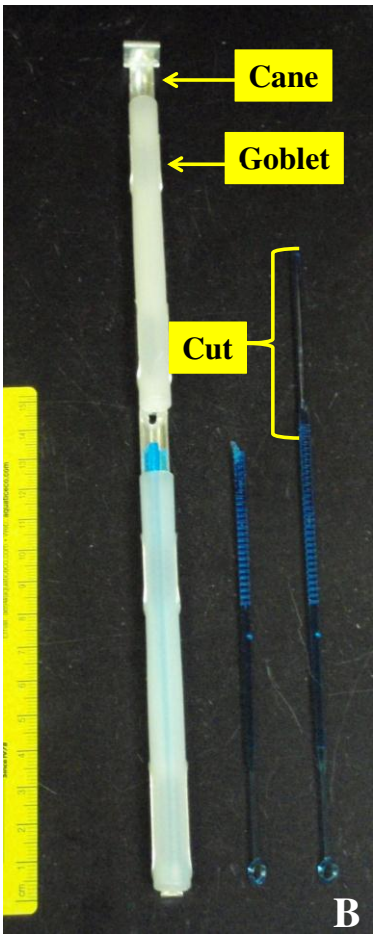
1. Assemble all the materials shown in Figure A.
2. Cut the loops to fit in the goblets (Figure B).
3. Label all canes using the four-letter codes for aquatic species used in Tiersch's laboratory: channel catfish CCFH, green swordtail GRSL, spotted seatrout SPST, red snapper RDSR, and red drum RDDM. After the four letter-codes, add the last two digits of the year followed by the letter M (M for males, F for females), and the male number. For example, red drum male 3 vitrified in 2010 should read as RDDM10M03. Add to the label the vitrification solution used. For example, 20% ethylene glycol + 20% glycerol could be abbreviated as 20%EG+20%Gly.
4. Add liquid nitrogen (LN2) to the Styrofoam box. Any Styrofoam box could be used. The box used in this dissertation had the following characteristics: interior L x W x H were 20 x 15 x 30 cm; wall thickness was 3 cm. This box allowed the complete submersion of canes and had a wall thickness that provided easy handling with liquid nitrogen.
5. Submerge the cane with the two goblets attached into liquid nitrogen.
6. Add 30 µL of the vitrification solution into a microcentrifuge tube.
7. Add 30 µL of the sperm suspension and flick the tube to mix the solution.
8. Pipet 30 µL of the solution and add it as a drop on the petri dish.
9. Using the loops, collect a film of the drop (Figure C).
10. Plung the loop into liquid nitrogen (Figure D).

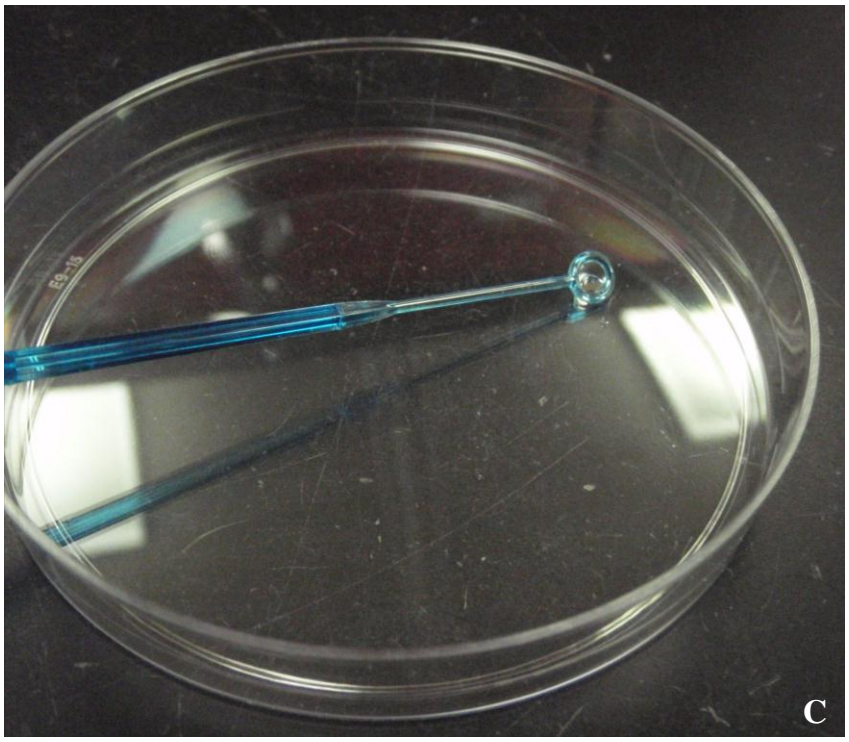
Note: the process time after the addition of the sperm suspension until the plunging of the loop should be less than one min.

11. Store the loop in a goblet. As many as four loops can be stored per goblet.
12. After vitrifying the loops for each treatment, transfer the cane into the dewar flask.
13. Record the location of the cane in the dewar flask.

Note: proper labeling of the samples will ensure identification of the samples. With poor labeling you project problems into the future.


Styrofoam box with LN2
Petri dish
Loops
Microcentrifuge tubes
A


Cane
Goblet
Cut
B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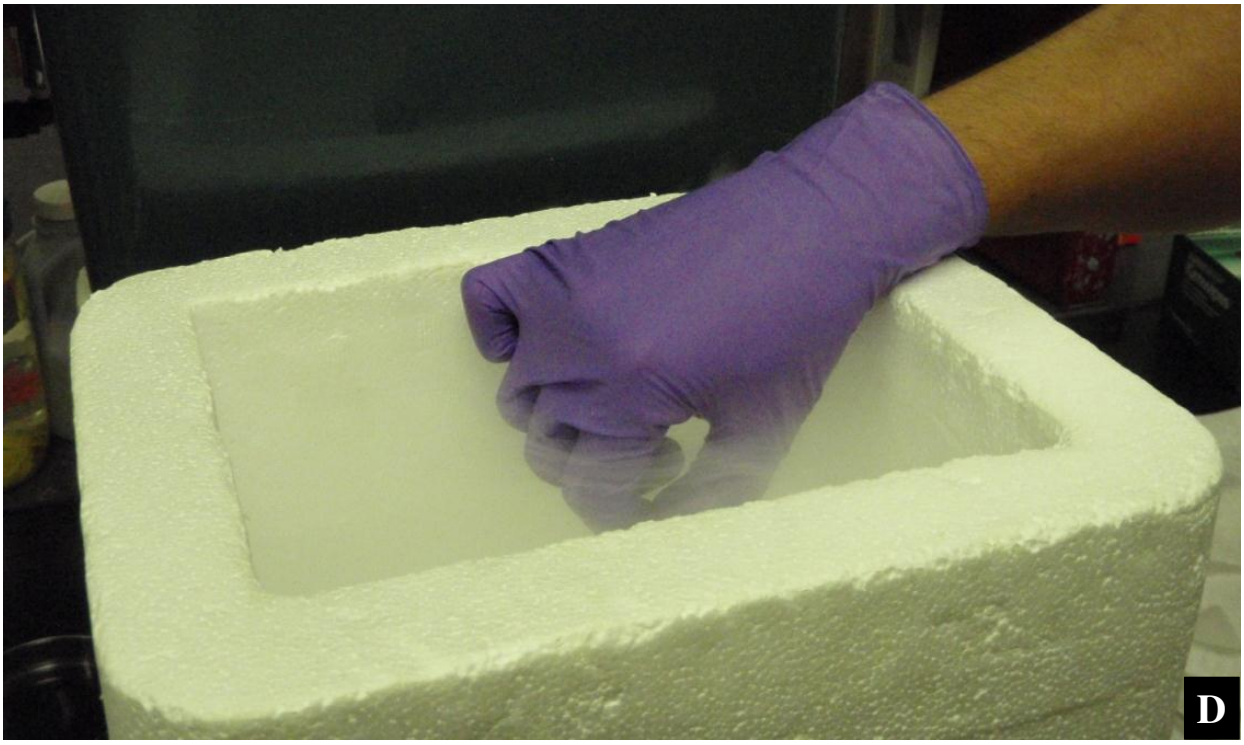

D

SOP-3. Warming Method

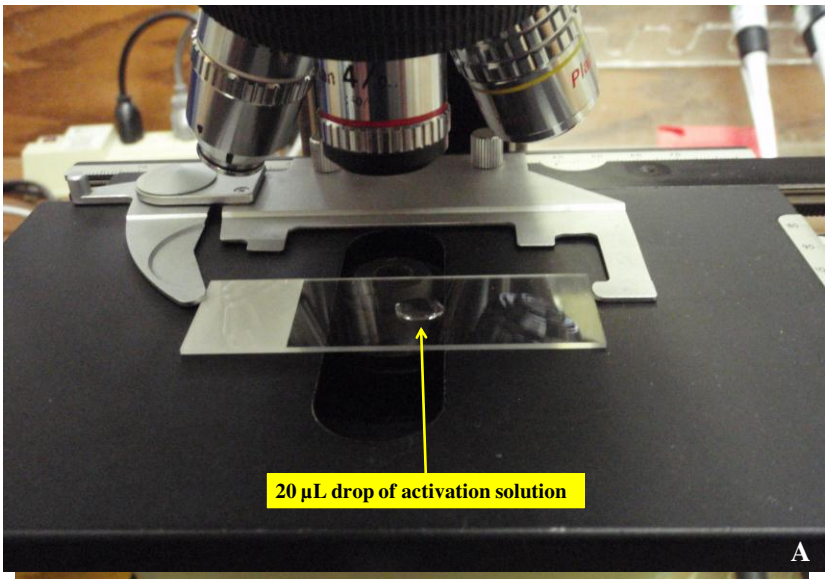
Materials needed:

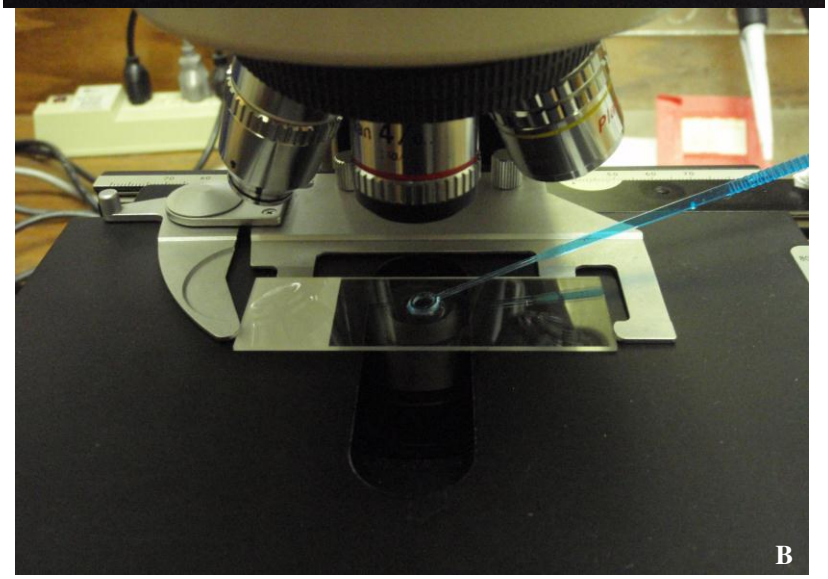
Microscope	Micropipette and tips (100 µL)
Glass slide	Long forceps
Laboratory notebook	Liquid nitrogen

Activation solutions: for channel catfish (deionized water), *Xiphophorus* (HBSS300), and marine species (seawater 30 – 35 ppt). Activation solution should be used at room temperature.

Procedure:

1. Place a 20 - 30 µL drop of the activation solution on microscope slide (Figure A).
2. Thaw the loop rapidly into the drop and mix gently (Figure B).
3. Estimate the percent of sperm that are vigorously swimming forward.





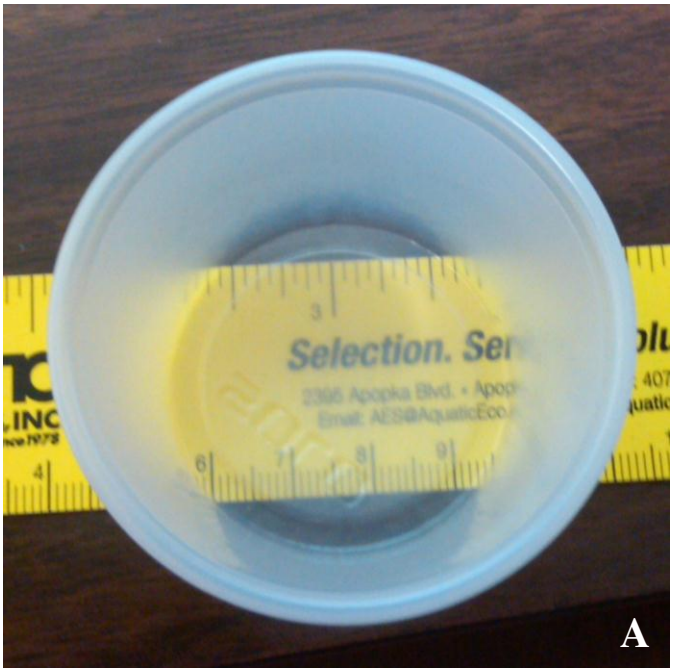
SOP-4. Warming for Fertilization Trials

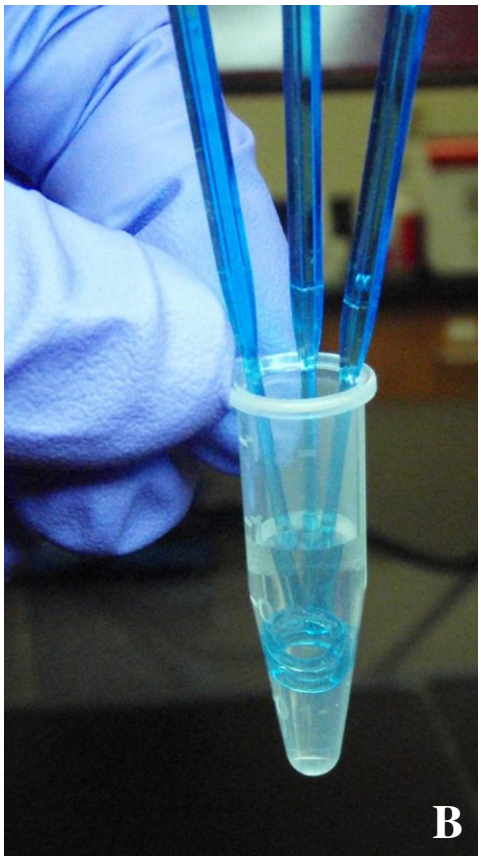
Material needed:

1.5-mL microcentrifuge tubes
Laboratory notebook
Permanent marking pen
Micropipette and tips (1000 µL)
Liquid nitrogen
Plastic container to place a monolayer of eggs. This could be a 100-mL tri-corner beaker for channel catfish or a 20-mL plastic container for marine species (Figure A).
Activation solutions: for channel catfish (deionized water), *Xiphophorus* (HBSS300), and marine species (seawater 30 – 35 ppt). Activation solution should be used at room temperature.

Procedures:

1. Add 1 mL of HBSS300 into centrifuge tubes for channel catfish or *Xiphophorus*. For marine species add 1 mL of seawater.
2. Label all the containers with the male number and treatment.
3. After collecting the eggs, place a monolayer ~100 eggs in the plastic containers.
4. Thaw the loops into the centrifuge tube and mix gently.
5. Immediately add the thawed sperm into the eggs.
6. Add 5 mL of the activation solution into the eggs and gently swirl.
7. Wait 10 min and add 10 mL of activation solution.
8. Allow fertilized eggs to incubate.





Appendix B - Estimation of Fish Sperm Concentration by Use of Spectrophotometry

Towards Harmonization: Some Lessons from Human Andrology Laboratories

A lack of standardization, wide variation among laboratories, and an urgent need for quality control led andrology laboratories worldwide to unify and developed standardized techniques and practices for semen analysis which were published by the World Health Organization (WHO) (Keel et al. 2002). The purpose of the WHO manual was to minimize variability among laboratories and to enable comparison of semen analysis results among laboratories. Standardized laboratory protocols and quality control are essential for meaningful comparisons of semen quality data from multiple sites (Brazil et al. 2004). Although the WHO manual is recognized globally as the "gold standard" for semen analysis, its acceptance has been less than universal. For example, most laboratories that participate in the German external quality control program for semen analysis do not use WHO-recommended methods (Cooper et al. 2007). Most inter-laboratory variation is attributed to the use of different techniques, and there is considerable disagreement regarding the relative accuracy and precision of various techniques (Brazil et al. 2004). Although improvements can be made in existing guidelines, protocols, and quality control systems, these current systems provide better tools than other non-standardized procedures (BjoRndahl et al. 2004).

New methods need to be validated for accuracy, repeatability, and precision before moving into standardization, which is a component of harmonization. To achieve harmonization, standards need to be developed for each method. For example, despite several efforts to standardize methods of semen analysis, sperm count is known to be subject to large inter-laboratory differences. Most variation is introduced through the use of different techniques (Jonckheere et al. 2005). Currently there is disparity in the equipment and procedural steps used for concentration measurements. This is because the standardization necessary for development of guidelines does not exist. After methods have been standardized, intercalibration comes into play. There is nothing inherently wrong in using different techniques as long as the results are accurate and consistent. Comparison by intercalibration is used to verify that values of a particular technique are correct. Thus the purpose of the intercalibration is not to harmonize the assessment method, but only the results (Buffagni and Furse 2006). Sometimes to ensure that the results of one technique are consistent with those of another, a transformation factor is applied to normalize the data (Poikane 2009). Criteria must be established to define the reliability of data for total allowable error specifications, and the extent of corrective measures that are acceptable. These criteria will define the procedures used for adjusting the data and compensation factors.

To minimize errors routine quality control needs to be established. Evaluation of an internal quality standard is essential to maintain accuracy, precision, and competence (Auger et al. 2000). Estimates of imprecision can be obtained from the internal quality control system. Imprecision can be reduced by regular training of personnel and by adopting best management practices. Workshops on standardization have been used to train andrology laboratory technicians (Toft et al. 2005). External quality control can also provide regular standardization checks and agreement among laboratories. External quality control programs should be directed

*The contents of this Appendix were published prior to the completion of this dissertation (Tiersch, T.R. and C.C. Green, editors. 2011. *Cryopreservation in Aquatic Species, 2nd edition*. World Aquaculture Society).

at tangible elements (e.g., staff, instrumentation, equipment, and supplies) and at intangible elements (protocols and techniques) (Castilla et al. 2010). Adherence to the same standard procedures and criteria for each method will allow separate laboratories to work in unison. Harmonization results in making the outcomes comparable, not necessarily in making every laboratory do things in an identical fashion (van Nieuwerburgh et al. 2007). Harmonization allows choices between alternatives, out of which one or several can be adopted depending on the given circumstances (Figure B-1).

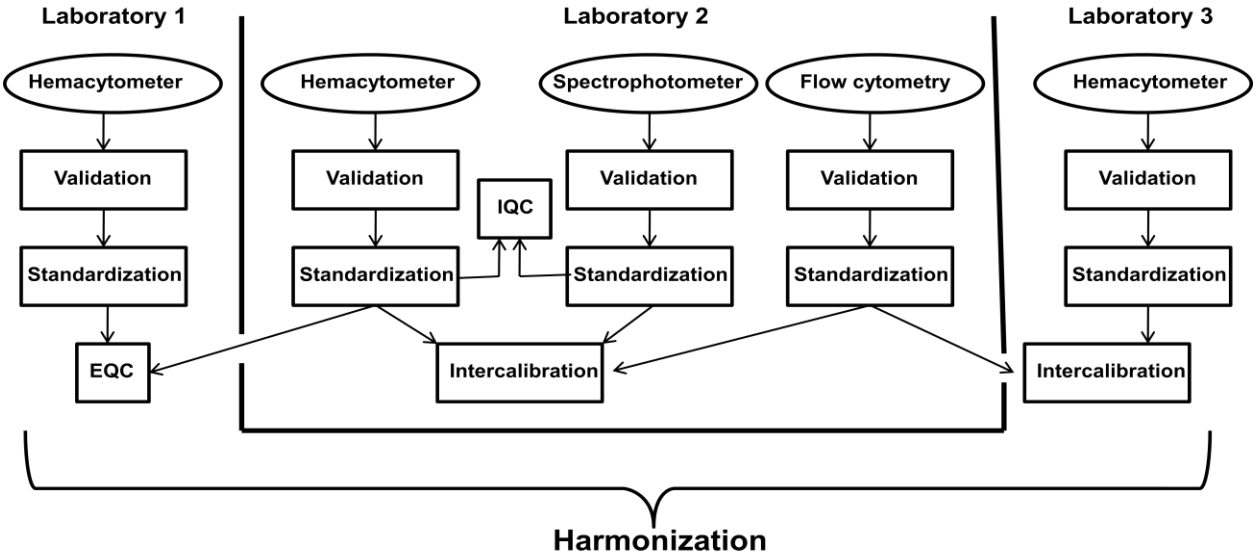


Figure B-1. Schematic overview of a harmonization process (rectangles) for sperm counting methods (ovals) across three laboratories. Method validation is the first step to ensure that results are reliable. Standardization follows and allows development of guidelines for uniformity of response (a "top-down" approach). Many laboratories have more than one instrument that can perform the same function which enables internal quality control (IQC). IQC and external quality control (EQC) are needed to test variability within and among technicians, and among laboratories. Intercalibration seeks consistency in classification of results of different methods, and is used to determine if results from methods are accurate, credible, and comparable. Following intercalibration a method can be trusted and can move toward harmonization, the process of making different standards compatible and providing choices among methods. Harmonization bridges existing variation to provide a state of comparability, consistency, and similarity.

The Current Status for Sperm Quality Analysis in Aquatic Species

Currently, the single largest problem for sperm quality analysis (and standardization in general) in aquatic species is the lack of control and reporting of sperm concentration (Dong et al. 2007a). We place this problem foremost because sperm concentration will directly affect analysis results even for assays that are otherwise standardized (Tiersch et al. 2007). The first step in dealing with this problem is to recognize the importance in controlling and reporting sperm concentration, and to adopt and ultimately harmonize methods to estimate concentration. A variety of methods exist, each with advantages and disadvantages, and only a few of these have established methods. As stated above, after choosing a method, validation is necessary to move into standardization (Figure B-1) which allows development of guidelines or standardized protocols for each method. After standardization, the next step is comparison and intercalibration

between and among laboratories to ensure that values are precise and accurate. Consistency, accuracy, and comparability of different methods are the keys for intercalibration (Poikane 2009). Harmonization can be addressed after the development of standardized protocols and establishment of an intercalibration process. At present, as stated above, there is a lack of standardization in the performance and reporting of sperm analyses in aquatic species. The study of sperm quality would greatly benefit from a standardization of analytical methods and tools (Fauvel et al. 2010). This would facilitate collaboration among laboratories with the aim to develop uniform (standardized) procedures and to derive similar accuracies through intercalibration exercises (Rosenthal et al. 2010) and eventual harmoniation. To facilitate making the first step in this process for aquatic species, the focus of this chapter will be on turbidimetric estimation of sperm concentration by use of spectrophotometry. This is not meant to suggest that this is the best or only technique available to measure sperm concentration, but it is widely accessible, has been widely applied for a variety of species and applications, and can serve as an overall representative model of the opportunities and problems inherent in other aspects of gamete quality analysis in aquatic species.

Principles of Turbidimetric Analysis

Whenever light strikes an object, the light can be scattered (reflected), absorbed, or passed through the object (refracted). The extent of light loss can be determined by measuring the amount scattered or reflected (nephelometry) or the amount of light transmitted (turbidimetry) (Csuros and Csuros 1999). In a turbidity measurement a spectrophotometer is used to measure the absorbance of light as a function of wavelength as it passes through a sample. The measurement of absolute absorbance depends in the separation of scattered light from the attenuation of light (absorbance). The sensitivity to measure light loss varies depending on the type, number, and position of detectors. For example, the sensitivity to measure absorbance increases if a detector is positioned far from the cuvette (sample container) (Figure B-2). For this reason there are differences in the accuracy of various instruments. This is why several authors avoid use of the term "absorbance" and refer to spectrophotometric output as "apparent absorbance", "optical density", or "turbidity" (Poole and Kalnenieks 2000).

