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High-throughput sperm cryopreservation of aquatic species

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HIGH-THROUGHPUT SPERM CRYOPRESERVATION OF AQUATIC SPECIES

A Dissertation

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In memory of

Yuhua Yao

My dear paternal grandmother, who devoted her life and love to raise and care for two generations, who has been the person I did everything to honor,

and

Jiaobao Lv

My dear maternal grandfather, who is a shining example for me as a dedicated scholar, visionary leader, and persevering human being.

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Abstract

The goal of this dissertation was to integrate multiple disciplines for application of high-throughput sperm cryopreservation to aquatic species. The blue catfish *Ictalurus furcatus* was selected for research due to its role in production of hybrid catfish with eggs from channel catfish *Ictalurus punctatus*. A high-throughput cryopreservation pathway was developed by systematically evaluating each process factor and using automated processing systems. Large quantities of cryopreserved sperm produced from this protocol were evaluated in commercial-scale hatcheries in cooperation with aquaculture producers. To develop production for commercial requirements, quality characteristics of materials and products were identified and recorded during processing and analyzed using industrial engineering methods to regulate quality. More than 1 million channel catfish eggs from 300 females were fertilized with thawed sperm, which was larger than any previous large-scale fertilization trial performed in fish. This demonstrated that the fertilization capability of cryopreserved sperm was equivalent to fresh sperm in production of hybrid fry. Quality regulation was presented within a quality assurance plan designed specifically for this process using quality standards (specifications) and quality variation data (quality control). This quality assurance plan is potentially the first of its kind for cryopreserved sperm of any species. After being scaled up by automation and standardized by the quality assurance plan, the cryopreservation process produced reliable products for hatcheries. Proper use of these products (i.e., as a dose of 3704 eggs/straw) can provide a basis for industry standards for artificial spawning. A quantitative evaluation was developed based on the defined dose and the production efficiency. To increase the efficiency for large-scale production, simulation model computing was used to create a virtual process. The simulation model was validated based on the existing process, and provides a valuable tool for future improvements.

This dissertation took the initiative to apply high-throughput cryopreservation in aquaculture and expand the process from planning to operation at the production line, to terminal use of products by customers. This enables cryopreserved sperm to become a practical form of genetic resources applicable for aquatic genetic improvement and conservation.

The Master standing by a stream, said, “It passes on just like this, not ceasing day or night!”

Confucian Analects, Book IX: Tsze Han, Chapter 16
Translated by James Legge, 1861, *In*: Confucian Analects, the Great Learning, and the Doctrine of the Mean. The Chinese Classics I. ISBN 978-1-60520-643-1.

Chapter 1
Background, Dissertation Overview and Achievements

From a broad perspective, this dissertation addresses the concept of time. Not only because the subject of this work —cryopreservation, a means of preservation that can be viewed as a form of time travel, —but more importantly, because the core of this dissertation, technology transformation from laboratory to production, was about progressively improving the utilization of time.

The value of genetic resources in forms such as frozen semen is essential to animal breeding for genetic improvement and conservation management. The semen of bulls (NAAB-CSS, 2011b) and human (Newton-Small, 2012) are shipped around the world in frozen form and remain functional. Frozen semen has resulted in human live births after 28 years of storage (Feldschuh et al., 2005) and calf births after 40 years (Bogren, 2010). In aquatic species, the potential of genetic value has often been overlooked. There are more than 32,000 species of fish (Barton, 2007), but only 95 species of fish, crustaceans, and mollusks serve as primary food resources for humans. The majority of aquatic species have not been accessed in any form of genetic resources. Among those 95 species, only 28 are cultured (FAO, 2011). The other 67 species have not been bred for genetic improvement, and therefore aquatic species have the potential to benefit from genetic material management tools such as cryopreservation.

After the development of sperm cryopreservation in aquatic species in the early 1950s (Blaxter, 1953), an increasing number of protocols have been reported in various journals (Tiersch, 2011). Because of the increasing attention on the value of brood fish, cryopreservation is beginning to be used for economically important species such as Atlantic salmon *Salmo salar* (NASCO, 2010). Meanwhile, hybridization between species places value on gametes as genetic resources. Hybrid striped bass (***striped bass*** *Morone saxatilis* female ***x white bass*** *M. chrysops*

male) (Rudacille and Kohler, 2000) and hybrid catfish (channel catfish *Ictalurus punctatus* female x blue catfish *I. furcatus* male) (Umali-Maceina, 2007) inherit superior features from the parental species. The application of hybridization using extracted sperm has also provided an opportunity for applying cryopreserved sperm in fertilization. However, before this project there were no true large-scale examples of cryopreservation application in assisting reproduction activities in aquatic species. Compared to the long-standing demands seen, for example, in frozen semen of farm-raised mammals and presently occurring in conservation of imperiled species (Fickel et al., 2007), the commercialization of sperm cryopreservation for fish and shellfish remains under development.

From the first cryopreservation of bull semen in 1952 (Polge and Rowson, 1952) to the development of trade networks and regulation of semen transport in 1976 (NAAB-CSS, 2011a), 24 years of nationwide efforts have made bull semen a billion dollar industry. Inspired by the progress made with bull semen, this 5-year dissertation project addressed the issue of how to convert experimental cryopreservation protocols into functional high-throughput processes with standard industrial features using blue catfish sperm as the primary research model.

Reinforcing the time concept prevalent throughout this dissertation, the approach of this project provides an example of technology transformation that could assist researchers and commercial-scale users with time management. The roots of this project go back 20-40 years with the rapid expansion of catfish aquaculture in the southern United States (USDA-NASS, 2005). The main research topic for cryopreservation was protocol development for channel catfish sperm to assist catfish culture (Guest et al., 1976, Tiersch et al., 1994, Christensen and Tiersch, 1997, Christensen and Tiersch, 2005). Unfortunately, US catfish culture has been depressed since 2001 (USDA-NASS, 2005). To regain the competitiveness of catfish farming,

hybrid catfish has become popular with farmers due to high growth rate and disease resistance (Dunham and Masser, 2012). Blue catfish sperm gained commercial level demand (based on killing of males to harvest sperm on demand), and cryopreservation was evaluated as a supporting technology for hybrid catfish hatcheries. With the funding of a US Department of Agriculture (USDA) - Small Business Innovation Research (SBIR) project in 2009 to Baxter Land Company and Dr. Tiersch of LSUAC, this dissertation work began to address fundamental research in high-throughput protocol development. In 2010, this USDA-SBIR proposal entered Phase 2 funding, and the research evolved to advance application through process engineering. There are four stages of progression in this dissertation: 1) standardization of cryopreservation protocols of blue catfish sperm, 2) large-scale application and evaluation, 3) process quality assurance, and 4) process simulation (Figure 1.1). The goal of this dissertation was to establish a high-throughput cryopreservation system to address the main issues relevant to the application of cryopreservation products and services to public use for aquatic species, and supporting development of new markets in germplasm and genetic resources (Figure 1.1). The objectives (individually addressed through Chapters 3 to 7) were to: 1) develop a high-throughput cryopreservation process for blue catfish sperm; 2) apply cryopreserved blue catfish sperm on commercial level; 3) establish an evaluation method for artificial spawning using cryopreserved sperm; 4) develop a quality assurance plan for high-throughput processing, and 5) build a useful simulation model for processing using simulation software.

The dissertation contained introduction chapters (Chapters 1 and 2), followed by research chapters (Chapters 3 to 7), ended with summary chapter (Chapter 8). All the supporting documents were repented in appendices. Chapter 1 described the general development of this dissertation. Chapter 2 introduced the related background information of research chapters.

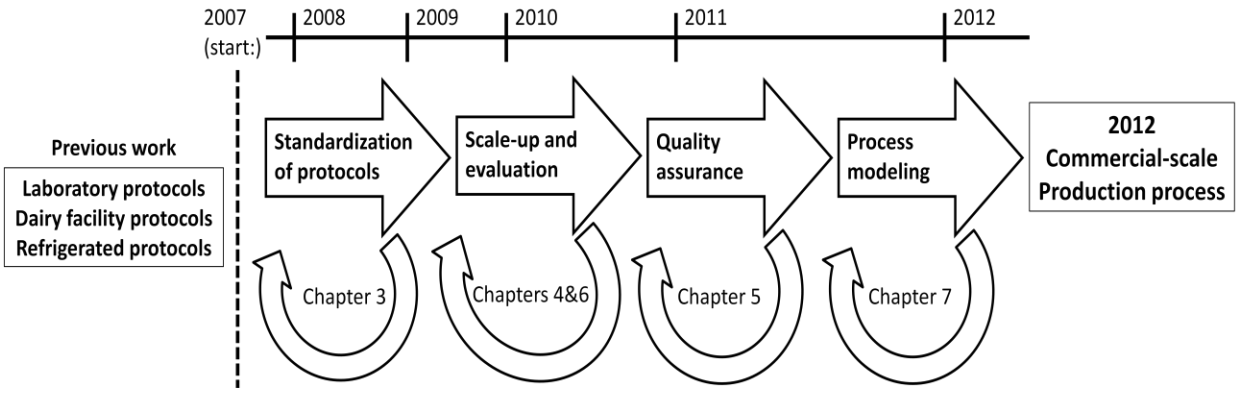

Figure 1.1. The structure of this dissertation work. The foundation of the dissertation (box to the left of dotted line) was previous studies on preservation of fish sperm. The straight arrows represent the progressive stages of the dissertation. The circled arrows represent continuous testing and improving during each respective stage, which is presented in the chapters identified in the center of the arrows. The dissertation comprises four stages (verifying and standardizing cryopreservation protocols, large-scale application and evaluation of fertilization, quality assurance throughout the process, and production behavior simulation) to achieve the final 2012 commercial-scale production. The timeline of this dissertation is presented above the stages.

Chapter 3 developed a fish-specific process with an automated packaging system adapted from commercial mammalian applications. Automated systems have been introduced previously to aquaculture to reduce the intensity of labor for activities such as feeding (Smolin, 1953), monitoring of dissolved oxygen (Bryan and Cushman, 1990), and maintaining pond temperature (Lang et al., 2003b). The cryopreservation procedures initially developed for fishes relied on the use of manual operations and highly skilled practitioners. When facing the challenges of large-scale production, laboratories could not reliably organize enough manpower for intensive manual processing and this has led to evaluation with aquatic species of automated systems designed for bull sperm (Lang et al., 2003a, Dong et al., 2007). Livestock sperm cryopreservation has automation available with multiple function modules: labeling to print sample information and barcodes; packaging to fill and seal large numbers of containers, and controlled-rate freezing to standardize cooling rates. The specific cryopreservation process for fish adapted automated systems developed for livestock and humans for use with fish reproductive features such as

extraction of sperm by crushing of dissected testes (Dunham and Masser, 2012) and refrigerated storage of sperm under isotonic conditions (Christensen and Tiersch, 2007). A fish-specific high-throughput cryopreservation protocol was established to fully utilize the automated capabilities. Laboratory-scale studies were used to improve the processing.

Chapter 4 represents the application of cryopreserved sperm in commercial hatcheries and indicates the potential capability of cryopreserved sperm. Before a new product enters the market, a series of surveys should be conducted to measure potential public acceptance. A survey of acceptance of improved genetics by use of germplasm cryopreservation showed positive feedback from aquaculture producers (Boever, 2006). However, at that time there were few deliverable cryopreservation products to address public demand. Without real-life application of cryopreserved sperm, data from small-scale tests could not support large information networks such as those utilized in the cattle sperm industry (NAAB-CSS, 2011a). Federal research grants to Dr. Tiersch at the ARS in 2008 (Louisiana Sea Grant College Program) and 2009 (SBIR) provided the opportunity for producers to apply and evaluate new products and services and provide commercial-level feedback (USDA, 2012). With this opportunity, from 2009 to 2011, cryopreserved blue catfish sperm were evaluated in this dissertation in the largest fertilization experiment to date in an aquatic species.

Chapter 5 demonstrates the establishment of specifications and quality assurance for the production line concept developed in the previous chapters. In scientific publications, experimental details have to be described so experiments are repeatable elsewhere. However, at least 21 major factors have been reported that need to be defined in publications addressing cryopreservation research in fishes (Yang et al., 2010). As such these factors had to be standardized (Tiersch et al., 2007) in this project as part of production line development. To

make the processing ready for real-life scenarios, this dissertation conceptualized the steps with tools from process engineering (Lee, 2000). Referring to the standardization needed for the process, the driving question was: how to operate high-throughput cryopreservation of blue catfish sperm to ensure reliable (functional) products of stable quality (minimum variation introduced by materials and operations)? From a manufacturing perspective, quality and efficiency are two essential aspects. An important standardization of quality in the process engineering literature is called a "specification" (or "tolerance"). With specification, each step can screen out materials that will produce nonfunctional products. Meanwhile, production lines under continuous inspection can use statistical quality control methods to monitor production behaviors such as quality variation and operational errors, that have been applied in human medicine (Westgard et al., 1981). An initial approach was taken in this dissertation to analyze and construct specifications and quality control using the 2010 and 2011 processing data of all quality characteristics. This study was the first application of statistical quality control in aquatic spcies cryopreservation.

Chapter 6 established evaluation methods for dose-based artificial spawning for future application of cryopreserved products. Work in Chapters 4 and 5 demonstrated that cryopreserved sperm could assist aquaculture as an independent resource. This new resource could serve as standard throughout artificial spawning activities that involve cryopreserved sperm products, but existing commercial-scale evaluation methods are not representative for individual males or females and as such were not useful for assessment at the level needed for genetic management. Existing methods were either limited to assessment based on testis mass (fry/g testis) (Dunham and Masser, 2012), or female mass (fry/kg female) (Phelps et al., 2007). With development of this new resource (cryopreserved sperm), evaluation of artificial spawning

became a new challenge yet offered new opportunities. Because of external fertilization in aquatic species, fertilization could be evaluated on a per-egg basis, and therefore, evaluation methods for cattle semen, which are based on the calf production for each female (Cook, 2009), cannot be directly adopted. As such, an evaluation method was developed based on the characteristics of fish reproduction and should be able to evaluate overall hatchery production with the availability of standardized cryopreservation products.

Chapter 7 developed a simulation model for the high-throughput cryopreservation production line. The process developed in this work could be further optimized or adapted to other application scenarios. However, given the inevitable limitations in space and labor, structural upgrades and larger-scale testing were not feasible in this work. As such, a simulation modeling approach was used to overcome these problems. The goal was that the modeled process could be reorganized and tested to meet different efficiency requirements. From the various commercially available software, ARENA (Rockwell Automation, Inc., Milwaukee, WI) was selected because of the objective-oriented user-interactive features. Measurements of each operation were built into the respective ARENA Model-Objects, and computer logic was developed to increase the artificial intelligence of the model. The developed model was able to perform realistic activities such as sample rejection, avoidance of waiting times, and finishing up remaining tasks before the close of an hpothtical 8-hr work day. Further, more complicated optimization scenarios were also able to be tested.

This dissertation work was supported in part by funding from the Louisiana Sea Grant College Program, USDA special grants, USDA-SBIR, and the National Institutes of Health — National Center for Research Resources. The results of this project represented collaborative efforts among several departments and institutions including the LSUAC – ARS; Department of

Industrial Engineering, LSU College of Engineering; Baxter Land Company, Inc, Dermott, AR; and the USDA Catfish Genetics Research Unit, Stoneville, MS. The high-throughput cryopreservation process was developed and operated at the ARS, and fertilization trials were performed at the ARS, Baxter Land Company, and USDA Catfish Genetics Research Unit. The process engineering was in collaboration with LSU Industrial Engineering Department.

During this project, research results have been presented at several scientific meetings (Table 1.1). In addition, at the time of this writing, three manuscripts related to this project, including Chapter 3 (Hu et al., 2011), Chapter 7, and Chapter 8 (Hu and Tiersch, 2011) have been published or submitted for publication in peer-reviewed outlets (Table 1.2). Chapters 4, 5, and 6 are also intended to be submitted for publication in peer-reviewed journals (Table 1.2). This dissertation also contains *Standard Operating Procedures* (Appendix A), a *Manual for Using Cryopreserved Sperm Stored in 0.5-ml Straws* (Appendix B), a *Component List for a Catfish Sperm High-throughput Cryopreservation Facility* (Appendix C), data recording spreadsheets during production (Appendix D), *Standard Operation Sheets* of blue catfish sperm high-throughput cryopreservation (Appendix E), the original data reported in the research chapters (3-7) (Appendix F), and the copyright permissions (Appendix G). In addition, five other manuscripts (3 published, 2 in preparation) have resulted from this project through initial application in other aquatic species and this work appears elsewhere (listed in Table 1.2). For internal consistency, all chapters of this dissertation have been prepared in the format of Harvard style with specific modifications as required to meet LSU dissertation format and style.

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

Year	Title	Conference	Location
2008	Development a protocol for high-throughput cryopreservation of channel catfish spermatozoa	Gulf Coast Conservation Biology Symposium	New Orleans, LA
2009	High-throughput cryopreservation for fish and shellfish	Annual meeting of Southern Division of the American Fisheries Society	New Orleans, LA
2009	High-throughput cryopreservation in Aquatic species: adapting mammalian technology to fish	Annual meeting of U.S. Aquaculture Society	Seattle, WA
2010	Detailed records on freezing conditions are necessary for standardization of sperm cryopreservation in aquatic species	Gulf Coast Conservation Biology Symposium	New Orleans, LA
2010	Commercial-scale blue catfish sperm cryopreservation for hybrid catfish production[1]	Louisiana Chapter of the American Fisheries Society	Baton Rouge, LA
2010	High-throughput cryopreservation of fish sperm: blue catfish protocol development and hatchery application	Triennial meeting of World Aquaculture Society	San Diego, CA
2011	Scaling of high-throughput cryopreservation for fishery and aquaculture applications[1]	Louisiana Chapter of the American Fisheries Society	Lafayette, LA
2011	Commercial-scale hybrid catfish production with high-throughput processing of cryopreserved sperm from individual males[2]	Annual meeting of U.S. Aquaculture Society	New Orleans, LA
2011	A throughput view for practical cryopreservation in aquatic species	Gulf Coast Conservation Biology Symposium	New Orleans, LA
2012	Process simulation, operation cost estimation and optimization for high-throughput cryopreservation of blue catfish sperm	Annual meeting of U.S. Aquaculture Society	Las Vegas, NV
2012	Commercial-scale sperm cryopreservation for blue catfish	Louisiana Chapter of the American Fisheries Society	Baton Rouge, LA

[1] Received Award for Best Abstract (1st place) from the Louisiana Chapter of the American Fisheries Society, 2009 and 2010.
[2] Received USAS Best Abstract/Travel Award in Aquaculture America 2011, Presentation Award of the Fish Culture Section of the American Fisheries Society and the US Aquaculture Society, and Aquaculture America 2011 Spotlight Presentation award.

Table 1.2. At the time of this writing, the research associated with this project or presented in this dissertation has resulted in five published papers and six manuscripts in review or preparation. Citations of published works are given in the References.

Title	Journal/Book	Status	Chapter
High-throughput cryopreservation of spermatozoa of blue catfish (*Ictalurus furcatus*): Establishment of an approach for commercial-scale processing	*Cryobiology*	Published	3
Commercial-scale hybrid production using cryopreserved blue catfish (*Ictalurus furcatus*) spermatozoa	*Aquaculture*	In preparation	4
Development of a quality assurance plan for aquatic species sperm high-throughput cryopreservation using blue catfish sperm as model	*Aquaculture Engineering*	In preparation	5
An evaluation method for artificial spawning using Dose-based cryopreserved sperm: commercial-scale sperm processing of blue catfish *Ictalurus furcatus* to produce hybrid catfish	*Journal of World Aquaculture Society*	In preparation	6
Simulation modeling of high-throughput cryopreservation of aquatic germplasm: a case study of blue catfish sperm processing	*Aquaculture Research*	In review	7
Development of high-throughput cryopreservation for aquatic species	*Cryopreservation in Aquatic Species*[1]	Published	8
Outlook for development of high-throughput cryopreservation for small-bodied biomedical model fishes[2]	*Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*	Published	--
High-throughput sperm cryopreservation of eastern oyster *Crassostrea virginica*[2]	*Aquaculture*	Published	--
Metaheuristic approaches to grouping problems in high-throughput cryopreservation operations for fish sperm[2]	*Applied Soft Computing*	Published	--
High-throughput cryopreservation of sperm from sex-reversed southern flounder *Paralichthys lethostigma*	*Journal of World Aquaculture Society*	In preparation	--
Development of a simplified and standardized protocol with potential for high throughput for sperm cryopreservation in Atlantic salmon (*Salmo salar*)[2]	*Cryobiology*	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] E Hu, Co-author (non-dissertation related)

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Chapter 2
Introduction

Studies on sperm cryopreservation of aquatic species have a history as long as similar research on domestic livestock animals (Blaxter, 1953), but the production scale and public acceptance for cryopreservation in fish and shellfish lags well behind (Tiersch, 2011a). For aquatic species, the past 60 years have dwelled on research methodology (Tiersch and Green, 2011). Therefore, this dissertation purposely shifted emphasis from revisiting the methodology of cryopreservation protocols to expansion of the thinking from the laboratory to commercial-scale production by combining insights from different disciplines ranging from animal science to industrial engineering (Figure 2.1). This dissertation can thus provide a template for the transition from research results to industrial application.