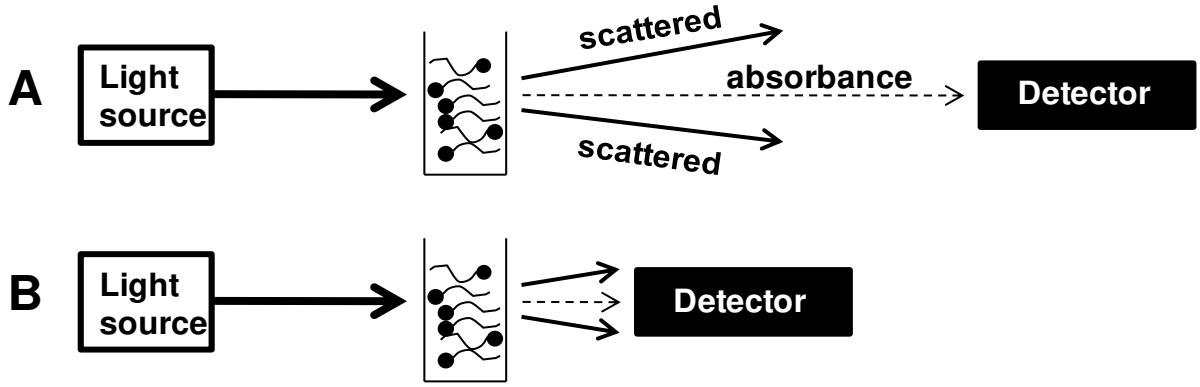


Figure B-2. The measurement of absorbance varies depending on position of the detector. For accurate measurement it is necessary to eliminate or minimize the effect of light scattering. A) Positioning of the detector far from the sample enhances sensitivity because it will detect less scattered light. B) When the detector is close to the sample, scattered light will cause an artifactual reduction of absorbance (modified from Poole and Kalnenieks 2000).

Turbidity is routinely used as a measure of biomass concentration because the amount of light lost or scattered is inversely proportional to the cell concentration or directly proportional to the absorbance (Csuros and Csuros 1999). The apparent absorbance of a cell suspension depends on the wavelength used, the pathlength, and the cell concentration of the suspension. When nephelometry is used, a wavelength is chosen to optimize sensitivity and dynamic range, and to minimize the effects of light absorption by the cells or the medium components. By choosing a wavelength where no light absorption is recorded (e.g., 320-800 nm for proteins), the light striking the detector will be result only from light scattering. As a rule, choosing lower wavelengths will provide greater sensitivity of optical density measurements, but longer wavelengths will provide greater linearity over the same range of cell suspensions (Poole and Kalnenieks 2000). When turbidimetry is used, the wavelength selected is based on the maximal absorbance peak (e.g., 260 nm for nucleic acids). Sperm concentration is directly proportional to the absorbance and indirectly proportional to percentage transmittance (Csuros and Csuros 1999). To quantify an absorbance reading, a quantitative enumeration must be made (e.g. hemacytometer and coulter counter) to correlate with the apparent absorbance.

Estimation of Sperm Concentration in Livestock

The importance of reporting sperm concentration due to individual male variation and to allow comparison of studies was recognized early as being essential by the bull sperm industry. This industry next focused on development of rapid methods for accurate estimation of sperm concentration which continues today (Table B-1). Different methods have been developed, ranging from comparison of sperm suspensions with opacity standards (Burbank 1935) to flow cytometric methods (Evenson et al. 1993). These methods vary in precision (Table B-1).

Table B-1. Coefficient of variation (repeatability) of different methods for estimation of sperm concentration reported in representative studies (arranged chronologically).

Animal studied	Hemacy-tometer	Sper-matocrit	Spectro-photometry	Coulter counter	Flow cytometry	Reference
Chicken	6.4	3.8	3.7	—	—	Taneja and Gowe 1961
Chicken	12.8	8.3	4.8	—	—	Taneja and Gowe 1961
Turkey	41.1	—	26.6	30.1	—	Brown et al. 1970
Chicken	17.9	9.9	2.2	1.6	—	Brillard and McDaniel 1985
Boar	12.3	—	2.9	2.3	—	Paulenz et al. 1995
Boar	7.1	—	10.4	—	2.7	Hansen et al. 2006
Bull	7.8	—	4.1	—	2.3	Prathalingam et al. 2006

The first record of using turbidity to measure sperm concentration in livestock dates back 70 years (Comstock and Green 1939). The National Association of Animal Breeders has developed guidelines for measurement of sperm concentration by turbidity (Foote 1972, Foote et al. 1978). A survey among laboratories was used to identify the sources of variation including the spectrophotometer used, wavelength used, method of calibration, type of diluents, and dilution rate used (Foote et al. 1978). From the laboratories interviewed, 80% were using the same spectrophotometer and most of them were using the same wavelength (550 nm). It was

concluded that the accuracy of estimating sperm concentration using spectrophotometer was dependent on the calibration and standard curve used to correlate absorbance values with hemacytometer sperm counts.

The Importance of Estimating Sperm Concentration in Aquatic Species

The urgent need to standardize sperm studies has been emphasized recently in a workshop (Rosenthal 2008), symposium (10^{th} International Symposium on Spermatology 2006,Tiersch et al. 2007), and publications (Dong et al. 2005a, Dong et al. 2007a). Standardization is required to reproduce and optimize reports and protocols. A lack of standardization of sperm concentration has led to variability in results and reporting of studies (Dong et al. 2005a, Tiersch et al. 2007). Accurate estimation of concentration is necessary for a wide variety of topics including standardization of cryopreservation (Dong et al. 2007a), determination of optimal sperm-to-egg ratios (Suquet et al. 1995), calibration of ultraviolet irradiation to induce gynogenesis (Mims et al. 1997), assessment of spermiation following hormonal stimulation (Miranda et al. 2005), assessment of sperm production following sex manipulation (Fitzpatrick et al. 2005), nutritional studies (Rinchard et al. 2003), estimation of sperm motility (Cosson 2008), optimization of staining with fluorescent dyes (Paniagua-Chavez et al. 2006), and reproductive toxicology (Aravindakshan et al. 2004).

Uncontrolled variation in sperm concentration is one of the main reasons for the inconsistency observed among various studies associated with cryopreservation and fertilization (Dong et al. 2007a), and as such determination of concentration should be considered as an essential parameter in the assessment of sperm quality (Aas et al. 1991, Cabrita et al. 2009). Sperm volume and concentration vary among species (Piironen and Hyvarinen 1983) and individuals within the same species, with reported concentrations ranging between 2×10^6 to 6.5×10^{10} cells/mL (Leung and Jamieson 1991, Vuthiphandchai and Zohar 1999, Alavi et al. 2008). These differences are due to factors such as the stage of spawning season (Munkittrick and Moccia 1987), seasonal variation (Alavi et al. 2008), strain and genetic backgrounds (Scott and Baynes 1980, Tiersch 2001), diet (Ciereszko and Dabrowski 2000), physicochemical and social environment (Fitzpatrick and Liley 2008), disease (Rurangwa et al. 2004), and age (Poole and Dillane 1998).

The time, effort, and expense involved in rearing or capturing mature fish requires efficient use of sperm samples, especially for imperiled species (Tiersch et al. 1994). Common hatchery practices, if described in reports, typically include the addition of volumetric measures of gametes (e.g. 20 mL of sperm per 2 - 3 L of salmonid eggs; Willoughby 1999) without estimating the concentration or motility of the sperm (Aas et al. 1991). Commercial success of hatchery effort depends upon efficient utilization of available gametes (Erdahl and Graham 1987) and sperm can be in short supply due to limited numbers of broodstock or due to the small body sizes of aquarium fish used as biomedical models such as zebrafish (*Danio rerio*) (Tiersch 2001, Tan et al. 2010). In addition, the success or failure of cryopreservation protocols can be dictated by sperm concentration (Dong et al. 2007a). For these and other reasons it is therefore essential to routinely adopt a rapid, efficient, and accurate method for estimation of sperm concentration in aquatic species.

Methods Used to Estimate Sperm Concentration

There are several techniques used to estimate sperm concentration as part of the process to estimate sperm quality (Figure B-3). Each technique has advantages and disadvantages, but no matter which technique is chosen it is essential to control, record, and report sperm concentration.

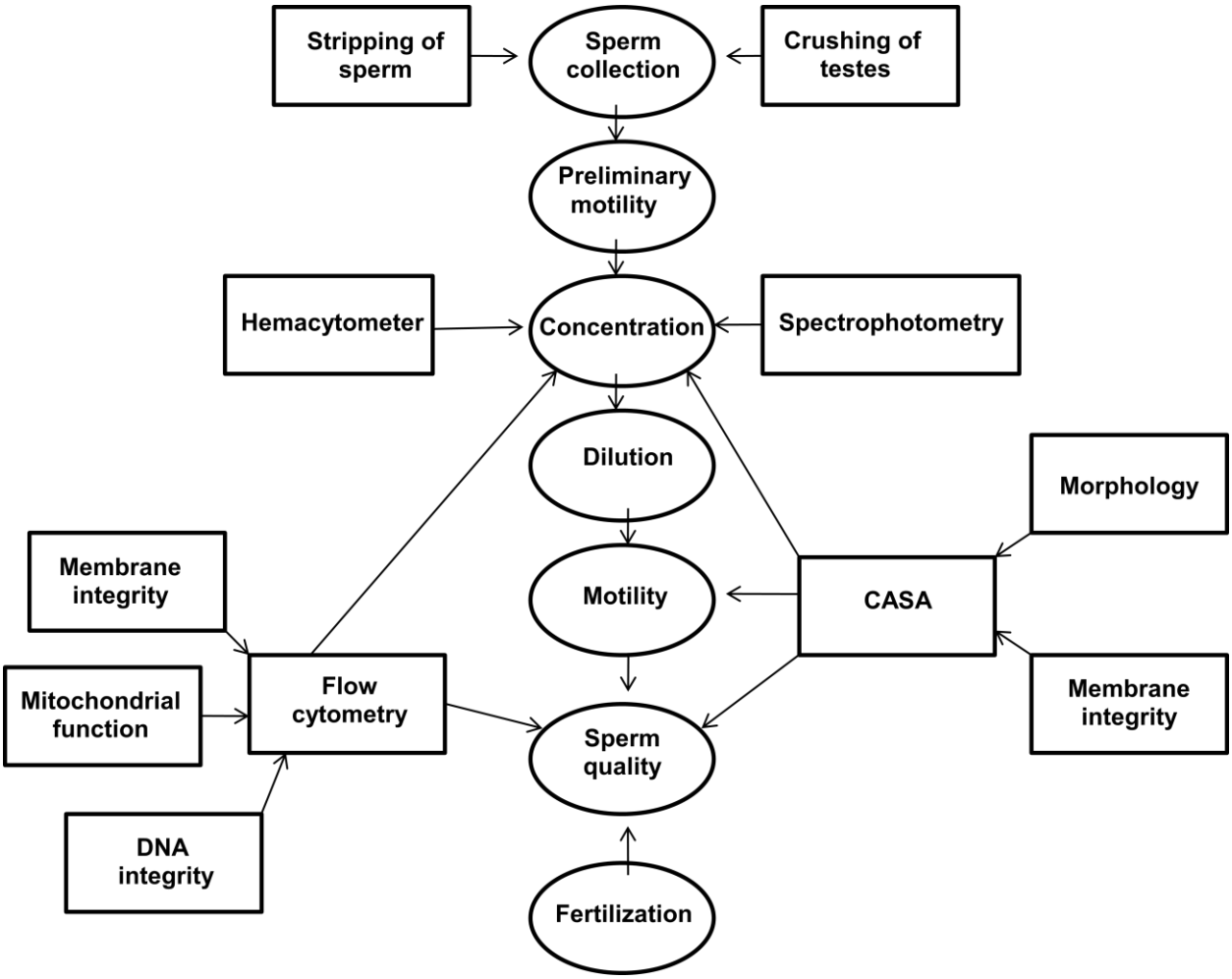


Figure B-3. Relevant steps (ovals) for sperm quality assessments. Traditional sperm quality parameters (rectangles) have included motility, morphology, concentration, and membrane integrity (viability), with fertilization providing an ultimate assessment. More than one instrument can perform different tests. For example, concentration can be measured by use of computer-assisted sperm analysis (CASA), flow cytometry, hemacytometer, or spectrophotometry.

Fish sperm concentration has been assessed by three main techniques: counting in a hemacytometer chamber, estimation of spermatocrit, and turbidity evaluation (for more details see reviews by Rurangwa et al. 2004, Alavi et al. 2008, and Fauvel et al. 2010). Hemacytometer counting is precise and reliable for fish sperm, but is time consuming and thus cumbersome for fertilization protocols involving precise timing or many males (Fauvel et al. 1999, Alavi et al.

2008, Cabrita et al. 2009). Spermatocrit determination, although widely used, requires centrifugation of the milt and only provides a relative measure expressed as the ratio of packed sperm volume to total volume of sample instead of the number of cells per mL. Because the handling time for samples should be minimized, establishment of a rapid and reliable method for sperm concentration estimation is required. Spectrophotometric determination of turbidity is an efficient and inexpensive method to estimate cell concentrations, given that an accurate initial calibration is established (Foote et al. 1978, Dong et al. 2005a). The advantages of using photometric measurement of sperm concentration in aquatic species has been known at least since 1949 (Rothschild 1950) and the application of this method to fish can be dated back at least to 1971 (Billard et al. 1971). Since then, the direct relationship between sperm concentration and absorbance has been established in approximately 41 species of fish (most within the past 10 years). Despite or because of this diversity of use, the application of turbidity to estimate sperm concentration has not been collectively studied, and remains unstandardized and variable in methods and reporting. By reviewing the literature addressing use of spectrophotometer to measure sperm concentration in fish (described below) we found that the majority (65%) of the studies did not describe how they established the absorbance-concentration standard curve, and just one-third of the studies (35%) gave a description of the calibration curve equations, or other spectrophotometric measurement protocols.

From this review it appeared that the necessity of developing separate calibration curves for each species or population was a significant hindrance to wider utilization of this method to determine concentration. Therefore, based on a previous study done in livestock which compared sperm concentrations from bull, boar, and stallion as determined by the use of a single calibration curve (Rondeau and Rouleau 1981), we decided to evaluate the feasibility of using a single calibration to measure sperm concentration across a range of fish species.

Thus, this chapter addresses two goals, the first was to review the literature on previous estimations on fish sperm concentration by the use of spectrophotometer. With this review our objectives were to: (1) provide an overview of the different types of uses for spectrophotometric analyses, and (2) highlight the sources of variations in the technology. The second goal was to evaluate the general utility of turbidity in determining sperm concentration in fish species. The research objectives were: (1) wavelength identification for sperm concentration assessment, (2) development of standard curves for turbidity estimations in seven species, (3) validation of regression models in estimations, (4) determination of the relationship of standard curves across various species, and (5) evaluation of the effects of other cell types such as blood on turbidity measurement of sperm concentration. To our knowledge this is the first review of this topic area and the first report that demonstrates the feasibility of a general curve (instrument-specific) that can be used to measure concentration in fishes where sperm is collected by stripping, and with further procedural modification could apply to aquatic species in which the testes are crushed.

Materials and Methods

Goal One: Literature Review

We sought to use the literature review to form a database and to describe the previous uses and application of turbidity to estimate the sperm concentration in fishes. The first problem that we encountered was the difficulty of identifying scientific publications that used

spectroscopy to measure sperm concentration. A simple search using the search terms "sperm concentration" and "fish spectrophotometer" in Google scholar (scholar.google.com) resulted in 296 publications. While compiling the publications that specifically used spectrophotometer to estimate the sperm concentration in fish, two truly influential articles were identified (Suquet et al. 1992a, Ciereszko and Dabrowski 1993) that most of the studies using this technology made reference to. Based on the citations listed for these articles and using ISI Web of Knowledge[SM], we found 52 citations for Suquet et al. (1992a) and 90 citations for Ciereszko and Dabrowski (1993). Overall between the year 1971 and 2009 we found a total of 71 articles (in 32 journals), 9 meeting abstracts, and 2 dissertations that specified use of spectrophotometer to measure sperm concentration. The two journals publishing the most articles were *Aquaculture* (Elsevier: www.elsevier.com) with 13 articles and *Aquaculture Research* (Wiley-Blackwell: www.wiley.com/WileyCDA/) with 11 articles. The categories within the database constructed were: country of research (based on address of first author), species of fish, purpose of the study, description of the turbidity method, wavelengths tested for maximal absorbance, optimal wavelength used, absolute determination method for the generation of standard curves, correlations between methods, and concentration ranges tested.

Goal Two: General Curve Development

Sperm Collection

The scientific name, common name, sources of fish, and collection methods were listed for the seven species studied (Table B-2). The studies were performed for tilapia during January to February of 2003 and the rest during March to August of 2004 at the Aquaculture Research Station of the Louisiana State University Agricultural Center in Baton Rouge.

Table B-2. Scientific and common names (arranged by phylogenetic order), sources, sperm collection methods, and published work reporting other results obtained for the same fish.

Common name	Species	Source	Collection method	Dilution ratio[a]	Extender solution	Reference
Paddlefish	*Polyodon spathula*	Forest Hill, LA[b]	Stripped	2:1	HBSS200[c]	Mims and Shelton 2005
Blue catfish	*Ictalurus furcatus*	Inverness, MS[d]	Crushed testis	10:1	HBSS300	Lang et al. 2003
Channel catfish	*Ictalurus punctatus*	Baton Rouge, LA[e]	Crushed testis	10:1	HBSS300	Bates et al. 1996
			Sliced testis	none	HBSS300	Riley et al. 2004
Striped bass	*Morone saxatilis*	San Diego, CA[f]	Stripped	5:1	C-F HBSS300[g]	Thirumala et al. 2006
White bass	*Morone chrysops*	San Diego, CA[f]	Stripped	5:1	C-F HBSS300	Thirumala et al. 2006
Red drum	*Sciaenops ocellatus*	Gulf of Mexico, LA	Stripped	5:1	C-F HBSS200	Wayman et al. 1998
Tilapia[h]	*Oreochromis* sp.	Tiltech Aquafarm, LA	Stripped	32	HBSS300	Segovia et al. 2000

a sperm : extender
b Louisiana Department of Wildlife and Fisheries, Booker Fowler Fish Hatchery
c Hanks' balanced salt solution at 200 or 300 mOsmol/kg
d Harvest Select Farms
e Louisiana State University Agricultural Center, Aquaculture Research Station
f Kent Sea Tech, now Kent BioEnergy Corporation
g C-F HBSS: calcium-free HBSS
h Nile tilapia O. niloticus, blue tilapia O. aureus, Mississippi commercial strain, Florida red tilapia

The fish were anesthetized using tricaine methane sulfonate (MS-222, Western Chemical Inc., Ferndale, WA) at 100-150 mg/l (Coyle et al. 2004) prior to stripping. They were removed from anesthesia and the genital papilla was dried with paper towels to avoid activation or contamination of the sperm by water. Samples were stripped carefully, to minimize contamination with urine or feces, and were diluted in Hanks' balanced salt solution (HBSS) or calcium-free HBSS (C-F HBSS) of appropriate osmotic strength (Table B-2). Because sperm cannot be stripped from ictalurid catfishes, testes were removed surgically and crushed in 300 mOsmol/Kg HBSS at a ratio of 1:10 (g testis:mL HBSS) to release sperm. This method yielded a mixture of cell types including mature and immature sperm cells, and somatic cells such as erythrocytes. Channel catfish testes were also sliced and squeezed to directly collect a relatively pure sample of sperm for research purposes.

Hemacytometer Counts and Dilution Preparation

An aliquot of each sperm sample was diluted before counts were made with a hemacytometer (Reichert bright-line, Hausser Scientific, Horsham, PA). A 1:32 dilution (sperm:HBSS) was used for all species, except for red drum (1:500) and white bass (1:1000) which produce highly concentrated sperm samples (> 10^{10} cells per mL). Sperm concentrations were calculated using the average of four replicate hemacytometer counts with the following equation:

(Mean of quadruplet counts x dilution factor) x 50,000 = cells/mL

After the initial sperm concentrations were calculated, the solutions were diluted to contain 10^9, 10^8, 10^7 and 10^6 sperm cells/mL, and these concentrations were validated again by hemacytometer counts.

Spectrophotometer Readings

A Spectronic 20 Genesys™ (Thermo Spectronic, Rochester, NY) was used to obtain the absorbance measurements except where otherwise stated. Disposable 1.5-mL polystyrene cuvets (Semimicro, Fisher Scientific, Pittsburg, PA) with a 10-mm pathlength were used for each sample. Blanks were set using 1.5 mL of the extender used for each species. Diluted sperm samples (1.5 mL) from all seven species were measured at five wavelengths (400, 450, 500, 550 and 600 nm). To determine the wavelength of maximum absorbance, a sperm concentration of 2.5 x 10^8 cells/mL was used in all fishes except for red drum for which 1 x 10^9 cells/mL was used.

Effects of Other Cells Types

To evaluate the influence of blood cells on photometric measurements, we collected blood, sperm from crushed testes, and a relatively pure sperm sample collected by pipet from sliced testes from three channel catfish. Three different concentrations were used (1 x 10^8, 1 x 10^7, and 1 x 10^6 sperm cells/mL) for the sperm samples collected directly from the testes. Blood was collected by caudal puncture using sodium heparin as an anticoagulant (from Becton Dickinson Vacutainer™, Franklin Lakes, NJ). In each of these sperm concentrations, whole blood (on the order of 10^9 blood cells per mL) was added to yield five different final volumetric proportions of blood and sperm (0.125%, 0.25%, 0.375%, 0.5%, and 1%). For example, to prepare one mL of sperm sample with 1% of blood, 10 µL of blood was added to 990 µL of

sperm sample. The absorbance of these samples was measured using a spectrophotometer (Spectronic 20) at five wavelengths (400, 450, 500, 550 and 600 nm). The absorbance values were measured by using a scanning microspectrophotometer (Nanodrop® ND-1000 Wilmington, DE) across wavelengths from 220 to 748 nm at 2-nm intervals.

Statistical Analysis

Simple linear regression was used for testing the correlation between absorbance and sperm concentration for each species at the five wavelengths. Data for sperm concentrations were logarithmically transformed (natural logarithm) prior to regression analysis (Berman et al. 1996). To test for significant differences ($P < 0.05$) among linear models, multisource regression with analysis of covariance was used. To test for correlation among species, and among blood percentages, multiple regression was used. Because R^2 values increase with the addition of new variables, the adjusted R^2 (Neter et al. 1996) was used to compensate for added explanatory variables. After determining that there were no differences among them ($P > 0.05$), the observations from Nile tilapia, blue tilapia, Mississippi commercial strain and Florida red tilapia were pooled to strengthen the sampling for tilapia species. The software used for all analyses was SAS® 9.1 (SAS Institute Inc., Cary, NC).

Results

Literature Review:
Overview of Uses for Turbidity Analyses

The 82 publications collected represented 18 countries. This reflected a wide diversity of research types and a wide variety of study purposes utilizing spectrophotometer (Table B-3). Estimations of sperm concentration by turbidity have been established in at least 41 species of fish and 3 species of mollusks. About 60% of the studies were done in the past 10 years.

Sources of Variation in the Reports

Approximately 65% of the publications did not report how they standardized the technique, which included the wavelengths tested, wavelengths selected, and sperm concentration ranges tested (Table B-3). Wavelengths between 260 and 660 nm have been used to determine sperm concentrations in fish. The most frequently used (20%) wavelength was 505 nm (Figure B-4). The most cited reference of technique (50%) was by Ciereszko and Dabrowski (1993), and Ciereszko was an author for 25% of these publications. Another source of variation was the type of spectrophotometer used, as less than half (31 of 75) of the studies named the model of the spectrophotometer. Of these, total of 17 different models were reported.

Establishment of General Curve:
Wavelength Identification

The absorbance spectra of sperm from the seven species were stable within the wavelengths tested (Figure B-5); there were no absorption peaks or discrete wavelengths of maximum absorbance within the range tested (400 – 600 nm). Therefore, any visible wavelength could be appropriate for use to generate a standard curve.

Table B-3. Previous studies that estimated sperm concentration of aquatic species by spectrophotometry (presented in chronological order). Standarization of the technique include: wavelength tested, absolute determination, coefficient of determination, and concentration range of the standard curve.