Cryopreservation and blue catfish

Cryopreservation is a combination of dehydration and low temperature preservation (Mazur, 2004). During cryopreservation, cells will first dehydrate in the hypertonic environment caused by cryoprotectants. Once ice crystal formation begins to reduce the free water outside of the cells, cells will further dehydrate before transitioning into the frozen state for storage (Cuevas-Uribe, 2011). To achieve high survival rates, the process involves consideration of factors beyond cryoprotectants and freezing (Tiersch et al., 2007). In aquatic species research, each step from gamete collection through fertilization assessment requires special attention (Tiersch, 2011b, Leibo, 2011).

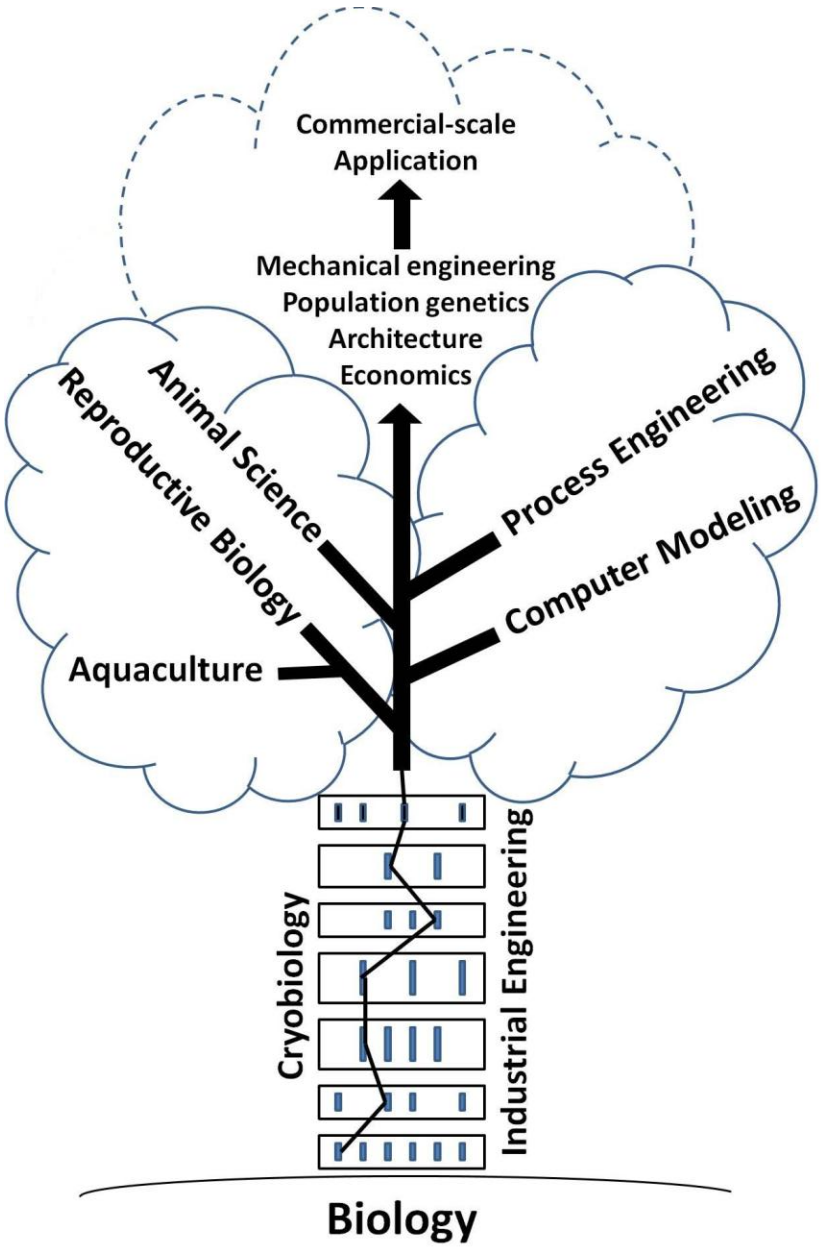

Figure 2.1. Relationships of the different disciplines bought together within this dissertation. The foundation was provided by the general biology of fishes. The cryopreservation protocol comprised a series of process steps (boxes) with identification and linkage of specific parameters (short bars) across boxes based on the principles of cryobiology and the needs for high-throughput and quality assurance. Large-scale fertilization tests were based on reproductive biology and the results were evaluated based on of the relevant factors in production aquaculture. To improve production, process engineering provided quality assurance concepts to ensure final product quality. The entire process was simulated by computer language to overcome space and capital limitations. Establishment of a specific pathway can be refined by inputs from other specific disciplines (bolded line) and lead to further research and application. Animal science in particular provided examples from decades of experience in scaling up, quality assurance, automation and business practices.

The selection of model animals is an important factor in any research project. Within aquatic organisms, there are more than 28,800 fish species distributed throughout the waters of the Earth (Barton, 2007). With different evolutionary strategies (Ricklefs and Miller, 2000), aquatic species have different reproductive features. Even within a single species, environmental variations can alter the features of sperm (Tiersch and Yang, 2012), gonadal maturation, and spawning (Chang and Yueh, 1990). The perceived need to address the uniqueness of each aquatic species has consumed most research resources in cryopreservation protocol development (Tiersch, 2011a), but this dissertation took the initiative to generalize different protocols into a production line concept using typical major aquaculture industry as an example. The outcome of this dissertation can bring direct economic value to catfish aquaculture, and can serve as a template for other aquatic species.

The research model in this dissertation, the blue catfish *Ictalurus punctatus*, is a popular recreational fishing species widely distributed in the Mississippi river basin (Graham, 1999). Blue catfish is not commonly cultured as food fish, but the hybrid catfish arising from the crossing of channel catfish females and blue catfish males is in increasing demand because of high growth rate and disease resistance (Dunham and Masser, 2012). Hybridization is performed by artificially combining eggs stripped from females with a sperm suspension (Dunham and Masser, 2012) prepared each day by killing of the male and extraction of sperm directly from dissected testes (Avery et al., 2005). This limits the practical resources of blue catfish males to a single-time use and requires year-round maintenance in ponds or collection from the wild to obtain live males for spawning. Cryopreservation can help distribute the genetic resources of the fish through space and time in the form of frozen sperm and bring better control to hybrid

production by enabling highly efficient use of samples collected from killed males (Lang et al., 2003).

High-throughput processing

Aquaculture is a product-driven business. To be accepted by the public, prototype products have to be tested in large-scale application. In this dissertation, development of reliable production capability that matched the scale of commercial hatcheries was a challenge as important as process development. There are a variety of ways to achieve large-scale cryopreservation: by increasing the size of packaging containers (Cabrita et al., 2001, Chen et al., 2004, Ji et al., 2004, Munkittrick and Moccia, 1984); by managing layout and setup for intensive use of labor (Draper and Moens, 2009), and by introducing automation (Dong et al., 2005, Hu et al., 2011, Lang et al., 2003). High-throughput cryopreservation addresses both the quantity and quality of products. Depending on different real-life scenarios, if labor resources are not a constraint, training and layout design can yield increases in output; if labor is limited, increases in the size of packaging (e.g., bags) can reduce sample handling and operational error and increase the volme of ouput. However, to maximize efficiency, reduce human error, and produce uniform and reliable products, stable industrial processes typically utilize automation. This is true for the industries associated with domestic livestock sperm.

The use of automation can be dependent on available resources. One type of adopted automation is the computer-controlled freezer. Before such freezers were widely available, exposure to dry ice (Draper and Moens, 2009) or liquid nitrogen (Leibo et al., 2007) were utilized for manual freezing. Comparatively, programmable freezers (Cabrita et al., 1998) which automatically control and monitor the cooling process are more suitable for large batch sizes. Another process step in cryopreservation that can benefit from automation is packaging. There

are automatic printing machines (e.g., MIA Model, reference number: 017615, IMV Technology USA, Maple Grove, MN) available to label containers (e.g., plastic straws), There are also automated platforms to fill and seal 0.25-ml or 0.5-ml straws (e.g., MRS1 Dual, reference number: 020052, IMV Technology USA, Maple Grove, MN). To fully integrate automatic packaging, there are platforms available that combine those operations with barcode labeling and verification (e.g., MAPI, CryoBioSystem, Paris, France or CombiSystem MPP Uno and MiniJet Printer, reference number: 13017/0002, Minitube of America, Inc., Verona, WI). Although automated sperm collection is available for farm animals such as swine (e.g., automatic boar collection system, reference number: 021036, IMV Technology USA, Maple Grove, MN), these do not exist for aquatic species. Given this background availablility, this dissertation focused on adapting a fully automatic packaging system (MAPI, CryoBioSystem, Paris, France) and programmable freezer (MicroDigitCool, CryoBioSystem, Paris, France) for use with blue catfish sperm, which yielded the highest automation level thus far achieved for aquatic species.

Process quality assurance

Through a series of transformations, industrial processes convert raw materials to final products. In this dissertation, the raw materials were male fish; the intermediate products were dissected testes, and sperm suspensions, and the final products were cryopreserved straws of sperm (Hu et al., 2011, Liao et al., 2012). From an industrial engineering perspective, a process is a system with uncontrolled inputs, such as environmental conditions and incoming material properties, and measurable outputs known as quality characteristics that are measurements of process and product quality (Montgomery, 2008). Being aware of the inputs and outputs of a process is the beginning of process control.

To assure the quality of a process, it is necessary to regulate the variations of every quality characteristic. During process repetition, some variation among products is inevitable (Montgomery, 2008). These variations are described by quality characteristics. The process should have established tolerances on extreme values of quality characteristics based on the customer's expectation (Griffin and Hauser, 1993). For example, if the customer wants at least 50% motile sperm as a post-thaw product, and the sperm had no motility before freezing, there would be no reason to continue processing and investing resources in that particular sample. Therefore, "50%-motile sperm" can serve as a tolerance for the quality characteristics used before freezing. Tolerances of quality characteristics are also known as "specifications", which are used to define the acceptance levels for particular quality characteristics. With specifications, the process can avoid wasting energy on high variation caused by less-functional or non-functional materials (Creveling, 1997).

After the process operates under these specifications, the basic customer expectation can be satisfied. However, low-quality products will still appear at some rate within the specification range. To monitor the variability of products and identify low-quality products (Montgomery, 2008), the quality control concept has been introduced as a part of quality assurance. Quality control calculates the desired range of variation (quality control limits) based on the acceptance of quality variation and monitors every inspection result for possible outliers (Westgard et al., 1981). The identified outliers indicate unreliable quality and possible operational errors that need to be investigated. Therefore, a properly designed quality assurance program can not only assure product quality, but can also be used to improve quality (Thor et al., 2007).

Evaluation of hatchery production with cryopreserved sperm

Sperm quality can be a major source of variation in artificial spawning and typically is evaluated by features such as swimming behavior (Rurangwa et al., 2004, Suquet et al., 1992). After adaption of automated systems and quality assurance in sperm cryopreservation, and application at the commercial level, the products should provide a reliable resource with minimum quality variation. Artificial spawning with cryopreserved sperm could then be evaluated excluding variable factors from males or sperm. In other words, the male component can at least be standardized. In the industry for cryopreserved cattle semen, insemination of one dose (a single straw) is defined as a "service" (Cook, 2009). With the availability of this service, other evaluation methods became available such as "Estimated Relative Conception Rate" (ERCR) (Smith, 1985), "Calving Success", and "Calving to First Insemination" (Donoghue et al., 2004). Later on, ERCR was replaced by the more effective (Kuhn and Hutchison, 2008, Kuhn et al., 2008) "Sire Conception Rate" (SCR) (Norman et al., 2008). Following evaluation method development in cattle semen, comparable evaluation methods for fish could begin from setting standards for sperm usage to make the evaluation more sensitive to factors such as female (egg) variability or hatchery management factors during fry production (Dunham and Masser, 2012).

Simulation for optimization

Process simulation refers to the methods and applications that mimic the behavior of real-world systems (Kelton et al., 2007). It is a relatively new tool for applications in aquaculture and fisheries, and therefore a detailed introduction is presented here. Simulation and modeling have attracted the attention of many engineers in the last decade (Costamagna et al., 2002, Bhattacharjee et al., 2007, Adenso-Diaz and Lozano, 2008, Bruno et al., 2010) and fish production has been simulated during this time (Halachmi et al., 2005). Currently, the

establishment of a simple simulation model can no longer satisfy the requirement for comprehensive statistics. Extensive information collection from details of the model operation and mathematical analysis are required for validation and efficiency assessment of newly established models. Among a number of simulation softwares available, the application of simulation optimization is possible with only a few commercial software packages: AutoStat, OptQuest, OPTIMIZ, SimRunner, WITNESS Optimizer (Fu, 2002), and eM-Plant (Yang et al., 2007). However, the disadvantage of commercial software packages is their typical functioning as a "black box": the feasible solutions provided by the software cannot be determined to be the optimal solution (Fu, 2002). Other than simulation optimization software, some simulation methods such as: performance metrics (Li and Wang, 2007), Monte-Carlo simulation (Gutjahr, 2003), steady-state simulation (Kleijnen et al., 2004), and Markovian and stochastic simulation models (Gourgand et al., 2003) can also function as "data generators".

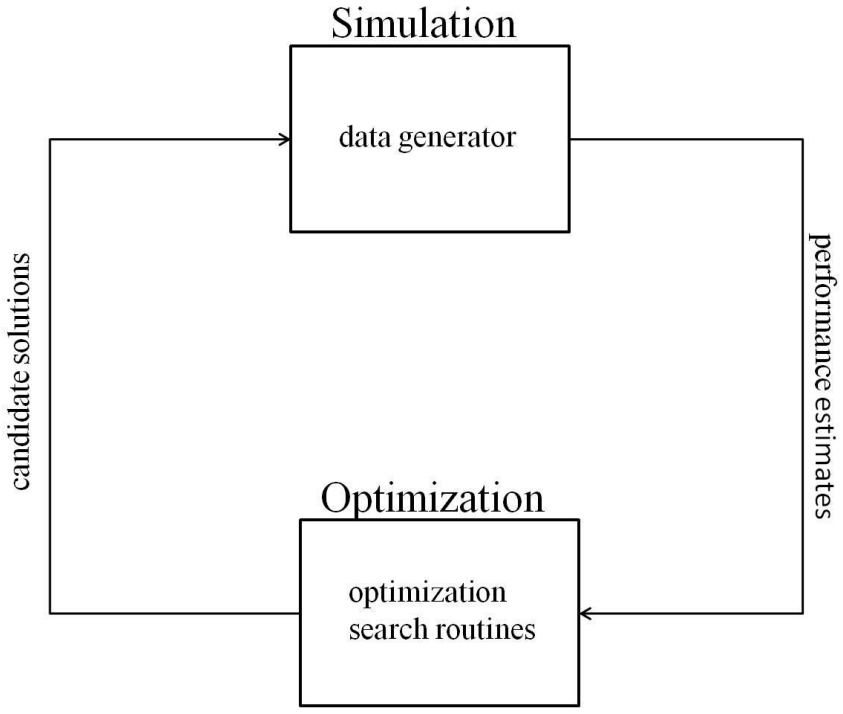

Figure 2.2. The simulation for optimization concept (modified from figures in Fu 2002). The performance estimates are based on statistical analysis.

The combination of optimization and simulation is considered as an efficient method because it provides a low-cost environment for comparison to physical experiments. In practice, this combination of capabilities performs as "optimization via simulation" (Fig. 2.2) (Fu, 2002). As mentioned above, the "data generator" provides performance estimates or statistical analysis based on the candidate solutions offered by the "optimization search routines". The performance estimates are used for evaluation and decision making for "optimization search routines" performed in further searches. In this cycle, commercial software focuses on the data generator so the optimum solution can be overlooked. In contrary, researchers rely on the existing simulation tools and models to select metaheuristic optimization which is a nontraditional, path-finding computing method (Berthiau and Siarry, 2001). Each solution from metaheuristic optimization goes through a control module in relative simulation model (Spieckermann et al., 2000). The balance between optimization and simulation is technology-dependent. The trade-off between the optimization level (number of metaheuristic optimizations involved, and more detailed constraints considered) and simulation feasibility (number of parameters involved, structural changes) (Fu, 2002), cannot be broken without significant improvement in computing capacity. Under current computing capabilities, an extra aspect can be attached to the data-generator-optimization cycle to optimize this trade-off: constant searching and comparison of input variables between the data generator and search routines, so the two sides of the cycle will be interactive and integrated (Fu, 2002).

Based on this description, the simulations in this dissertation are at the beginning stage. The process to be simulated was the entire high-throughput cryopreservation of blue catfish sperm, from transporting fish to the processing site through storage of cryopreserved products in a cryopreservation facility. The simulation software ARENA (Rockwell Automation, Inc.,

Milwaukee, WI) was chosen for this project, and contains a sub-program named OptQuest for advanced optimization (Kelton et al., 2007). The basic structure of the ARENA simulation model is a Create Model-Object, followed by a Process Model-Object, ending with a Dispose Model-Object (Fig. 2.3). The Create Model-Object simulates entities one by one and then entities go through Process Model-Object. Process Model-Object contains resources for process entities, and it defines the time for entities to move through the process. Frequency and time are recorded for each entity, and the entities are disposed at Dispose Model-Object to end the process. The recorded frequency and time create the basis of simulation results and provide information on system performance.

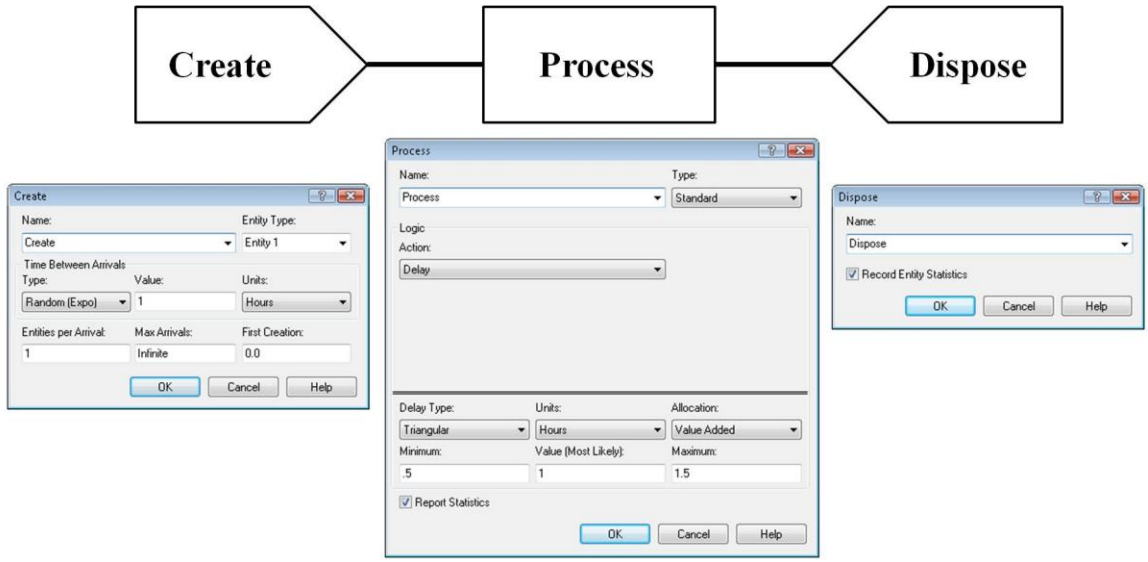

Figure 2.3. Basic structure of the ARENA simulation model. The simulation starts from Create Model-Objects to Process Model-Objects to Dispose Model-Objects. The setting boxes are presented under respective Model-Objects.

Supporting aspects for newly established production lines

The growth of a business requires a good publicity base. Unfortunately, high-throughput fish sperm cryopreservation is not known or understood by the majority of fish producers. According to theoretical interactions among social trust, benefit, risk, and willingness to buy,

increases in social trust can help with development of potential markets (Siegrist et al., 2007). A recent survey indicated that after the availability of the potential value of cryopreservation, producers were willing to pay for the transaction of this technology (Boever, 2006). Therefore, educating the public or specific potential markets becomes an important task. During this dissertation research, public communication was conducted in oral presentations (Table 1.1), personal discussions, site visits, on-farm trials, and written documentation to increase awareness among the public and private sectors about the availability and utility cryopreserved blue catfish sperm (Appendix B).