Species	Scientific name	Purpose of study	Method described	Wavelength tested (nm)	Optimal wavelength	Absolute determination	R^2	Sperm concentration range tested	Citation
Rainbow trout	*Oncorhynchus mykiss*	Spermatogenesis	Yes	200 - 600	410	hemacytometer	0.99	1×10^{10} to 2.8×10^{10}	Billard et al. 1971
Rainbow trout	*Oncorhynchus mykiss*	Spermiation	ND*	ND	410	ND	ND	ND	Billard 1974
Common carp	*Cyprinus carpio*	Spermiation	ND	ND	410	ND	ND	ND	Takashima et al. 1984
Turbot	*Psetta maxima*	Sperm concentration	Yes	300 - 750	420	hemacytometer	0.94	5×10^{9} to 8×10^{10}	Suquet et al. 1992a
Turbot	*Psetta maxima*	Spermiation	ND	ND	420	ND	ND	ND	Suquet et al. 1992b
Rainbow trout	*Oncorhynchus mykiss*	Sperm concentration	Yes	400 - 700	505	hemacytometer	0.95	1.9×10^{9} to 2.1×10^{10}	Ciereszko & Dabrowski 1993
Lake whitefish	*Coregonus clupeaformis*	Sperm concentration	Yes	400 - 700	505	hemacytometer	0.71	3.4×10^{9} to 1.4×10^{10}	Ciereszko & Dabrowski 1993
Yellow perch	*Perca flavescens*	Sperm concentration	Yes	400 - 700	505	hemacytometer	0.94	3.7×10^{10} to 4.7×10^{10}	Ciereszko & Dabrowski 1993
Turbot	*Psetta maxima*	Sperm characterization	ND	ND	420	ND	ND	ND	Suquet et al. 1993
Eastern oyster	*Crassostrea virginica*	Fertilization trials	ND	ND	650	hemacytometer	ND	ND	Gaffney et al. 1993
Lake whitefish	*Coregonus clupeaformis*	Sperm storage	ND	ND	505	ND	ND	ND	Ciereszko & Dabrowski 1994
Rainbow trout	*Oncorhynchus mykiss*	Sperm storage	ND	ND	505	ND	ND	ND	Ciereszko & Dabrowski 1994
Rainbow trout	*Oncorhynchus mykiss*	Sperm quality	ND	ND	ND	ND	ND	ND	Ciereszko & Dabrowski 1995
Eurasian perch	*Perca fluviatilis*	Sperm characterization	ND	ND	ND	hemacytometer	ND	ND	Lahnsteiner et al. 1995
Turbot	*Psetta maxima*	Fertilization trials	ND	ND	420	ND	ND	ND	Suquet et al. 1995
Bleak	*Alburnus alburnus*	Sperm physiology	ND	ND	ND	hemacytometer	ND	ND	Lahnsteiner et al. 1996a
Blue mussel	*Mytilus edulis*	Larval culture	Yes	200 - 800	320	hemacytometer	0.98	1.3×10^{9} to 1.3×10^{10}	Del Rio-Portilla 1996
Yellowtail flounder	*Limanda ferruginea*	Sperm physiology	Yes	300 - 900	420	hemacytometer	0.92	2.7×10^{9} to 2.7×10^{10}	Clearwater 1996
Muskellunge	*Esox masquinongy*	Sperm characterization	Yes	400 - 800	610	hemacytometer spermatocrit	0.79	7.5×10^{9} to 3×10^{10}	Lin et al. 1996a
Muskellunge	*Esox masquinongy*	Cryopreservation	ND	ND	610	ND	ND	ND	Lin et al. 1996b
Rainbow trout	*Oncorhynchus mykiss*	Cryopreservation	Yes	ND	505	hemacytometer	ND	ND	Conget et al. 1996
Rainbow trout	*Oncorhynchus mykiss*	Cryopreservation	ND	ND	ND	hemacytometer	ND	ND	Lahnsteiner et al. 1996b
Rainbow trout	*Oncorhynchus mykiss*	Reproductive performance	ND	ND	ND	ND	ND	ND	Dabrowski & Ciereszko 1996
Rainbow trout	*Oncorhynchus mykiss*	Sperm storage	ND	ND	ND	hemacytometer	ND	ND	Lahnsteiner et al. 1997
Shovelnose sturgeon	*Scaphirhynchus***	Gynogenesis	ND	ND	ND	ND	ND	ND	Mims et al. 1997
Shovelnose sturgeon	*Scaphirhynchus***	Gynogenesis	ND	ND	ND	ND	ND	ND	Mims & Shelton 1998
Common carp	*Cyprinus carpio*	Gynogenesis	Yes	ND	360	hemacytometer	0.78	$21.2 \pm 12.8 \times 10^{6}$	Porter 1998
Bluegill	*Lepomis macrochirus*	Gynogenesis	Yes	ND	360	hemacytometer	0.84	$6.9 \pm 2.7 \times 10^{6}$	Porter 1998
Brown trout	*Salmo trutta*	Sperm concentration	Yes	ND	505	hemacytometer	0.94	2.2×10^{9} to 2.7×10^{10}	Poole & Dillance 1998
Rainbow trout	*Oncorhynchus mykiss*	Sperm characterization	ND	ND	ND	hemacytometer	ND	ND	Lahnsteiner et al. 1998

Table B-3. Continued.

Species	Scientific name	Purpose of study	Method described	Wavelength tested (nm)	Optimal wavelength	Absolute determination	R^2	Sperm concentration range tested	Citation
Atlantic salmon	*Salmo salar*	Reproductive performance	ND	ND	ND	ND	ND	ND	Estay et al. 1999
Bream	*Abramis brama*	Cryopreservation	Yes	ND	530	hemacytometer	0.97	6×10^9 to 2.1×10^{10}	Glogowski et al. 1999
Muskellunge	*Esox masquinongy*	Cryopreservation	ND	ND	ND	ND	ND	ND	Ciereszko et al. 1999
European bass	*Dicentrarchus labrax*	Fertilization trials	Yes	200 - 500	260	hemacytometer	0.97	4×10^7 to 2×10^8	Fauvel et al. 1999
Paddlefish	*Polyodon spathula*	Spermiation	Yes	ND	450	hemacytometer	0.86	1×10^8 to 1.6×10^9	Linhart et al. 2000
Rainbow trout	*Oncorhynchus mykiss*	Cryopreservation	ND	ND	ND	ND	ND	ND	Glogowski et al. 2000
Common carp	*Cyprinus carpio*	Sperm characterization	Yes	ND	610	hemacytometer	ND	$1\text{-}2 \times 10^5$	Dzuba et al. 2001
Argentinian silverside	*Odontesthes bonariensis*	Spermiation	Yes	ND	600	hemacytometer	ND	ND	Miranda et al. 2001
Zebra mussel	*Dreissena polymorpha*	Sperm characterization	Yes	ND	500	hemacytometer	0.98	2.2×10^6 to 1.8×10^7	Ciereszko et al. 2001
African catfish	*Clarias gariepinus*	Spermiation	ND	ND	505	hemacytometer	0.69	5×10^8 to 9×10^9	Viveiros et al. 2001
African catfish	*Clarias gariepinus*	Spermiation	ND	ND	505	hemacytometer	0.88	ND	Viveiros et al. 2002
Sterlet	*Acipenser ruthenus*	Sperm characterization	ND	ND	530	hemacytometer	ND	ND	Piros et al. 2002
Siberian sturgeon	*Acipenser baerii*	Sperm characterization	ND	ND	530	hemacytometer	ND	ND	Piros et al. 2002
Siberian sturgeon	*Acipenser baerii*	Fertilization trials	ND	ND	ND	ND	ND	ND	Glogowski et al. 2002
Rainbow trout	*Oncorhynchus mykiss*	Gamete and embryo storage	ND	ND	ND	ND	ND	ND	Babiak & Dabrowski 2003
African catfish	*Clarias gariepinus*	Spermiation	Yes	ND	505	hemacytometer	0.85	2×10^8 to 1.2×10^{10}	Viveiros et al. 2003
African catfish	*Clarias gariepinus*	Sperm characterization	ND	ND	650	hemacytometer	ND	3×10^9 to 9×10^9	Mansour et al. 2004
Rainbow trout	*Oncorhynchus mykiss*	Sperm characterization	ND	ND	ND	ND	ND	ND	Ciereszko et al. 2004
Siberian sturgeon	*Acipenser baerii*	Cryopreservation	ND	ND	530	ND	ND	ND	Sarosiek et al. 2004
Sterlet	*Acipenser ruthenus*	Cryopreservation	ND	ND	530	ND	ND	ND	Sarosiek et al. 2004
Atlantic cod	*Gadus morhua*	Sperm quality	Yes	ND	260	hemacytometer	0.99	1×10^9 to 5×10^{10}	Suquet et al. 2005
Blue catfish	*Ictalurus furcatus*	Sperm concentration	Yes	400 - 600	600	hemacytometer	0.77	1.1×10^7 to 1×10^8	Campbell et al. 2005a
Channel catfish	*Ictalurus punctatus*	Sperm concentration	Yes	400 - 600	500	hemacytometer	0.53	1×10^6 to 3×10^8	Campbell et al. 2005b
Zebrafish	*Danio rerio*	Cryopreservation	Yes	460, 560, 660	All	hemacytometer	0.97	ND	Yang & Tiersch 2005
Argentinian silverside	*Odontesthes bonariensis*	Spermiation	ND	ND	410	ND	ND	ND	Miranda et al. 2005
Paddlefish	*Polyodon spathula*	Aquaculture	ND	ND	450	hemacytometer	ND	2×10^8 to 1.7×10^9	Mims & Shelton 2005
Brown trout	*Salmo trutta* Linnaeus	Toxicology	ND	ND	405	hemacytometer	ND	ND	Lahnsteiner et al. 2005a
Rainbow trout	*Oncorhynchus mykiss*	Toxicology	ND	ND	ND	ND	ND	ND	Lahnsteiner et al. 2005b
Rainbow trout	*Oncorhynchus mykiss*	Sperm characterization	ND	ND	ND	ND	ND	ND	Dietrich et al. 2005b
Pacific oyster	*Crassostrea gigas*	Sperm concentration	Yes	380 - 780	550, 581	hemacytometer	0.99	2×10^7 to 2×10^9	Dong et al. 2005a
Pacific oyster	*Crassostrea gigas*	Cryopreservation	ND	ND	581	ND	ND	ND	Dong et al. 2005b
Pacific oyster	*Crassostrea gigas*	Cryopreservation	ND	ND	581	ND	ND	ND	Dong et al. 2005c
Pacific oyster	*Crassostrea gigas*	Cryopreservation	ND	ND	581	ND	ND	ND	Dong et al. 2006
Eurasian perch	*Perca fluviatilis*	Sperm characterization	ND	ND	ND	ND	ND	ND	Krol et al. 2006
European bass	*Dicentrarchus labrax*	Spermiation	ND	ND	260	ND	ND	ND	Schiavone et al. 2006

Table B-3. Continued.

Species	Scientific name	Purpose of study	Method described	Wavelength tested (nm)	Optimal wavelength	Absolute determination	R^2	Sperm concentration range tested	Citation
Pacific oyster	*Crassostrea gigas*	Cryopreservation	ND	ND	581	ND	ND	ND	Dong et al. 2007a
Pacific oyster	*Crassostrea gigas*	Cryopreservation	ND	ND	ND	ND	ND	ND	Dong et al. 2007b
Caspian brown trout	*Salmo trutta caspius*	Sperm concentration	Yes	ND	480	hemacytometer spermatocrit	0.91	7×10^8 to 6.5×10^9	Hatef et al. 2007
Brown trout	*Salmo trutta*	Sperm characterization	ND	ND	ND	ND	ND	ND	Dietrich et al. 2007a
Common carp	*Cyprinus carpio*	Sperm characterization	ND	ND	ND	ND	ND	ND	Dietrich et al. 2007a
Common carp	*Cyprinus carpio*	Sperm characterization	ND	ND	ND	ND	ND	ND	Wojtczak et al. 2007
Rainbow trout	*Oncorhynchus mykiss*	Fertilization trials	ND	ND	ND	ND	ND	ND	Dietrich et al. 2007b
Rainbow trout	*Oncorhynchus mykiss*	Spermiation	Yes	ND	410	spermatocrit	0.98	ND	Fitzpatrick & Liley 2008
Rainbow trout	*Oncorhynchus mykiss*	Fertilization trials	ND	ND	ND	ND	ND	ND	Tuset et al. 2008
Rainbow trout	*Oncorhynchus mykiss*	Sperm characterization	ND	ND	ND	ND	ND	ND	Dietrich et al. 2008
Arctic char	*Salvelinus alpinus*	Fertilization trials	ND	ND	ND	ND	ND	ND	Mansour et al. 2008a
Arctic char	*Salvelinus alpinus*	Cryopreservation	ND	ND	ND	ND	ND	ND	Mansour et al. 2008b
Blue mussel	*Mytilus edulis*	Sperm concentration	Yes	200 - 800	320	hemacytometer coulter counter	0.99	9.4×10^5 to 1.1×10^7	Del Rio Portilla & Beaumont 2008
Powan	*Coregonus lavaretus*	Cryopreservation	ND	ND	ND	ND	ND	ND	Ciereszko et al. 2008
Brook trout	*Salvelinus fontinalis*	Sperm concentration	ND	ND	ND	NucleoCounter	0.96	8.2×10^9 to 1.8×10^{10}	Nynca & Ciereszko 2009
Zebrafish	*Danio rerio*	Sperm concentration	Yes	200 - 780	380 - 700	hemacytometer	0.92	2.2×10^7 to 5.9×10^8	Tan et al. 2010
Swordtail	*Xiphophorus helleri*	Sperm concentration	Yes	200 - 780	380 - 700	hemacytometer	0.94	ND	Tan et al. 2010
Medaka	*Oryzias latipes*	Sperm concentration	Yes	200 - 780	380 - 700	hemacytometer	0.93	ND	Tan et al. 2010
Powan	*Coregonus lavaretus*	Sperm characterization	ND	ND	530	ND	ND	ND	Hliwa et al. 2010
European bass	*Dicentrarchus labrax*	Sperm quality	ND	200 - 500	260	hemacytometer	0.97	ND	Fauvel et al. 2010
Gilthead bream	*Sparus aurata*	Sperm quality	ND	200 - 500	260	hemacytometer	0.96	ND	Fauvel et al. 2010
Turbot	*Psetta maxima*	Sperm quality	ND	200 - 500	260	hemacytometer	0.95	ND	Fauvel et al. 2010
Wreckfish	*Polyprion americanus*	Sperm quality	ND	200 - 500	260	hemacytometer	0.98	ND	Fauvel et al. 2010
Bluefin tuna	*Thunnus thynnus*	Sperm quality	ND	200 - 500	260	hemacytometer	0.84	ND	Fauvel et al. 2010
Striped catfish	*Pangasianodon hypophthalmus*	Sperm quality	ND	200 - 500	260	hemacytometer	0.86	ND	Fauvel et al. 2010
Atlantic cod	*Gadus morhua*	Sperm quality	ND	200 - 500	260	hemacytometer	0.99	1×10^6 to 5×10^7	Fauvel et al. 2010

*ND: not described; ***Scaphirhynchus platorynchus*

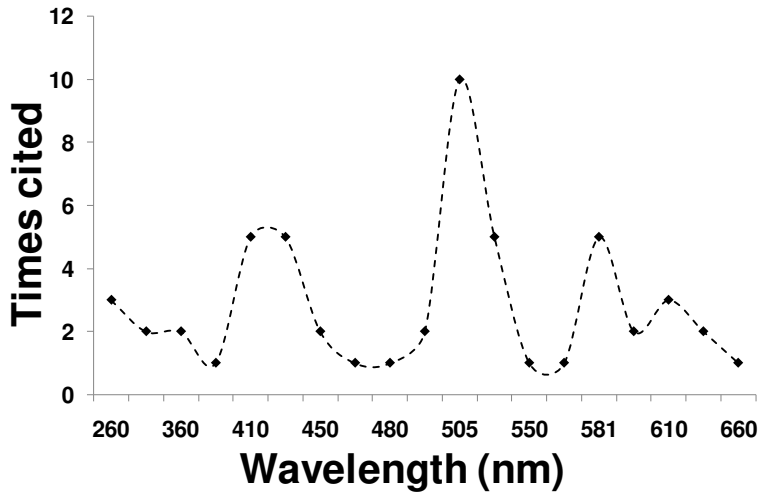


Figure B-4. Frequency of use of various wavelengths evaluated in 54 previous studies for the development of standard curves to estimate sperm concentration in fishes.

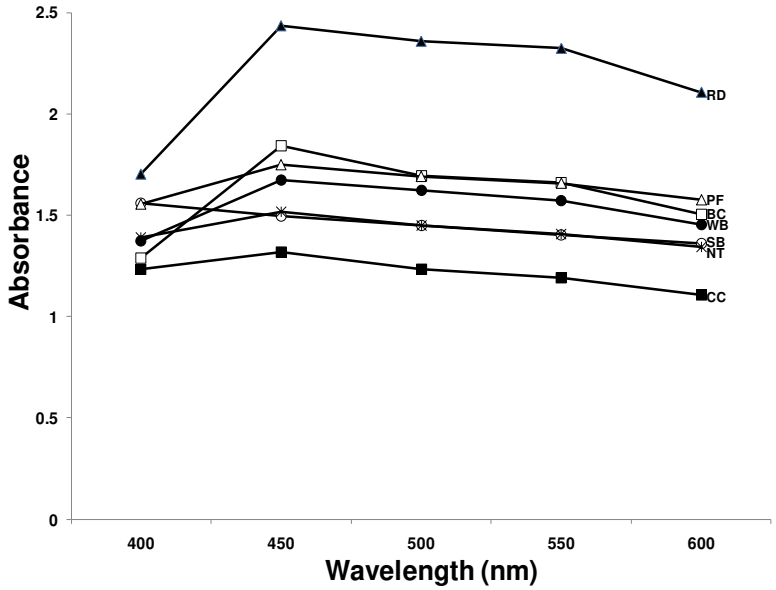


Figure B-5. Absorbance spectra measured in this study for sperm of red drum (RD: filled triangles), paddlefish (PF: open triangles), blue catfish (BC: open squares), channel catfish (CC: filled squares), white bass (WB: filled circles), striped bass (SB: open circles), and Nile tilapia (NT: asterisks). Absorbance values were based on using 2.5 x 10^8 cells/mL for all species, except red drum at 1 x 10^9 cells/mL.

Development of Standard Curves

A linear relationship (R^2 values from 0.586 to 0.904) was found between the natural logarithm of sperm concentration (1 x 10^6 to 6 x 10^{10} cells/mL) assessed by hemacytometer counting and the corresponding absorbance in the different wavelengths used (Table B-4). The

strongest correlations at the different wavelengths remained stable for the majority of the species, except at 400 nm for red drum (adjusted $R^2 = 0.040$). This indicated that there was a wide range of wavelengths that could be used to estimate sperm concentration.

Table B-4. Standard curves, linear regression equations, and coefficient of determination from the logarithmic regression of sperm counts and absorbance at the different wavelengths tested.

Common name	N[1]	Obs[2]	Wavelengths (nm)				
			400	450	500	550	600
Blue catfish	15	72	y= -2.994 + 0.215 lnX Adj R^2 = 0.810	y= -3.418 + 0.243 lnX Adj R^2 = 0.739	y= -3.130 + 0.222 lnX Adj R^2 = 0.730	y= -2.977 + 0.212 lnX Adj R^2 = 0.694	y= -2.678 + 0.190 lnX Adj R^2 = 0.703
Channel catfish	47	202	y= -2.067 + 0.162 lnX Adj R^2 = 0.587	y= -2.450 + 0.185 lnX Adj R^2 = 0.601	y= -2.348 + 0.176 lnX Adj R^2 = 0.603	y= -2.233 + 0.167 lnX Adj R^2 = 0.586	y= -2.118 + 0.158 lnX Adj R^2 = 0.592
Paddlefish	4	11	y= -6.799 + 0.446 lnX Adj R^2 = 0.901	y= -8.663 + 0.553 lnX Adj R^2 = 0.889	y= -8.567 + 0.544 lnX Adj R^2 = 0.885	y= -8.442 + 0.535 lnX Adj R^2 = 0.881	y= -8.106 + 0.513 lnX Adj R^2 = 0.877
Red drum	5	14	y= 1.495 + 0.010 lnX Adj R^2 = 0.040	y= -2.702 + 0.243 lnX Adj R^2 = 0.777	y= -3.246 + 0.265 lnX Adj R^2 = 0.805	y= -3.040 + 0.253 lnX Adj R^2 = 0.784	y= -1.228 + 0.157 lnX Adj R^2 = 0.791
Striped bass	12	45	y= -5.626 + 0.349 lnX Adj R^2 = 0.850	y= -6.457 + 0.397 lnX Adj R^2 = 0.860	y= -6.285 + 0.386 lnX Adj R^2 = 0.853	y= -6.129 + 0.376 lnX Adj R^2 = 0.846	y= -5.914 + 0.362 lnX Adj R^2 = 0.840
Tilapia[3]	69	114	y= -5.207 + 0.336 lnX Adj R^2 = 0.843	y= -6.523 + 0.410 lnX Adj R^2 = 0.820	y= -6.379 + 0.400 lnX Adj R^2 = 0.816	y= -6.214 + 0.389 lnX Adj R^2 = 0.812	y= -5.895 + 0.369 lnX Adj R^2 = 0.814
White bass	21	75	y= -4.476 + 0.289 lnX Adj R^2 = 0.871	y= -6.588 + 0.411 lnX Adj R^2 = 0.904	y= -6.525 + 0.406 lnX Adj R^2 = 0.902	y= -6.476 + 0.402 lnX Adj R^2 = 0.901	y= -5.879 + 0.366 lnX Adj R^2 = 0.903

1N = number of fish
2Obs = number of observations, each observation is a different concentration
3 Nile tilapia *Oreochromis niloticus*, blue tilapia *O. aureus*, Mississippi commercial strain, Florida red tilapia

Validation of Regression Models

When validating the equation generated with the concentrations provided by hemacytometer counts, there were no significant differences between the observed values by counts and the predicted values of the standard curves, except when comparing the predicted concentrations from the general curve of all species (combining the data for crushed testis and stripped sperm) ($P = 0.001$).

Relationship of Standard Curves among Species

When comparing the regression curves across species within individual genera (i.e. *Ictalurus, Morone,* and *Oreochromis)*, there were no differences, except for blue and channel catfish at 400 and 450 nm, and white and striped bass at 400 nm (Table B-5). When comparing the curves across all species (independent of the genus), there were significant differences for all the wavelengths ($P < 0.001$). However, when catfishes were removed from the model, there was no difference in the curves of paddlefish, red drum, striped bass, and tilapias for all the wavelengths except 400 nm (Table B-5). Overall, the linear relationship of catfishes followed a pattern different from the values of the other species studied (Figure B-6). When the absorbance values of catfishes were compared with those for other fishes within the absorbance range of 0.1 to 2.5 at concentrations of lower than 1×10^9 cells/mL, there was an overestimation of absorbance for the catfishes at any given concentration. This difference was likely because suspensions from crushed testes were contaminated with somatic cells such as erythrocytes which increased the absorbance values. Overestimation was greater at lower sperm

concentrations due to the higher relative proportion of somatic cells in relation to sperm cells (Figure B-6).

Table B-5. Comparison of standard curves among species. The curves were compared by an analysis of covariance (ANCOVA) at each wavelength. Multiple regression was used to correlate among the species the absorbance, and natural logarithmic of sperm concentration.

Common name	N[1]	Obs[2]	Wavelengths (nm)				
			400	450	500	550	600
Blue & channel catfish	2	274	p-value = 0.022	p-value = 0.028	p-value = 0.061	p-value = 0.068	p-value = 0.149
White & striped bass	2	120	p-value = 0.019	p-value = 0.633	p-value = 0.497	p-value = 0.379	p-value = 0.889
Tilapia[3]	4	114	p-value = 0.057	p-value = 0.296	p-value = 0.252	p-value = 0.248	p-value = 0.239
All species	8	533	R^2 = 0.774 p-value = <.001	R^2 = 0.806 p-value = <.001	R^2 = 0.811 p-value = <.001	R^2 = 0.805 p-value = <.001	R^2 = 0.805 p-value = <.001
All species without catfish	6	259	R^2 = 0.875 p-value = <.001	R^2 = 0.899 p-value = 0.358	R^2 = 0.896 p-value = 0.401	R^2 = 0.895 p-value = 0.334	R^2 = 0.890 p-value = 0.183
All species without blood	8	282	R^2 = 0.879 p-value = <.001	Adj R^2 = 0.899 p-value = 0.079	Adj R^2 = 0.896 p-value = 0.041	Adj R^2 = 0.894 p-value = 0.023	Adj R^2 = 0.900 p-value = 0.013
Blood	1	108	p-value = <.001	p-value = 0.208	p-value = 0.067	p-value = 0.188	p-value = 0.149

1N = number of species
2Obs = number of observations, each observation is a different concentration
3 Nile tilapia *O. niloticus*, blue tilapia *O. aureus*, Mississippi commercial strain, Florida red tilapia

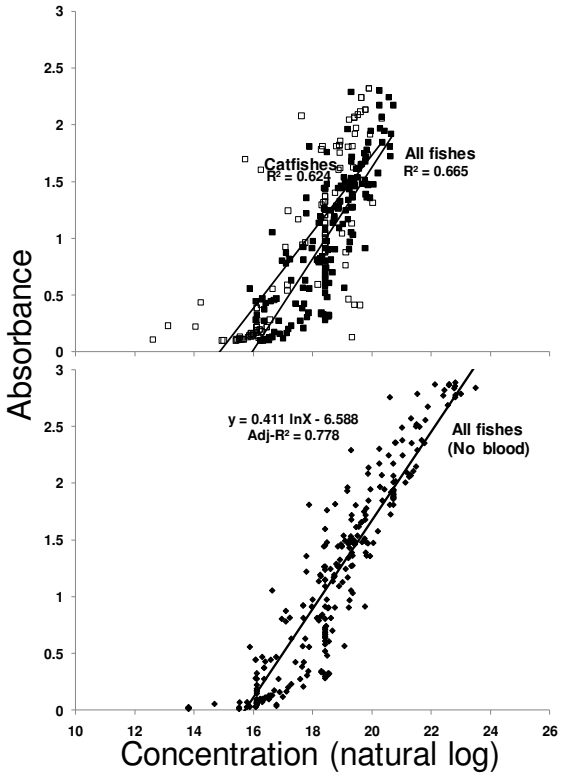


Figure B-6. Relationship between absorbance at 450 nm and sperm counts by hemacytometer. Upper panel: regression lines for all fishes without catfishes, and catfishes only. The absorbance range was 0.1 to 2.5, and the maximum sperm concentration was 1 x 10^9 cells/mL. Lower panel: standard curve for all fishes, including catfish, with no blood.