From the facility perspective, supporting information is also important for consistent production, such as facility layout (Kusiak and Heragu, 1987), supply lists, and Production Line Operation Sheets (Appendix E). As mentioned above, facility layout plays an important role in the management of production. During production, supplies must be accessible at all times to prevent unnecessary interruptions (Appendix C). Meanwhile, workers have to be trained according to specifications contained in the operation sheet to reduce human factors (Sanders and McCormick, 1987). Therefore, with the real-life goal of facilitating actual development and establishment of commercial-scale cryopreservation and enabling development of genetic resource management, this dissertation has provided a comprehensive ("one-stop shopping") approach to this work.

High-throughput cryopreservation will bring new challenges to inventory management. In laboratory-scale or local conservation-orientated cryopreservation, small six-canister storage dewars could provide sufficient storage capacity (Wayman, 2003). However, with larger scales such as dairy bull processing, the products require commercial-level inventory space and practices (Lang et al., 2003). Meanwhile, with expansion of conservation programs (Bart, 2002,

Holt and Moore, 1988), cryogenic storage demands a database that can crosslink biological and process information (Wayman, 2003) across wide expanses of time and distance. The data recording of processing and products has to be formatted for inventory management and as such, this was also addressed in this dissertation (Appendix D).

From laboratory to commercial-scale production

The cryopreservation of blue catfish sperm must be developed and evaluated biologically, and be enhanced by engineering to achieve reliable high-throughput production. The transition from laboratory to production facilities requires major changes in perspective. For example, all of the many research parameters must be condensed to a few straightforward indices so that workers can operate the process at maximum efficiency without comprehensive scientific training. However, pursuing large-scale production is not contradictory to aquaculture or cryobiology research. With the new availability of large amounts of newly generated data, aquatic genetic research can evolve to a new quantitative level, and provide much greater value to society.

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Chapter 3*
High-Throughput Cryopreservation of Spermatozoa of Blue Catfish (*Ictalurus furcatus*): Establishment of an Approach for Commercial-Scale Processing

Fish sperm is, in general, different from bull sperm in many ways. For example, the length of the bull sperm head is ~9 μm (Gravance et al., 1998), while the length of the fish sperm head in most common teleost species is 1 to 3 μm (Jamieson, 1991). Bull sperm held at ambient temperature in the presence of carbon dioxide can remain viable for days upon ejaculation (Chandler, 2000), while sperm of most freshwater fishes (such as of channel catfish and blue catfish) can remain motile for only 1 min or less after activation by hypoosmotic solutions (<150 mOsmol/kg) (Bates et al., 1996). Bull sperm function in an isotonic environment (~270 mOsmol/kg), but fish sperm, due to various spawning environments, become activated and motile across osmotic pressures ranging from 25 mOsm/kg to 1000 mOsm/kg depending on species. However, despite these differences, the sperm of livestock and fishes share enough features to provide opportunities for adaptation of mammalian cryopreservation technology and equipment for use with aquatic species.

Catfish production, despite problems with global competition and rising prices of feed and fuel in the last decade, remains the largest foodfish aquaculture industry in the United States (2005). Hybrid catfish created by crossing of female channel catfish (*Ictalurus punctatus*) and male blue catfish (*Ictalurus furcatus*) are in high demand by the industry because of their fast and uniform growth rate, disease resistance and efficient food conversion (Dunham and Masser, 2012). However, this hybrid is available only in limited numbers because blue catfish do not readily hybridize with channel catfish naturally (Dunham and Masser, 2012) due to biological

* The contents of this chapter were published prior to the completion of this dissertation (Hu et al. 2011. *Cryobiology* 62:74-82). Reprinted by permission.

differences including non-coincident times of peak spawning, and limited commercial availability of blue catfish males. Because induced spawning is required to produce the hybrid, sperm cryopreservation can be used to provide blue catfish sperm when female channel catfish are in peak spawning condition. To date, sperm cryopreservation of blue catfish has been studied in the laboratory; initial studies have been done to establish the feasibility of using commercial facilities dedicated to cryopreservation of dairy bull sperm for blue catfish sperm (Bart et al., 1998, Lang et al., 2003), and a basic approach to commercial-scale application has been generated (Fig. 3.1). However, to meet the large industrial demand for hybrid catfish fingerlings, an approach for high-throughput production of cryopreserved sperm is needed for use at facilities dedicated to aquatic species.

High-throughput cryopreservation has been widely applied in the dairy industry for decades (Pickett and Berndtson, 1974). Cryopreserved germplasm constitutes an independent industry used for animal breeding, preservation of genetic diversity, and medical research. However, there is currently no system for large-scale production of cryopreserved germplasm of aquatic species. Recently, a survey of fish culturists revealed a high demand for genetic improvement of the type that can be provided by cryopreservation (Boever, 2006), especially among hatcheries that produce hybrid catfish. Therefore, this study focused on the development of a high-throughput sperm cryopreservation approach for fishes, specifically addressing the problems of blue catfish.

Development of a high-throughput cryopreservation approach for fish using existing equipment requires several modifications. For example, an automated system for loading, sealing,

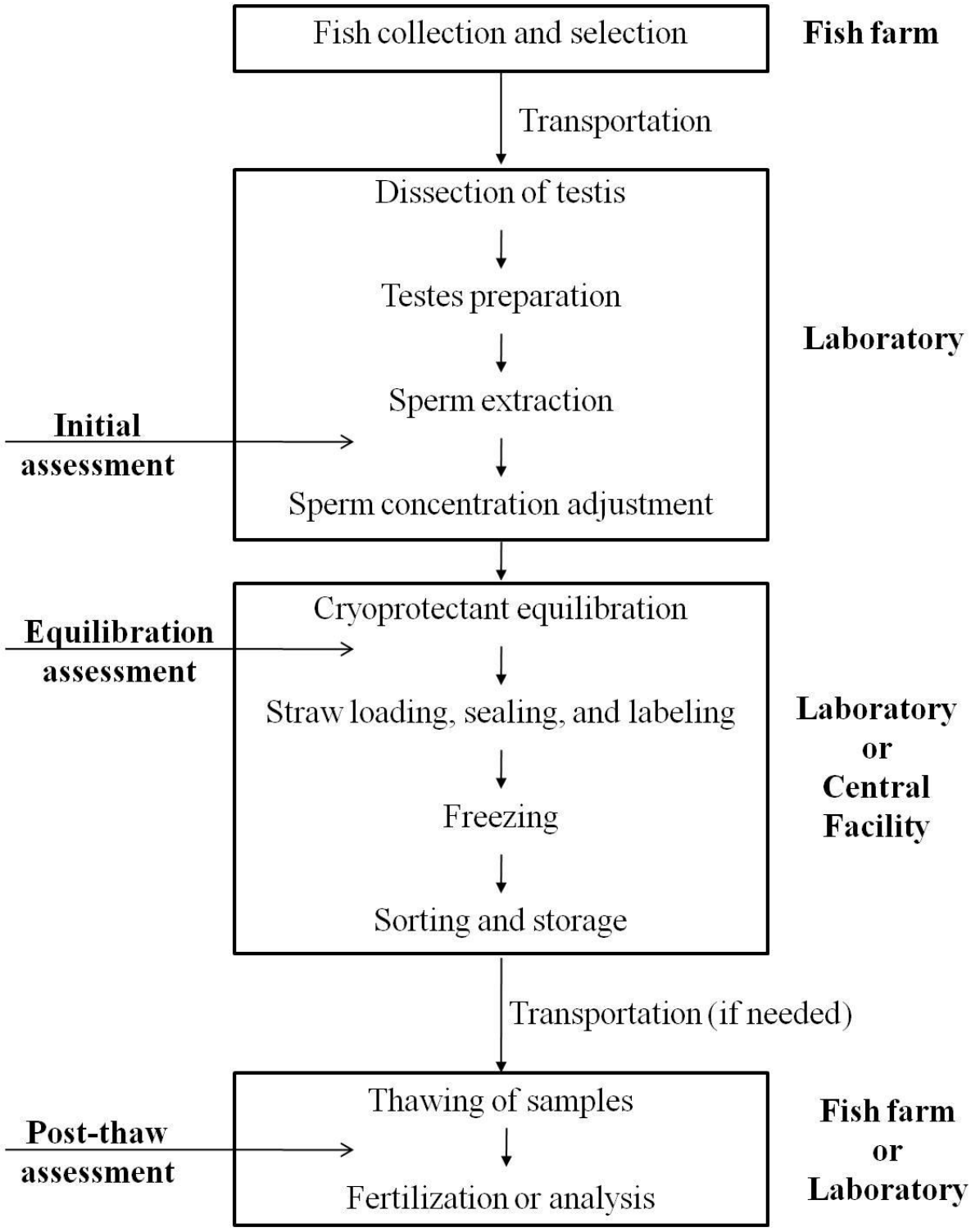

Figure 3.1. Overview of the sperm cryopreservation process used for blue catfish (*Ictalurus furcatus*). The motility assessment points are indicated on the left, and the locations of activities are shown on the right.

labeling, and reading of straws has been developed (MAPI system, CryoBioSystem Co. Paris, France) for sperm cryopreservation of livestock and humans. The sperm packaging required by the MAPI system is a specially designed 0.5-ml (CBS) straw with an external polyvinyl chloride identification jacket for labeling. The CBS straw is made from an ionomeric resin different from the traditional 0.5-ml French straw (IMV Technologies, Paris, France) which is made from polyvinyl chloride and polyethylene terephthalate glycol (Shaw and Jones, 2003). Thus, the cooling rate, and cryoprotectant type and concentration, require refinement for the CBS straw because the identification jacket and other differences in thermal properties can affect heat transfer compared to the 0.5-ml French straw used in previous trials (Christensen and Tiersch, 1997). Second, sperm concentration is an extremely important factor in sperm cryopreservation (Dong et al., 2007), but cell concentration has not been reported in previous publications on blue catfish (or for the vast majority of aquatic species studied) (Tiersch et al., 2007). In addition, from a commercial point of view, higher concentrations of sperm loaded into a single straw (the "product unit"), will lower the costs of packaging, storage, shipping, and use. Third, the thawing process is a critical factor that can affect the viability of cryopreserved sperm (Muldrew et al., 2004). Due to potential differences in thermal properties deriving from the use of the CBS straw for the MAPI system, thawing methods need to be evaluated as well.

The overall purpose of this research is to establish general protocols for high-throughput cryopreservation for aquatic species. The goal of this study was to develop practical approaches for commercial-scale sperm cryopreservation of blue catfish by use of an automated high-throughput system (MAPI, CryoBioSystem Co.). The objectives were to: 1) refine cooling rate and cryoprotectant concentration, and evaluate their interactions; 2) evaluate the effect of sperm concentration on cryopreservation; 3) refine cryoprotectant concentration based on the highest

effective sperm concentration; 4) compare the effect of thawing samples at 20 ℃ or 40 ℃; 5) evaluate the fertility of thawed sperm at a research scale by fertilizing with channel catfish eggs; 6) test the post-thaw motility and fertility of sperm from individual males in a commercial setting, and 7) test for correlation of cryopreservation results with biological indices used for male evaluation. In this study, a practical protocol for sperm cryopreservation of blue catfish was established by use of the high-throughput system. This is the first example of true commercially relevant production of cryopreserved sperm designed specifically for aquatic species rather than by adapting facilities designed for mammals, and opens the door for potential large-scale production of cryopreserved sperm for hybrid catfish production and for aquatic species in general.

Materials and methods

Fish

The male blue catfish (D&B strain, original from Crockett, TX) and channel catfish (current commercial stocks) used in this study came from Baxter Land Company Fish Farm (Arkansas City, Arkansas: 33°34’58.64”N, 91°15’18.45”W). Males with readily observable secondary sexual characteristics (e.g. well-muscled head, and dark coloration) were selected at the farm. After transport to the Aquaculture Research Station of the Louisiana State University Agricultural Center (Baton Rouge, 30°22’07.32”N, 91°10’27.90”W) in an oxygenated hauler in February, 2009, the fish were maintained in aerated outdoor 0.1-acre ponds and fed commercial diets (Aquaxcel, CargillTM, 45% protein). Two days before the start of experiments in April, the fish were captured by seining and moved into indoor tanks with a recirculating system. The bubble-washed bead filters were back-flushed every 2 d. The water quality parameters were: pH

7.0 – 8.0, ammonia 0.1 – 0.8 mg/l, nitrite 0.04 – 0.30 mg/l, alkalinity 39 – 125 mg/l, hardness 44 – 126 mg/l, temperature 28 ± 1 ℃, and dissolved oxygen 4.3 – 6.5 mg/l. Cryopreservation experiments were performed at the Aquaculture Research Station, and commercial-scale fertilization tests of individual male variance were performed at the hatchery of Baxter Land Co. Guidelines from the Institutional Animal Care and Use Committees (IACUC) of Louisiana State University were followed for animal care in this study.

Sperm Collection

Before dissection, fish were killed by a sharp blow to the head, and the body was rinsed with Hanks' balanced salt solution at an osmolality of 300 mOsmol/kg (HBSS300) to prevent fresh water on the fish from inadvertently activating the sperm during dissection (Avery et al., 2005). After measuring of the body weight and standard length, the fish were dissected and the testes were removed and placed into HBSS300 in a tared weigh boat (catalog number: 02-203-501, Fisher Scientific). After removal of blood and attached tissue, the testes were blotted with a paper towel and weighed. The anterior portion of each testis was separated and weighed for sperm collection (Sneed and Clemens, 1963). To suspend the sperm, the anterior portion of the testis was crushed in a quart Ziploc freezer bag ™ (S.C. Johnson & Son, Inc., Racine, WI) after addition of HBSS300 with a volume (ml) of two times the mass (g) of the anterior testis (Sneed and Clemens, 1963). 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-µ mesh filter. Samples from each male were processed separately. None of the samples were pooled.

Determination of sperm concentration

Cell concentrations were determined by measuring absorbance of the sperm suspensions with a microspectrophotometer (NanoDrop 1000, Thermo Scientific, Wilmington, DE), and calculation from an equation generated from our standard curve between the absorbance reading of serially diluted sperm suspensions and the sperm concentration as determined by hemocytometer (Tan et al., 2010) (Dong et al., 2007). Measurements were made using 2-µl aliquots at a wavelength of 601 nm. The equation used was:

$$\text{sperm concentration (cells/ml)} = \text{absorbance} \times 5.12 \times 10^{8} - 4.07 \times 10^{7} \ (R^2 = 0.960).$$

Motility estimation

Sperm motility was estimated by viewing through a dark-field microscope (Olympus CX41RF, Japan) at 200-× magnification. Fresh sperm suspension (1 µl) was placed on the slide, and was activated by mixing with 20 µl of filtered deionized distilled water (25 mOsmol/kg). Motility estimation was based on an observation of 3-5 different fields within 20 s after activation, and expressed as the percentage of sperm swimming progressively forward within the sample. The motility of sperm suspensions estimated within 2 hr of testis collection (without cryoprotectant) was considered to be the "initial motility", the motility of the sperm suspension at the end of the 30-min equilibration process (after addition of cryoprotectant) was the "equilibration motility" (Figure 1), and the motility of sperm after thawing was "post-thaw motility." Sperm suspensions with an initial motility of less than 40% were not included in these experiments (based on this, samples from 4 of the 21 males were excluded).

Sample preparation for cryopreservation using the automated system

After motility was estimated, the cryoprotectant was prepared in HBSS300 at twice the target concentration, and was mixed 1:1 (v/v) with the sperm samples to yield the desired final concentrations of cryoprotectant (5% and 10%) and desired sperm concentrations (ranging from 1×10^8 to 1.7×10^9 sperm/ml depending on experimental design). Upon mixing with cryoprotectant, the sperm samples were placed on the MAPI system, and filling, sealing and labeling of the straws were controlled by a proprietary computer program (SIDE, CBS). Samples were drawn into 0.5-ml CBS straws by vacuum applied from the cotton end of the straw, and the straws were continuously transferred to the sealing platform to seal both ends by application of 158 ℃ heat clamps. The sealed straws were labeled with alphanumeric information on the identification jacket with an ink printer (A400, Domino, IL, USA) before being transferred to the collection area for label verification, and quality control evaluation. For freezing, the straws were arrayed on horizontal racks (40 straws per rack) and placed in a commercial-scale programmable freezer (Micro Digitcool, IMV, France) with a capacity of 280 straws per freezing cycle. We did not attempt to equalize the thermal mass at every freezing (e.g. by adding "dummy" straws) and the number of straws in each freezing cycle ranged from 180 to 240 straws. At 30 min after addition of cryoprotectant to the sperm suspension, the cooling program was initiated. The cooling rate for this freezer can be programmed from 1 ℃ /min to 40 ℃/min (based on chamber temperature) and two rates were studied for this research (detailed below). When the final target temperature (-80 ℃) was reached and held for 5 min the frozen samples were removed and placed into liquid nitrogen. The individual straws were sorted under liquid nitrogen into 12-compartment storage containers (Daisy goblets, reference number: 015144, Cryo Bio System) for long-term storage in liquid nitrogen.

Egg collection and artificial fertilization

Channel catfish eggs were used for artificial fertilization. Two days before egg collection, the females were injected intraperitoneally with 100 μg/kg of luteinizing hormone-releasing hormone analog (LHRHa, Syndel Laboratories Ltd.) at the Aquaculture Research Station, or with 10 mg/kg common carp pituitary (lot numbers: 031109, 032209, 033109, Stoller Fisheries) at Baxter Land Co. based on existing techniques at each facility (Avery et al., 2005). Eggs were collected into HBSS300 in a greased (high vacuum grease, Dow Corning®, USA) bowl by gentle squeezing of the abdominal area. Individual eggs with full yellowish yolk and limited or no blood contamination were considered to be of good quality and were selected for artificial fertilization.

Small-scale (research) studies were performed at the Aquaculture Research Station. Eggs were placed into 100-ml tri-cornered polypropylene beakers (catalog number: 14-955-111B, Fisher Scientific) to form a monolayer at the bottom (yielding ~100 eggs). Frozen straws were removed from the liquid nitrogen storage Dewar and plunged into a 40 ℃ water bath for 20 s, and diluted to 1×10^8 sperm/ml by addition of 4.5 ml of HBSS300, and 1 ml of this sperm suspension was used to fertilize the 100 eggs in each beaker. To activate the gametes, 10 ml of water from the hatchery system were added to the beakers producing a final sperm concentration of 9×10^6 sperm/ml, and a sperm-to-egg ratio of 1×10^8 sperm/egg. An additional 10 ml of water was added 10 min later to assist final water-hardening (expansion) of the egg chorion. After an additional 10 min, the eggs were transferred into individual fully screened cups in a recirculating system at 27 to 29 ℃ for incubation and development (Bates and Tiersch, 1998). For fertilization evaluation, three beakers from each female were used for sperm samples from each individual male, and two females were used for each male.

Commercial-scale studies were performed at the Baxter Land Co. Fish Hatchery. Before sample transport, all frozen straws were sorted into Daisy goblets and grouped by males at the Aquaculture Research Station. All Daisy goblets were placed into shipping Dewars that had been filled at least twice with liquid nitrogen (AR1000, Cryoport, USA), and transported to the hatchery. During transportation, the Dewars were tightly positioned at the back of the vehicle. In the hatchery during spawning, batches of eggs collected from individual females were divided into two aliquots with a volume of 150-200 ml each (average 40 eggs/ml): one for thawed sperm, and one for fresh blue catfish sperm (used to evaluate egg quality). Frozen sperm were thawed at 40 ℃ for 20 s, and four straws (a total of 2 ml suspension with 1×10^9 sperm/ml) were used to fertilize each aliquot of eggs. Each day, fresh sperm samples were collected from male blue catfish that came from the same population used for the frozen sperm samples, and were stored in a refrigerator (4 ℃) at a concentration of 1×10^9 sperm/ml. The same volume (2 ml) of sperm was used to fertilize each aliquot of the eggs. The final sperm concentrations were therefore $1 - 1.3 \times 10^7$ sperm/ml, and the sperm-to-egg ratio was $2.5 - 3.3 \times 10^5$ sperm/egg. The eggs were held in 25-cm metal pie pans, and after addition of the sperm, water (1 L) from the hatchery system was added to the pans to activate the gametes for fertilization. After 20 min, eggs that had adhered normally into masses were moved into individual mesh baskets in a flow-through system for embryo development and incubation following routine hatchery procedures (Steeby and Avery, 2005). For sperm samples from each blue catfish male, eggs from 5 to 8 females were used as replicates. A total of 71 females were tested, and more than 21 L of eggs were used for fertilization trials.

At 27 to 30 hr after fertilization at 27 to 29 ℃, the neurula stage covers the yolk sac and segmentation of trunk mesoderm is initiated in the embryo (Stage V) (Saksena et al., 1961,

Makeeva and Emel'yanova, 1993, Small and Bates, 2001). The neurulated embryos were counted by viewing with the naked eye and 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"). This number was determined for both aliquots of eggs from each female representing the control (fertilized with fresh sperm) and thawed sperm. The Stage VI embryos (initiation of embryonic mobility) (Saksena et al., 1961, Makeeva and Emel'yanova, 1993, Small and Bates, 2001) were counted at 48-50 hr after fertilization. Because there was no way to maintain separate groups of hatched larvae (sacfry) in the commercial hatchery, hatch rate was estimated as the percentage of Stage VI embryos in relation to the total number of eggs.

Study I: Refinement of cooling rate and cryoprotectant

Two concentrations of methanol (5 and 10%; v/v) were tested, because methanol has previously been used in our laboratory for sperm cryopreservation of blue catfish (Lang et al., 2003) and channel catfish (Christensen and Tiersch, 1997, Christensen and Tiersch, 2005). For each concentration of methanol, two cooling rates 5 ℃/min, and 40 ℃/min) were used to bring the samples from 5 to -80 ℃. For these experiments, the final concentration of the sperm suspensions was held within the range of 1×10^8 sperm/ml to 1×10^9 sperm/ml. Three replicates were produced from sperm of each of three individual males. Three straws from each treatment and each male were thawed in a water bath at 40 ℃ for 20 s and post-thaw motility was evaluated as described above.

Study II: Effect of sperm concentration on cryopreservation

In this experiment, sperm samples were frozen with 10% methanol as cryoprotectant and a cooling rate of 5 ℃/min. In the first trial, two sperm concentrations (1×10^8 sperm/ml and $1 \times$

10^9 sperm/ml) were tested, using sperm for three individuals as replicates. In the second trial, two concentrations (1×10^9 sperm/ml and 1.7×10^9 sperm/ml) were tested from another three individuals as replicates. From these, three straws from each treatment were thawed as described in Study I and motility was assessed as described above.

Study III: Refinement of methanol concentration based on the maximum sperm concentration

Sperm samples from six individuals were used for this experiment with a final sperm concentration of 1×10^9 sperm/ml (based on Study II). The methanol concentrations tested were 5% (1.23 mol/L) and 10% (2.46 mol/L). All samples were frozen at a cooling rate of 5 ℃/min from 5 to -80 ℃. From these, three straws from each treatment were thawed as described in Study I and motility was assessed as described above.

Study IV: Effect of thawing temperature on post-thaw motility

Sperm samples from three individuals were used in this experiment at a final concentration of 1×10^9 sperm/ml and were frozen with 10% methanol as cryoprotectant and a cooling rate of 5 ℃/min. To test the effect of thawing temperature, three straws from each male were thawed in a water bath at 40 ℃ for 20 s, or at 20 ℃ for 40 s. Post-thaw motility was assessed as described above.

Study V: Fertilization of cryopreserved blue catfish sperm by crossing with channel catfish eggs

Sperm from three blue catfish were cryopreserved at a final concentration of 1×10^9 sperm/ml with 10% methanol as cryoprotectant and a cooling rate of 5 ℃/min from 5 ℃ to -80 ℃. In addition, sperm from three channel catfish were frozen using the same protocol. Frozen samples from both species were thawed at 40 ℃ for 20 s, and used to fertilize eggs from

two female channel catfish as described above for research-scale artificial fertilization at the Aquaculture Research Station.

Study VI: Effect of male-to-male variation on post-thaw motility and fertility

Sperm were collected from ten blue catfish in May, 2009. Sperm samples at a final concentration of 1×10^9 sperm/ml were cryopreserved with 10% methanol at a cooling rate of 5 ℃/min from 5 ℃ to -80 ℃. Frozen samples (N = 276 straws) were thawed at 40 ℃ for 20 s, and used at Baxter Land Co. to fertilize eggs collected from five to eight blue catfish females for each male as described above for artificial fertilization. The thawed samples were held at 4 ℃ and used within 12 hr after thawing. Previous studies have held thawed sperm of channel catfish at 4 ℃ for more than 7 d [13].

Study VII: Correlation within biological indices

For the 10 males used in Study VI, correlation analysis was performed among the biological characteristics (body weight, body length, testis weight, anterior testis weight), gonadosomatic index (GSI) body condition factor (BCF), initial motility of fresh sperm, post-thaw motility, and post-thaw fertility (neurulation percentage). The GSI was calculated as the percentage of testis weight (g) in relation to body weight (g) (Testis weight (g) / Body weight (g) x 100%), and the BCF was calculated as the percentage of body weight (g) in relation to the cube of the body length (cm) (Body weight (g) / Body length^3 (cm) x 100%).

Data analysis

Data were organized using Microsoft Office Excel 2007, and analyzed with the GLM, CORR program and ANOVA t-test (Statistical Analysis System, version 9.0, 2002). Percentage

data were arcsin-square-root transformed before analysis. Correlation analysis was performed using SAS 9.0. Differences were considered significant at $P < 0.050$.

Results

Study I: Refinement of cooling rate and cryoprotectant

The initial motility of fresh sperm from 5 males was 69 ± 5% (mean ± SD). There were no significant differences between initial motility and equilibration motility (after 30 min with cryoprotectant) ($P = 0.915$). Post-thaw motility decreased significantly compared to equilibration motility ($P < 0.001$). The concentration of cryoprotectant ($P < 0.001$) and cooling rate ($P < 0.001$) each significantly affected post-thaw motility. The highest post-thaw motility was achieved using 10% methanol (v/v) with a cooling rate of 5 ℃/min (24 ± 8%), followed by 5% methanol at 5 ℃/min (16 ± 5%), 10% methanol at 40 ℃/min (4 ± 2%), and finally 5% methanol at 40 ℃/min (1 ± 1%).

Study II: Effect of sperm concentration on cryopreservation

The initial motility of fresh sperm from 6 males was 59 ± 11%. In the first trial, no significant difference in post-thaw motility was found between sperm cryopreserved at 1×10^8 cell/ml (17 ± 7%) and sperm cryopreserved at 1×10^9 sperm/ml (22 ± 8%) ($P = 0.240$). In the second trial, post-thaw motility of sperm cryopreserved at 1×10^9 sperm/ml (30 ± 10%) was significantly higher than that at 1.7×10^9 sperm/ml (4 ± 5%) ($P < 0.001$). In the first trial there was no significant difference ($P > 0.050$) between initial motility and equilibration motility, but there was for the second trial ($P = 0.041$).

Study III: Refinement of methanol concentration based on the maximum sperm concentration

The initial motility of fresh sperm from 5 males was 69 ±5%. With the selected sperm concentration of 1×10^9 sperm/ml, post-thaw motility was higher when sperm were cryopreserved with 10% methanol (35 ±6%) than that with 5% methanol (16 ±8%) ($P < 0.001$). There was no significant difference in equilibration motility between 5% methanol (59 ±2%) and 10% methanol (63 ±5%) ($P = 0.160$).

Study IV: Effect of thawing temperature on post-thaw motility

The initial motility of fresh sperm from 3 males was 50 ±7%. The temperatures of 20 ℃ and 40 ℃ did not produce a significant difference in post-thaw motility of cryopreserved sperm (32 ±9% vs. 28 ±11%) ($P = 0.415$). Post-thaw motility at each thawing temperature was significantly lower than initial motility (51 ±6%) ($P < 0.001$).

Study V: Fertilization of channel catfish eggs with cryopreserved blue catfish sperm

The initial motility of fresh sperm from 3 males was 52 ±1%. Fresh blue catfish sperm yielded 17 ±10% neurulation (Stage V), and 53 ±37% initiation of embryo motility (Stage VI); fresh channel catfish sperm yielded 20 ±16% neurulation, and 41 ±32% Stage VI embryos. No differences were found between the fresh sperm of blue catfish and channel catfish, indicating that the fertilization potential of blue catfish sperm with channel catfish eggs is comparable to that of channel catfish sperm. With the selected protocol, the post-thaw motility of blue catfish sperm was 28 ±1%. After fertilization, the percentage of neurulation was 80 ±21%, and percentage of Stage VI embryos was 51 ±22% after normalizing by considering the fertility with fresh sperm as 100% (not all eggs are fertilizable). For a parallel comparison, there were no differences between cryopreserved blue catfish sperm and cryopreserved channel catfish sperm

in post-thaw motility (12 ±11%) ($P = 0.155$), percentage of neurulation (75 ±21%) ($P = 0.476$), and percentage of Stage VI embryos (67 ±29%) ($P = 0.169$).

Study VI: Effect of male-to-male variation on post-thaw motility and fertility

The initial motility of fresh sperm from 10 individuals was 52 ±9% (Table 3.1). After thawing, the post-thaw motility was 31 ±12% (Table 3.1), which was significantly lower than the initial motility ($P = 0.040$). Among these individual males, there was no significant difference in initial motility ($P \geq 0.560$) or post-thaw motility ($P \geq 0.207$). Fertilization of thawed sperm from these 10 males with eggs from channel catfish showed an average neurulation of 38 ±17% (absolute numbers) (Fig. 3.2), which was significantly lower than that for the egg quality test with fresh sperm (45 ±16%) ($P = 0.009$). Among the 10 individuals, the fertility of thawed sperm (in terms of percentage of neurulation) was not different ($P = 0.767$).

Study VII: Correlations with biological indices

The correlation analysis of initial motility, post-thaw motility, fertility, and the biological indices showed significant relationships among these factors (Tables 3.1 and 3.2). Significant correlations were found between initial motility and post-thaw motility ($P = 0.048$), and between post-thaw motility and neurulation ($P = 0.005$). The initial motility and neurulation were not correlated ($P = 0.059$). Anterior testis weight was correlated with total testis weight ($P < 0.001$), and anterior testis weight ($P = 0.014$) and total testis weight ($P = 0.005$) were each correlated with body condition factor. As would be expected, body length was correlated with body weight ($P < 0.001$).

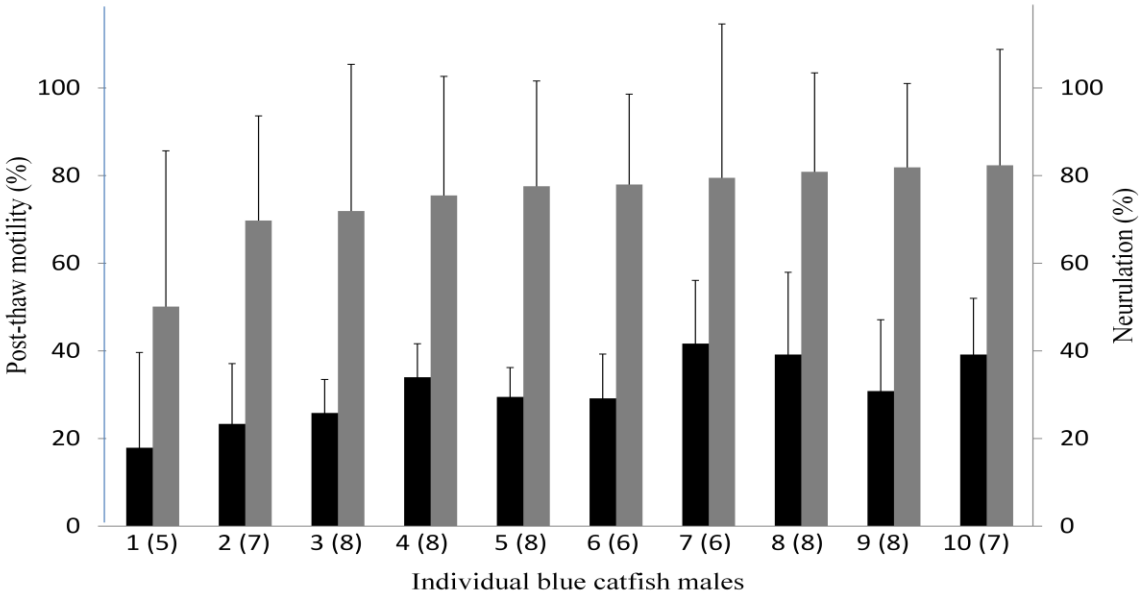

Figure 3.2. Sperm from ten blue catfish (*Ictalurus furcatus*) cryopreserved using 1×10^9 sperm/ml with 10% methanol with a 5 ℃/min cooling rate. Sperm were thawed and used to fertilize eggs from 5 to 8 females, (the number of females is indicated within parentheses). Post-thaw motility (dark bars) and neurulation (light bars) were used to evaluate male-to-male variation. The error bars represented standard deviations. Among the 10 individuals, the post-thaw motility ($P \geq 0.207$) and the fertility of thawed sperm (percent neurulation) ($P = 0.767$) were not significantly different.

Discussion

Development of protocols for high-throughput sperm cryopreservation in fish

Cryopreservation of sperm was first achieved 60 yr ago for livestock (Polge and Rowson, 1952) and fish (Blaxter, 1953). Since then a multi-billion dollar global industry has developed for cryopreserved livestock germplasm, but no corresponding industry exists for aquatic species which currently rely on live populations to provide genetic improvement. Specialized equipment and facilities that have been developed for high-throughput processing of livestock and human sperm are available for adoption with aquatic species.

Table 3.1. Values (mean ± SD) and indices measured from ten blue catfish (*Ictalurus furcatus*) males. Body condition factor (BCF) was calculated as the percentage of body weight (g) to the cube of the body length (cm). Gonadosomatic index (GSI) was calculated as the percentage of testis weight to the body weight. The initial concentration of the sperm suspension was measured after crushing and filtering*. "Initial motility": motility of the sperm suspension prior to addition of cryoprotectant; "equilibration motility": motility of the sperm suspension after equilibration time with cryoprotectant (30 min); "post-thaw motility": motility after thawing at 40 °C for 20 s; Neurulation: the percentage of Stage V embryo produced with thawed sperm normalized by comparing to the percentage of Stage V embryos produced with fresh sperm.

Male	BCF	GSI	Initial concentration (sperm/ml)	Initial motility	Equilibration motility	Post-thaw motility	Neurulation
1	0.012	0.288	1.68×10^9	43%	43%	30 ± 7%	78 ± 24%
2	0.011	0.226	1.72×10^9	55%	58%	34 ± 8%	75 ± 27%
3	0.011	0.196	9.13×10^9	40%	40%	18 ± 22%	50 ± 35%
4	0.012	0.213	3.73×10^9	45%	45%	29 ± 10%	78 ± 21%
5	0.009	0.205	3.10×10^9	53%	43%	39 ± 13%	82 ± 26%
6	0.011	0.377	2.15×10^9	63%	45%	42 ± 14%	79 ± 35%
7	0.011	0.191	1.93×10^9	50%	45%	26 ± 8%	72 ± 33%
8	0.011	0.117	2.42×10^9	55%	45%	39 ± 19%	81 ± 23%
9	0.011	0.243	2.26×10^9	48%	38%	23 ± 14%	70 ± 24%
10	0.011	0.340	1.15×10^9	68%	58%	31 ± 16%	82 ± 19%

* Sperm were collected by crushing the testis in a 2:1 ratio of extender (ml):anterior testis weight (g).

Table 3.2. Pearson correlation coefficients among body and gonadal indices of blue catfish (*Ictalurus furcatus*) males (n = 10). The values listed in each cell (except for those with a single number) are correlation coefficient (above) and *P* value (below). BCF: body condition factor was calculated as the percentage of body weight (g) to the cube of the body length (cm); GSI: gonadosomatic index was calculated as the percentage of testis weight to the whole body weight. The initial concentration of the sperm suspensions was measured after crushing and filtering.

	weight	Length	Anterior testis	Testis	BCF	GSI	Initial concentration	Initial motility	Post-thaw motility	Neurulation
		0.966	0.131	0.266	-0.533	-0.236	0.165	-0.395	-0.366	-0.393
Weight	1.000	<.001 *	0.717	0.458	0.113	0.512	0.648	0.259	0.298	0.261
			0.011	0.163	-0.497	-0.321	0.06	-0.388	-0.378	-0.49
Length		1.000	0.975	0.652	0.144	0.367	0.87	0.268	0.281	0.151
				0.985	-0.74	0.362	0.12	0.515	0.463	0.336
Anterior testis			1.000	<.001 *	0.014*	0.304	0.741	0.127	0.178	0.343
					-0.807	0.294	0.084	0.456	0.384	0.242
Testis				1.000	0.005 *	0.409	0.817	0.185	0.274	0.5
						0.162	-0.127	-0.275	-0.353	-0.13
BCF					1.000	0.655	0.727	0.442	0.318	0.721
							-0.275	0.487	0.171	0.241
GSI						1.000	0.442	0.154	0.636	0.503
Initial concentration								-0.112	0.401	0.489
							1.000	0.757	0.251	0.152
									0.635	0.614
Initial motility								1.000	0.048*	0.059**
Post-thaw motility										0.807
									1.000	0.005 *
Neurulation										1.000

* values with $P < 0.050$; ** values with $0.050 < P < 0.100$.

To adapt high-throughput cryopreservation technology to fishes, the first task was to establish a reliable and practical protocol for sperm cryopreservation by use of an automated system. In this study, the system selected for sample filling, sealing, and labeling requires a specifically designed high-biosecurity straw (CBS straw) which possesses an outer identification jacket and is manufactured using different materials compared to the traditional 0.5-ml French straw (used in previous catfish publications) which results in differences in flexibility and thermal properties between the straw types. Structurally, use of the CBS straw for sperm cryopreservation interferes with heat transfer, and consequently affects the cooling and thawing rates of samples, which are two critical factors for sperm viability that are interact with cryoprotectant type and concentration (Yang et al., 2010). Also, in comparison with previous publications addressing cryopreservation of catfish sperm (Guest et al., 1976, Withler, 1982, Tiersch et al., 1994, Bart and Dunham, 1996, Christensen and Tiersch, 1997, Bart et al., 1998, Viveiros et al., 2000, Lang et al., 2003, Christensen and Tiersch, 2005, Linhart et al., 2005, Bobe and Labbe, 2008), only one (Tiersch et al., 1994) reported the estimated sperm concentration. To develop a high-throughput protocol, sperm concentration is a required factor to consider because it controls the yield in straw production; dictates the time and space requirements for storage, shipping, and use of samples, and the sperm concentration can directly affect sperm viability during cryopreservation (Dong et al., 2005b, Tiersch et al., 2007). Therefore, at a minimum, development of protocols for high-throughput sperm cryopreservation must address the cooling rate within the container (i.e. specific straw type), type and concentration of cryoprotectant, and sperm concentration.

Faced with the multi-factorial interactions among cooling rate, sperm loading concentration, and cryoprotectant concentration, we chose in this study to use previous information from our laboratory and to sequentially tease apart the interactions rather than utilize a large multifactorial experimental matrix. In this study, the cooling rate was determined first, followed by the loading concentration, and then the cryoprotectant concentration. The differences between cryoprotectant concentrations of 5% and 10% methanol became statistically significant when these results were integrated. Sperm motility values at the end of the 30-min equilibration period were reported to differentiate the effect of the freezing and thawing processes (rather than combining them with potential toxicity effects encountered before freezing). However, in this study, the lack of a significant difference between initial motility (at collection) and equilibration motility (after addition of cryoprotectant) indicated that methanol at either concentration produced little or no toxic effects (at least in regard to motility) prior to freezing.

Channel catfish and blue catfish are closely related members of the North American genus *Ictalurus* (Nelson et al., 2004). Both species possess 58 chromosomes (Zhang et al., 1999) and the hybrid of these species is fertile and can produce F_2 and subsequent generations, or back cross to either parental species (Tiersch and Goudie, 1993). Mature blue catfish are larger than channel catfish and are considered to be too large to serve as routine broodstock for catfish aquaculture. Otherwise the gametes of these species are very similar in structure and function (Tiersch et al., 1994, Lang et al., 2003), and our unpublished observations]. In a previous study of channel catfish, sperm cryopreservation with 0.5-ml French straws showed that freezing at 3 ℃/min and 45 ℃/min each yielded satisfactory post-thaw motilities (Christensen and Tiersch, 2005). In the present study, freezing at 5 ℃/min retained higher post-thaw motility than

40 ℃/min with CBS straws. Also, interaction effects of cryoprotectant concentration and cooling rate were detected in this study. In a previous study, 5% methanol and French straws were used for channel catfish cryopreservation. However, the combination of 5% methanol and the CBS straw was not as effective as the combination of 10% methanol and CBS straw at either cooling rate. This could be because: 1) higher sperm concentrations were used for freezing in the present study, which required the use of a higher concentration of cryoprotectant; 2) 10% methanol would be more effective than 5% methanol in decreasing the freezing point of the solution (Muldrew et al., 2004), and 3) a lower freezing point provided more protection from intracellular ice formation during freezing, especially when coupled with slower cooling rates (Mazur, 2004).