Subsequent plotting of data for catfish sperm collected without blood (relatively pure samples) were combined with the data for paddlefish, red drum, tilapia, white bass and striped bass, and there was no significant difference for each wavelength except for 400 nm (Table B-5). Also, the estimated values from the standard curve of all fish samples without blood had no significant differences ($P = 0.181$ at 450 nm) when compared with the hemacytometer counts. Moreover, plotting of the observed values of all species without blood against the standard curve generated from readings at 450 nm indicated a strong relationship ($R^2 = 0.778$) (Figure B-6).

Effect of Blood

The interaction of the different blood percentages was significant for the 400 nm wavelength (Table B-5). As the volumetric proportions of blood increased, the absorbance values also increased (Figure B-7).

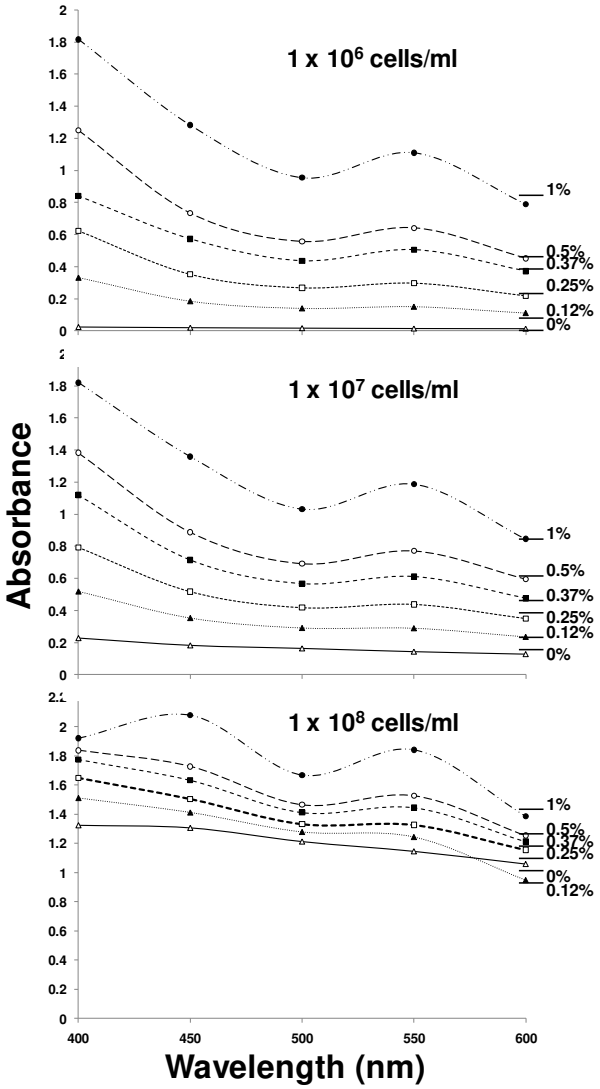


Figure B-7. Absorbance values measured at wavelengths between 400 nm and 600 nm for three concentrations of channel catfish sperm with different volumetric proportions of blood. Upper panel: absorbance values using 1 x 10^6 sperm cells/mL with different percentages of blood; middle panel: 1 x 10^7 cells/mL; lower panel: 1 x 10^8 cells/mL.

A broad absorbance peak did not occur at low volumetric proportions of blood but the peak increased with higher proportions of blood. This is consistent with the absorbance spectra for pure blood samples. When the absorbance of blood was measured at different wavelengths, there was an increase in absorbance at 450 nm, with a maximum at 500 to 550 nm (Figure B-8).

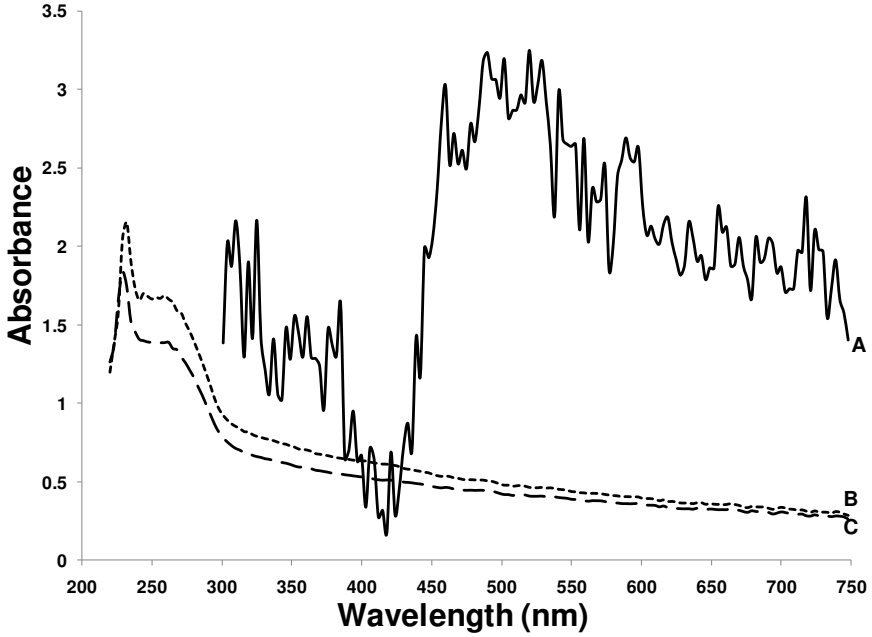


Figure B-8. Absorbance values measured at wavelengths between 220 nm to 748 nm for: A) channel catfish blood, B) sperm collected from crushed testis, and C) sperm without blood. Absorbance values were the average of three males with a sperm concentration of 1 x 10^8 cells/mL. There was no maximal absorbance peak within the visible spectrum for the samples. The maximal absorption of blood was between 500 to 550 nm. Absorbance values of samples below 300 nm were variable.

Absorbance values of blood at wavelengths lower than 300 nm were inconsistent. However, the absorbance of sperm from crushed testes and sperm without blood remained constant. Although a maximum absorbance was detected at 230 nm, the absorbance values (greater than 1.2) yielded high variability (Figure B-8). Within the visible wavelengths (390 – 750 nm) there was no absorbance peak. In general there was a gradual linear reduction in absorbance as the wavelength increased. Therefore, at low sperm concentrations (< 1 x 10^6 cells/mL) the absorbance values were influenced more by the presence of blood. As the sperm concentrations increased (> 1 x 10^7 cells/mL), there was an increase of absorbance caused by the sperm. When no blood was added the absorbance values were directly related to the sperm concentration (Figure B-7).

Discussion

Sperm concentration is rarely reported during studies of aquatic species, and typically when reported does not include a description of the methods used. This leads to problems for comparison and reproducibility of published studies especially for activities such as

cryopreservation and fertilization trials. The estimation of sperm concentration is essential for a range of activities including standardization of cryopreservation, optimization of fertilization, calibration of ultraviolet irradiation to induce gynogenesis, and study of spermiation following hormonal stimulation. Despite measurements in more than 40 species, currently there is a lack of specific information regarding sperm concentration determination and how it relates to cryopreservation and fertilization in essentially all aquatic species (Tiersch et al. 2007). Traditionally in livestock species, sperm concentrations have been estimated by the use of cell counting devices such as the hemacytometer or other specialized counting chambers (Foote et al. 1978, Prathalingam et al. 2006). Although by observing the sperm using a microscope, other parameters such as morphology could be determined. But counting chambers are time consuming, and require the use of microscopes and trained technicians typically not available in hatchery settings. As such, most aquaculture work does not include sperm concentrations (Campbell et al. 2005a, Dong et al. 2005a).

In the search for faster and more practical ways to estimate relative or absolute sperm concentration, centrifugation (to determinate spermatocrit) and spectrophotometer (to determine turbidity) have been used. Because spermatocrit and absorbance are easy to measure, the choice of methods has generally been based on access to equipment (Tvedt et al. 2001). Spermatocrit is an indirect method which is expressed as the volume of sperm in relation to the total volume of sample (packed cell volume divided by total sample volume). One of the common problems in spermatocrit estimation is the lack of a clear separation between the packed sperm cells and the seminal fluid; this can lead to false estimations of spermatocrit. To avoid this problem, prolonged centrifugation times are needed, usually more than 10 min, but as long as 45 min in species with dense sperm samples such as striped bass (Vuthiphandchai and Zohar 1999). An additional problem is the relatively large volume (at least 0.1 mL) needed (Lin et al. 1996b).

Sperm evaluation should be rapid and effective so that samples can be processed efficiently to preserve initial quality and fertility (Foote 1980). Spectrophotometric determination of turbidity is recognized as a reliable, efficient, and rapid technique to estimate the concentration within semen samples in farm animals (Brillard and McDaniel 1985). From the previous work that used spectrophotometer to determine sperm concentration in aquatic species, there has been no attempt to evaluate the feasibility of generating a general calibration curve.

Literature Review:

Previous studies that estimated sperm concentration by spectrophotometry in fishes are characterized by a lack of description of the methodology used (Table B-3). This failure of reporting and in defining procedures limits reproducibility, weakens results, and makes direct comparisons among studies problematic or impossible. There is a pressing need for development of standardized protocols. Less than 20% of the studies in our literature review tested different wavelengths to identify the wavelength of maximum absorbance (this may or may not be a large problem depending on the instrument used). Our review also found that one third of the studies used wavelengths between 500 to 550 nm which can be affected by the presence of blood in the samples (Figure B-7). The reference cited most (18 of 54, 33%) in the methods sections of published papers was by Ciereszko and Dabrowski (1993). This publication addressed comparison of three methods for sperm concentration determination (i.e., spectrophotomer,

hemacytometer, and spermatocrit). Almost all of these publications (16 of 18) did not state which of these three methods were actually used (e.g., Rinchard et al. 2001, Kowalski et al. 2006). In an attempt to compare previous studies with the present study, we estimated the sperm concentration from the equations described in six studies and plotted them (Figure B-9). The differences among studies can be explained by the difference in instruments (see below) and the ranges of sperm concentration tested.

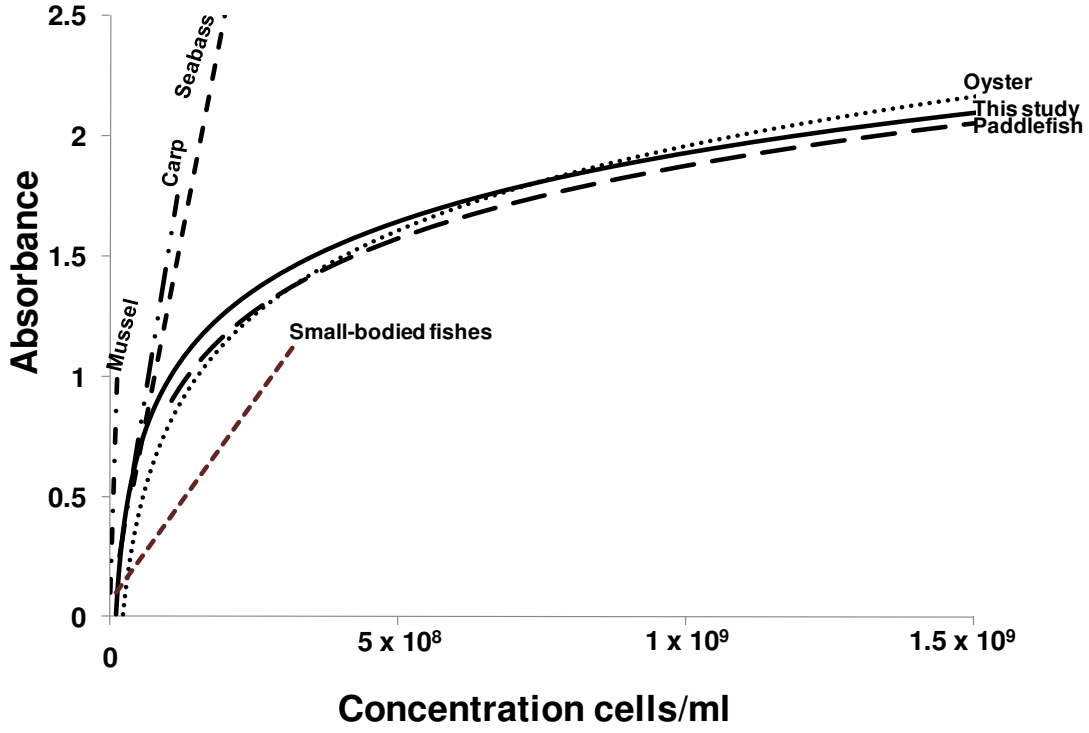


Figure B-9. Relationships between absorbance and sperm concentration in previous studies: blue mussel (Del Rio-Portilla and Beaumont 2008), common carp (Takashima et al. 1984), seabass (Fauvel et al. 1999), Pacific oyster (Dong et al. 2005a), paddlefish (Linhart et al. 2000), small-bodied fishes (zebrafish, medaka, and green swordtail) (Tan et al. 2010), and a multi-species curve (this study).

A spectrophotometer is generally composed of one or more light sources, a wavelength selector, sample container, detector, signal processor, and readout devices. In a turbidity measurement a spectrophotometer is used to measure the attenuation of light as a function of wavelength as it passes through a sample. Because previous studies used different wavelengths and different spectrophotometers, the instrument characteristics (such as light transmission properties of the sample cell, aperture size, distance between sample cell and detector, and pathlengths) were different. For example, cuvettes made of plastic or glass can be used to measure within the visible spectrum, while a cuvette made of quartz glass or fused silica should be used for the UV region (i.e., below 350 nm). In addition, there are several types of detectors such as photovoltaic cells, vacuum photodiodes, photomultiplier tubes, and silicon photodiodes (Cole and Levine 2003). Each detector has a differential sensitivity of wavelength range (Cole

and Levine 2003). All of these components and configurations are instrument specific and constitute some of the known and unknown sources of variation in previous studies.

Previous studies in livestock compared the estimation of sperm concentration using different spectrophotometers. Comparison of duplicate instruments yielded nearly identical results in bull sperm (Foote 1972, Foote et al. 1978) while the use of different models of spectrophotometers resulted in different readings for the same sperm samples from boar (Knox et al. 2002, Knox 2004). It is important to note that one study compared three spectrophotometers for the sperm of boar, bull, and stallion (Rondeau and Rouleau 1981). Two of the instruments yielded no difference in standard curves, and the authors concluded that if spectrophotometers have the same characteristics in terms of spectral bandwidth, the calibration curves need not be statistically different. Even so, variable maintenance and calibration of instruments, different types and quality of cuvettes, and the lifespan of instrument lamps could introduce differences in responsivity and accuracy across time for a single instrument or among duplicate instruments.

Feasibility of Establishing a General Curve:

Determination of an absorption spectrum for analyzing a given sample material is the first step of turbidimetry analysis to identify the maximum sensitivity for measurements. This is typically done by plotting the absorbance measurement as a function of wavelength (Dong et al. 2005a, Tan et al. 2010). Wavelengths in the range of 260-660 nm have been used to determine sperm concentrations in fishes. The livestock industry has used wavelengths in the range of 275 to 630 nm (Foote et al. 1978). Wavelengths in the range of 550 to 576 nm appear most sensitive for white suspensions (color induced absorbance) such as for sperm, and a wavelength of 550 nm is recommended and mostly used (Foote et al. 1978, Knox 2004). In the present study, we tested five wavelengths (400, 450, 500, 550 and 600 nm) with seven species. There was no single wavelength that yielded maximum absorbance. This indicates that any of these wavelengths could be appropriate to generate a standard curve.

Other studies found that lower wavelengths yield greater variation. For example, for zebrafish the maximum absorbance of sperm was around 265 nm but the absorbance profiles at wavelengths below 380 nm (UV) varied widely for single samples and the use of wavelengths above 400 nm was recommended (Tan et al. 2010). In a different study with blue mussels, the maximum absorbance was at 216 nm but the absorbance values had a large variation and the use of wavelengths above 320 nm was recommended (Del Rio-Portilla and Beaumont 2008). In contrast, another study recommended the use of 260 nm based on the hypothesis that differences in optical density using this wavelength in different fishes were due to the DNA content. This report compared the light absorption of sperm of turbot (*Psetta maxima*), seabream (*Sparus auratus*), and seabass (*Dicentrarchus labrax*) but without a standardized concentration (Fauvel et al. 2010). This hypothesis and other claims such as that volume changes in sperm can be tracked by their absorbance (Dzuba and Kopeika 2002), should be addressed in future research. In general, lower wavelengths yield higher transmittance values and thus have been recommended for use (e.g., Del Rio-Portilla and Beaumont 2008). Absorbance values below 0.1 and above 1.0 represent 10% and 20% transmittance, respectively, while absorbance values above 2.0 represent ≤1% transmittance. In earlier spectrophotometers, this low transmittance could result in inaccurate readings, although this is not normally a problem with current instruments (Mantle and Harris 2000). Earlier spectrophotometers used vacuum photodiodes while current spectrophotometers use silicon photodiodes and optical filters with higher resolution.

Overall, there are three main types of spectrophotometers: 1) Visible spectrophotometers that have inexpensive glass components, use tungsten lamps as the light source, and operate across a range of 325 to 1,000 nm. Older instruments of this type rely on blue- and red-sensitive phototubes. 2) Ultraviolet-Visible spectrophotometers that measure absorbance in the 200 to 1,000 nm range. For instruments of this type the most common source of radiation is the hydrogen-discharge lamp, but if more intensity is desired (3-5 times) a deuterium-discharge lamp is used. 3) Infrared spectrophotometers that use a heat source (i.e. Globar and Nernst glower) and the spectra result from molecular vibrational transitions in the range of 750–15,000 nm (Csuros 1997). These have been applied for multi-component analyses of fish meat (Elvingson and Sjaunja 1992) but have not been used for estimation of sperm concentrations.

In this study, we evaluated wavelengths within the visible spectrum (390–750 nm). This is the first study that attempted to evaluate a general standard curve to measure sperm concentration for fish species. Although there is a publication in which a single formula was used to measure sperm concentration in the Siberian sturgeon (*Acipenser baerii*) and sterlet (*A. ruthenus*), there was no explanation of the reasoning behind this usage, or if both species were validated for that equation (Sarosiek et al. 2004). It has been stated as a common belief that different calibration curves are required for different species because of the specificities of the sperm (Foote et al. 1978). However, as stated above, a general standard curve has been established for bulls, boars, and stallions (Rondeau and Rouleau 1981), and there were no differences among the slopes of the calibration curves for these livestock species. Whether for mammals or for fish, seminal samples consist of seminal plasma (or seminal fluid) and spermatozoa. Fish seminal plasma contains mainly mineral compounds and low concentrations of organic substances (Ciereszko et al. 2000a). The absorbance of seminal plasma (< 0.1) was measured in seabass and yellowtail flounder (*Limanda ferruginea*), which led to the conclusion that seminal plasma did not interfere within the wavelength range (200–900 nm) tested (Clearwater 1996, Fauvel et al. 1999). In fact, the effect of light scattering and light absorption by spermatozoa has been shown to dominate the effect of light absorption by seminal plasma (Rothschild 1950, Taneja and Gowe 1961). Therefore, in the present study there was little justification to remove the seminal plasma before estimating the concentration by the use of spectrophotometer. We hypothesized that a single calibration curve could be used to determine the concentration of spermatozoa for most fish species.

In this study, after comparing the data of all fishes, the correlation coefficient of the standard curve at all wavelengths was higher than 0.77. But the validations of the observed values against the spectrophotometric estimation were significantly different. This was resolved however when the collection methods (stripped and crushed) were separated. The correlation coefficients for all species without catfish were higher ($R^2 = 0.87$) and there was no statistical difference between observed and estimated values at wavelengths higher than 450 nm. Differences in absorbance between sperm collection methods (stripped and crushed) have been reported in zebrafish and green swordtail (*Xiphophorus helleri*) (Tan et al. 2010), although a robust applicability was reported in that study among all of the curves generated across species (zebrafish, swordtail, and medaka *Oryzias latipes*), and different collection techniques (stripped and crushed). For relatively pure, homogenous sperm samples, if the sperm size and shape among species are similar, the changes in light absorbed among samples will primarily be due to a difference in sperm cell concentration (Rondeau and Rouleau 1981). In addition, studies have shown that dilute suspensions of most bacteria have nearly the same absorbance per unit of dry

weight concentration, regardless of the variation of cell size and shape (Omstead 1990). Therefore, based on our results, we postulate that a general standard curve for any single instrument should be able to measure concentration for most fishes from which pure samples of sperm can be collected.

To evaluate the effect of other cell types such as those in whole blood, sperm with no overt blood contamination was collected in catfishes. When these absorbance values were combined with the data for all species without blood, the resulting correlation value was the same as that observed for all fishes collected by stripping. When there was no addition of blood to the samples, the absorbance was directly related to the sperm concentration. Therefore blood cells and other cell types that are mixed with sperm during crushing of the testis can interfere with accurate estimation of sperm concentration. Depending on the timing in relation to the spawning season, the ratio of somatic cells and germ cells can vary considerably. Crushing of the testes can release a mixture of cells types such as spermatogonia, spermatocytes, spermatids, spermatozoa, and Sertoli-like cells (Viveiros 2003). Failure to properly clean the testes before crushing could also contaminate samples with connective tissues that contain Leydig-like cells, nerve fibers, fibroblasts, collagen fibers, smooth muscle cells, and endothelial cells (Grier and Uribe 2009). In addition, the cytoplasm of epithelial cells of the main testicular ducts and spermatic ducts contains lipid vacuoles, and the seminal fluids also contain lipids during interspawning periods (Lahnsteiner and Patzner 2009). Based on our observations contamination of this sort can lead to a systematic overestimation of sperm concentration in direct relationship to the volume of blood or other contaminants present as described above (Figure B-7). Other studies found similar effect, when debris present in the raw semen, such as cytoplasmic droplets, affected the accuracy of the spectrophotometric method (Christensen et al. 2004). Thus, depending on spawning condition the correlation between absorbance and sperm concentration could be affected by somatic contamination, and more work needs to be done to evaluate measurements of samples collected from crushed testes at different times of the year. It should be noted that microscopic observations can be used to assess the level of contamination of samples before measuring the absorbance (Figure B-3).

This is not the first publication to mention that other cell types such as blood can disturb absorbance measurements. In fact, the presence of other cells in the sperm of the landlocked sea lamprey (*Petromyzon marinus*) disrupted the use of spectrophotometry (Ciereszko et al. 2000b). Stripped samples of the African catfish (*Clarias gariepinus*) were contaminated with blood and the turbidity estimation for sperm concentration could not be applied (Viveiros et al. 2003). And, in African catfish two types of sperm samples, “white” and “grey”, were collected by dissection and stripping of the testes. The white samples had a higher sperm cell concentration and absorbance values (650 nm) than did grey samples (Mansour et al. 2004). The white samples were characterized by high sperm densities and a low number of spermatids, while the grey samples contained numerous germinal cysts with spermatids in addition to sperm. In another study, different levels of blood contamination of rainbow trout (*Oncorhynchus mykiss*) milt were obtained in relation to sampling period and method of milt collection (Ciereszko et al. 2004). In an attempt to measure the influence of blood in sperm samples, blood was added to a pure sample (final volumetric proportion of blood was 0.2%) of rainbow trout sperm (sperm concentration 9×10^9 to 1.4×10^{10} cells/mL) (Ciereszko et al. 2004). Sperm quality parameters (osmolality, protein concentration, lactate dehydrogenase activity) were not affected by the contribution from this small amount of blood and although this study used turbidity methods it

did not report any influence of blood on the estimation of sperm concentration (we presume due to the high proportion of sperm cells in relation to the erythrocytes).

In the present study, five different volumetric proportions of blood were tested with three concentrations of sperm. Although the absorbance values for the different volumes of blood were different, there was no statistical difference in the standard curves for wavelengths higher than 450 nm. This corresponded with the sperm collected by crushed testes in which higher correlations were obtained at wavelengths from 500 to 600 nm. Contamination with as much as 1% blood, did not affect the standard curve for concentrations as low as 1×10^6 cells/mL. This indicates that blood contamination might not be a major factor of concern when measuring concentration by spectrophotometer in samples with a high proportion of sperm cells in relation to blood cells (Figure B-7, lower panel). It could however be expected that higher proportions of blood to sperm (> 1%) at low sperm concentrations would influence the absorbance, especially at the wavelengths of maximum absorbance of blood (> 450 nm) which corresponds to the peaks of maximum absorbance of oxyhemoglobin (540 and 575 nm) (Stryer 1995).