Sperm concentration is an important factor for cryopreservation, and is commonly ignored by most researchers for aquatic species (Tiersch et al., 2007); (Dong et al., 2007). In this study, comparison of sperm concentrations showed significant differences in post-thaw motility. To maintain a high sample capacity for each straw, a sperm concentration of 1×10^9 sperm/ml was chosen. Also, determination of sperm concentration is a necessary component for standardizing protocols to yield reliable products especially for high-throughput production. More importantly, control and refinement of sperm number can greatly improve the efficiency of use of cryopreserved sperm during artificial fertilization. At the same time, choice of the concentration of sperm in straws depends on a balance between the biological characteristics of the species and the demands of practical utility and economics. For sperm of Pacific oyster *Crassostrea gigas*, the fertility decreased because of increased sperm concentration at given cryoprotectant concentrations (Dong et al., 2007), and a concentration of 1×10^8 fresh sperm/ml was chosen in that study as the maximum concentration to maintain highest biological function by avoiding sperm agglutination (head-to-head aggregations elicited by an acrosome reaction)

(Dong et al., 2007). A previously recommended sperm-to-egg ratio for production of catfish hybrids was 1.25×10^5 fresh sperm per egg (Bart and Dunham, 1996). In the present study, best results were obtained with 1.35×10^5 motile sperm per egg using post-thaw sperm (Tiersch et al., 1994). When freezing a large volume of samples, the use of higher loading concentration in each straw is an efficient way to reduce the costs of straw packaging, storage space, and shipping. Also, because the condensed sperm can easily be diluted to a final working concentration without forming agglutination in blue catfish, a relatively high straw loading concentration (1×10^9 cell/ml) was used in this study. Most of the previous research of catfish have yielded satisfactory results in the laboratory based on estimated concentrations of around 1×10^8 sperm/ml (our estimations of the mass of testis per volume of extender reported) (Tiersch et al., 1994). In this study we tested the maximum loading concentration, and found that 1×10^9 sperm/ml was not different from 1×10^8 sperm/ml in post-thaw motility, but motility dropped significantly with less than a doubling of this maximum concentration to 1.7×10^9 sperm/ml. The sperm solutions became viscous, perhaps decreasing the capacity for molecular movement (Dong et al., 2007) or simply exceeding the cryoprotectant capability per unit concentration of the available cryoprotectant. Based on these results, 1×10^9 sperm/ml was selected as the optimal loading concentration for blue catfish sperm for high-throughput cryopreservation.

The effect of thawing temperature

Thawing rate can be as important as the cooling rate, in terms of being critical factors that affect the viability of cryopreserved sperm (Christensen and Tiersch, 2005). In previous publications on blue catfish, the thawing method used for samples in 0.5-ml French straws was 40 ℃ for 7 s (Lang et al., 2003, Christensen and Tiersch, 2005). This method was originally applied for mammalian sperm (Pickett and Berndtson, 1974). In the present study, samples

within CBS straws required 20 sec for thawing at 40 ℃. When samples were heated in a 20 ℃ water bath, complete thawing required 40 s. For practical purposes, it is more convenient and economical to use city (tap) water rather than heating in a water bath for thawing. The results showed that thawing at 20 ℃ or 40 ℃ did not affect post-thaw motility, but if time is limited, 40 ℃ should be considered, especially when large numbers of samples need to be processed because of time constraints in the hatchery.

Quality management of sperm for high-throughput cryopreservation

Motility is a direct and convenient index to evaluate sperm quality and viability. In this study, the viability of sperm after cryopreservation was evaluated by post-thaw motility and the percentages of neurulated (Stage V) and actively mobile (Stage VI) embryos (which spin within the chorion). Although cryopreservation decreased the fertility of sperm from blue catfish and channel catfish, the difference between the species was not significant. This suggested that by using the same cryopreservation techniques for sperm for artificial fertilization, the same number of fertilized (hybrid) eggs could be produced with blue catfish sperm as when fertilizing eggs with channel catfish sperm. In addition, the estimated hatching rates (Stage VI embryos) were not different when making hybrids using cryopreserved sperm or using fresh sperm. Thus cryopreserved sperm could serve as an alternative when fresh sperm is not available, and the results demonstrate the potential commercial application of cryopreserved blue catfish sperm for production of hybrid catfish.

Male-to-male variation in post-thaw motility and fertility

Male-to-male variation for cryopreserved sperm is commonly observed in species studied thus far including mammals, fishes, and invertebrates (Christensen and Tiersch, 2005, Yang et al.,

2007, Mazur et al., 2008, Yang et al., 2009), although the underlying mechanisms remain unclear. Unlike previous reports, no significant variation was observed in post-thaw motility and fertility among the ten males used in this study to evaluate the final protocol. This is a desirable result for high-throughput technology because it can provide products with consistent and predictable output. Possible explanations for the lack of male-to-male variability in this study include: 1) a small sample size of ten males (although comparable to previous studies); 2) unknown attributes of the biology of blue catfish; 3) tight standardization of sperm concentration for each straw which limited variation in response to toxicity or cryopreservation, and 4) the setting of threshold of initial motilities (>40%) at the beginning of the process which reduced the variability of fresh sperm quality (although comparable to previous studies). Although these factors alone or in combination could explain the low observed variability we propose that the use of a tightly standardized protocol (especially for sperm concentration given that the majorly of previous studies in aquatic species did not control this critical variable) would be consistent with production of a predictable and consistent range of post-thaw quality.

Future work should address selection of broodstock for sperm collection. Broodstock blue catfish males are large (typically 3 - 20 kg) and are at least 4-5 yr old. As such, they are individually valuable and it is highly desirable to only kill males with well-developed testes. In this study, blue catfish body length and weight were highly correlated. The negative coefficient between body condition factor and testis weight indicated that long thin fish had proportionately bigger testes than did rounder fish, and perhaps there is an energetic trade-off between somatic growth and reproductive fitness. The correlations among initial motility, post-thaw motility and neurulation indicated that fresh sperm suspensions with higher percentages of motile sperm would be more tolerant to cryopreservation, and have higher post-thaw motilities and fry

production rates. In a previous study of cryopreservation of channel catfish sperm (Christensen and Tiersch, 2005) there was no relationship between pre-freeze motility and post-thaw motility among 50 males with initial motilities of 70% or greater (high quality for catfish sperm collected by crushing of the testis). This seems to be in conflict with the correlation found between initial and post-thaw motility in the present study. However, close examination of the methods reveals that despite using only high-quality sperm samples, the previous study used hand-processing of straws and undetermined and uncontrolled sperm concentrations. From this perspective, the conclusion from previous publications supports the results from this study: standardization of protocols, especially sperm numbers, will yield consistent and predictable quality. Further studies focusing on hatchery management for hybrid catfish could establish quantified relationships among factors such as motility before freezing and after thawing, neurulation rates, and hatching, which would be of value for commercial use in sample selection.

It is ironic that although there is considerable potential similarity between the processing of bull semen and fish sperm in principles, procedures, and technology, there has been little effort to adapt the livestock model for automated processing to aquatic species (Dong et al., 2005a). Cryopreserved bull semen has been far more widely accepted within agricultural production than cryopreserved fish sperm. Bull owners were willing to use cryopreserved sperm because they historically had breeder's clubs for example, and shared access to high-value sires (Chandler, 2000). Fish breeders have typically utilized mass selection of populations or families (Tave, 2000) disregarding the breeding value of individuals and this has delayed application of cryopreserved products especially for species that can produce sufficient offspring each year simply by natural pond spawning (such as channel catfish) to meet demands for seedstock. As such, commercial-scale cryopreservation facilities do not yet exist for aquatic species, but with

development of high-throughput facilities, sperm samples or fish can be transported for cryopreservation, and be shipped back to the original farm hatchery or laboratory, a third-party customer, or to germplasm repositories for storage or use (Tiersch et al., 2004). The economics of establishing or integrating these capabilities into an existing fish hatchery have been investigated (Caffey and Tiersch, 2000a, Caffey and Tiersch, 2000b), and germplasm repositories such as the USDA National Animal Germplasm Program are in operation (http://www.ars.usda.gov).

Conclusions

In summary, this study established a reliable and practical protocol for sperm cryopreservation of blue catfish by use of a commercial-scale automated straw processing system, and demonstrated the feasibility for high-throughput sperm cryopreservation for catfish. The detailed protocol was: 1) collection of sperm by crushing of dissected anterior testis in HBSS300 at a volume (ml) of 2 times the testis weight (g); 2) filtration of the sperm suspensions (down to 200-μm mesh size) to remove large pieces of tissue; 3) determination of sperm concentration and adjustment to 2×10^9 sperm/ml; 4) mixing of 20% methanol in HBSS300 with an equal volume of sperm suspension to yield a final concentration of 10% methanol and 1×10^9 sperm/ml; 5) filling, sealing, and labeling of 0.5-ml CBS straws by the automated MAPI system; 6) freezing of samples in straws on horizontal racks in a programmable freezer at 5 ℃/min from 4 ℃ to -80 ℃ (programmed using chamber temperature) and holding for 5 min; 7) removal of frozen samples and plunging them into liquid nitrogen for sorting into multi-compartment goblets under liquid nitrogen; 8) storage of the frozen samples in liquid nitrogen in storage Dewars; 9) transportation of the samples if necessary in vapor-phase shipping Dewars to the site of usage;

10) thawing of straws at 40 ℃ for 20 s in a water bath, and 11) release of thawed samples from the straws for use in fertilization or analysis within 12 hr of thawing. In addition to post-thaw motility, channel catfish eggs provide an alternative option to test cryopreserved sperm quality because blue catfish eggs are typically not available. After testing of post-thaw motility and fertilization with channel catfish eggs, thawed sperm showed fertility comparable to fresh sperm, which indicates that the high-throughput process is feasible for commercial application. The correlations among body and gonadal indices showed for sexually mature male blue catfish during the channel catfish spawning season, that the body length and weight were related and that relatively thinner fish had larger testes (and larger anterior portions of the testis). And, importantly, that sperm with higher initial motility yielded higher post-thaw motility and higher fertility when conditions such as sperm concentration were controlled.

Based on the results of this study, processing for cryopreservation based on an automated system can be used for mass production in catfish. Compared to other aquatic species, blue catfish is an atypical species, because sperm cannot be stripped from live males (Bart et al., 1998, Lang et al., 2003). Dissection of the testis requires extra time and labor, and usually killing of the male. Other large-bodied aquatic species that allow non-lethal sperm collection by stripping such as salmon or trout (Scott and Baynes, 1980, Stoss, 1983) would be easier to adapt to high-throughput cryopreservation processes. The potential value of cryopreserved aquatic germplasm is essentially unexploited at present. Meanwhile, more studies are needed to increase the efficiency of cryopreserved sperm to equal or exceed the current potential production of using fresh sperm in commercial settings. This work would involve hatchery management and technique improvement outside of the laboratory. If cryopreserved sperm can be used to replace or serve as an alternative to live males, fish germplasm banks can be established based on this

technology. In that way, hatchery operations and facilities will change; live males will be reduced or eliminated at the hatchery; fish diversity can be preserved; breeding programs will be more efficient, and in the event of disease or other catastrophic problems in the industry, specific fish populations can be reconstituted in a short time. Overall blue catfish can serve as a model fish species to develop high-throughput techniques for sperm cryopreservation including small-bodied aquarium fishes such as zebrafish used in biomedical research (Tiersch, 2001, Yang et al., 2007). Thus, this study can serve as a template for development of high-throughput cryopreservation technology for application across most aquatic species.

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Chapter 4
On-Site Evaluation of Commercial-Scale Hybrid Catfish Production Using Dose-Based Cryopreserved Blue Catfish Sperm

The dairy industry has used high-throughput sperm cryopreservation since the 1940s (Pickett and Berndtson, 1974). There is a billion-dollar global market specifically for cryopreserved cattle germplasm according to the National Association of Animal Breeders – Certified Semen Services (www.naab-css.org). Sperm cryopreservation technology currently serves the purposes of animal breeding, preservation of genetic diversity, and medical research. In human reproduction applications, US sperm banks export frozen semen of human donors to more than 60 countries to help infertile parents, and this industry grew from less than $1 billion in 1988 to more than $4 billion in 2012 (Newton-Small, 2012). However, large-scale application of cryopreserved germplasm of aquatic species is not currently utilized. A survey among fish culturists indicated common interest in genetic improvement by adopting cryopreservation into existing procedures (Boever, 2006), such as among hybrid catfish hatcheries.

As the largest foodfish aquaculture industry in the United States (Harvey, 2006), the catfish industry has been challenged recently with global competition and increased costs of feed and fuel (USDA-NASS, 2011). A number of efforts, including hybrid production, are being implemented by catfish farmers to deal with those challenges. By fertilizing eggs of channel catfish (*Ictalurus punctatus*) with sperm from blue catfish (*Ictalurus furcatus*), hybrid catfish can be produced with improvements in growth rate, disease resistance and food conversion (Dunham and Masser, 2012). Although hybrid catfish are currently in high demand by the industry, the production capacity for hybrids is constrained primarily due to the lack of natural hybridization between these species and the consequent need for artificial spawning (Dunham and Masser, 2012). In addition, limited availability of blue catfish males can constrain hybrid production. The

use of artificial spawning and incorporation of cryopreserved sperm collected from blue catfish in peak spawning condition enables fertilization of channel catfish eggs while they are at peak spawning condition, overcoming biological limitations and maximizing hybrid production. Initial studies tested the feasibility of using a commercial dairy bull facility for cryopreservation of blue catfish sperm (Lang et al., 2003) and a high-throughput cryopreservation process has been established for this species and is ready for testing at a commercial level (Hu et al., 2011).

During the cryopreservation process, from before freezing to after thawing, sperm undergo biological, chemical, and physical stresses (Leibo, 2011). The frequency of damaged sperm is often estimated by the difference between initial motility and post-thaw motility (Rurangwa et al., 2004). The differences in motility between fresh and thawed can affect the final ratio of motile sperm to eggs (Tiersch et al., 1994) which can in turn affect fertilization rates (Saksena et al., 1961, Makeeva and Emel'yanova, 1993, Small and Bates, 2001, Hu et al., 2011). Using additional thawed sperm at fertilization to compensate for losses in motility is a potential solution to reduced fertility. However, due to the cost and availability of blue catfish males, the efficient use of limited sperm must be carefully managed (Avery et al., 2005) to avoid unnecessary costs in fry production. A previous study has established a working ratio of a single 0.5-ml straw (1×10^9 sperm/ml) to fertilize 3704 eggs as a standard dose (Chapter 6). The question from practice exists (i.e., coming from our cooperating commercial hatcheries) if increasing the dose would assist fertility?

A protocol for high-throughput cryopreservation of blue catfish sperm was developed with the assistance of automated equipment (Hu et al., 2011). When the number of straws that could be produced reached a commercial level, quality assurance became the most critical aspect

for blue catfish sperm cryopreservation (Chapter 5). Laboratory-scale and hatchery-scale neurulation (fertilization) testing have shown that the cryopreserved blue catfish sperm had consistent quality that was minimally influenced by individual variation in males (Chapter 7). However, evaluation is needed at the commercial production level to realistically demonstrate feasibility and efficiency for the use of cryopreserved blue catfish sperm in hybrid fry production.

With large-scale application of cryopreserved sperm, the practical and genetic value of sperm is greatly increased. Proper handling and utilization of sperm also become essential to practice. Because thawed cryopreserved sperm had a larger variation in motility among individuals than did fresh sperm (Hu et al., 2011), and testing of the individual fertility of males is typically not feasible in commercial hatcheries through separate fertilization trials due to time and space limitations, sperm samples from several blue catfish males are routinely pooled in commercial hatcheries (Avery et al., 2005). The effects of pooling sperm on subsequent fertilization are not known and could have important implications for commercial use of cryopreserved blue catfish sperm. Although the influence of factors such as motility and concentration on fertility of sperm from individual males is becoming more studied (Rurangwa et al., 2004), it will be important to understand how the pooling of sperm samples can influence commercial applications. For example, will aggregate fertilization of the pool be characterized by a few outstanding individuals, and should sperm of similar quality be pooled together selectively? The overall goal of this study was to address high-throughput sperm cryopreservation of blue catfish at a commercial level. The objectives were to: 1) evaluate double-dosage of thawed sperm in hybrid fry production; 2) evaluate commercial application of high-throughput cryopreserved sperm with standard hatchery techniques, and 3) evaluate the fertility relationship between individuals and pooled samples.

Material and methods

Fish

Blue catfish (D&B strain) males were obtained from Baxter Land Company Fish Farm (Arkansas City, Arkansas; 33°34'58.64"N, 91°15'18.45"W). The males were 4-to-6 years old, and ranged from 61 to 97 cm, 2.8 to 9.8 kg. In early April, they were selected before transportation at the farm based on observable secondary sexual characteristics indicative of maturity (e.g. well-muscled head and dark coloration) (Avery et al., 2005). An oxygenated hauler was used for transport of fish from the fish farm to the Aquaculture Research Station of the Louisiana State University Agricultural Center (Baton Rouge; 30°22'07.32"N, 91°10'27.90"W) in February of 2010 and 2011. The males were first held in aerated outdoor 405-m^2 ponds and fed commercial diets (Aquaxcel, Cargill^{TM}, 45% protein) for 3 to 4 weeks until early May. Fish were collected by seining and moved into indoor tanks within a recirculating system 2 d before processing. The system used bubble-washed bead filters that were back-flushed every 2 d. The water quality parameters were: pH 7.0 – 8.0, total ammonia-nitrogen 0.1 – 0.8 mg/l, nitrite 0.04 – 0.30 mg/l, alkalinity 39 – 125 mg/l, hardness 44 – 126 mg/l, temperature 28 ± 1 ℃, and dissolved oxygen 4.3 – 6.5 mg/l. Guidelines from the Institutional Animal Care and Use Committees (IACUC) of Louisiana State University were followed for animal care in this study.

Sperm collection

At the beginning of channel catfish spawning season, blue catfish males were killed by a sharp blow to the head, and were rinsed with Hanks' balanced salt solution at an osmolality of 300 mOsmol/kg (HBSS300) to remove low osmolality fluids that could cause activation of sperm during dissection (Bates et al., 1996). The body weight and standard length were measured, after which the testes were removed by dissection. Tared weigh boats (catalog number:

02-203-501, Fisher Scientific) were used as containers for testes. HBSS300 was added to the weigh boat to prevent desiccation and sperm activation. The testes were blotted on a paper towel to remove blood and adherent tissues. The entire testis was weighed and the anterior portion of each testis was collected and weighed separately. Only the anterior portion (Sneed and Clemens, 1963) was used for sperm collection by crushing in HBSS300. The volume of HBSS300 (ml) used was two times the mass (g) of the crushed testis. The suspension was 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 to screen out tissues. Sperm suspensions were processed and labeled for each male fish.

Determination of sperm concentration

Fish sperm concentration has been found to be highly correlated with absorbance readings (Dong et al., 2007, Tan et al., 2010, Cuevas-Uribe and Tiersch, 2011) and a microspectrophotometer (NanoDrop 1000, Thermo Scientific, Wilmington, DE) was used to measure the absorbance of serially diluted sperm suspensions with 2-ul aliquots using a wavelength of 600 nm to estimate sperm concentration. The concentration of sperm suspensions was also calculated using a hemocytometer to count sperm concentration directly. A linear relationship was applied to the absorbance and respective concentration measurements. The conversion equation between absorbance and concentration was:

$$\text{sperm concentration (cells/ml)} = \text{absorbance} \times 5.12 \times 10^{8} - 4.07 \times 10^{7} \quad (R^2 = 0.960)$$

Motility estimation

Sperm samples were placed on glass slide and viewed with dark-field microscope (Olympus CX41RF, Japan) at 200-× magnification. Sperm were activated with a ratio of 1 µl of

sperm suspension to 20 µl of filtered deionized distilled water (25 mOsmol/kg). Motility was estimated within 20 s after activation with the use of 3-5 different fields by visually comparing progressively swimming sperm to non-motile sperm. A percentage was used to express the proportion of motile sperm (Hu et al., 2011). The motility of each sperm suspension when estimated within 2 hr of collection (without cryoprotectant) was considered to be the fresh sperm motility and was designated as the "initial motility". Motility of sperm suspensions estimated within 30 min of thawing was considered as the "post-thaw motility". Samples from individual males were discarded before cryopreservation if initial motility was less than 40%.

Sample preparation and cryopreservation using an automated system

Based on previous work, sperm concentration within straws was adjusted to 1×10^9 cells/ml with 10% methanol as cryoprotectant (Hu et al., 2011). The sperm samples and methanol solution were each prepared in HBSS300 at twice the final concentration, before mixing at 1:1 (v/v). The cooling process was started after 30 min of equilibration with cryoprotectant. During equilibration, the mixtures were placed on the automated MAPI packaging system (CryoBioSystem Co. Paris, France) which automatically filled, sealed and labeled straws under the control of a proprietary computer program (SIDE, CryoBioSystem Co. Paris, France). Commercially available 0.5-ml CBS straws (reference number: 014657, CryoBioSystem Co. Paris, France) were used as the standard container for packaging (Fig. 4.1). After packaging, the straws were arrayed on racks (40 straws per rack) and placed in a commercial-scale programmable freezer (Micro Digitcool, IMV, France). The capacity of this freezer was 280 straws per freezing cycle, and the number of straws in the freezer for each cycle ranged from 180 to 240 straws. No additional steps were taken to equalize the thermal mass at every cycle (e.g. using "dummy" straws). The temperature of the freezer chamber dropped from

5 ℃ to -80 ℃ at a cooling rate of 5 ℃/min. At the end of the programmed cooling cycle, the samples were stabilized at -80 ℃ for another 5 min, and then plunged into liquid nitrogen. The straws remained under liquid nitrogen during sorting, and were placed for final storage into 12-compartment containers (Daisy goblets, reference number: 015144, CryoBioSystem). The products of cryopreserved sperm were stored at the Aquaculture Research Station, and large-scale application was performed at the hatchery of Baxter Land Company.

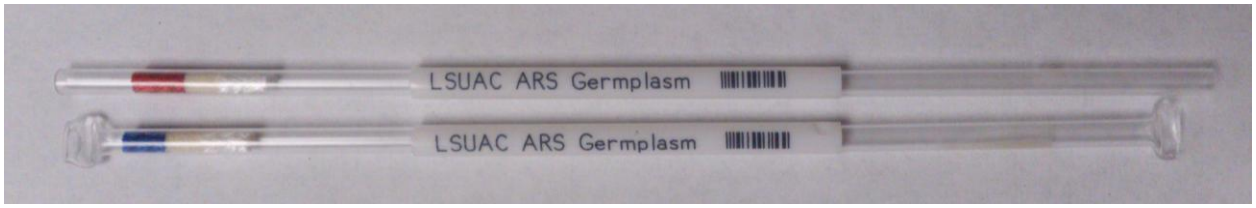

Figure 4.1. Example of unsealed (top) and sealed (bottom) 0.5-ml CBS straws (CryoBioSystem, Paris, France) with barcode and alphanumerical label.

Egg collection and artificial fertilization in the commercial hatchery

All frozen straws were grouped for use and inventoried at the Aquaculture Research Station before transport to the commercial hatchery. All Daisy goblets were placed into shipping dewars (MVE Cryo Moover, Chart Industries Inc., OH, USA) which were filled at least twice with liquid nitrogen before transportation.

During artificial spawning, eggs from individual females were divided into aliquots of 100 to 150 ml for each batch (40 ± 7 eggs/ml). Samples of fresh sperm from fish of the same blue catfish strain were collected each day at the hatchery (Avery et al., 2005). After processing the testes, the fresh sperm suspensions (~1×10^9 sperm/ml) were stored in a refrigerator (4 ℃). The containers used for holding of eggs during fertilization were 25-cm diameter metal pie pans (reference number: 004400126, Walmart, Bentonville, AR). For fertilization, 2 ml of sperm were

used for each batch (except Experiment 1). After the sperm suspension was added to the eggs, 1 L of water from the hatchery system was added to initiate gamete activation and fertilization. After water hardening (i.e. 10 min) the egg masses were moved into a flow-through system for incubation, following routine hatchery procedures (Steeby and Avery, 2005). The incubation temperature was 27 to 29 ℃. After 27 to 30 hr, normal embryo development would be at the neurula stage (Stage V) (Saksena et al., 1961, Makeeva and Emel'yanova, 1993) with development of the yolk sac and segmentation of the trunk mesoderm (Small and Bates, 2001). The neurulated embryos were counted and their proportion of the total number of eggs was calculated to estimate percent fertilization (referred as percent neurulation). After 10 d, the number of swim-up fry was estimated for treatments and controls for each day of production. Because of commercial practices egg masses were pooled in hatching troughs within a treatment, therefore fry numbers could not be estimated for individual spawns and instead represent the number of fry for the entire hatching trough.

Experiment 1: Evaluation of a double-dosage of thawed sperm

Sperm samples from 9 males were processed individually at the Louisiana State University Agricultural Center, 180 straws representing all 9 males were produced and shipped to Baxter Land Co. Fish Hatchery. All frozen straws were thawed at 40 ℃ for 20 s, and pooled in a 500-ml Tupperware container. Concentrations of fresh and thawed sperm were estimated using a hemocytometer (Hausser Bright-Line 3100, Hausser Scientific Company, Horsham, PA). Eggs pooled from 15 females were divided equally into three groups of 150-ml pans. One group was treated with 2 ml of thawed sperm in each pan; one was treated with 4 ml of thawed sperm in each pan; and the rest were fertilized with 2 ml of fresh sperm in each pan as positive control group to represent normal fertilization (no cryopreserved sperm) in the commercial hatchery.

Experiment 2: Evaluation of the commercial application of high-throughput cryopreserved sperm

In 2010 and 2011, testes from 57 males were processed individually at the Louisiana State University Agricultural Center, and more than 3,000 straws representing all 57 males were shipped to Baxter Land Co. Fish Hatchery. 316 females were used in 7 d of artificial fertilization at the hatchery. Each day, sperm were thawed at 40 ℃ for 20 s and pooled in a 500-ml Tupperware container. Fresh sperm were prepared within 24 hr before use based on the procedures described above. Eggs were pooled and divided equally into two aliquots of 150-ml batches as described above. One set was fertilized with thawed sperm, and the other set was fertilized with freshly prepared sperm as positive control group to represent normal fertilization (no cryopreserved sperm) at the commercial hatchery. If the batches were an odd number, the extra batch was included in positive control group.

Experiment 3: Evaluation of the fertility relationship between individual and pooled samples

A total of 51 males from Experiment 2 were tested. Straws from each male were arranged and recorded at the Aquaculture Research Station before transport to USDA Catfish Genetics Research Unit (Stoneville, MS). All Daisy goblets were placed into shipping dewars (MVE Cryo Moover, Chart Industries Inc., OH, USA), which were filled at least twice with liquid nitrogen before transportation.

The containers used for holding eggs were 9-cm plastic petri dishes (Catalog number: 50-894-833, Fisher Scientific, Waltham, WA). During artificial spawning, eggs from three females were pooled and divided into 10 - 12 ml aliquots for each petri dish (475 ± 28 eggs/petri dish). Samples of fresh sperm were collected the same day for egg quality controls (Avery et al., 2005). During fertilization 0.25 ml of thawed sperm was used for each petri dish, the sperm samples

from each male were used to fertilize the eggs in two petri dishes. After the sperm suspension was added to the eggs, water from the hatchery system was added to fill the petri dish, followed by manual water exchange frequently for 5 min using a 50-ml syringe (Catalog number: NC0213999, Fisher Scientific, Waltham, WA). The petri dishes were covered with 1-mm window screen and moved into a flow-through system for incubation, following routine hatchery procedures (Steeby and Avery, 2005). The incubation temperature was 27 to 29 ℃. After 27 to 30 hr, the neurulated embryos were photographed and counted. Their proportion of the total number of eggs was calculated to estimate percent neurulation, and the percentage was divided by the value for the fresh sperm control to eliminate the female (egg quality) effect.

Individual-weighted proportions (male identification number and number of straws per male) of 6 pooled samples were recorded and the motility of the pooled sample and resulting neurulation were measured during Experiment 2. The motility and neurulation were divided by the corresponding values derived from the positive control group to eliminate variations associated with quality differences among egg aliquots. Each pooled sample contained sperm from 6 to 11 males. With the male identification number and respective straw number, theoretical post-thaw motility and neurulation were calculated for the pooled sample by applying weighted means. For example, in individual tests, Male 1 yielded post-thaw motility M1, and Male 2 yielded post-thaw motility M2; while the pooled sample contained 100 straws of Male 1 and 200 straws of Male 2, the theoretical post-thaw motility should be (100*M1 +200*M2)/(100+200). The calculation of theoretical neurulation followed the same pattern. The theoretical post-thaw motility and neurulation were compared with the measured values for all 6 pooled samples by paired T-test.

Data analysis

Microsoft Excel 2007 was used for data organization. All percentage data were normalized by arcsin-square-root-transformation before statistical analysis. Data sets were tested for normality by SAS 9.2 UNIVARIATE program, before comparisons in General Linear Model GLM and Analysis of Variance ANOVA programs. When the calculated *P* value was less than 0.05, the difference was considered to be significant.

Results

Experiment 1: Evaluation of the double dose usage of thawed sperm

The post-thaw motility of thawed sperm was 45% (N = 9) and initial motility of fresh sperm was 60%. The thawed sperm concentration ($1.3 \pm 0.1 \times 10^9$ sperm/ml) and the freshly prepared control sperm concentration ($0.9 \pm 0.4 \times 10^9$ sperm/ml) were not different ($P = 0.089$). Due to different sperm volumes (2 ml and 4 ml) used among the treatments, average outputs on a per-male or per-sperm basis were calculated (Table 4.1). The percent neurulation among all groups was not significantly different ($P = 0.136$).

Experiment 2: Evaluation of the commercial application of high-throughput cryopreserved sperm

After 7 d of fertilization trials, there were 50.9 L of eggs fertilized with thawed sperm that produced 1,044,222 hybrid swim-up fry and 66.2 L of eggs fertilized with freshly prepared control sperm that produced 1,476,000 hybrid swim-up fry. The thawed sperm motility (33 ± 10%) was lower ($P = 0.029$) than the fresh sperm motility ($43 \pm 4\%$). On a per-day basis, for each liter of eggs yielded $23{,}737 \pm 9{,}518$ fry using thawed sperm. Using the same volume at a similar concentration for fresh sperm, $26{,}318 \pm 8{,}510$ fry were produced per liter of eggs each

day. There was no difference ($P = 0.603$) in fry production between the same volume of fresh and thawed sperm when fertilizing the same volume of eggs.

Table 4.1. Fresh or thawed sperm were used to fertilize batches of channel catfish eggs (150 – 200 mL). Before fertilization, the concentrations of all samples were not significantly different ($P = 0.089$). There were no significant differences in percent neurulation for the three groups. The commercial hatchery could not provide evaluation of fry production from single batches, therefore standard deviation could not be calculated.

Source	Sperm volume /batch of eggs	Neurulation (%)	Sperm /egg ($\times 10^5$)	Motile sperm /egg ($\times 10^4$)	Swim-up fry/ 1×10^9 sperm
Fresh	2 ml	67 ± 16	1.46	9.00	1430
Thawed	2 ml (4 straws)	65 ± 13	1.35	6.08	1238
	4 ml (8 straws)	61 ± 15	2.70	12.15	900

Experiment 3: Evaluation of the fertility relationship between individual and pooled samples

The individual post-thaw motility ranged from 0 to 45%, and the individual normalized neurulation (against the respective egg quality control) ranged from 19 to 127%. The measured motility for pooled samples (36 ± 8%) was higher ($P = 0.030$) than the theoretical post-thaw motility (24 ± 11%) and there was no correlation between the two values ($P = 0.259$). The measured neurulation for pooled samples (88 ± 7%) was not different ($P = 0.208$) from the theoretically predicted neurulation (68 ± 35%).

Discussion

A previous study established a practical sperm-to-egg ratio of 2.5 - 3.3 $\times 10^5$ sperm/egg and derived an optimal equilibration concentration of 1 $\times 10^9$ cells/ml for cryopreserved blue catfish sperm (Hu et al., 2011). With respect to motile-sperm-to-egg ratios the double-volume group was higher than the control group (Table 4.1), but doubling of the sperm volume did not increase neurulation percentage. In fact, the 4-ml treatment yielded the lowest swim-up fry

production per billion sperm which indicated that the doubled volume was not beneficial at the concentrations used in this study. Previous studies have presented different sperm-to-egg ratios in different catfish species (Table 4.2). These values reinforce the observation in this study that a doubling of sperm volume in each fertilization pan when using the established sperm-to-egg ratio ($2.5 - 3.3 \times 10^5$ sperm/egg) was unnecessary during hybrid catfish production. The fertility response to different sperm-to-egg ratios has been found to follow a logistic curve (Bart and Dunham, 1996, Linhart et al., 2004), and therefore, determination of an optimal sperm-to-egg ratio for commercial level use requires further study. However, at this point it cannot be concluded that thawed sperm functions in the same fashion as fresh sperm when using equal volumes. More comprehensive experiments will be required to fully refine the performance of cryopreserved sperm.

Table 4.2. Survey of sperm-to-egg ratios used in cultured catfish species. Only one studied specified the ratio as being for motile sperm.

Species	Sperm type	Sperm-to-egg ratio (sperm/egg)	Citation
African catfish *Clarias Gariepinus*	Cryopreserved	4.90×10^4	(Steyn and Van Vuren, 1987)
	Fresh	1.50×10^4	(Rurangwa et al., 1998)
	Cryopreserved	1.13×10^4	(Viveiros et al., 2000)
Striped catfish *Pangasius hypophthalmus*	Cryopreserved	6.90×10^7	(Kwantong and Bart, 2003)
	Fresh	1.89×10^6	(Kwantong and Bart, 2009)
	Cryopreserved	6.94×10^6	(Kwantong and Bart, 2009)
Channel catfish *Ictalurus punctatus*	Cryopreserved	2.00×10^5 *	(Tiersch et al., 1994)
Hybrid catfish (channel catfish *Ictalurus punctatus* × blue catfish *I. furcatus*)	Fresh	1.25×10^5	(Bart and Dunham, 1996)
	Cryopreserved	1.33×10^7	(Bart et al., 1998)
	Cryopreserved	2.50×10^5	(Hu et al., 2011)

* motile sperm concentration

Commercial hatcheries are effective for large-scale production but have limited flexibility for experimental trials. The hatchery where the commercial-scale trials were performed has the capability of fertilizing eggs from more than 40 female channel catfish per day. The traditional catfish egg hatching trough (Dunham and Masser, 2012) in the hatchery provided 67 ±16% neurulation during Experiment 1, and a 66% hatching rate (26,318 ±8,510 fry/L eggs divided by 40 ±7 eggs/ml) during Experiment 2, which represented reasonable results for a commercial hatchery (Ligeon et al., 2004). The fry produced from each fertilized egg mass were not traceable back to individual females due to the pooling of batches within the hatching trough (Steeby and Avery, 2005), and each trough could therefore only provide a single estimated value for numbers of fry. Therefore, comprehensive hatching records would require large-scale fertilization trials over an entire spawning season (e.g. 1-2 months of daily spawning). For the same reason, the number of treatments was also restricted by the practices within the facility. Neurulation estimates could serve as an alternative index to gain access to more information during commercial-level fertilization trials, however, hatching results (fry production) remains the most used index available at a commercial facility.

Previous studies have begun to address the integration of cryopreserved sperm into aquatic species production applications. Several studies (Cabrita et al., 2001, Chen et al., 2004, Ji et al., 2004) have examined increases in the volume of cryopreserved sperm through use of larger packaging. Others have examined incorporation of industrial-scale protocols from dairy semen approaches to aquatic species. (Lang et al., 2003, Dong et al., 2005a, Dong et al., 2005b). However, the current study is the first and largest experiment of cryopreserved fish sperm at a true commercial scale. We demonstrated that cryopreserved sperm could be effectively used in commercial-scale production of hybrid catfish without changes in existing hatchery protocols or

equipment other than the activities associated with the collection, freezing and transport of cryopreserved sperm that occur outside of the hatchery. The results from this study indicate that the utility of cryopreserved sperm was the same as that of sperm collected from fresh-killed blue catfish males.

The results of this study and a previous study with channel catfish (Tiersch et al., 1994) demonstrate that even though motility is reduced in thawed samples there was no negative effect on fertility at the sperm concentrations typically used in commercial hatcheries. Hybrid fry output suggested that thawed sperm could be a reliable alternative sperm source. Furthermore, with individual male identification and minimized storage requirements (i.e., cryogenic tanks instead of ponds), cryopreserved sperm offers benefits not possible with fresh sperm including improved hatchery management (e.g., by avoiding waste of excess sperm), more efficient use of pond resources and implementation in selective breeding programs.

The adoption of cryopreserved sperm for blue catfish males can lead to improvement of hatchery management, particularly more efficient use of sperm. Daily variations in factors such as the percentage of females that ovulate, and the volume of eggs produced, result in variability in the volume of sperm used. Typically at hatcheries using fresh sperm, males are killed and testes prepared daily based on projected needs. However, projected and actual needs are frequently mismatched resulting in carryover or waste of excess sperm or interruption of egg collection to prepare more sperm. Use of cryopreserved sperm would eliminate problems associated with providing sperm on-demand. Blue catfish typically mature at 5 years of age and testis development is highly variable even in mature males, therefore farmers must devote considerable pond resources to maintaining several age classes of males and keeping excess fish

to ensure that they will have adequate sperm resources. This cost is intensified by the risk of losing males due to disease or water quality problems (e.g., low dissolved oxygen) in the ponds. Use of cryopreserved sperm would allow the use of ponds currently devoted to blue catfish males to other uses (e.g. fingerling production) and improve resource utilization. Currently, methodology to non-lethally collect sufficient sperm from live blue catfish males for routine fertilization does not exist and males are killed and testes removed. This approach severely limits the ability to implement genetic improvement programs in hybrid catfish, a key component to improve profitability of catfish farming. Use of cryopreserved sperm is crucial to development and implementation of genetic improvement of economically important traits in hybrid catfish. Data from this trial demonstrated that 1 million hybrid catfish fry could be produced from 48.7 L of eggs, with a swim-up fry survival rate of 51%. Fertilization of this volume of eggs required 650 ml of thawed sperm which was contained in 1,300 straws. With the high-throughput process reported previously (Hu et al., 2011), we demonstrated that 1,300 straws can be produced from 20 blue catfish males in about 8.5 hr (Chapter 7).

Differences between measured and predicted post-thaw motility indicated that pooled samples were not characterized by extreme values. Motilities were neutralized among individuals. Naked-eye motility estimates are not as precise as more objective methods such as computer-assisted sperm analysis (Yang and Tiersch, 2011) which could help further identify the relationship between pooled and individual samples. In addition, there are other candidate parameters such as membrane integrity (Rurangwa et al., 2004) that could be tested for this use. The similarity between measured and predicted neurulation indicated that the fertility of pooled samples reflected the expected cumulative effects of the individual males composing the pool and that fertilizations by particular males might contribute proportionately in pooled samples.

Domination by certain males in pools could be caused by sperm competition (Pizzari and Parker, 2009) and has been observed in various species such as Atlantic salmon *Salmo salar* (Gage et al., 2004), bluegill *Lepomis macrochirus* (Burness et al., 2004), and swordtails *Xiphophorus nigrensis* (Morris et al., 1992). Sperm competition among males involves not only relative sperm numbers but also can involve cell size, longevity, viability, and mobility (Snook, 2005). There are few quantitative studies that have addressed the relationship between such sperm traits and fertility, or competition in samples from pooled males. The potential occurrence and effects of sperm competition in hybrid production requires further study.

In practice, the fertilizations were performed with a small excess of sperm. Cryopreserved sperm from the standard process contains sufficient motile sperm for each fertilization trial. Therefore, the cryopreserved product can yield consistent production. Our finding that pooled samples reflected the fertilizing capability of the individuals composing the pool is useful for predicting fertilization results with current methods (i.e. a constant small excess of sperm). When all of the straws were from individuals with good individual fertilization (e.g., 50% or higher), the pooled thawed sperm produced 50% or higher fertility. In contrast, pooling of sperm from individual males with poor (e.g., 20% or lower) fertilization led to pools with 20% or lower fertilization. The use of cryopreserved sperm offers managers the ability to select males that will produce pooled sperm samples with optimal fertility based on predictive quality parameters (e.g., motility) or retrospective individual fertility results (preliminary fertilization trials to characterize large batches). In the long term, use of cryopreserved catfish sperm will allow assignment of economic value based on sperm quality and genetic value similar to the practices employed in other animal industries. (NAAB-CSS, 2011).

Genetic resource management

The present study demonstrated that cryopreserved sperm can be used in hybrid catfish hatcheries as an alternative to fresh sperm. In addition, the application of cryopreserved sperm leads to genetic resource management concepts that can be integrated to future hybrid catfish production. The genetic resource management concept is based on the traditional phases of production (Fig. 4.2). The core of genetic management is to consider the broodfish as genetic resources instead of live fish or simply as gamete dispensers. There are three aspects in management: banking, application, and enhancement.

Banking: cryopreservation technology allows storage of genetic resources indefinitely in the frozen form, reducing pond facilities otherwise required to maintain live broodfish. Therefore, more ponds and other resources will be free for other production requirements. The removal of the need for pond facilities and the relatively small footprint and low cost of storing frozen catfish sperm allows storage of a large variety of germplasm from multiple sources over time. Currently little is known about the genetic value of existing blue catfish germplasm sources. Therefore, sperm from a wide variety of sources could be frozen and serve as a "savings account" until it has been evaluated. When evaluations reveal which strains or individuals are genetically superior, sperm from these fish can be distributed and used for blue catfish broodstock development, hybrid catfish production, or temporary storage for the coming spawning season as a backup sperm source. The higher frequency of access makes this type of storage the equivalent of a "checking account" linked to a "savings account".