The effects caused by crushing of testes with respect to contamination of the samples are not simple or straightforward. Errors in absorbance or sperm concentration can result in overestimations or underestimations. For example, use of a calibration curve developed during the peak of the spawning season using pure sperm when applied to crushed testis samples could yield overestimations of concentration (based on the inflated absorbance values of the samples). Or, conversely use of a curve developed early in the spawning season when testes did not contain large volumes of mature sperm would result in an upward-shifted curve (based on higher somatic absorbances) and could yield underestimates in concentration later in the spawning season when sperm production peaks. Thus, the types of errors are affected by variations of cell types in relation (proportion) to one another and this relationship varies over time due to reproductive seasonality. As such, the observed effects can sometimes be small between crushed testis and pure sperm samples (e.g., Figure B-8), but the potential contribution of cells such as erythrocytes to absorbance should not be overlooked. It is also important to note that the patterns observed in the absorbance profiles generated by blood addition (Figure B-7) do not exactly match the absorbance profile of pure blood (Figure B-8) likely due to the relative contributions of the various components in contaminated samples (as described above) to the aggregate profile. Hemoglobin itself can exist in a variety of forms with different absorbance profiles based on interactions with atmospheric gases (Stryer 1995).

The testes of ictalurid catfishes in particular present an even more complicated picture with regard to contamination caused by crushing. The testes possess two recognizable portions: anterior and posterior (Figure B-10) (Sneed and Clemens 1963). These two portions are characterized by different cells types, and their relative sizes and color vary depending on the spawning period. The anterior portion is considered to be spermatogenic in function and is small, flat and transparent outside of the spawning season, but expands dramatically, turning white with finger-like extensions, and composing 2/3 of the testis, as sperm production fills the tubules and lumen (Guest et al. 1976). This portion contains the majority of available sperm and is sometimes the only portion of the testis to be harvested while the posterior portion is discarded (Tiersch et al. 1994). The posterior portion has been reported to function as an accessory glandular organ that secretes a mucopolysaccharide-protein-lipid-rich fluid (seminal vesicular fluid) believed to contribute to the seminal volume and participate in prolongation and stabilization of sperm viability (Chowdhury and Joy 2007). The posterior testis is composed of interstitial cells, fibroblasts, blood capillaries, and nerve elements. During spawning the

epithelial cells of the posterior portion contain an abundance of rough endoplasmic reticulum, Golgi apparatus, secretory vacuoles, and electron-dense secretion products (Chowdhury and Joy 2007). The role of the posterior portion is most likely involved in maturation and nutrition of sperm, although it possesses a similar sperm concentration (based on cells per wet weight of tissue) as the anterior (Guest et al. 1976, Jaspers et al. 1978). The size and color of the posterior portion is more variable than the anterior portion (our unpublished observation) and it can be larger or smaller than the anterior portion, although it is almost darker (pink to light red to brown).

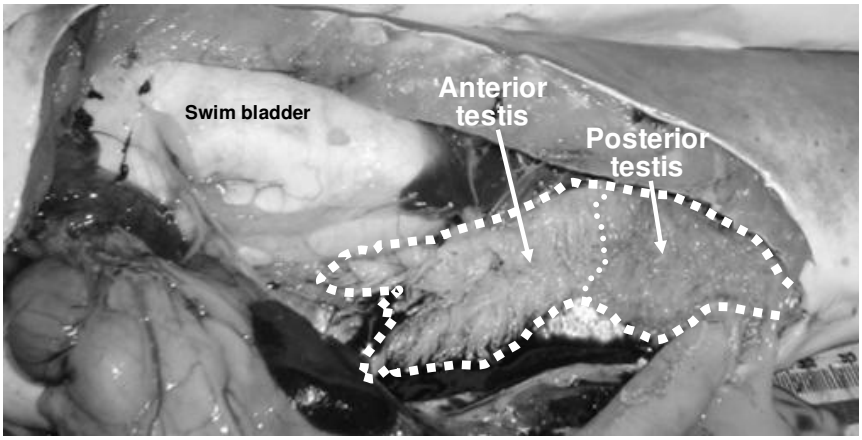


Figure B-10. View of channel catfish testes within the body cavity during the spawning period (April to May in Southern Louisiana). The anterior portion is considered to be spermatogenic and the posterior portion to serve glandular functions. Dissection and crushing of the testis to collect sperm can involve both portions or only the anterior portion, and can contribute a variety of somatic cells and other contaminants to samples.

As indicated above, sperm collection can proceed by crushing of the anterior portion alone, or as an admixture with the posterior portion yielding a complex collection of cell types and compounds within the sample with potential for considerable effects on the aggregate absorbance values. In this study, we chose to crush and mix both portions of the testes to fully capture the variation encountered in different protocols. From the previous discussion it should be evident that development of calibration curves from either portion or their combination would present considerable variation throughout reproductive seasonality and offers great opportunity for standardization of protocols and reporting to reduce or minimize variation within and among studies and hatchery operations. Future studies are needed to more fully evaluate these portions of the ictalurid testis in terms of biological function and the methods appropriate for their use in practical spawning protocols (including cryopreservation).

Protocols describing methodology to use turbidity to measure sperm concentrations have been published previously for livestock (Foote 1972, Foote et al. 1978), and a recent study was performed to illustrate development and standardization of photometric measurement of sperm concentration in Pacific oysters (*Crassostrea gigas*) (Dong et al. 2005a). Procedurally, care should be taken when collecting sperm, because contamination with other substances such as urine could affect calibration and concentration estimates (Clearwater 1996). The viscosity of the

sperm often makes it difficult to obtain a homogeneous dilution of the sample, and depending on the pipette and tips used for sample handing, different values could be obtained from the same sample. Therefore thorough mixing of sperm suspensions before measurement is essential for accurate readings (Cabrita et al. 2009). The presence or absence of small aggregations of spermatozoa in an aliquot of dilution could affect the accuracy and precision of sperm concentration estimates (Rakitin et al. 1999). These types of errors are magnified when working with the limited sperm samples (2-4 µL) collected from small-bodied fish such as zebrafish (Tan et al. 2010). Standard curves should be established for each spectrophotometer and regular calibrations are needed to ensure accurate estimation of sperm concentration (Knox et al. 2002). In the present study, standard curves generated at wavelengths from 450 to 550 nm, within the range of 1×10^6 to 6×10^{10} cells/mL were effective for determination of the concentration of sperm from paddlefish, red drum, tilapias, white bass, and striped bass, and would likely be useful for other fish species. Data for sperm concentration should be logarithmically transformed prior to application of simple linear regression. Such log transformation has been suggested previously (Berman et al. 1996, Handelsman 2002) and applied in Pacific oysters (Dong et al. 2005a), yellowtail flounder (Clearwater 1996), paddlefish (Linhart et al. 2000), and blue catfish (Campbell et al. 2005a). It is important to note that the generation of a standard curve will be specific for each type of spectrophotometer or instrument and even different laboratories, because not all conditions can be expected to be the same among locations (Knox et al. 2002). Accurate and comprehensive reporting of methods is thus necessary to accurately evaluate and compare studies.

Although the hemacytometer is considered as the "gold standard" for measurement of sperm concentration, there are variations among different designs and operators (Seaman et al. 1996, Christensen et al. 2005). Estimation of sperm concentration from a spectrophotometric determination of turbidity is routinely used in artificial insemination of mammals because it is fast and precise (Prathalingam et al. 2006). The precision of turbidity estimation derives from standard curves produced from multiple hemacytometer counts. Although some have considered spectrophotometer to be costly and time consuming (Powell 2002), there is little evidence to support this observation. Compared to a hemacytometer there is a higher initial cost for the spectrophotometer (Table B-6). However, the same spectrophotometer can be used for other purposes such as measuring water quality or general laboratory analyses. There are portable spectrophotometers that are used in the daily routine of fish farming, and could provide an inexpensive and rapid method for sperm concentration determination in field conditions (Dietrich et al. 2005a, Dong et al. 2005a). The use of a portable spectrophotometer (Eppendorf, Germany) has been reported to estimate the sperm concentration in whitefish (Ciereszko et al. 2008). Other options are microspectrophotometers that work with microliter sample volumes. These microspectrophotometers can be essential to measure sperm concentration for small-bodied fishes such as zebrafish that only yield 2-4 µL total of sperm sample (Tan et al. 2010).

Other techniques available to estimate sperm concentration include computer-assisted sperm analysis (CASA) instruments (e.g., Hamilton Thorne, Beverly, MA; SQA-V Medical Electronic Systems, Los Angeles, CA), flow cytometry, and fluorescence microscopy, and Coulter counter. These techniques require specialized instruments that are prohibitively expensive if they are used only to measure sperm concentration. A disadvantage for using CASA is that it often requires a specific disposable chamber in which non-uniform distribution of the spermatozoa can lead to false estimations (Lu et al. 2007). There is no replacement for a direct observation of the sperm to detect other sperm quality parameters such as morphology and the

presence of clumps or other types of contaminants. There is image analysis software available for free downloading distributed by the National Institutes of Health (rsbweb.nih.gov/ij) that has been used to estimate sperm concentration in Atlantic bluefin tuna *Thunnus thynnus thynnus* (Mylonas et al. 2007).

Table B-6. Comparison of price, volume requirements, and wavelengths available for different spectrophotometers.

Spectrophotometer	Cost (US$)	Volume needed	Wavelength range (nm)	Web address
Ultrospec 10, GE HealthCare[1]	742	1.5 mL	600	www.gelifesciences.com
Biowave, Biochrom WPA[1]	762	10 µL	190 - 900	www.biochrom.co.uk
YSI 9300[1]	807	10 mL	450 - 650	www.ysi.com
Smart2, LaMotte[1]	909	10 mL	350 - 1000	www.lamotte.com
DR890 Colorimeter, Hach[1]	1,177	2.5 mL	420 - 610	www.hach.com
Genesys 20, Thermo Scientific[1]	1,862	1 mL	325 - 1100	www.thermo.com
590b Densimeter, ARS[2]	1,895	200 µL	ND[3]	www.arssales.com
BioPhotometer, Eppendorf[1]	4,585	50 µL	230 - 650	www.eppendorf.com
GeneQuant, BioChrom[1]	5,071	7 µL	190 - 900	www.gelifesciences.com
Epoch, BioTek[1]	8,950	2 µL	200 - 999	www.biotek.com
NanoDrop, Thermo Scientific[1]	8,950	0.5 µL	190 - 840	www.nanodrop.com

[1] Price from Fisher Scientific as October 2009 (www.fishersci.com)
[2] Price from animal reproduction systems
[3] ND: not described

The use of flow cytometry to measure sperm concentration has yielded variable results (Lu et al. 2007, Anzar et al. 2009) and depends on how the counts are performed (i.e., typically in relation to a known concentration of a fluorescent bead internal standard), the concentration range tested, and whether the emphasis is on precision or accuracy (Haugen 2007). Future research needs to be conducted to compare newer designs of flow cytometers (such as the Accuri C6®, Ann Arbor, MI) which measure the actual volume that is pulled from the sample and can directly estimate cell counts without the need of fluorescent bead standards. Another new flow cytometry device (S-FCM, Kobe, Japan) was developed to measure sperm concentration. This device has been reported to be suitable for measurement of human sperm concentration (Tsuji et al. 2002). A recent publication demonstrated that computer-aided fluorescent microscopy (NucleoCounter SP-100, Denmark) could be used to measure sperm concentration in brook trout (*Salvelinus fontinalis*) (Nynca and Ciereszko 2009). Coulter counters have long been accepted as a reliable technique for particle sizing and counting (Brillard and McDaniel 1985) and are available in some fish hatcheries for ploidy determination (Wattendorf 1986), but are expensive for sperm counting only. Other techniques such as cell-UV chambers, and packed cell volumes (e.g., VoluPAC, Sartorius, Germany) need to be evaluated for aquatic species. Potentially, a general standard curve for fishes could be incorporated into analysis-specific spectrophotometers (e.g., 590B Densimeter, Animal Reproduction System, Chino, CA). Such densimeters are designed to measure sperm concentrations of stallions, bulls, boars, canines, roosters, and turkeys.

Conclusions

We conclude that estimation of sperm concentration is essential for many studies in aquatic species and that reports using sperm estimations from spectrophotometric determination of turbidity should include at a minimum: the spectrophotometer model and type, cuvette description, wavelengths used, absolute determination method (e.g., hemacytometer) used, range of sperm concentrations tested, absorbance range tested, the standard curve, linear regression equation, and the coefficient of determination. In this study, a single general (instrument-specific) standard curve generated at wavelengths of 450 to 600 nm within the range of 1×10^6 to 6×10^{10} cells/mL was developed for determining the concentration of sperm from seven different fish species where sperm was collected by stripping. With further study or procedural modifications this could apply to fishes for which the testes are crushed. This would have broad applicability in reproductive studies and is essential for standardization of cryopreservation.

The importance of sperm concentration determination in livestock has been recognized since 1939 (Comstock and Green 1939). Sperm concentration is vital in artificial insemination because the number of sperm determines how many females can be inseminated (Foote 1972). The livestock industry sells packaged semen for artificial insemination of livestock in "frozen doses" that supply a specific number of sperm. This requires a high level of strict quality control and standardization for all of the parameters involved in the dose, including sperm concentration. Standardization and reproducibility are key factors for the success of this industry for livestock (Thibier and Wagner 2002).

Sperm cryopreservation is a proven technique for developing, maintaining, and distributing genetic improvement in livestock, and provides great unexploited potential for fish breeding. In addition, the availability of frozen sperm allows the creation of genetic resource repositories and conservation programs to increase the potential breeding population size to ensure that proper genetic combinations are produced in breeding of endangered species (Tiersch 2008). The future development and utility of technologies such as cryopreservation will rely on standardization and control of major variables such as sperm concentration. The results presented in this chapter call attention to the need for standardization and suggest that variation in sperm concentration results can be influenced more by the instrument used than the species studied.

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Appendix C
Original Data in Research Chapters

Chapter 3

Table C-1. The biological and sperm characteristics of males channel catfish (*Ictalurus punctatus*) used in this project.

Male	Weight (kg)	Length (cm)	Testis weigth (gr)	Initial concentration (sperm/ml)	Sperm Motility (%)
Males used for acute toxicity					
CCFH08M39	1.79	60	7.11	1.14E+09	60
CCFH08M40	2.11	60	6.6	1.03E+09	50
CCFH08M41	1.59	57	9.53	7.45E+08	60
CCFH08M43	1.87	56	5.27	8.80E+08	50
Males used for vitrification studies					
CCFH08M27	2.03	58.0	11.11	5.00E+08	40
CCFH08M31	1.43	54.0	6.38	5.00E+08	40
CCFH08M34	1.76	56.0	10.64	7.35E+08	35
CCFH08M37	1.52	53.0	10.15	6.35E+08	30
BCFH09M26	3.73	69.9	14.04	2.15E+09	60
BCFH09M28	6.99	86.4	8.2	2.42E+09	55
BCFH09M30	3.84	71.1	13.06	1.15E+09	65
BCFH09M36	3.64	64.8	9.13	9.94E+08	75
BCFH09M37	3.45	69.9	6.97	9.96E+08	80
BCFH09M38	3.49	72.4	9.9	1.27E+09	90
CCFH10M04	2.6	66.0	9.4	1.00E+09	80
CCFH10M05	2.2	61.0	11.5	2.02E+09	70
CCFH10M06	2.04	58.4	10	2.85E+09	75
BCFH10M38	7.8	91.4	9.44	2.50E+09	60
BCFH10M39	6.22	82.6	9.92	2.00E+09	50
BCFH10M40	10.45	100.3	12.31	3.30E+09	50

Table C-2. Acute toxicity of channel catfish

			Motlity (%)							
			Time (min)							
Replicate	Cryoprotectant	Concentration (%)	< 1	5	10	15	20	25	30	60
1	Methyl glycol	5	60	60	65	55	50	50	55	45
1	Methyl glycol	10	50	60	60	60	50	35	40	60
1	Methyl glycol	20	40	10	10	20	30	20	5	3
1	Methyl glycol	30	35	0	0	0	0	0	0	0
1	Methanol	5	60	65	55	60	60	60	50	50
1	Methanol	10	60	55	50	55	55	45	50	45
1	Methanol	20	45	35	45	25	30	35	20	15
1	Methanol	30	2	0	0	0	0	0	0	0
1	DMSO	5	10	15	15	30	40	60	60	45
1	DMSO	10	5	5	25	5	5	5	5	5
1	DMSO	20	0	0	0	0	0	0	0	0
1	DMSO	30	0	0	0	0	0	0	0	0
1	Propanediol	5	40	40	35	20	50	30	60	30
1	Propanediol	10	15	3	10	3	3	5	3	0
1	Propanediol	20	0	0	0	0	0	0	0	0
1	Propanediol	30	0	0	0	0	0	0	0	0
1	DMA	5	60	50	50	50	20	50	50	35
1	DMA	10	65	70	45	10	5	7	10	3
1	DMA	20	50	0	0	0	0	0	0	0
1	DMA	30	0	0	0	0	0	0	0	0
1	Control	—	65	70	70	70	65	60	55	55
2	Methyl glycol	5	50	40	50	45	60	45	35	40
2	Methyl glycol	10	55	40	45	50	50	55	50	40
2	Methyl glycol	20	45	35	15	5	30	10	10	3
2	Methyl glycol	30	10	0	0	0	0	0	0	0
2	Methanol	5	60	40	50	60	55	45	50	50
2	Methanol	10	50	40	50	35	50	40	50	50
2	Methanol	20	40	35	25	25	15	10	10	5
2	Methanol	30	3	0	0	0	0	0	0	0
2	DMSO	5	50	50	55	55	35	45	45	50
2	DMSO	10	45	40	45	45	45	40	50	25
2	DMSO	20	40	25	20	10	15	10	5	3

			Motlity (%)							
			Time (min)							
Replicate	Cryoprotectant	Concentration (%)	< 1	5	10	15	20	25	30	60
2	DMSO	30	3	0	0	0	0	0	0	0
2	Propanediol	5	10	30	30	40	20	35	20	40
2	Propanediol	10	5	5	5	5	5	5	5	5
2	Propanediol	20	0	0	0	0	0	0	0	0
2	Propanediol	30	3	0	0	0	0	0	0	0
2	DMA	5	50	50	40	35	35	35	35	45
2	DMA	10	50	30	15	5	30	10	5	0
2	DMA	20	45	5	5	0	0	0	0	0
2	DMA	30	0	0	0	0	0	0	0	0
2	Control	—	50	60	60	60	45	60	50	50
3	Methyl glycol	5	50	60	45	60	30	55	35	30
3	Methyl glycol	10	20	55	50	45	30	40	45	30
3	Methyl glycol	20	35	20	10	25	10	10	5	5
3	Methyl glycol	30	10	0	0	0	0	0	0	0
3	Methanol	5	35	50	45	55	35	40	45	60
3	Methanol	10	40	70	60	50	40	40	40	50
3	Methanol	20	30	30	30	15	5	15	5	0
3	Methanol	30	5	0	0	0	0	0	0	0
3	DMSO	5	5	25	40	25	40	50	40	30
3	DMSO	10	0	0	0	0	0	0	5	3
3	DMSO	20	0	0	0	0	0	0	0	0
3	DMSO	30	0	0	0	0	0	0	0	0
3	Propanediol	5	10	30	35	50	40	30	40	20
3	Propanediol	10	5	5	5	5	5	5	5	5
3	Propanediol	20	0	0	0	0	0	0	0	0
3	Propanediol	30	0	0	0	0	0	0	0	0
3	DMA	5	50	60	60	45	50	35	35	35
3	DMA	10	40	50	40	50	35	35	25	10
3	DMA	20	35	10	0	0	0	0	0	0
3	DMA	30	0	0	0	0	0	0	0	0
3	Control	—	60	65	65	60	70	60	70	60

Table C-3. The biological and sperm characteristics of males from green swordtail (*Xiphophorus hellerii*) used in this study.

Male	Weight (g)	Length (cm)	Testis weigth (mg)	Initial concentration (sperm/ml)	Sperm Motility (%)	Pool
Males used for acute toxicity						
GRSL08M01	0.977	3.8	9.4	1.60E+08	70	1
GRSL08M02	1	4	5.5	1.90E+08	75	1
GRSL08M04	0.99	3.9	3.2	2.30E+08	80	1
GRSL08M05	1.52	4.3	9.2	2.40E+08	80	1
GRSL08M06	1.07	3.8	5.9	2.15E+08	90	1
GRSL08M07	1.2	3.8	6.3	2.00E+08	60	1
GRSL08M08	1.3	3.8	11.2	1.55E+08	60	1
GRSL08M09	1.3	4.1	9.5	2.55E+08	70	1
GRSL08M10	0.95	3.7	3	2.65E+08	50	2
GRSL08M11	1.02	3.8	3.9	1.65E+08	50	2
GRSL08M14	1.81	4	7	2.80E+08	90	2
GRSL08M15	1.5	4.5	4.8	2.90E+08	65	2
GRSL08M16	1.9	4.8	7.4	5.30E+08	90	2
GRSL08M18	0.89	3.6	1.1	2.85E+08	80	2
GRSL08M19	0.87	3.5	6.5	1.10E+08	50	2
GRSL08M34	1.63	4.3	7.1	3.45E+08	70	3
GRSL08M37	1.8	4.2	16.7	1.95E+08	60	3
GRSL08M38	1.99	4.5	21.7	2.65E+08	60	3
GRSL08M47	0.64	3.2	7.4	1.30E+08	50	4
GRSL08M48	1.46	3.9	11.2	1.70E+08	50	4
GRSL08M49	1.73	4.1	9.9	1.70E+08	50	4
GRSL08M50	2.1	4.6	13.9	2.00E+08	50	4
GRSL08M51	2.09	4.8	14.4	3.90E+08	50	5
GRSL08M52	1.2	3.9	10.3	3.10E+08	70	5
GRSL08M53	1.2	4.1	9.4	2.70E+08	50	5
GRSL08M55	1.64	4.6	18.3	2.45E+08	50	5
GRSL08M60	1.54	4.2	9.7	1.90E+08	60	6
GRSL08M61	1.29	3.9	17.5	1.48E+08	50	6
GRSL08M62	1.06	3.8	10.5	2.38E+08	65	6
GRSL08M63	1.32	4.1	17.6	1.75E+08	55	6
GRSL08M64	0.98	3.5	10.1	2.90E+08	45	7
GRSL08M65	1.58	4.2	13.7	4.13E+08	50	7
GRSL08M67	1.51	4.2	16	1.93E+08	50	7
GRSL08M69	1.31	4.1	7.6	2.25E+08	50	8
GRSL08M70	1.12	3.6	9.6	2.15E+08	50	8
GRSL08M72	0.9	3.5	11.9	2.35E+08	65	8

Male	Weight (g)	Length (cm)	Testis weigth (mg)	Initial concentration (sperm/ml)	Sperm Motility (%)	Pool
GRSL08M75	1.87	4.3	14.4	1.95E+08	55	8
GRSL08M76	3.54	5.5	19.8	2.10E+08	50	9
GRSL08M79	1.47	4.2	12.1	2.43E+08	50	9
GRSL08M80	1.27	4	9.3	3.83E+08	70	9
GRSL08M81	1.96	4.5	18.2	2.90E+08	55	10
GRSL08M82	2.9	5	50.5	2.55E+08	55	10
GRSL08M84	2.7	4.7	14.4	1.93E+08	50	11
GRSL08M85	1.87	4.5	19.2	2.03E+08	60	11
GRSL08M86	0.95	3.7	6.2	1.23E+08	60	11
GRSL08M87	0.81	3.3	6.2	1.73E+08	60	11
GRSL08M88	1.47	4	21.3	2.53E+08	50	12
GRSL08M89	1.47	3.9	13.5	2.00E+08	50	12
GRSL08M92	1.44	4.1	16.2	2.23E+08	50	12
GRSL08M93	1.95	4.5	33.9	2.10E+08	50	13
GRSL08M96	1.43	4	15.9	1.33E+08	50	14
GRSL08M97	1.68	4.3	27.6	1.23E+08	50	14
GRSL08M99	2.44	4.9	34.7	2.18E+08	50	15
GRSL08M101	1.31	3.8	16.6	1.53E+08	55	16
GRSL08M102	1.6	4.3	23.8	1.28E+08	50	16
GRSL08M103	3.04	5.3	42.6	1.55E+08	50	17
GRSL08M104	1.66	4.3	31.5	2.23E+08	50	18
GRSL08M105	3.08	5	56.5	1.83E+08	50	18
GRSL09M02	1.08	5	10	2.40E+07	50	19
GRSL09M03	3.41	7.4	31	1.15E+09	70	19
GRSL09M05	1.59	5.9	10.8	1.79E+09	70	20
GRSL09M07	3.61	7.6	45	2.85E+08	70	21
Males used for vitrification/thawing studies						
GRSL08M123	2.18	4.7	27.1	5.85E+08	70	
GRSL08M124	2.62	5	34.7	9.45E+08	80	
GRSL09M09	1.43	5.5	25	1.35E+08	50	
GRSL09M10	0.852	5	7	8.50E+07	50	
GRSL09M11	1.21	5.5	10	2.05E+08	70	
Males used for vitrification/insemination studies						
GRSL08M135	1.24	4.1	10.1	5.90E+08	60	
GRSL08M139	0.48	3.2	9.1	6.60E+08	60	
GRSL08M141	0.83	3.5	11.3	7.50E+08	50	
GRSL08M145	0.54	3	10.5	1.72E+09	60	
GRSL08M146	0.52	3.2	13.4	1.49E+09	70	
GRSL08M147	0.5	3	6.8	1.32E+09	80	
GRSL08M148	0.84	3.5	10	9.35E+08	80	
GRSL08M149	0.49	2.9	12	1.15E+09	90	

Male	Weight (g)	Length (cm)	Testis weigth (mg)	Initial concentration (sperm/ml)	Sperm Motility (%)
Males used for vitrification/insemination studies					
GRSL08M150	0.74	3.4	8.7	8.10E+08	90
GRSL08M151	0.49	3.0	4.7	1.43E+09	70
GRSL09M25	0.706	4.5	11	3.67E+08	65
GRSL09M26	0.633	4.3	6	6.98E+08	75
GRSL09M27	0.508	4.2	5	3.02E+08	60
GRSL09M28	0.698	4.4	2.4	8.88E+08	80
GRSL09M30	0.607	4.5	15	7.86E+08	85
GRSL09M31	0.816	4.9	7	2.66E+08	50
GRSL09M34	0.548	4.2	12	4.72E+08	85
GRSL09M35	0.293	3.6	5	5.55E+08	80
GRSL09M36	0.658	4.6	6	1.75E+08	70
GRSL09M38	0.483	4.2	7	3.44E+08	70
GRSL09M39	0.573	4.3	5	4.69E+08	75
GRSL09M40	0.398	3.8	3	7.53E+08	80
GRSL09M41	0.435	3.9	9	7.81E+08	80
GRSL09M42	0.505	4.1	8	5.05E+08	60
GRSL09M43	0.566	4.4	9	5.15E+08	70
GRSL09M44	0.509	4.4	6	1.05E+08	70
GRSL09M46	0.783	4.6	10.1	4.06E+08	70
GRSL09M47	0.856	5	18.3	1.83E+109	60
GRSL09M48	0.95	5.2	9.2	1.36E+09	80
GRSL09M49	0.707	4.8	15.8	1.70E+09	70
GRSL09M50	0.364	4.1	3.4	9.60E+109	60
Males used for flow cytometry analysis					
GRSL10M02	1.606	4.2	29.4	1.42E+08	50
GRSL10M03	1.723	4.5	20.4	2.31E+08	40
GRSL10M04	1.31	4	13.3	2.15E+08	50

Table C-4. Acute toxicity of green swordtail. Cryoprotectants used were 2-methyl-2,4-pentanediol (MPD), 1-methoxy-2-propanol (MP), methyl glycol (MG), polyethylene glycol (PEG MW 200), ethylene glycol (EG), 2,3-butanediol (BD), glycerol (Gly), 1,2-propanediol (PrOH), dimethyl sulfoxide (DMSO), VitriFreeze™ Medium 1 and 2, V_{EG}, VM3, X-1000™, and Z-1000™ .