Application: with the banking repository model, all hatcheries can access their own "savings" and "checking" accounts regardless of distance and time. For example, if a farm decided to

start a new hatchery across the country in 2 years, there would be no need to transport populations of live blue catfish to the new site, or waiting until young fish were ready for spawning. Instead, the cryopreserved sperm could be shipped to the new hatchery for fertilization when needed. Furthermore, repository materials can be exchanged or transferred between hatcheries based on trade agreements. Therefore, due to public preference for high quality genetic resources, specific genetic improvements (e.g., in growth rate or disease resistance) could be profitable to the owners of the germplasm as well as other users (e.g., customers).

Enhancement: hybrid catfish are prohibitively difficult to spawn naturally (Dunham and Argue, 2000), and blue catfish selection programs would proceed slowly due to the long sexual maturation period (Graham, 1999). Cryopreservation provides an efficient method for blue catfish selection, because cryopreserved products can have detailed records for individuals, and the parental sperm of high performance hybrid offspring are traceable. Selection can be repeated annually by making hybrid fish using the same sperm sources that produced improved offspring last season. This can also be coupled with molecular genetics and genome mapping for marker-assisted selection (Tiersch et al., 2004).

Conclusions

High-throughput cryopreservation of blue catfish sperm for use in hybrid catfish production has been successfully applied at a commercial-scale level. Cryopreserved sperm can serve as an alternative to live blue catfish males during hybrid production. This study demonstrates the feasibility of aquatic germplasm cryopreservation at a commercial-scale application for aquaculture production and addresses the concept of genetic resources management for genetic improvement. Use of cryopreserved sperm could increase the

competitive of the US catfish aquaculture in the long term, and offers opportunities for development of new markets and industries in genetic resources and germplasm.

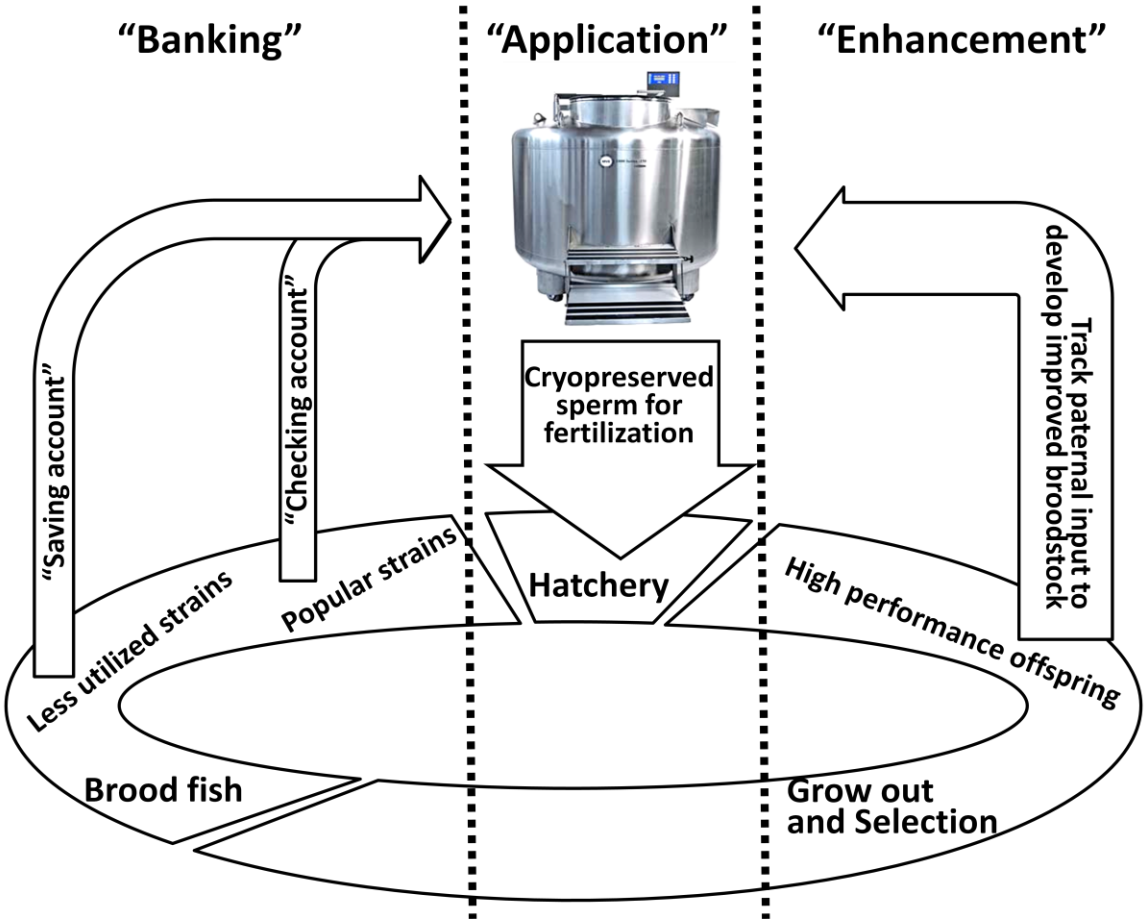

Figure 4.2. A summary of genetic resource management concepts integrated into hybrid catfish production. During hybrid catfish production, all activities can be grouped into three broad categories: broodfish, hatchery, and grow out and selection. Genetic resource management can be grouped into three aspects (separated by dotted lines): banking, application, and enhancement. In broodfish management, "Banking" can store the less-utilized strains to liberate farm resources as a long-term "savings account", and store popular strains to increase their accessibility. In the hatchery, "Application" uses cryopreserved blue catfish sperm to fertilize channel catfish eggs to produce hybrid catfish fry. During grow out and selection, "Enhancement" collects information about hybrid offspring performance and links it with the sperm donors. Broodfish and the sperm are considered as genetic resource in this concept.

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Chapter 5
A Quality Assurance Initiative for Commercial-Scale Production in High-Throughput Cryopreservation of Blue Catfish Sperm

Cryopreservation can retain biological function of animal spermatozoa for years or decades under very low temperatures (usually <-100 ℃). Cryopreservation technology has been developed in mammals, birds, amphibians, fish, and some invertebrate species (Mazur et al., 2008). The development of fish sperm cryopreservation dates back 60 yr (Blaxter, 1953), and in the past decade alone, there were some 300 publications on fish sperm cryopreservation (Tiersch, 2011). With growing interest and applications in cryopreserved fish sperm, laboratory-scale cryopreservation cannot satisfy the demands from aquaculture farms or fish hatcheries. Therefore, cryopreservation of fish sperm at a commercial scale with high-throughput has become an increasingly important research topic (Tiersch et al., 2011).

Catfish sperm cryopreservation has been studied for more than 35 yr (Guest et al., 1976) but mostly in the laboratory environment. Previous large-scale trials have been performed in a dairy improvement center using processing specialized for bull semen (Lang et al., 2003), although these products were not tested at a commercial level. During the past 4 years, automation of the cryopreservation process has been developed at laboratory and commercial scales (Hu et al., 2011). A demonstration production line was developed for cryopreserved blue catfish sperm, but was operated without the quality assurance guidelines necessary to produce reliable products at a scale relevant to commercial application..

The quality assurance concept has been integrated previously into aquaculture research. These include qualitative activities, such as sex identification (Kelly, 2004) and separating spawning females after collection from ponds (Dunham and Masser, 2012), and quantitative

activities, such as selective breeding (Bondari, 1983). However, those activities and their details were seldom placed in written form or uniform formats. From an engineering perspective, all criteria used for decision making in those activities could be described as quality characteristics, and all quality assurance can be documented within two aspects: specification design and quality control. Specification design regulates the acceptance of materials with certain quality characteristics, so that waste and high variation in the process due to less-functional or non-functional parts can be avoided (Creveling, 1997). For example, during selection and injection females for artificial spawning, if the quality characteristics are defined by observable body features (Dunham and Masser, 2012), the specification would be a flat belly (indicating poor potential for spawning); if the quality characteristic is the ultrasonic image features of gonads (Novelo and Tiersch, 2012), the specification would be the absence of visible mature eggs. These specifications could guarantee that the final products would meet the basic expectation of farmers (customers). Quality control is used to monitor the variability of products during processing, and to provide suggestions for reduction of variability (Montgomery, 2008). In the same example of selecting catfish females, the ultrasound pictures can provide quantified quality characteristics such as the size of eggs within the ovaries (Guitreau et al., 2012). When statistical quality control is applied, control charts of egg size developed from previous egg size data can be used to check every new sample and identify any samples that exceed the control limits. Once the data set exceeds control limits, corrective measurements can be taken to rectify out-of-control products or operation steps by using quality control tools such as a cause-and-effect diagrams (Montgomery, 2008).

In this study, an initial quality assurance plan for high-throughput cryopreservation of blue catfish sperm was developed and evaluated in practice. The objectives were to identify: 1)

the main quality characteristics; 2) the process features for quality assurance; 3) the internal quality characteristics and their specifications designs; 4) the quality control and process capability evaluation methods, and 5) the directions for further improvements and applications.

Main quality characteristics

Voice of customers

The voice of customers represents the demands for product quality which provide the initial point of quality assurance. Properly satisfying the requirements of customers can improve profitability (Griffin and Hauser, 1993). The "Voice of Customers" data are collected from large comprehensive surveys, and represent the customer tolerances on the product. Engineers and designers would convert these data into a production engineering matrix using quality function deployment (Griffin and Hauser, 1993). Commercial-scale high-throughput cryopreservation in blue catfish sperm is a newly developed process. Customers have not yet provided quantitative expectations on its production. Without comprehensive surveys and quality function deployment support, in this study, the voice of customers was generalized as an overall pursuit of high product quality.

Fertility quality characteristics

The product of this process was cryopreserved sperm contained in sealed plastic straws. With the purpose of making hybrid catfish fry, the quality of product was directly related to the fertility of the sperm cells. In commercial practice with fresh sperm, the number of swim-up fry is the most commonly used evaluation method (Avery et al., 2005). In laboratories, the ratio of the initial number of eggs to different stages of embryo development is often used for evaluations (Bart et al., 1998, Lang et al., 2003, Hu et al., 2011). Although fry and embryo development are reliable indices for sperm fertility, the evaluation takes 1 – 10 days and

introduces other variations such as female condition (Dorman and Torrans, 1987, Kelly, 2004) and hatchery management (Steeby and Avery, 2005). Specifically, after sperm is used to fertilize eggs, neurulation is assessed after 27 -30 hr (at 27 – 29 ℃); embryonic mobility is observed after 48-50 hr (Hu et al., 2011); hatching percent or sacfry numbers are assessed after 5 d, and swim-up fry are counted after 10 d (Avery et al., 2005). In addition, channel catfish females only spawn within a strict temperature range (Steeby and Avery, 2005). Therefore, high quality eggs for performing fertility evaluations are limited.

Some of the measureable indices for thawed sperm are motility, membrane integrity and cell concentration. These indices are frequently used in fish sperm research. Motility represents the sperm swimming behavior at a population level (Suquet et al., 1992, Rurangwa et al., 2004). Membrane integrity represents the overall viability of sperm cell (Rurangwa et al., 2004). Concentration indicates sperm numbers after freezing. Although, there has been no significant correlation between these three indices of thawed sperm and the numbers of fry produced (most likely due to variation at the hatchery level during development such as water quality), studies suggested that motility of sperm is correlated with embryo development (Guest et al., 1976, Ciereszko and Dabrowski, 1994, Vermeirssen et al., 2004, Hu et al., 2011). In this study, motility, membrane integrity and cell concentration of thawed sperm were considered as main quality characteristics for the final product.

Specification design

Blue catfish (D&B strain) males were from Baxter Land Company Fish Farm (Arkansas City, Arkansas; 33°34’58.64”N, 91°15’18.45”W). The males were selected at the farm based on observable secondary sexual characteristics (e.g. well-muscled head, and dark coloration) (Avery et al., 2005) before transportation. An oxygenated hauler was used for transport from Baxter

Land Company Fish Farm to the Aquaculture Research Station of the Louisiana State University Agricultural Center (Baton Rouge; 30°22'07.32"N, 91°10'27.90"W) in spring of 2009 to 2011. The males were first held for 2 – 4 wks in aerated outdoor 0.1-acre ponds and fed commercial diets (Aquaxcel, CargillTM, 45% protein), then captured and moved into indoor tanks with a recirculating system 2 d before processing. The system used bubble-washed bead filters that were back-flushed every 2 d. The water quality parameters were: pH 7.0 – 8.0, total ammonia-nitrogen 0.1 – 0.8 mg/L, nitrite 0.04 – 0.30 mg/L, alkalinity 39 – 125 mg/L, hardness 44 – 126 mg/L, temperature 28 ± 1 ℃, and dissolved oxygen 4.3 – 6.5 mg/L. Guidelines from the Institutional Animal Care and Use Committees (IACUC) of Louisiana State University were followed for animal care in this study.

Sperm from 27 males were cryopreserved individually in 2010 following the high-throughput protocol (Hu et al., 2011). Thawing of straws was performed in a 40 ℃ water bath for 20 sec. Motility assessment (Appendix A, SOP-4) and flow cytometer analysis (Appendix A, SOP-6) were performed for the individual thawed samples (Cuevas-Uribe, 2011). The process period overlapped with the hybrid catfish fry production season (April – June) and the males in this study were representative of the blue catfish male populations used commercially in hybrid catfish hatcheries.

The ideal specifications for industrial processing are often set with the three-sigma rule which equals a 99.7% confidence interval (Montgomery, 2008). However, with the biological products in this study, a 95% confidence interval was applied, which equaled to two sigmas (1.96 exactly) from the mean value. All data were first checked to identify outliers, and tested for normality. The range was calculated according to the data distribution and practical considerations (Table 5.1).

Table 5.1. Specifications for the main quality characteristics in blue catfish sperm high-throughput cryopreservation. LSL: lower specification limit; USL: upper specification limit.

Quality characteristic	Unit	LSL	USL
post-thaw motility	%	7	100
post-thaw membrane integrity	%	70	100
post-thaw concentration	Cells/ml	9,880	307,511

Because sperm with low post-thaw motility (e.g., 18 ± 22%) can fertilize eggs (Hu et al., 2011), there were no quantitative studies available to define the acceptable level of post-thaw motility. Based on our practical experience, this study used 10% as a threshold. After discarding values under 10%, Kolmogorov-Smirnov normality testing showed the post-thaw motility followed normal distribution ($P = 0.127$) with a mean of 25% and standard deviation of 11%. The 95% confidence interval was from 7 - 44%. However, a positive correlation with embryo development suggested that the higher the motility the better (Hu et al., 2011), so the adjusted specification for post-thaw motility was from 7 - 100%.

The post-thaw membrane integrity dataset contained a single outlier based on the data distribution. After eliminating the outlier, Kolmogorov-Smirnov normality testing showed the data followed a normal distribution ($P = 0.291$) with a mean of 80%, and a standard deviation of 6%. The 95% confidence interval was from 70 - 89%. Because the biological definition of membrane integrity represents viable cells, high membrane integrity was not considered as a negative impact. Therefore, the adjusted specification for post-thaw membrane integrity was 70 – 100%.

The post-thaw sperm concentration dataset contained a single outlier based on the data distribution. After eliminating the outlier, Kolmogorov-Smirnov normality testing showed the data did not follow a normal distribution ($P < 0.005$) with a mean of 1.04×10^5 cells/ml, a standard deviation of 1.34×10^5/ml, and a skewness of 1.12 and kurtosis of -0.16. The best fit was 3-parameter Weibull distribution ($P < 0.005$) with shape 0.53, scale of 5.92×10^4, and a threshold 4.75×10^3. A large number of random data (500 data sets) were simulated from the same distribution and sorted repeatedly. From the probability density function of these simulated data, the 95% confidence interval was from 9,880 – 307,511 cells/ml.

High-throughput cryopreservation process structure

The entire high-throughput cryopreservation process was divided into 11 operations (Table 5.2). Each operation could be carried out separately as an assembly line; or a single worker could finish the job by executing the operations in sequence. To ensure product quality, detailed quality assurance activities had to be established and documented. The sperm cells were kept in different forms in different operations during the process. The purpose of the process was to transfer the sperm cells from the fish body to a well-controlled artificial condition for long-term preservation. For Operations 1 to 3, sperm were kept inside the fish body cavity; for Operation 4, sperm were kept inside the extracted gonad; for Operations 5 to 7, sperm were kept in tubes of Hanks' balanced salt solution at 300 mOsmol/kg (HBSS300) with or without cryoprotectant (5% methanol); for Operation 8, sperm were kept in straws with HBSS300 and cryoprotectant; for Operations 9 to 11, sperm were frozen in straws, and for Operation 11 large numbers of straws (e.g., 144 straws) were sorted and grouped into 12-compartment daisy goblets.

The current process had two distinguishing features. First, it was a continuous process specifically designed for processing of blue catfish sperm whenever the process was running. No

other fish species were applied in this process, and process setup remained unchanged through time. Secondly, the process was built based on 100% inspection. In each operation, all samples were inspected. The inspection type affected the methods of quality control. For variable (or quantitative) data, this study applied cumulative sum x (CUSUM x) for mean monitoring and cumulative sum x^2 (CUSUM x^2) for variance monitoring (Reynolds et al., 2004), and for attribute (or qualitative) data, binomial cumulative sum (CUSUM) (Bourke, 2001) has been recommended.

Table 5.2. The list of operations in blue catfish sperm high-throughput cryopreservation process.

Operation number	Operation name	Material used
1	Fish handling	Blue catfish males
2	Fish dissection	Blue catfish males
3	Testes processing	Blue catfish testes
4	Filtering sperm suspension	Crushed testes
5	Inspection	Sperm suspensions
6	Preparing for freezing	Sperm suspensions
7	Packaging	Sperm suspensions
8	Freezing and off-line inspection	Unfrozen straws
9	Sorting and storage	Frozen straws
10	Off-line quality check	Frozen straws
11	Transportation preparation	Daisy goblets

Related internal quality characteristics and specification design

The major quality characteristics of the final products were influenced by the many internal quality characteristics along the operations of processing. As part of the quality

assurance plan, identifying and managing these internal quality characteristics helped to control the process and reduce the variation in product quality.

Defects

The high-throughput cryopreservation process was identified as a batch process with several material transformations during the process (Table 5.2). Batch processing meant that from fish materials were handled individually to sperm suspension. After packaging, the individual materials were distributed into smaller units (straws), but they were still identified by their sources as individual fish and were handled together. Throughout the processing, every step of the operation could incur defects that might lead to wastes, scraps or low quality (Figure 5.1). Some of these defects were related to incoming material quality while others could be caused by operational errors throughout the process.

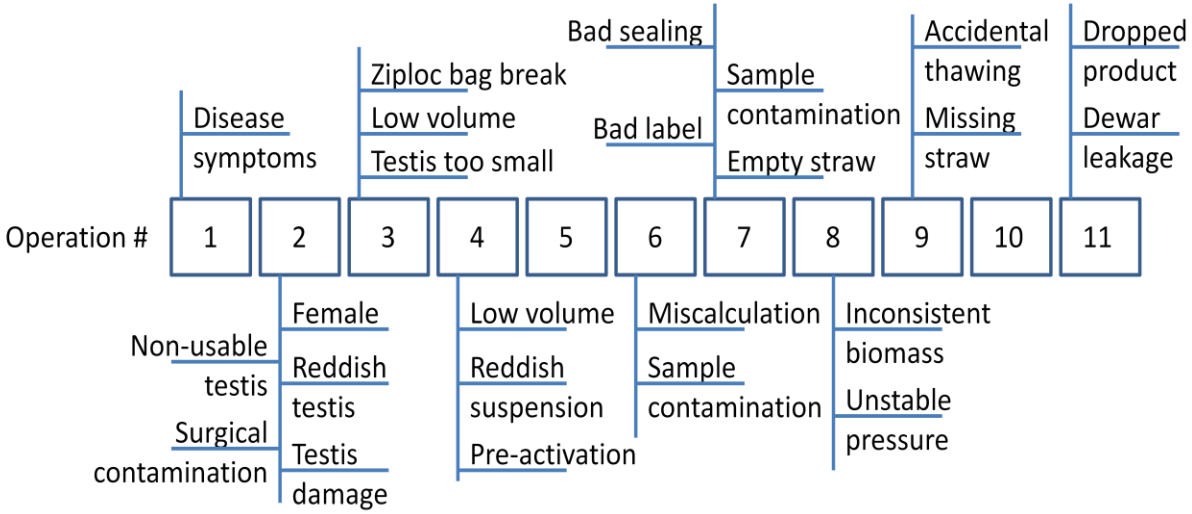

Figure 5.1. Common defects along the entire 11-step process of blue catfish sperm high-throughput cryopreservation.