				Motlity (%)							
				Time (min)							
Pool	Cryoprotectant	Concentration (%)	Temp.	< 1	5	10	15	20	25	30	60
1	MPD	5	cold	30	25	15	7	10	3	3	1
1	MPD	10	cold	50	25	15	7	3	2	0	1
1	MPD	15	cold	10	10	25	1	0	0	0	0
1	MPD	20	cold	20	5	0	0	0	0	0	0
1	MPD	25	cold	0	0	0	0	0	0	0	0
1	MPD	30	cold	0	0	0	0	0	0	0	0
1	MP	5	cold	30	55	45	35	45	5	5	20
1	MP	10	cold	50	40	25	15	40	35	20	20
1	MP	15	cold	45	10	5	3	5	0	0	0
1	MP	20	cold	45	10	0	0	0	0	0	0
1	MP	25	cold	0	0	0	0	0	0	0	0
1	MP	30	cold	0	0	0	0	0	0	0	0
1	MG	5	cold	50	50	45	35	7	5	18	10
1	MG	10	cold	55	45	40	50	8	20	20	40
1	MG	15	cold	30	45	35	10	3	15	10	5
1	MG	20	cold	25	50	50	7	5	5	0	0
1	MG	25	cold	5	5	10	0	0	0	0	0
1	MG	30	cold	30	0	0	0	0	0	0	0
1	PEG	5	cold	30	10	10	20	5	15	15	25
1	PEG	10	cold	1	30	40	5	20	5	5	5
1	PEG	15	cold	25	0	0	0	0	0	0	0
1	PEG	20	cold	0	0	0	0	0	0	0	0
1	PEG	25	cold	0	0	0	0	0	0	0	0
1	PEG	30	cold	0	0	0	0	0	0	0	0
1	EG	5	cold	50	40	15	40	30	10	8	30
1	EG	10	cold	40	30	30	10	20	50	10	15
1	EG	15	cold	40	40	30	10	10	10	5	25
1	EG	20	cold	60	40	10	20	40	10	45	40
1	EG	25	cold	40	30	30	10	20	50	10	15
1	EG	30	cold	25	10	35	5	5	10	50	5

				Motlity (%)							
				Time (min)							
Pool	Cryoprotectant	Concentration (%)	Temp.	< 1	5	10	15	20	25	30	60
1	BD	5	cold	10	60	20	40	35	15	10	15
1	BD	10	cold	60	25	15	35	5	5	5	5
1	BD	15	cold	10	40	25	50	50	25	5	0
1	BD	20	cold	25	50	10	20	50	20	5	0
1	BD	25	cold	5	0	0	0	0	0	0	0
1	BD	30	cold	0	0	0	0	0	0	0	0
1	Gly	5	cold	10	40	35	35	25	20	50	30
1	Gly	10	cold	10	50	35	35	10	20	40	10
1	Gly	15	cold	40	50	35	45	50	10	35	10
1	Gly	20	cold	45	10	40	50	10	30	40	5
1	Gly	25	cold	10	5	20	5	35	55	15	20
1	Gly	30	cold	5	5	7	50	20	30	10	10
1	PrOH	5	cold	40	25	50	10	45	35	40	25
1	PrOH	10	cold	50	15	15	10	10	40	30	20
1	PrOH	15	cold	5	35	5	40	60	10	10	10
1	PrOH	20	cold	5	5	40	5	15	5	5	5
1	PrOH	25	cold	15	30	10	5	5	0	0	0
1	PrOH	30	cold	5	2	5	5	0	0	0	0
1	DMSO	5	cold	45	20	15	10	15	10	15	10
1	DMSO	10	cold	20	20	40	10	30	40	10	45
1	DMSO	15	cold	10	10	25	45	5	15	10	5
1	DMSO	20	cold	20	10	30	10	5	5	0	0
1	DMSO	25	cold	5	20	5	5	0	0	0	0
1	DMSO	30	cold	0	0	0	0	0	0	0	0
1	Control	—	cold	30	60	60	70	45	65	50	40
1	Control	—	cold	70	70	70	50	50	50	50	45
1	Control	—	cold	60	60	50	55	20	45	35	60
7	MPD	5	cold	30	30	20	5	10	15	40	3
7	MPD	10	cold	0	0	0	0	0	0	0	0
7	MPD	15	cold	0	0	0	0	0	0	0	0
7	MPD	20	cold	5	0	0	0	0	0	0	0
7	MPD	25	cold	0	0	0	0	0	0	0	0
7	MPD	30	cold	0	0	0	0	0	0	0	0

				Motlity (%)							
				Time (min)							
Pool	Cryoprotectant	Concentration (%)	Temp.	< 1	5	10	15	20	25	30	60
7	MP	5	cold	15	5	10	5	15	20	5	20
7	MP	10	cold	10	25	35	25	10	10	0	0
7	MP	15	cold	15	0	0	0	0	0	0	0
7	MP	20	cold	10	0	0	0	0	0	0	0
7	MP	25	cold	0	0	0	0	0	0	0	0
7	MP	30	cold	20	0	0	0	0	0	0	0
7	MG	5	cold	10	5	30	10	10	15	10	15
7	MG	10	cold	25	7	5	25	35	5	10	5
7	MG	15	cold	10	30	10	20	10	15	35	0
7	MG	20	cold	0	5	0	0	0	0	0	0
7	MG	25	cold	20	0	0	0	0	0	0	0
7	MG	30	cold	0	0	0	0	0	0	0	0
7	PEG	5	cold	20	15	5	45	40	35	35	45
7	PEG	10	cold	0	40	5	3	5	3	0	10
7	PEG	15	cold	10	0	0	0	0	0	0	0
7	PEG	20	cold	20	0	0	0	0	0	0	0
7	PEG	25	cold	0	0	0	0	0	0	0	0
7	PEG	30	cold	0	0	0	0	0	0	0	0
7	Control	—	cold	50	50	50	50	50	45	45	45
7	Control	—	cold	40	35	45	45	50	50	55	50
7	Control	—	cold	40	50	45	40	45	45	45	40
11	EG	5	cold	60	20	40	50	45	60	40	50
11	EG	10	cold	30	60	60	50	55	45	40	50
11	EG	15	cold	40	50	50	45	40	50	45	55
11	EG	20	cold	40	40	45	20	50	40	35	50
11	EG	25	cold	15	35	10	50	50	55	40	60
11	EG	30	cold	0	0	20	20	10	15	7	40
11	BD	5	cold	50	20	50	20	50	30	25	15
11	BD	10	cold	45	45	40	10	10	10	45	10
11	BD	15	cold	50	50	40	10	7	15	60	0
11	BD	20	cold	0	2	40	5	5	5	3	0
11	BD	25	cold	0	0	0	0	0	0	0	0
11	BD	30	cold	0	0	0	0	0	0	0	0

				Motlity (%)							
				Time (min)							
Pool	Cryoprotectant	Concentration (%)	Temp.	< 1	5	10	15	20	25	30	60
11	Gly	5	cold	45	55	40	40	35	50	50	40
11	Gly	10	cold	45	45	60	10	45	45	50	15
11	Gly	15	cold	40	50	50	60	15	55	35	50
11	Gly	20	cold	20	35	10	55	40	40	10	45
11	Gly	25	cold	20	40	20	55	40	5	60	45
11	Gly	30	cold	50	40	30	45	55	10	30	35
11	PrOH	5	cold	50	50	40	35	50	15	45	10
11	PrOH	10	cold	45	45	40	55	5	50	20	45
11	PrOH	15	cold	35	40	20	35	50	50	35	55
11	PrOH	20	cold	25	15	30	20	25	50	35	20
11	PrOH	25	cold	35	50	50	30	25	20	40	5
11	PrOH	30	cold	10	0	3	3	5	15	0	0
11	DMSO	5	cold	35	60	25	20	40	55	50	20
11	DMSO	10	cold	40	45	45	25	40	55	45	40
11	DMSO	15	cold	45	50	50	30	50	35	10	40
11	DMSO	20	cold	30	45	15	15	30	50	10	10
11	DMSO	25	cold	30	20	30	20	40	10	10	0
11	DMSO	30	cold	5	3	3	3	0	0	0	0
11	Control	—	cold	60	60	60	60	45	60	50	60
11	Control	—	cold	65	55	55	55	60	60	55	60
8	MPD	5	cold	5	10	5	50	20	15	5	45
8	MPD	10	cold	25	20	10	30	5	5	0	0
8	MPD	15	cold	35	0	0	0	0	0	0	0
8	MPD	20	cold	5	0	0	0	0	0	0	0
8	MPD	25	cold	25	35	0	0	0	0	0	0
8	MPD	30	cold	35	0	0	0	0	0	0	0
8	MP	5	cold	10	0	5	10	10	20	10	10
8	MP	10	cold	25	20	10	30	5	5	0	0
8	MP	15	cold	20	20	15	10	10	5	30	0
8	MP	20	cold	15	5	5	0	0	0	0	0
8	MP	25	cold	10	0	0	0	0	0	0	0
8	MP	30	cold	0	0	0	0	0	0	0	0
8	MG	5	cold	25	50	35	10	40	30	10	10

				Motlity (%)							
				Time (min)							
Pool	Cryoprotectant	Concentration (%)	Temp.	< 1	5	10	15	20	25	30	60
8	MG	10	cold	20	10	5	35	5	5	10	10
8	MG	15	cold	5	35	20	10	50	35	40	0
8	MG	20	cold	40	5	15	15	10	5	0	0
8	MG	25	cold	20	10	0	0	0	0	0	0
8	MG	30	cold	0	0	0	0	0	0	0	0
8	PEG	5	cold	50	40	30	50	45	10	10	10
8	PEG	10	cold	50	20	40	45	20	30	40	30
8	PEG	15	cold	0	3	0	0	0	0	0	0
8	PEG	20	cold	10	20	0	0	0	0	0	0
8	PEG	25	cold	10	3	0	0	0	0	0	0
8	PEG	30	cold	0	0	0	0	0	0	0	0
8	Control	—	cold	50	55	65	50	50	50	55	45
8	Control	—	cold	45	50	45	55	55	25	50	50
12	EG	5	cold	50	45	40	45	20	40	50	40
12	EG	10	cold	45	50	40	45	45	40	40	40
12	EG	15	cold	50	35	45	35	30	40	35	40
12	EG	20	cold	40	40	30	45	10	40	35	45
12	EG	25	cold	20	45	20	40	40	15	5	5
12	EG	30	cold	5	3	0	20	40	40	30	20
12	BD	5	cold	25	45	55	15	15	40	40	40
12	BD	10	cold	40	5	25	5	5	5	5	3
12	BD	15	cold	45	35	5	40	40	40	15	0
12	BD	20	cold	3	15	10	10	5	5	0	0
12	BD	25	cold	0	0	0	0	0	0	0	0
12	BD	30	cold	0	0	0	0	0	0	0	0
12	Gly	5	cold	25	50	20	45	50	50	45	40
12	Gly	10	cold	55	40	45	30	35	45	25	35
12	Gly	15	cold	10	40	35	10	15	50	40	5
12	Gly	20	cold	45	10	25	60	45	35	45	40
12	Gly	25	cold	50	30	10	20	5	5	10	45
12	Gly	30	cold	0	5	35	45	50	5	10	10
12	PrOH	5	cold	45	35	40	40	10	10	20	50
12	PrOH	10	cold	35	40	15	35	15	35	40	30

				Motlity (%)							
				Time (min)							
Pool	Cryoprotectant	Concentration (%)	Temp.	< 1	5	10	15	20	25	30	60
12	PrOH	15	cold	10	40	20	10	10	10	30	30
12	PrOH	20	cold	15	25	10	5	20	5	45	40
12	PrOH	25	cold	30	15	30	40	35	15	10	3
12	PrOH	30	cold	25	30	10	5	0	0	0	0
12	DMSO	5	cold	20	40	5	40	45	35	20	20
12	DMSO	10	cold	40	10	5	40	30	10	15	15
12	DMSO	15	cold	10	35	10	7	15	5	35	3
12	DMSO	20	cold	10	35	10	15	15	0	10	3
12	DMSO	25	cold	10	5	5	3	5	3	0	0
12	DMSO	30	cold	5	0	3	0	0	0	0	0
12	Control	—	cold	50	55	50	50	45	55	55	55
12	Control	—	cold	60	60	55	65	60	45	50	50
2	MPD	5	room	10	10	10	50	15	3	10	0
2	MPD	10	room	50	5	5	0	0	0	0	0
2	MPD	15	room	15	0	0	0	0	0	0	0
2	MPD	20	room	0	0	0	0	0	0	0	0
2	MPD	25	room	0	0	0	0	0	0	0	0
2	MPD	30	room	40	0	0	0	0	0	0	0
2	MP	5	room	60	65	65	60	10	50	40	5
2	MP	10	room	35	55	40	35	30	35	25	0
2	MP	15	room	40	35	0	0	0	0	0	0
2	MP	20	room	0	0	0	0	0	0	0	0
2	MP	25	room	10	0	0	0	0	0	0	0
2	MP	30	room	0	0	0	0	0	0	0	0
2	MG	5	room	60	40	20	40	45	60	50	70
2	MG	10	room	35	35	30	50	40	40	20	40
2	MG	15	room	50	50	10	50	50	5	0	0
2	MG	20	room	60	10	0	0	0	0	0	0
2	MG	25	room	20	0	0	0	0	0	0	0
2	MG	30	room	50	0	0	0	0	0	0	0
2	PEG	5	room	60	50	40	50	40	50	45	60
2	PEG	10	room	50	50	50	5	5	35	40	45
2	PEG	15	room	5	0	5	0	10	0	0	0

				Motlity (%)							
				Time (min)							
Pool	Cryoprotectant	Concentration (%)	Temp.	< 1	5	10	15	20	25	30	60
2	PEG	20	room	45	5	0	0	0	0	0	0
2	PEG	25	room	40	0	0	0	0	0	0	0
2	PEG	30	room	45	0	0	0	0	0	0	0
2	Control	—	room	60	50	15	45	60	30	40	55
2	Control	—	room	70	50	60	60	60	40	45	50
4	EG	5	room	55	60	65	40	45	60	65	65
4	EG	10	room	60	70	70	70	55	80	55	75
4	EG	15	room	65	85	75	80	80	80	80	90
4	EG	20	room	60	75	75	70	80	65	60	60
4	EG	25	room	75	85	50	65	60	0	65	3
4	EG	30	room	55	70	30	50	50	50	30	25
4	BD	5	room	80	55	55	60	50	50	20	3
4	BD	10	room	55	40	40	20	10	10	20	0
4	BD	15	room	50	50	5	5	0	0	0	0
4	BD	20	room	20	0	0	0	0	0	0	0
4	BD	25	room	0	0	0	0	0	0	0	0
4	BD	30	room	0	0	0	0	0	0	0	0
4	Gly	5	room	60	65	60	65	55	50	70	70
4	Gly	10	room	60	60	65	60	75	70	75	85
4	Gly	15	room	45	85	65	70	75	85	75	85
4	Gly	20	room	55	80	80	75	80	65	70	75
4	Gly	25	room	60	60	80	80	80	80	40	40
4	Gly	30	room	15	80	50	60	45	75	50	20
4	PrOH	5	room	55	65	65	70	60	65	55	80
4	PrOH	10	room	50	70	65	60	55	60	60	40
4	PrOH	15	room	60	55	45	55	60	65	25	50
4	PrOH	20	room	50	20	70	70	60	40	25	10
4	PrOH	25	room	35	35	0	0	0	0	0	0
4	PrOH	30	room	65	0	0	0	0	0	0	0
4	DMSO	5	room	45	65	50	60	55	70	60	75
4	DMSO	10	room	80	65	75	75	70	60	70	80
4	DMSO	15	room	30	30	50	60	60	60	55	50
4	DMSO	20	room	50	65	60	45	5	40	70	3

				Motlity (%)							
				Time (min)							
Pool	Cryoprotectant	Concentration (%)	Temp.	< 1	5	10	15	20	25	30	60
4	DMSO	25	room	10	10	0	0	0	0	0	0
4	DMSO	30	room	50	5	0	0	0	0	0	0
4	Control	—	room	50	20	25	50	50	45	50	50
4	Control	—	room	40	50	50	50	50	40	40	55
4	Control	—	room	40	50	50	50	50	40	40	55
5	MPD	5	room	5	5	5	5	0	0	0	0
5	MPD	10	room	5	0	0	0	0	0	0	0
5	MPD	15	room	10	0	0	0	0	0	0	0
5	MPD	20	room	20	0	0	0	0	0	0	0
5	MPD	25	room	10	0	0	0	0	0	0	0
5	MPD	30	room	0	0	0	0	0	0	0	0
5	MP	5	room	10	10	5	5	5	5	5	20
5	MP	10	room	20	5	5	0	0	0	0	0
5	MP	15	room	5	0	0	0	0	0	0	0
5	MP	20	room	15	0	0	0	0	0	0	0
5	MP	25	room	5	0	0	0	0	0	0	0
5	MP	30	room	10	0	0	0	0	0	0	0
5	MG	5	room	10	5	5	5	40	25	30	30
5	MG	10	room	10	10	5	5	3	3	5	5
5	MG	15	room	5	0	0	0	0	0	0	0
5	MG	20	room	10	0	0	0	0	0	0	0
5	MG	25	room	5	0	0	0	0	0	0	0
5	MG	30	room	0	0	0	0	0	0	0	0
5	PEG	5	room	5	40	5	5	10	30	30	30
5	PEG	10	room	20	5	5	30	5	15	5	5
5	PEG	15	room	5	20	5	0	5	3	0	0
5	PEG	20	room	0	0	0	0	0	0	0	0
5	PEG	25	room	0	0	0	0	0	0	0	0
5	PEG	30	room	0	0	0	0	0	0	0	0
5	Control	—	room	50	40	50	50	65	50	40	50
5	Control	—	room	60	45	50	45	45	40	40	50
9	EG	5	room	65	70	60	65	35	50	70	50
9	EG	10	room	55	45	25	55	20	5	10	5

				Motlity (%)							
				Time (min)							
Pool	Cryoprotectant	Concentration (%)	Temp.	< 1	5	10	15	20	25	30	60
9	EG	15	room	65	65	55	65	50	40	70	50
9	EG	20	room	55	65	20	25	55	60	40	65
9	EG	25	room	50	35	20	75	5	30	50	50
9	EG	30	room	50	35	40	50	40	55	25	0
9	BD	5	room	55	50	15	45	10	5	15	5
9	BD	10	room	20	50	25	15	5	25	0	0
9	BD	15	room	40	10	0	0	0	0	0	0
9	BD	20	room	0	0	0	0	0	0	0	0
9	BD	25	room	0	0	0	0	0	0	0	0
9	BD	30	room	0	0	0	0	0	0	0	0
9	Gly	5	room	50	40	65	60	45	60	60	60
9	Gly	10	room	50	55	55	65	55	40	60	55
9	Gly	15	room	55	80	60	40	40	70	65	65
9	Gly	20	room	15	65	60	70	40	55	55	60
9	Gly	25	room	35	70	65	60	55	60	70	25
9	Gly	30	room	45	50	35	5	35	35	45	5
9	PrOH	5	room	40	45	40	45	40	50	45	60
9	PrOH	10	room	60	65	50	50	20	45	40	40
9	PrOH	15	room	65	35	70	65	35	50	55	55
9	PrOH	20	room	25	25	35	20	60	35	3	0
9	PrOH	25	room	40	15	0	0	0	0	0	0
9	PrOH	30	room	0	0	0	0	0	0	0	0
9	DMSO	5	room	60	50	50	50	50	40	40	40
9	DMSO	10	room	60	50	50	40	15	35	35	40
9	DMSO	15	room	65	50	45	50	35	40	30	25
9	DMSO	20	room	60	60	50	40	50	25	40	5
9	DMSO	25	room	60	50	10	0	0	0	0	0
9	DMSO	30	room	0	10	0	0	0	0	0	0
9	Control	—	room	60	65	60	60	60	60	60	60
9	Control	—	room	70	50	50	60	60	60	60	65
6	MPD	5	room	10	10	10	50	15	3	10	0
6	MPD	10	room	50	5	5	0	0	0	0	0
6	MPD	15	room	15	0	0	0	0	0	0	0

				Motlity (%)							
				Time (min)							
Pool	Cryoprotectant	Concentration (%)	Temp.	< 1	5	10	15	20	25	30	60
6	MPD	20	room	0	0	0	0	0	0	0	0
6	MPD	25	room	0	0	0	0	0	0	0	0
6	MPD	30	room	40	0	0	0	0	0	0	0
6	MP	5	room	60	65	65	60	10	50	40	5
6	MP	10	room	35	55	40	35	30	35	25	0
6	MP	15	room	40	35	0	0	0	0	0	0
6	MP	20	room	0	0	0	0	0	0	0	0
6	MP	25	room	10	0	0	0	0	0	0	0
6	MP	30	room	0	0	0	0	0	0	0	0
6	MG	5	room	60	40	20	40	45	60	50	70
6	MG	10	room	35	35	30	50	40	40	20	40
6	MG	15	room	50	50	10	50	50	5	0	0
6	MG	20	room	60	10	0	0	0	0	0	0
6	MG	25	room	20	0	0	0	0	0	0	0
6	MG	30	room	50	0	0	0	0	0	0	0
6	PEG	5	room	60	50	40	50	40	50	45	60
6	PEG	10	room	50	50	50	5	5	35	40	45
6	PEG	15	room	5	0	5	0	10	0	0	0
6	PEG	20	room	45	5	0	0	0	0	0	0
6	PEG	25	room	40	0	0	0	0	0	0	0
6	PEG	30	room	45	0	0	0	0	0	0	0
6	Control	—	room	70	70	65	70	70	70	70	70
6	Control	—	room	70	75	70	60	65	75	65	65
10	EG	5	room	55	60	65	40	45	60	65	65
10	EG	10	room	60	70	70	70	55	80	55	75
10	EG	15	room	65	85	75	80	80	80	80	90
10	EG	20	room	60	75	75	70	80	65	60	60
10	EG	25	room	75	85	50	65	60	0	65	3
10	EG	30	room	55	70	30	50	50	50	30	25
10	BD	5	room	80	55	55	60	50	50	20	3
10	BD	10	room	55	40	40	20	10	10	20	0
10	BD	15	room	50	50	5	5	0	0	0	0
10	BD	20	room	20	0	0	0	0	0	0	0

				Motlity (%)							
				Time (min)							
Pool	Cryoprotectant	Concentration (%)	Temp.	< 1	5	10	15	20	25	30	60
10	BD	25	room	0	0	0	0	0	0	0	0
10	BD	30	room	0	0	0	0	0	0	0	0
10	Gly	5	room	60	65	60	65	55	50	70	70
10	Gly	10	room	60	60	65	60	75	70	75	85
10	Gly	15	room	45	85	65	70	75	85	75	85
10	Gly	20	room	55	80	80	75	80	65	70	75
10	Gly	25	room	60	60	80	80	80	80	40	40
10	Gly	30	room	15	80	50	60	45	75	50	20
10	PrOH	5	room	55	65	65	70	60	65	55	80
10	PrOH	10	room	50	70	65	60	55	60	60	40
10	PrOH	15	room	60	55	45	55	60	65	25	50
10	PrOH	20	room	50	20	70	70	60	40	25	10
10	PrOH	25	room	35	35	0	0	0	0	0	0
10	PrOH	30	room	65	0	0	0	0	0	0	0
10	DMSO	5	room	45	65	50	60	55	70	60	75
10	DMSO	10	room	80	65	75	75	70	60	70	80
10	DMSO	15	room	30	30	50	60	60	60	55	50
10	DMSO	20	room	50	65	60	45	5	40	70	3
10	DMSO	25	room	10	10	0	0	0	0	0	0
10	DMSO	30	room	50	5	0	0	0	0	0	0
10	Control	—	room	65	70	85	75	80	85	80	85
10	Control	—	room	85	80	85	80	85	80	80	85
10	Control	—	room	70	80	80	80	80	80	85	90
16	EG	30	room	50	40	30	5	5	5	0	0
16	EG	35	room	30	25	10	0	3	0	0	0
16	EG	40	room	30	15	3	0	0	0	0	0
16	Gly	30	room	40	30	10	5	15	7	10	0
16	Gly	35	room	40	10	3	5	0	3	3	0
16	Gly	40	room	10	5	15	5	5	5	3	0
16	PrOH	30	cold	55	55	25	10	5	5	0	0
16	PrOH	35	cold	20	15	3	0	0	0	0	0
16	PrOH	40	cold	0	0	0	0	0	0	0	0
16	Control	—	room	60	50	60	60	60	60	65	60

				Motlity (%)							
				Time (min)							
Pool	Cryoprotectant	Concentration (%)	Temp.	< 1	5	10	15	20	25	30	60
16	Control	—	room	70	60	65	65	65	65	60	65
17	EG	30	room	50	50	10	10	5	5	0	0
17	EG	35	room	50	40	5	0	0	0	0	0
17	EG	40	room	30	5	0	0	0	0	0	0
17	Gly	30	room	30	20	10	10	10	5	10	0
17	Gly	35	room	40	5	5	5	5	5	5	0
17	Gly	40	room	10	5	10	10	10	5	10	3
17	PrOH	30	cold	50	40	20	5	0	0	0	0
17	PrOH	35	cold	30	0	0	0	0	0	0	0
17	PrOH	40	cold	35	0	0	0	0	0	0	0
17	Control	—	room	50	50	60	50	50	45	50	40
17	Control	—	room	55	55	50	50	60	50	45	40
18	EG	30	room	50	35	35	5	5	0	0	0
18	EG	35	room	60	35	20	10	0	0	0	0
18	EG	40	room	15	5	0	0	0	0	0	0
18	Gly	30	room	50	40	5	15	30	10	30	0
18	Gly	35	room	10	40	30	5	0	5	5	0
18	Gly	40	room	10	5	20	20	0	5	15	0
18	PrOH	30	cold	40	55	40	40	5	0	0	0
18	PrOH	35	cold	5	0	0	0	0	0	0	0
18	PrOH	40	cold	0	0	0	0	0	0	0	0
18	Control	—	room	60	60	60	65	65	60	60	60
18	Control	—	room	60	60	50	65	60	60	65	60
13	VEG	10	room	60	30	30	5	5	5	5	0
13	VEG	20	room	15	0	0	0	0	0	0	0
13	VEG	30	room	70	0	0	0	0	0	0	0
13	VEG	40	room	0	0	0	0	0	0	0	0
13	VEG	50	room	0	0	0	0	0	0	0	0
13	X-1000	10	room	75	70	70	70	70	70	70	70
13	X-1000	20	room	60	70	75	75	70	60	70	70
13	X-1000	30	room	75	55	70	80	70	80	75	65
13	X-1000	40	room	20	60	40	25	30	10	7	20
13	X-1000	50	room	3	20	15	10	10	5	3	0

				Motlity (%)							
				Time (min)							
Pool	Cryoprotectant	Concentration (%)	Temp.	< 1	5	10	15	20	25	30	60
13	Z-1000	10	room	65	5	5	5	5	3	5	5
13	Z-1000	20	room	65	5	5	3	3	7	5	5
13	Z-1000	30	room	3	0	0	0	0	0	0	0
13	Z-1000	40	room	0	0	0	0	0	0	0	0
13	Z-1000	50	room	50	3	0	0	0	0	0	0
13	Vitrifreeze M1	10	room	80	60	75	60	55	70	70	85
13	Vitrifreeze M1	20	room	60	60	70	55	60	65	75	70
13	Vitrifreeze M1	30	room	50	70	65	75	70	70	80	75
13	Vitrifreeze M1	40	room	60	60	60	80	70	75	65	70
13	Vitrifreeze M1	50	room	55	60	70	70	60	60	70	50
13	Vitrifreeze M2	10	room	55	40	40	30	40	50	45	45
13	Vitrifreeze M2	20	room	50	50	35	35	30	25	20	20
13	Vitrifreeze M2	30	room	50	40	20	20	10	15	10	10
13	Vitrifreeze M2	40	room	60	40	10	5	5	5	5	0
13	Vitrifreeze M2	50	room	50	25	5	5	3	3	0	0
13	VM3	10	room	65	60	70	60	60	65	60	60
13	VM3	20	room	75	20	10	5	0	0	0	0
13	VM3	30	room	70	0	0	0	0	0	0	0
13	VM3	40	room	10	0	0	0	0	0	0	0
13	VM3	50	room	0	0	0	0	0	0	0	0
13	Control	—	room	80	50	75	70	75	75	70	75
13	Control	—	room	85	80	80	75	75	75	75	80
13	Control	—	room	80	80	70	80	80	80	80	85
14	VEG	10	room	50	55	20	25	20	25	15	20
14	VEG	20	room	40	5	0	0	0	0	0	0
14	VEG	30	room	10	0	0	0	0	0	0	0
14	VEG	40	room	0	0	0	0	0	0	0	0
14	VEG	50	room	0	0	0	0	0	0	0	0
14	X-1000	10	room	50	60	60	50	60	50	50	65
14	X-1000	20	room	50	40	30	25	25	55	35	60
14	X-1000	30	room	40	40	30	30	30	40	30	30
14	X-1000	40	room	10	25	15	15	10	3	5	0
14	X-1000	50	room	20	40	10	10	15	10	5	0

				Motlity (%)							
				Time (min)							
Pool	Cryoprotectant	Concentration (%)	Temp.	< 1	5	10	15	20	25	30	60
14	Z-1000	10	room	45	5	5	5	5	5	5	0
14	Z-1000	20	room	60	7	5	5	7	10	5	5
14	Z-1000	30	room	3	0	0	0	0	0	0	0
14	Z-1000	40	room	0	0	0	0	0	0	0	0
14	Z-1000	50	room	20	0	0	0	0	0	0	0
14	Vitrifreeze M1	10	room	50	55	50	50	50	35	50	60
14	Vitrifreeze M1	20	room	60	30	40	40	30	40	40	65
14	Vitrifreeze M1	30	room	50	55	50	45	45	55	50	60
14	Vitrifreeze M1	40	room	60	65	60	60	60	45	60	70
14	Vitrifreeze M1	50	room	30	45	40	30	45	45	45	55
14	Vitrifreeze M2	10	room	55	55	55	50	40	55	45	50
14	Vitrifreeze M2	20	room	55	55	45	25	20	20	20	20
14	Vitrifreeze M2	30	room	60	55	25	10	5	10	5	10
14	Vitrifreeze M2	40	room	60	50	40	50	55	45	40	5
14	Vitrifreeze M2	50	room	60	40	10	15	10	10	10	3
14	VM3	10	room	40	15	30	10	5	25	10	15
14	VM3	20	room	50	10	5	5	0	0	0	0
14	VM3	30	room	50	5	0	0	0	0	0	0
14	VM3	40	room	5	0	0	0	0	0	0	0
14	VM3	50	room	0	0	0	0	0	0	0	0
14	Control	—	room	65	65	60	60	55	60	65	50
14	Control	—	room	55	55	50	45	40	50	50	50
14	Control	—	room	70	65	65	70	65	65	65	50
15	VEG	10	room	55	20	40	25	5	15	15	15
15	VEG	20	room	50	5	0	0	0	0	0	0
15	VEG	30	room	0	5	0	0	0	0	0	0
15	VEG	40	room	3	0	0	0	0	0	0	0
15	VEG	50	room	0	0	0	0	0	0	0	0
15	X-1000	10	room	40	50	55	50	55	50	50	60
15	X-1000	20	room	60	55	55	45	50	45	50	50
15	X-1000	30	room	50	55	45	50	50	50	55	45
15	X-1000	40	room	55	10	5	5	3	3	3	5
15	X-1000	50	room	50	10	10	10	3	0	0	0

				Motlity (%)							
				Time (min)							
Pool	Cryoprotectant	Concentration (%)	Temp.	< 1	5	10	15	20	25	30	60
15	Z-1000	10	room	60	10	10	15	5	10	10	3
15	Z-1000	20	room	40	0	0	0	0	0	0	0
15	Z-1000	30	room	10	0	0	0	0	0	0	0
15	Z-1000	40	room	0	0	0	0	0	0	0	0
15	Z-1000	50	room	45	0	0	0	0	0	0	0
15	Vitrifreeze M1	10	room	60	55	45	45	40	50	45	40
15	Vitrifreeze M1	20	room	60	50	55	40	40	55	40	40
15	Vitrifreeze M1	30	room	60	55	30	45	55	50	50	50
15	Vitrifreeze M1	40	room	50	50	40	40	55	40	15	45
15	Vitrifreeze M1	50	room	50	55	50	50	50	50	55	40
15	Vitrifreeze M2	10	room	65	60	50	55	45	50	50	40
15	Vitrifreeze M2	20	room	55	35	20	50	40	35	15	25
15	Vitrifreeze M2	30	room	65	50	10	30	10	10	10	7
15	Vitrifreeze M2	40	room	40	10	0	3	0	3	3	0
15	Vitrifreeze M2	50	room	50	10	10	5	3	5	5	3
15	VM3	10	room	55	30	20	50	40	40	30	20
15	VM3	20	room	50	5	3	0	0	5	3	0
15	VM3	30	room	55	0	0	0	0	0	0	0
15	VM3	40	room	20	0	0	0	0	0	0	0
15	VM3	50	room	0	0	0	0	0	0	0	0
15	Control	—	room	60	60	60	40	55	50	55	40
15	Control	—	room	60	60	65	60	60	60	45	60
15	Control	—	room	60	50	60	55	55	65	55	55

Table C-5. Acute toxicity of green swordtail to combined cryoprotectants. Cryoprotectants used were: ethylene glycol (EG), glycerol (Gly), dimethyl sulfoxide (DMSO), 0.45 M trehalose dihydrate (Tre), 1,2-propanediol (PrOH), methanol (MeOH), methyl glycol (MG), 2,3-butanediol (BD), 1% X-1000™ (X), 1% Z-1000™ (Z), polyethylene glycol (PEG MW 200), and 15% acetamide (Ace).

		Motlity (%)		
		Time (min)		
Pool	Vitrification solution	< 1	5	10
19	20%EG+20%Gly	5	0	0
19	30%EG+10%DMSO+Tre	5	0	0
19	30%EG+10%PrOH	0	0	0
19	20%MeOH+20%MG	0	0	0
19	40%Gly+Tre	50	5	3
19	30%EG+10%BD	0	0	0
19	30%EG+10%Gly	20	10	0
19	40%Gly	20	20	20
19	30%EG+15%Gly	0	0	0
19	15%EG+10%Gly+15%DMSO+XZ	50	3	0
19	20%EG+20%Gly+Tre	0	1	3
19	30%EG+10%MeOH	0	0	0
19	20%DMSO+10%PrOH+6%PEG+Ace	0	0	0
19	40%EG+Tre	20	0	0
19	40%EG	20	0	0
19	control1	60	50	50
19	control 2	30	50	40
19	control 3	40	40	40
20	20%EG+20%Gly	10	3	0
20	30%EG+10%DMSO+Tre	50	0	0
20	30%EG+10%PrOH	5	0	0
20	20%MeOH+20%MG	20	0	35
20	40%Gly+Tre	20	20	5
20	30%EG+10%BD	5	0	0
20	30%EG+10%Gly	10	10	0
20	40%Gly	40	20	40
20	30%EG+15%Gly	10	0	0
20	15%EG+10%Gly+15%DMSO+XZ	40	40	3
20	20%EG+20%Gly+Tre	0	0	0
20	30%EG+10%MeOH	3	0	0
20	20%DMSO+10%PrOH+6%PEG+Ace	0	0	0
20	40%EG+Tre	20	5	0
20	40%EG	10	0	0
20	control1	60	60	60

		Motlity (%)		
		Time (min)		
Pool	Vitrification solution	< 1	5	10
20	control 2	70	60	60
20	control 3	60	70	70
21	20%EG+20%Gly	60	5	5
21	30%EG+10%DMSO+Tre	35	10	0
21	30%EG+10%PrOH	40	5	1
21	20%MeOH+20%MG	0	0	0
21	40%Gly+Tre	70	15	5
21	30%EG+10%BD	5	0	0
21	30%EG+10%Gly	5	5	5
21	40%Gly	20	20	40
21	30%EG+15%Gly	1	20	5
21	15%EG+10%Gly+15%DMSO+XZ	20	10	10
21	20%EG+20%Gly+Tre	5	15	5
21	30%EG+10%MeOH	0	0	0
21	20%DMSO+10%PrOH+6%PEG+Ace	0	0	0
21	40%EG+Tre	70	15	5
21	40%EG	5	40	20
21	control1	80	80	80
21	control 2	70	80	70
21	control 3	60	80	80

Table C-6. Post-thaw sperm motility of green swordtail vitrified with different treatments. Cryoprotectants used were: glycerol (Gly), ehtylene glycol (EG), 0.45 M trehalose (Tre), dimethyl sulfoxide (DMSO), 1% X-1000™ (X), and 1% Z-1000™ (Z).

			Motility (%)	
Male	Treatment	Temperature	Vibration	Progressive
GRSL08M123	40%Gly	room	30	
GRSL08M123	40%Gly	room	40	
GRSL08M123	40%Gly	room	40	
GRSL08M124	40%Gly	room	60	
GRSL08M124	40%Gly	room	60	
GRSL08M124	40%Gly	room	60	
GRSL08M123	40%Gly	40 C	60	
GRSL08M123	40%Gly	40 C	50	
GRSL08M123	40%Gly	40 C	40	
GRSL08M124	40%Gly	40 C	60	
GRSL08M124	40%Gly	40 C	70	
GRSL08M124	40%Gly	40 C	60	
GRSL08M123	20%EG+20%Gly	room	40	
GRSL08M123	20%EG+20%Gly	room	40	
GRSL08M123	20%EG+20%Gly	room	60	
GRSL08M124	20%EG+20%Gly	room	60	
GRSL08M124	20%EG+20%Gly	room	40	
GRSL08M124	20%EG+20%Gly	room	70	
GRSL08M123	20%EG+20%Gly	40 C	50	
GRSL08M123	20%EG+20%Gly	40 C	60	
GRSL08M123	20%EG+20%Gly	40 C	30	
GRSL08M124	20%EG+20%Gly	40 C	70	
GRSL08M124	20%EG+20%Gly	40 C	60	
GRSL08M124	20%EG+20%Gly	40 C	70	
GRSL09M09	40%Gly+Tre	room		3
GRSL09M10	40%Gly+Tre	room		1
GRSL09M11	40%Gly+Tre	room		13
GRSL09M09	40%Gly+Tre	40 C		3
GRSL09M10	40%Gly+Tre	40 C		0
GRSL09M11	40%Gly+Tre	40 C		13
GRSL09M09	20%EG+20%Gly	room		3
GRSL09M10	20%EG+20%Gly	room		5
GRSL09M11	20%EG+20%Gly	room		20
GRSL09M09	20%EG+20%Gly	40 C		0
GRSL09M10	20%EG+20%Gly	40 C		7
GRSL09M11	20%EG+20%Gly	40 C		13
GRSL09M09	15%EG+10%Gly+15%DMSO+XZ	room		3
GRSL09M10	15%EG+10%Gly+15%DMSO+XZ	room		0

			Motility (%)	
Male	Treatment	Temperature	Vibration	Progressive
GRSL09M11	15%EG+10%Gly+15%DMSO+XZ	room		7
GRSL09M09	15%EG+10%Gly+15%DMSO+XZ	40 C		3
GRSL09M10	15%EG+10%Gly+15%DMSO+XZ	40 C		5
GRSL09M11	15%EG+10%Gly+15%DMSO+XZ	40 C		10
GRSL09M09	30%EG+10%DMSO+Tre	room		1
GRSL09M10	30%EG+10%DMSO+Tre	room		3
GRSL09M11	30%EG+10%DMSO+Tre	room		7
GRSL09M09	30%EG+10%DMSO+Tre	40 C		3
GRSL09M10	30%EG+10%DMSO+Tre	40 C		3
GRSL09M11	30%EG+10%DMSO+Tre	40 C		7
GRSL09M09	10%EG+20%Gly+5%DMSO+XZ	room		0
GRSL09M10	10%EG+20%Gly+5%DMSO+XZ	room		1
GRSL09M11	10%EG+20%Gly+5%DMSO+XZ	room		3
GRSL09M09	10%EG+20%Gly+5%DMSO+XZ	40 C		0
GRSL09M10	10%EG+20%Gly+5%DMSO+XZ	40 C		3
GRSL09M11	10%EG+20%Gly+5%DMSO+XZ	40 C		3

Table C-7. The biological and sperm characteristics of marine fish males used in this study. Fishes used were: spotted seatrout (SPST), red snapper (RDSR), and red drum (RDDM).

Male	Length (cm)	Testis weigth (g)	Initial concentration (sperm/ml)	Sperm Motility (%)
Males used for acute toxicity				
SPST09M03	36	6.6	3×10^9	50
SPST09M16	39	13.8	6.15×10^9	80
SPST09M26	31	3.2	2.7×10^9	80
SPST09M38	44	11.6	4×10^9	50
SPST09M74	43	12	5.73×10^9	90
RDSR09M07	73	84.9	4.9×10^9	70
RDSR09M08	67	79.2	4.68×10^9	80
RDSR09M10	76	59.1	7.6×10^9	80
Males used for vitrification/thawing studies				
SPST09M68	37	3.4	3.45×10^9	70
SPST09M69	36	4.2	4.45×10^9	70
SPST09M74	43	12	5.73×10^9	90
RDSR09M08	67	79.2	4.68×10^9	80
RDSR09M09	69	24.7	6.55×10^9	70
RDSR09M10	76	59.1	7.6×10^9	80
RDDM09M01	68	9.3	3.68×10^{10}	70
RDDM09M02	102	103.6	1.58×10^{10}	90
RDDM09M03	96	70.6	8.0×10^9	85
RDDM09M04	97	199.6	1.45×10^{10}	90

Table C-8. Acute toxicity of spotted seatrout (*Cynoscion nebulosus*). Cryoprotectants used were: methanol (MEOH), ethylene glycol (EG), 1,2-propanediol (PROH), dimethyl sulfoxide (DMSO), 2,3-butanediol (BD), glycerol (Gly), polyethylene glycol (PEG MW 200), and methyl glycol (MG).

			Motlity (%)			
			Time (min)			
Male	Cryoprotectant	Concentration (%)	< 1	5	10	15
3	MEOH	15	50	40	40	40
3	MEOH	20	50	40	5	0
3	EG	10	40	50	50	50
3	EG	15	50	50	50	50
3	EG	20	45	40	45	40
3	EG	25	40	35	35	20
3	EG	30	40	35	5	5
3	PROH	10	40	50	50	40
3	PROH	15	50	50	50	15
3	PROH	20	40	50	10	1
3	DMSO	15	50	50	60	55
3	DMSO	20	50	40	40	25
3	DMSO	25	50	20	5	3
3	BD	10	50	50	40	40
3	Gly	10	50	15	0	1
3	Gly	15	30	5	0	5
3	PEG	10	50	40	0	0
3	MG	15	60	50	60	50
3	MG	20	50	40	30	10
3	Control	—	50	50	50	50
3	Control	—	50	50	50	45
3	Control	—	50	50	50	50
3	Control	—	50	50	50	40
16	MEOH	15	80	70	30	20
16	MEOH	20	80	40	25	0
16	EG	10	70	80	80	80
16	EG	15	80	80	80	80
16	EG	20	80	70	70	50
16	EG	25	70	50	60	70
16	EG	30	80	70	40	5
16	PROH	10	80	70	50	70
16	PROH	15	80	70	55	15

			Motlity (%)			
			Time (min)			
Male	Cryoprotectant	Concentration (%)	< 1	5	10	15
16	PROH	20	80	80	50	3
16	DMSO	15	80	80	80	50
16	DMSO	20	70	70	80	50
16	DMSO	25	80	70	50	30
16	BD	10	80	70	50	70
16	Gly	10	80	50	25	25
16	Gly	15	50	30	30	30
16	PEG	10	80	60	5	0
16	MG	15	80	80	80	80
16	MG	20	80	80	80	60
16	Control	—	70	80	70	80
16	Control	—	60	70	80	70
16	Control	—	80	80	80	80
16	Control	—	80	80	80	80
26	MEOH	15	70	50	50	50
26	MEOH	20	70	60	5	3
26	EG	10	50	70	60	60
26	EG	15	60	35	40	40
26	EG	20	50	60	60	70
26	EG	25	50	50	40	20
26	EG	30	20	10	10	10
26	PROH	10	60	70	50	35
26	PROH	15	70	50	5	1
26	PROH	20	70	20	5	0
26	DMSO	15	60	70	60	50
26	DMSO	20	60	70	70	70
26	DMSO	25	70	70	60	20
26	BD	10	60	60	70	60
26	Gly	10	40	50	10	1
26	Gly	15	70	60	5	5
26	PEG	10	50	5	0	0
26	MG	15	70	60	60	40
26	MG	20	70	60	60	40
26	Control	—	60	70	70	70
26	Control	—	60	70	70	70
26	Control	—	60	60	60	60
26	Control	—	60	60	60	60

Table C-9. Acute toxicity of red snapper (*Lutjanus campechanus*). Cryoprotectants used were: methanol (MEOH), ethylene glycol (EG), 1,2-propanediol (PROH), dimethyl sulfoxide (DMSO), 2,3-butanediol (BD), glycerol (Gly), polyethylene glycol (PEG MW 200), and methyl glycol (MG).

			Motlity (%)			
			Time (min)			
Male	Cryoprotectant	Concentration (%)	< 1	5	10	15
7	MEOH	15	80	80	70	70
7	MEOH	20	70	70	65	70
7	EG	10	70	70	80	70
7	EG	15	80	80	70	70
7	EG	20	60	70	80	80
7	EG	25	70	80	70	60
7	EG	30	80	70	50	65
7	PROH	10	80	65	70	65
7	PROH	15	80	75	80	80
7	PROH	20	80	80	80	70
7	DMSO	15	70	80	65	80
7	DMSO	20	80	80	80	70
7	DMSO	25	80	70	65	45
7	BD	10	70	60	70	70
7	Gly	10	80	5	3	0
7	Gly	15	80	0	0	0
7	PEG	10	80	80	50	50
7	MG	15	75	80	50	40
7	MG	20	70	70	80	50
7	Control	—	70	80	70	70
7	Control	—	70	80	70	70
7	Control	—	80	80	80	80
7	Control	—	80	70	70	70
8	MEOH	15	80	70	80	70
8	MEOH	20	80	60	60	60
8	EG	10	70	80	80	80
8	EG	15	80	80	70	80
8	EG	20	80	70	70	70
8	EG	25	80	70	70	70
8	EG	30	70	50	70	60
8	PROH	10	80	70	80	70
8	PROH	15	80	40	70	60

			Motlity (%)			
			Time (min)			
Male	Cryoprotectant	Concentration (%)	< 1	5	10	15
8	PROH	20	80	80	80	70
8	DMSO	15	80	70	80	70
8	DMSO	20	80	80	80	80
8	DMSO	25	80	50	50	40
8	BD	10	70	60	60	60
8	Gly	10	80	5	5	5
8	Gly	15	80	5	3	3
8	PEG	10	80	70	70	70
8	MG	15	80	80	80	80
8	MG	20	80	80	80	80
8	Control	—	80	70	70	70
8	Control	—	80	80	80	80
8	Control	—	80	70	80	80
8	Control	—	70	80	70	70
10	MEOH	15	85	80	80	75
10	MEOH	20	75	75	70	65
10	EG	10	80	80	80	80
10	EG	15	80	70	75	80
10	EG	20	80	80	70	70
10	EG	25	80	80	70	80
10	EG	30	70	50	50	40
10	PROH	10	70	80	70	75
10	PROH	15	80	80	80	80
10	PROH	20	80	80	80	70
10	DMSO	15	70	80	80	70
10	DMSO	20	80	80	80	80
10	DMSO	25	80	70	65	5
10	BD	10	75	70	70	65
10	Gly	10	80	0	0	0
10	Gly	15	70	1	1	1
10	PEG	10	75	70	70	65
10	MG	15	80	80	80	70
10	MG	20	80	80	70	70
10	Control	—	80	75	80	70
10	Control	—	70	75	80	80
10	Control	—	80	80	75	70
10	Control	—	80	80	80	80

Table C-10. Acute toxicity of spotted seatrout to combined cryoprotectants. Cryoprotectants used were: ethylene glycol (EG), glycerol (Gly), dimethyl sulfoxide (DMSO), methanol (MeOH), methyl glycol (MG), 2,3-butanediol (BD), 1,2-propanediol (PrOH), Trehalose (Tre), Acetamide (Ace), polyvinyl alcohol (PVA), and polyethylene glycol (PEG).

		Motlity (%)		
		Time (min)		
Male	Vitrification solution	< 1	5	10
16	20%EG + 20%Gly	80	0	0
16	15%EG + 25%DMSO	10	0	0
16	20%MeOH + 20%MG	0	0	0
16	10%BD + 30%EG	10	5	0
16	10%MeOH + 30%EG	50	0	0
16	20%DMSO + 20%MG	0	0	0
16	20%DMSO + 20%PrOH	30	0	0
16	20%EG + 20%MG	0	0	0
16	20%EG + 20%DMSO	50	0	1
16	25%DMSO + Tre	90	70	50
16	10%Gly + 15%EG + 15%DMSO	80	0	1
16	20%DMSO + 15%Ace + 10%PrOH + 6%PEG	50	0	0
16	35%PrOH + 3%PVA	80	5	0
16	30%EG + 0.25 M Tre	90	80	60
16	20%EG + 15% Ace	60	40	10
16	30%DMSO + 0.25 M Tre	80	5	1
16	35%DMSO + 0.25 M Tre	80	0	0
16	35%EG + 0.25 M Tre	80	50	50
16	35%PrOH + 0.25 M Tre	60	0	0
16	20%DMSO + 15%EG + 0.25 M Tre	90	60	50
16	20%DMSO + 10%MG + 10%PrOH	1	1	1
16	20%DMSO + 10%PrOH + 10%EG	30	0	0
16	10%BD + 30%DMSO	0	1	0
16	40%EG + 0.25 M Tre	70	5	5
16	40%DMSO + 0.25 M Tre	0	0	0
16	10%DMSO + 30%EG + 0.25 M Tre	50	15	10
16	20%Gly + 20%DMSO	20	0	0
16	25%DMSO + 15%EG + 15%Ace	30	0	0
16	10%BD + 25%DMSO + 15%Ace	0	0	0
16	30%DMSO	50	1	0
16	Control	80	80	80
16	Control	80	70	80
16	Control	70	80	80

		Motlity (%)		
		Time (min)		
Male	Vitrification solution	< 1	5	10
16	Control	70	70	80
16	Control	80	80	80
38	20%EG + 20%Gly	40	0	1
38	15%EG + 25%DMSO	10	0	1
38	20%MeOH + 20%MG	0	0	0
38	10%BD + 30%EG	5	0	1
38	10%MeOH + 30%EG	5	0	0
38	20%DMSO + 20%MG	5	0	0
38	20%DMSO + 20%PrOH	0	1	0
38	20%EG + 20%MG	0	0	1
38	20%EG + 20%DMSO	0	0	0
38	25%DMSO + 0.25 M Tre	50	10	0
38	10%Gly + 15%EG + 15%DMSO	50	0	0
38	20%DMSO + 15%Ace + 10%PrOH + 6%PEG	20	0	0
38	35%PrOH + 3%PVA	40	0	3
38	30%EG + 0.25 M Tre	40	30	25
38	20%EG + 15% Ace	45	5	15
38	30%DMSO + 0.25 M Tre	50	3	0
38	35%DMSO + 0.25 M Tre	35	0	0
38	35%EG + 0.25 M Tre	30	10	10
38	35%PrOH + 0.25 M Tre	40	0	0
38	20%DMSO + 15%EG + 0.25 M Tre	35	30	5
38	20%DMSO + 10%MG + 10%PrOH	0	0	0
38	20%DMSO + 10%PrOH + 10%EG	10	0	0
38	10%BD + 30%DMSO	0	0	0
38	40%EG + 0.25 M Tre	20	3	3
38	40%DMSO + 0.25 M Tre	0	0	0
38	10%DMSO + 30%EG + 0.25 M Tre	30	5	1
38	20%Gly + 20%DMSO	20	0	1
38	25%DMSO + 15%EG + 15%Ace	0	0	0
38	10%BD + 25%DMSO + 15%Ace	20	0	0
38	30%DMSO	35	1	1
38	Control	50	50	50
38	Control	50	50	50
38	Control	50	50	50
38	Control	50	50	50
38	Control	50	50	50
74	20%EG + 20%Gly	40	0	0

		Motlity (%)		
		Time (min)		
Male	Vitrification solution	< 1	5	10
74	15%EG + 25%DMSO	0	0	0
74	20%MeOH + 20%MG	0	0	0
74	10%BD + 30%EG	1	0	0
74	10%MeOH + 30%EG	0	0	0
74	20%DMSO + 20%MG	0	0	0
74	20%DMSO + 20%PrOH	5	1	3
74	20%EG + 20%MG	0	0	0
74	20%EG + 20%DMSO	1	0	0
74	25%DMSO + 0.25 M Tre	40	5	1
74	10%Gly + 15%EG + 15%DMSO	60	3	1
74	20%DMSO + 15%Ace + 10%PrOH + 6%PEG	5	0	0
74	35%PrOH + 3%PVA	40	0	1
74	30%EG + 0.25 M Tre	60	60	50
74	20%EG + 15%Ace	50	5	3
74	30%DMSO + 0.25M Tre	60	0	0
74	35%DMSO + 0.25M Tre	10	0	0
74	35%EG + 0.25M Tre	40	5	3
74	35%PrOH + 0.25M Tre	55	0	3
74	20%DMSO + 15%EG + 0.25M Tre	60	5	5
74	20%DMSO + 10%MG + 10%PrOH	1	0	0
74	20%DMSO + 10%PrOH + 10%EG	5	0	0
74	10%BD + 30%DMSO	0	0	0
74	40%EG + 0.25M Tre	10	5	0
74	40%DMSO + 0.25M Tre	0	0	0
74	10%DMSO + 30%EG + 0.25M Tre	35	0	1
74	20%Gly + 20%DMSO	5	0	0
74	25%DMSO + 15%EG + 15%Ace	0	0	0
74	10%BD + 25%DMSO + 15%Ace	0	0	0
74	30%DMSO	15	3	0
74	Control	60	50	60
74	Control	50	50	50
74	Control	50	50	50
74	Control	50	50	50
74	Control	55	50	50

Table C-11. Acute toxicity of red snapper to combined cryoprotectants. Cryoprotectants used were: ethylene glycol (EG), glycerol (Gly), dimethyl sulfoxide (DMSO), methanol (MeOH), methyl glycol (MG), 2,3-butanediol (BD), 1,2-propanediol (PrOH), Trehalose (Tre), Acetamide (Ace), polyvinyl alcohol (PVA), and polyethylene glycol (PEG).

		Motlity (%)		
		Time (min)		
Male	Vitrification solution	< 1	5	10
7	20%EG + 20%Gly	40	1	1
7	15%EG + 25%DMSO	60	0	0
7	20%MeOH + 20%MG	0	0	0
7	10%BD + 30%EG	70	30	15
7	10%MeOH + 30%EG	40	0	0
7	20%DMSO + 20%MG	0	0	0
7	20%DMSO + 20%PrOH	30	0	5
7	20%EG + 20%MG	10	0	0
7	20%EG + 20%DMSO	70	0	1
7	25%DMSO + 0.25M Tre	70	60	20
7	10%Gly + 15%EG + 15%DMSO	60	5	3
7	20%DMSO + 15%Ace + 10%PrOH + 6%PEG	40	0	0
7	35%PrOH + 3%PVA	80	60	0
7	30%EG + 0.25M Tre	70	60	60
7	20%EG + 15% Ace	70	50	40
7	30%DMSO + 0.25M Tre	60	1	0
7	35%DMSO + 0.25M Tre	30	0	0
7	35%EG + 0.25M Tre	80	50	25
7	35%PrOH + 0.25M Tre	70	3	5
7	20%DMSO + 15%EG + 0.25M Tre	70	30	30
7	20%DMSO + 10%MG + 10%PrOH	20	0	0
7	20%DMSO + 10%PrOH + 10%EG	60	0	0
7	10%BD + 30%DMSO	1	0	0
7	40%EG + 0.25M Tre	70	25	50
7	40%DMSO + 0.25M Tre	5	0	1
7	10%DMSO + 30%EG + 0.25M Tre	70	50	50
7	20%Gly + 20%DMSO	5	0	0
7	25%DMSO + 15%EG + 15%Ace	1	0	0
7	10%BD + 25%DMSO + 15%Ace	0	0	0
7	30%DMSO	70	15	3
7	Control	70	70	70
7	Control	70	70	70
7	Control	70	80	70
7	Control	70	70	70

		Motlity (%)		
		Time (min)		
Male	Vitrification solution	< 1	5	10
7	Control	70	70	60
8	20%EG + 20%Gly	50	0	3
8	15%EG + 25%DMSO	60	0	0
8	20%MeOH + 20%MG	0	0	0
8	10%BD + 30%EG	10	35	5
8	10%MeOH + 30%EG	40	0	0
8	20%DMSO + 20%MG	0	0	0
8	20%DMSO + 20%PrOH	5	1	0
8	20%EG + 20%MG	5	0	0
8	20%EG + 20%DMSO	70	15	3
8	25%DMSO + 0.25M Tre	80	50	30
8	10%Gly + 15%EG + 15%DMSO	80	5	1
8	20%DMSO + 15%Ace + 10%PrOH + 6%PEG	40	0	1
8	35%PrOH + 3%PVA	70	3	0
8	30%EG + 0.25M Tre	80	80	50
8	20%EG + 15% Ace	80	70	70
8	30%DMSO + 0.25M Tre	80	40	5
8	35%DMSO + 0.25M Tre	35	0	0
8	35%EG + 0.25M Tre	80	80	50
8	35%PrOH + 0.25M Tre	80	3	3
8	20%DMSO + 15%EG + 0.25M Tre	80	25	40
8	20%DMSO + 10%MG + 10%PrOH	0	3	0
8	20%DMSO + 10%PrOH + 10%EG	50	0	0
8	10%BD + 30%DMSO	0	0	0
8	40%EG + 0.25M Tre	80	70	50
8	40%DMSO + 0.25M Tre	3	0	0
8	10%DMSO + 30%EG + 0.25M Tre	80	60	50
8	20%Gly + 20%DMSO	5	0	0
8	25%DMSO + 15%EG + 15%Ace	0	0	0
8	10%BD + 25%DMSO + 15%Ace	0	0	0
8	30%DMSO	70	40	5
8	Control	80	80	80
8	Control	80	80	80
8	Control	80	70	80
8	Control	80	80	80
8	Control	80	80	70
10	20%EG + 20%Gly	80	0	0
10	15%EG + 25%DMSO	70	1	0

		Motlity (%)		
		Time (min)		
Male	Vitrification solution	< 1	5	10
10	20%MeOH + 20%MG	0	0	0
10	10%BD + 30%EG	60	15	0
10	10%MeOH + 30%EG	50	0	0
10	20%DMSO + 20%MG	0	0	0
10	20%DMSO + 20%PrOH	0	0	0
10	20%EG + 20%MG	0	0	0
10	20%EG + 20%DMSO	50	0	0
10	25%DMSO + 0.25M Tre	70	5	3
10	10%Gly + 15%EG + 15%DMSO	80	20	3
10	20%DMSO + 15%Ace + 10%PrOH + 6%PEG	0	0	0
10	35%PrOH + 3%PVA	75	3	0
10	30%EG + 0.25M Tre	80	70	35
10	20%EG + 15% Ace	75	70	70
10	30%DMSO + 0.25M Tre	70	0	0
10	35%DMSO + 0.25M Tre	40	0	0
10	35%EG + 0.25M Tre	75	60	15
10	35%PrOH + 0.25M Tre	80	0	0
10	20%DMSO + 15%EG + 0.25M Tre	80	5	0
10	20%DMSO + 10%MG + 10%PrOH	0	0	0
10	20%DMSO + 10%PrOH + 10%EG	60	0	0
10	10%BD + 30%DMSO	0	0	0
10	40%EG + 0.25M Tre	80	65	15
10	40%DMSO + 0.25M Tre	0	0	0
10	10%DMSO + 30%EG + 0.25M Tre	75	30	20
10	20%Gly + 20%DMSO	40	0	0
10	25%DMSO + 15%EG + 15%Ace	5	0	0
10	10%BD + 25%DMSO + 15%Ace	0	0	0
10	30%DMSO	80	40	0
10	Control	80	80	75
10	Control	75	80	70
10	Control	70	75	70
10	Control	70	75	70
10	Control	70	75	70

Table C-12. Parameters of southern flounder (SOFR) received and used in experiments.

ID Male				
NCSU Tag #	LSU	Osmolality mOsmol/kg	Arrival motility (%)	Initial concentration (sperm/ml)
Arrive at LSU 4/02/09				
4A761806D1	SOFR09M25	---	50	1.70×10^9
ID #6	SOFR09M28	---	60	2.73×10^9
4A71DE122D	SOFR09M29	---	40	2.32×10^9
Arrive at LSU 3/25/10				
4A7468545F	SOFR10M03	317	60	1.4×10^{10}
4A7255151A	SOFR10M05	472	70	1.29×10^{10}
4B1864570F	SOFR10M07	348	50	1.12×10^{10}
Arrive at LSU 8/27/10				
4B0062132B	SOFR10M21	---	50	5.99×10^9
4B18531E17	SOFR10M23	---	50	8.01×10^9
4B1840664A	SOFR10M24	---	70	7.24×10^9

Table C-13. Post-thaw sperm motility of southern flounder (*Paralichthys lethostigma*) vitrified with different vitrification solutions, and thawed at different temperatures. Cryoprotectants used were: dimethyl sulfoxide (DMSO), glycerol (Gly), 1,2-propanediol (PrOH), ehtylene glycol (EG), X-1000™ (X), and Z-1000™ (Z).

Male	Treatment	Temperature	Motility (%)
SOFR09M25	20% DMSO + 20% Gly	21	35
SOFR09M25	20% DMSO + 20% Gly	21	30
SOFR09M25	20% DMSO + 20% Gly	37	30
SOFR09M25	20% PrOH + 20% Gly	21	30
SOFR09M25	20% PrOH + 20% Gly	21	20
SOFR09M25	20% PrOH + 20% Gly	37	25
SOFR09M25	20% EG + 20% Gly	21	20
SOFR09M25	20% EG + 20% Gly	37	35
SOFR09M25	20% EG + 20% Gly	37	30
SOFR09M25	20% DMSO + 20% PrOH	21	0
SOFR09M25	20% DMSO + 20% PrOH	37	0
SOFR09M25	20% DMSO + 20% PrOH	37	0
SOFR09M25	20% DMSO + 20% EG	21	0
SOFR09M25	20% DMSO + 20% EG	37	1
SOFR09M25	20% DMSO + 20% EG	37	5
SOFR09M25	20% EG + 20% PrOH	21	10
SOFR09M25	20% EG + 20% PrOH	21	5
SOFR09M25	20% EG + 20% PrOH	37	7
SOFR09M28	20% DMSO + 20% Gly	21	10
SOFR09M28	20% DMSO + 20% Gly	21	15
SOFR09M28	20% DMSO + 20% Gly	37	20
SOFR09M28	20% PrOH + 20% Gly	21	5
SOFR09M28	20% PrOH + 20% Gly	37	10
SOFR09M28	20% PrOH + 20% Gly	37	15
SOFR09M28	20% EG + 20% Gly	21	20
SOFR09M28	20% EG + 20% Gly	21	20
SOFR09M28	20% EG + 20% Gly	37	20
SOFR09M28	20% DMSO + 20% PrOH	21	1
SOFR09M28	20% DMSO + 20% PrOH	21	3
SOFR09M28	20% DMSO + 20% PrOH	37	1
SOFR09M28	20% DMSO + 20% EG	21	3
SOFR09M28	20% DMSO + 20% EG	37	0
SOFR09M28	20% DMSO + 20% EG	37	0
SOFR09M28	20% EG + 20% PrOH	21	1
SOFR09M28	20% EG + 20% PrOH	37	3
SOFR09M28	20% EG + 20% PrOH	37	0
SOFR09M29	20% DMSO + 20% Gly	21	10
SOFR09M29	20% DMSO + 20% Gly	37	20

Male	Treatment	Temperature	Motility (%)
SOFR09M29	20% DMSO + 20% Gly	37	15
SOFR09M29	20% PrOH + 20% Gly	21	5
SOFR09M29	20% PrOH + 20% Gly	37	5
SOFR09M29	20% PrOH + 20% Gly	37	7
SOFR09M29	20% EG + 20% Gly	21	25
SOFR09M29	20% EG + 20% Gly	21	10
SOFR09M29	20% EG + 20% Gly	37	20
SOFR09M29	20% DMSO + 20% PrOH	21	3
SOFR09M29	20% DMSO + 20% PrOH	37	3
SOFR09M29	20% DMSO + 20% PrOH	37	3
SOFR09M29	20% DMSO + 20% EG	21	3
SOFR09M29	20% DMSO + 20% EG	37	3
SOFR09M29	20% DMSO + 20% EG	37	1
SOFR09M29	20% EG + 20% PrOH	21	0
SOFR09M29	20% EG + 20% PrOH	21	0
SOFR09M29	20% EG + 20% PrOH	37	3
SOFR10M03	20% EG + 20% Gly	24	20
SOFR10M03	20% EG + 20% Gly	24	20
SOFR10M03	10% DMSO + 30% EG + 0.25 M Trehalose	24	5
SOFR10M03	10% DMSO + 30% EG + 0.25 M Trehalose	24	5
SOFR10M03	15%DMSO + 15%EG + 10%Gly + 1%X + 1%Z	24	20
SOFR10M03	15%DMSO + 15%EG + 10%Gly + 1%X + 1%Z	24	15
SOFR10M05	20% EG + 20% Gly	24	30
SOFR10M05	20% EG + 20% Gly	24	40
SOFR10M05	20% EG + 20% Gly	24	40
SOFR10M05	10% DMSO + 30% EG + 0.25 M Trehalose	24	10
SOFR10M05	10% DMSO + 30% EG + 0.25 M Trehalose	24	10
SOFR10M05	15%DMSO + 15%EG + 10%Gly + 1%X + 1%Z	24	10
SOFR10M05	15%DMSO + 15%EG + 10%Gly + 1%X + 1%Z	24	30
SOFR10M07	20% EG + 20% Gly	24	20
SOFR10M07	20% EG + 20% Gly	24	25
SOFR10M07	10% DMSO + 30% EG + 0.25 M Trehalose	24	5
SOFR10M07	10% DMSO + 30% EG + 0.25 M Trehalose	24	5
SOFR10M07	15%DMSO + 15%EG + 10%Gly + 1%X + 1%Z	24	5
SOFR10M07	15%DMSO + 15%EG + 10%Gly + 1%X + 1%Z	24	5

Table C-14. Sperm motility and membrane-integrity of fresh sperm from southern flounder before the addition of vitrification solutions and after vitrification. Cryoprotectants used were: dimethyl sulfodixde (DMSO), glycerol (Gly), and ehtylene glycol (EG).

		Fresh		Post-thaw	
Male	Treatment	Motility (%)	Membrane-intact (%)	Motility (%)	Membrane-intact (%)
SOFR10M21	20% DMSO + 20% Gly	50	88.68	1	9.62
SOFR10M21	20% DMSO + 20% Gly	50	88.68	5	11.19
SOFR10M21	20% DMSO + 20% EG	50	89.05	0	3.17
SOFR10M21	20% DMSO + 20% EG	50	89.05	0	2.29
SOFR10M21	20% EG + 20% Gly	50	89.21	10	15.01
SOFR10M21	20% EG + 20% Gly	50	89.21	20	16.79
SOFR10M23	20% DMSO + 20% Gly	50	87.31	5	2.39
SOFR10M23	20% DMSO + 20% Gly	50	87.31	5	3.05
SOFR10M23	20% DMSO + 20% EG	50	88.25	1	1.50
SOFR10M23	20% DMSO + 20% EG	50	88.25	0	1.45
SOFR10M23	20% EG + 20% Gly	50	87.22	5	7.37
SOFR10M23	20% EG + 20% Gly	50	87.22	10	12.56
SOFR10M24	20% DMSO + 20% Gly	70	89.32	20	4.44
SOFR10M24	20% DMSO + 20% Gly	70	89.32	5	3.82
SOFR10M24	20% DMSO + 20% EG	70	89.32	1	2.01
SOFR10M24	20% DMSO + 20% EG	70	89.32	0	1.54
SOFR10M24	20% EG + 20% Gly	70	89.49	15	5.39
SOFR10M24	20% EG + 20% Gly	70	89.49	20	8.67

Vita

Rafael Cuevas-Uribe, son of Jorge Luis and Aurora Guadalupe, was born November 5, 1976, in Guadalajara, Mexico. He is an alumnus of the Colegio Salesiano Anahuac Chapalita at Zapopan, Mexico. In September 1995, he entered the Universidad de Guadalajara, Mexico, and in 2001 he received a Bachelor of Science in Biology with a concentration in genetics and aquaculture. His undergraduate thesis project addressed hormonal masculinization of tilapia in Teocaltiche, Jalisco, Mexico. As an undergraduate he did a professional internship in the fish biology department at the Technical University of Munich, Germany where he was mentored by Dr. Herbert Stein, introducing him to the field of fish sperm cryopreservation. In June 2001 he began working as an aquaculture technician at the Aquaculture and Fisheries Institute of Jalisco State agency, Mexico. As an aquaculture technician, Rafael lived in a fish hatchery producing fingerlings of common carp, channel catfish, and tilapia. Working there convinced him to go back to school and pursue a graduate degree in aquaculture. He entered the Aquaculture Graduate Program at Kentucky State University, Frankfort, Kentucky, in August 2003 under the supervision of Dr. Boris Gomelsky. He received in 2006 a Master of Science in Aquaculture/Aquatic Science with a thesis titled "Advancements on hormonal sex reversal and genetic sex control in black crappie." While at Kentucky, Rafael received the outstanding aquaculture graduate student award and was one of the founders and the first president of the "Aquabreds" student club. In January 2006, he began the degree of Doctor of Philosophy in the School of Renewable Natural Resources at Louisiana State University under the supervision of Dr. Tiersch. His research focused on developing protocols for sperm vitrification in aquatic species. While at LSU, Rafael served on the Executive Committee of the Aquaculture and Fisheries club. In 2008 and 2010, he received the Best Abstract Award, at the annual meeting of the Louisiana Chapter of the American Fisheries Society. In 2009, he received the Best Abstract/Travel Award at Aquaculture America, and in 2010 Rafael was awarded Best Abstract at the Triennial Conference of the World Aquaculture Society. In August 2011, Rafael accepted a postdoctoral Co-investigator position at Kentucky State University to work on reservoir ranching and bioenergetics of paddlefish. Rafael is a candidate for the degree of Doctor of Philosophy in wildlife and fisheries science in December 2011.