Identification of quality characteristics

Quality characteristics are identifiable characteristics that can affect product quality. Generally, all possible defects can be considered as quality characteristics. However, tracking

too many characteristics at one time will increase work, and the cost of inspection. Therefore, only key quality characteristics were targeted to be regularly checked. In the process, three inspections were included as routine tasks. Sperm motility, membrane integrity, and sperm concentration were measured through inspections in Operations 5, 8, and 10. Sperm motility and sperm cell membrane integrity reflected sperm quality (Rurangwa et al., 2004); concentration reflected sample amounts (Suquet et al., 1992). Any defects (Figure 5.1) that directly led to changes in those three characteristics, which included all sorts of contamination, low volume of sperm, or freezing and sorting errors, were eliminated as secondary characteristics. The independent defects (Figure 5.2) along with motility, membrane integrity, and concentration were key quality characteristics of this process (more discussion on these characteristics is provided in following sections).

Key internal qualitative quality characteristics

Among the internal quality characteristics, some were measurable or quantifiable, others were not. In general, those non-measurable quality characteristics were defined by qualitative specifications. Even measurable characteristics can use qualitative specifications according to manufacturer's recommendations (Creveling, 1997). Qualitative specifications set standards for defect identification, such as phenomenon, appearance, or a certain numerical level. In this study, six key quality characteristics were applied with qualitative specifications (Table 5.3).

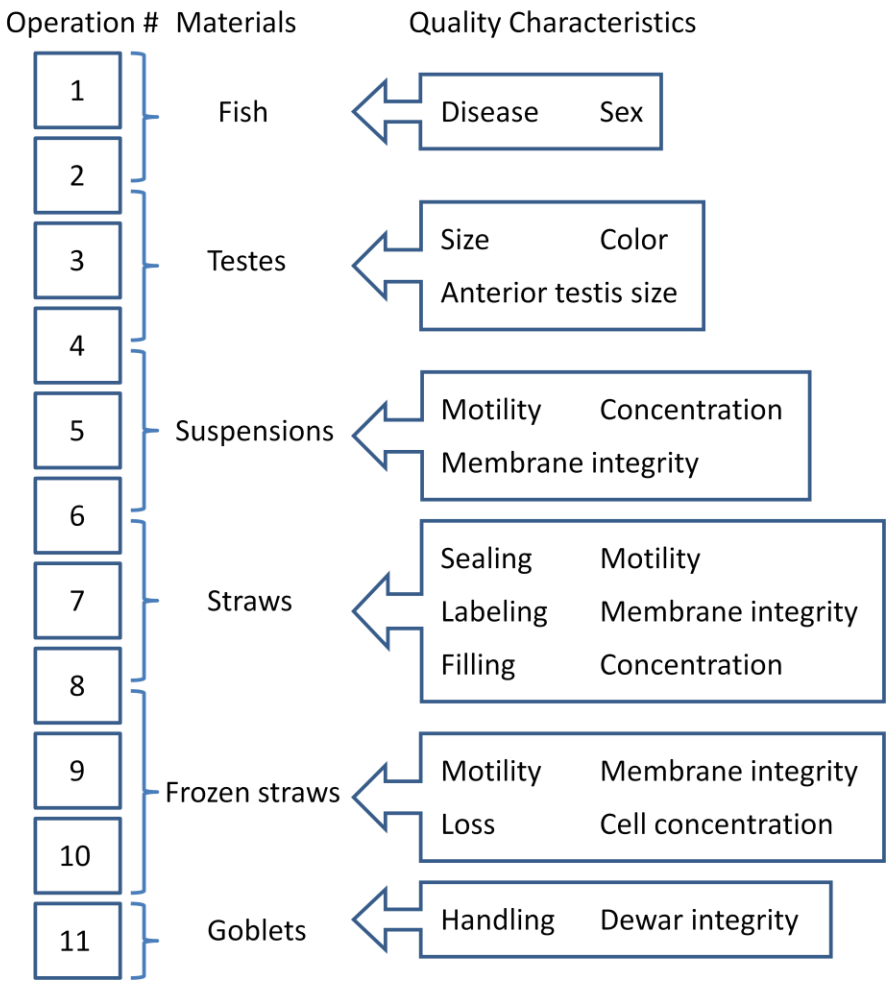

Figure 5.2. Key quality characteristics with their target materials in entire 11-step process of blue catfish sperm high-throughput cryopreservation.

Key internal quantitative quality characteristics

To further reduce the number of quality characteristics needed to be inspected, a correlation was performed among quantitative quality characteristics. The sperm samples taken from intact testes were labeled as “intact”; the sperm samples taken from crushed sperm suspension were labeled as “crushed sample”; the sperm samples taken from sperm suspensions after 30-minute equilibration with cryoprotectant were labeled as “equilibration”; and the samples taken from thawed sperm suspensions were labeled as “post-thaw”. The membrane

Table 5.3. Qualitative specifications for key quality characteristics.

Inspection level	Quality characteristic	Standard for defect	Justification
Fish	Disease	Signs, Symptoms	Health issues, biosecurity
Fish	Sex	Female	No sperm
Testis	Size	No usable testis	No sperm
Testis	Color	Pink or reddish	Undeveloped gonad
Anterior testis	Size	< 3 grams	Too small to work with
Concentration	Initial concentration	$< 1 \times 10^9$ cells/ml	Does not match required density
Motility	Initial motility	< 40%	Low motility, low quality
Shipping	Handling	Dropped products	Damaged products
Shipping	Dewar integrity	Leakage	Damaged products

integrity measurements used in correlation were from Operation 3 (intact membrane integrity), Operation 5 (crushed sample membrane integrity), Operation 8 (equilibration membrane integrity), and Operation 10 (post-thaw membrane integrity). The motility measurements used in correlation analysis were from Operation 5 (initial motility), Operation 8 (equilibration motility), and Operation 10 (post-thaw motility). The concentration measurements used in correlation analysis were from Operation 3 by flow cytometer (intact concentration), Operation 5 by spectrophotometer (initial concentration), Operation 5 by flow cytometer (crushed sample concentration), Operation 8 by flow cytometer (equilibration concentration), and Operation 10 by flow cytometer (post-thaw concentration). To reduce the size of the correlation matrix, related indices were combined: because concentration represented the amount of live cells in every ml of suspension, concentration combined with motility indicated the amount of motile sperm per ml;

concentration combined with membrane integrity indicated the amount of viable cells (membrane-intact cells) per ml (Table 5.4). Those new indices were justifiable with biological meanings. All statistics were performed by use of Minitab (version 14.12.0, State College, PA, USA).

Table 5.4. List of indices combinations for correlation testing in blue catfish sperm high-throughput cryopreservation.

Operation number	Index name	Combined index	New index abbreviation
3	intact membrane integrity	intact concentration	Int.live/ml
5	crushed sample membrane integrity	crushed sample concentration	Cr.live/ml
5	initial motility	initial concentration	Ini.mot/ml
8	equilibration membrane integrity	equilibration concentration	Eq.live/ml
8	equilibration motility	equilibration concentration	Eq.mot/ml
10	post-thaw membrane integrity	post-thaw concentration	Po.live/ml
10	post-thaw motility	post-thaw concentration	Po.mot/ml

A total of 7 combined indices were tested (Table 5.5). The Int.live/ml (intact membrane integrity divided by intact concentration) had no correlation with other indices. The fractions of testis sampled for this index "intact sample" were largely impacted by sampling technique due to small sample size, therefore high variation occurred comparing to other inspections later in the process. The Ini.mot/ml (initial motility divided by initial concentration) was correlated with Cr.live/ml (crushed sample membrane integrity divided by crushed sample concentration); however, the equipment required for measuring Cr.live/ml (flow cytometer) was more complex than use of a microscope or spectrophotometer. In addition, at Operation 5, motility and

concentration testing were built as on-line inspections, but flow cytometer testing was an off-line inspection. To reduce inspection costs, the flow cytometer testing at Operation 5 could be optional. The same reasoning applied to Operation 8 off-line inspection. Because Eq.mot/ml (equilibration motility divided by equilibration concentration) and Eq.Live/ml (equilibration membrane integrity divided by equilibration concentration) were correlated, motility test results were sufficient to represent the quality in that operation. Reduction of the flow cytometer inspection at Operation 8 was based on economics. Operation 10 off-line inspection had two correlated indices. To measure the main quality characteristics more accurately, the flow cytometer was still recommended. With suggestions from the correlation results, the key internal quality characteristics for quantitative specifications design were initial motility, initial concentration, and equilibration motility.

The calculation of the specifications was performed following the method of the main quality characteristics described previously:

The initial motility data were screened and values lower than 40% were discarded based on qualitative quality characteristics specifications. Kolmogorov-Smirnov normality testing showed that the remaining initial motility data did not follow a normal distribution (P=0.007) with a mean of 56% and a standard deviation of 9%. The best fit distribution was Weibull ($P <$ 0.010) with a shape of 7.74, and scale of 60.05. A large number of random data (500 data sets) were simulated from the same distribution and sorted repeatedly. From the probability density function of those simulated data, the 95% confidence interval was from 43 - 70%. In addition, this characteristic was considered to be a one-sided specification, so the adjusted specification was set at 43 – 100%.

Table 5.5. Correlations among combined indices. Int.Live/ml: live cell concentration in testis fractions; Cr.Live/ml: live cell concentration in sperm suspension; Ini.mot/ml: motile cell concentration in sperm suspension; Eq.Live/ml: live cell concentration in equilibrated sperm; Eq.mot/ml: motile cell concentration in equilibrated sperm; Po.Live/ml: live cell concentration in post-thaw sperm; Po.mot/ml: motile sperm concentration in post-thaw sperm.

	Int.Live/ml	Cr.Live/ml	Ini.mot/ml	Eq.Live/ml	Eq.mot/ml	Po.Live/ml
Cr.Live/ml	-0.151					
	0.387					
Ini.mot/ml	-0.309	0.436				
	0.071	0.006				
Eq.Live/ml	0.274	0.725	0.137			
	0.123	0.000	0.407			
Eq.mot/ml	0.014	0.750	0.270	0.837		
	0.943	0.000	0.122	0.000		
Po.Live/ml	0.161	-0.193	-0.260	-0.016	-0.166	
	0.423	0.334	0.158	0.934	0.398	
Po.mot/ml	0.078	-0.046	-0.245	0.188	0.084	0.807
	0.725	0.835	0.219	0.369	0.688	0.000

The initial concentration data were screened and values lower than 1×10^9 cells/ml were discarded based on qualitative quality characteristics specifications. Eight outliers based on the data distribution were identified from the data sets of initial concentration. After the outliers were eliminated, Kolmogorov-Smirnov normality testing showed that the data did not follow a normal distribution ($P = 0.006$) with a mean of 2.68×10^9 cells/ml, a standard deviation of 1.13×10^9 cells/ml, a skewness of 0.579 and a kurtosis of -0.549. The best fit distribution was Lognormal ($P = 0.338$) with location of 21.62, and a scale of 0.43. A large number of random data (500 data sets) were simulated from the same distribution and sorted repeatedly. From the probability density function of those simulated data, the tolerance ranged from 1.23×10^9 - 5.00×10^9 cells/ml.

Kolmogorov-Smirnov normality testing showed that equilibration motility followed a normal distribution ($P = 0.231$) with the mean of 48% and standard deviation 10%. The 95%

confidence interval was from 32 - 64%. With initial motility, this characteristic was considered to be a one-sided specification, so the adjusted specification was 32 – 100%.

Design specifications

The specifications for each internal quality characteristic defined the foundations for quality assurance (Table 5.6). They defined the quality at the accepted level for each operation, such that unnecessary rejection of the final product could be avoided based on the main quality characteristics (Table 5.1). Those specifications also determined the Quality Control Points for inspections for the key quality characteristics during the process. Therefore quality assurance actions took place at each Quality Control Point to collect and monitor quality characteristics.

Table 5.6. Internal quality characteristics and specifications in blue catfish sperm high-throughput cryopreservation.

Quality characteristics	Specification type	Reject condition
Disease	Qualitative	Symptoms, signs
Sex	Qualitative	Female
Size	Qualitative	No usable testis
Color	Qualitative	Pink or reddish (blood contamination)
Anterior testis size	Qualitative	< 3 g
Initial concentration	Quantitative	$< 1.23 \times 10^9$ or $> 5 \times 10^9$ cells/ml
Initial motility	Quantitative	< 40%
Handling	Qualitative	Dropped products
Dewar integrity	Qualitative	Leakage
Equilibration motility	Quantitative	< 32%

Control Charts and Process Capability

Besides designing specifications for quality characteristics, quality control is the other important part of a quality assurance plan. Quality control can monitor each quality characteristic and identify the out-of-control samples based on shifts of mean and variation (Lee, 2000). Quality control started from the manufacturing industry (Montgomery, 2008), and was applied to many other fields such as food production (Grigg, 1998) and hospital management (Sego, 2006, Howley et al., 2009). This study was the first application of statistical quality control in aquatic species sperm cryopreservation and potentially the first of its kind for any species.

Quality control of a high-throughput cryopreservation process has its unique features. Firstly, the biological material has inconsistent quality variation. If the quality characteristics were statistically stable over years, one quality control chart generated from a beginning stage could continuously monitor the process. However, if the quality characteristics vary year by year, each year should be monitored on its own by setting a new chart at the beginning of each year. Secondly, the process has a mixture of qualitative and quantitative quality characteristics with 100% inspection. The quality control chart setup depended on the data type and inspection sample size. Use of 100% inspection is a special case in quality control. Recent research indicates that the cumulative sum (CUSUM) control charts performed better than Shewhart control charts in 100% inspection cases (Reynolds et al., 2004). Generally, qualitative characteristics should apply binomial CUSUM (Bourke, 2001, Sego, 2006) and quantitative characteristics should apply ordinary or robust CUSUM (insensitive to normal distribution assumptions) (Reynolds et al., 2004, Reynolds and Stoumbos, 2010). In this study, one qualitative characteristic and two quantitative characteristic were selected to demonstrate the use of the above-mentioned control charts using collected data.

Data collection and analysis

High-throughput sperm cryopreservation was operated in 2010 and 2011 (Hu et al., 2011). Data were collected from inspections throughout the process (Table 5.2). There were 53 sets of records from 2010, and 59 sets from 2011. The shipping inspection (for quality characteristics "handling" and "dewar") was performed at research separate from this dissertation, so the data were not included in this study. Microsoft Excel 2007 was used for data organization and calculation. The development of quality control charts included two phases: Phase 1 was to use normal group data (in-control data in QC nomenclature) to produce a control chart; Phase 2 was to test data of interest (e.g., data from processes under monitoring) using the established chart.

Case study: qualitative data

The testes weight data were presented as:

$$Vi = \begin{cases} 1 \ \ if\ the\ testis\ weights\ less\ than\ 3\ g \\ 0 \ \ if\ the\ testis\ weights\ more\ than\ 3\ g \end{cases}$$

The CUSUM statistics were:

$S_0 = 0$

$Si = \ max(0, S_{i-1} + V_i - k)$ i = 1,2,… (Chang and Gan, 2001),

Where k= $\left.-\log\left(\frac{1-p1}{1-p0}\right)\right/\log\left(\frac{p1(1-p0)}{p0(1-p1)}\right)$, h is the control limit, P0 is the original occurrence probability, and P1 is the shifted occurrence probability.

In Phase 1, from the 2010 data, P0 = 0.08. Based on field experience, if one fish of ten had a testis that was not usable, the population of fish had questionable gonad development. Therefore, assume P1 = 0.1, then k = 0.090. Because the 2010 data were considered as a normal group, the highest CUSUM value for the 2010 data was set as the h value at 3.11 (Figure 5.3).

After the 2011 data were applied to the CUSUM chart in Phase 2, each time out-of-control signals appeared, the first out-of-control data point was eliminated and the CUSUM chart was reset (calculated from the first data point again) until all data sets were in-control. After elimination of 3 out-of-control data sets, all processing was in-control. Those three datasets were identified and the products were separated for further quality inspection and investigation of causes contributing to the out of control status. All of the out-of-control datasets were within the lowest 5% of testis weight. Two of these had no sign of testes. The CUSUM chart accurately detected extreme quality variations.

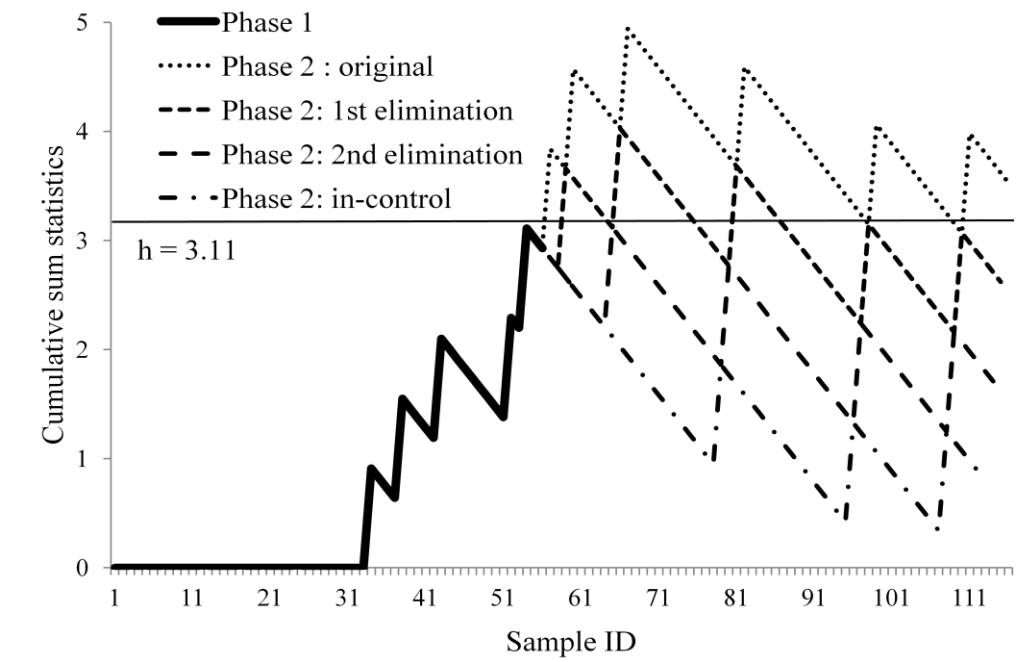

Figure 5.3. Binomial cumulative sum control charts for 2010 and 2011 testis weight data. By 100% inspection, all testes that weighed less than 3 g were assigned a value of 1; all others were assigned a value of 0. The chart was set up with 2010 data as Phase 1 and the control limit (straight line) kept all Phase 1 in-control (no data set exceed the line). The tested 2011 data were Phase 2. The chart presented the process of eliminating each first out-of-control point utill all Phase 2 data were in-control. h: control limit.

Case study: quantitative data indifferent from year to year

T-testing showed that the post-thaw motility was not different from 2010 to 2011 (P = 0.398). In practice, a motility of lower than 10% post-thaw was not recommended for

production. However, even applying the 10% threshold to both years, there was still no difference (P = 0.064). Because high post-thaw motility was not considered to be a negative impact (Table 5.1), the one-sided CUSUM x chart statistics (C_i^{X-}) were more appropriate:

$C_i^{X-} = \min\{0, C_{i-1}^{X-}\} + (\overline{Xi} - \mu 0 + \frac{C_\mu \sigma 0}{2})$, i = 1,2,…, (Reynolds et al., 2004, Reynolds and Stoumbos, 2010)

Where $C_0^{X-} = 0$, $C_\mu = |\mu 1 - \mu 0|/\sigma 0$, and out-of-control signal will be triggered if $C_i^{X-} < -h_{cx}\, \sigma 0/\sqrt{n}$;

For variance monitoring, the statistics of CUSUM x^2 chart (C_i^{X2}) were calculated as:

$C_i^{X2} = max\{0, C_{i-1}^{X2}\} + (\sum_{k=1}^{n} \left(\frac{(Xik-\mu 0)^2}{n}\right) - (\frac{2InC_\sigma}{1-C_\sigma^{-2}})\sigma_0^2)$, i =1, 2,…, (Reynolds et al., 2004, Reynolds and Stoumbos, 2010)

Where $C_0^{X2} = 0$, $C_\sigma = \sigma 1/\sigma 0$, n=1 and out-of-control signal will be triggered if $C_i^{X2} > \frac{h_{cx^2}\, \sigma_0^2}{n} = h_{cx^2}\sigma_0^2$.

In Phase 1, 2010 data had a mean μ0 of 27, a standard deviation $\sigma 0$ of 10.12. To identify the mean shift to the lowest acceptable value μ1 was set at 10, whereas to identify the variation shift that possibly reached the lowest acceptable value $\sigma 1$ was set at 17. To keep all 2010 data in-control (they were a "normal group" by experience), $\min\{C_i^{X-}\} = -h_{cx}\frac{\sigma 0}{\sqrt{n}} = -8$ and $\max\{C_i^{X2}\} = h_{cx^2}\sigma_0^2 = 410.13$. Therefore, the critical parameters of both control charts, and the control limits, were h_{cx}=0.79 and h_{cx^2}=4.00. After being applied to the 2011 data in Phase 2, one data set was identified as out-of-control in the CUSUM x chart (Figure 5.4). In the CUSUM x^2 chart, there were several out-of-control points appearing in the first trial. After eliminating the

first out-of-control dataset, there were in total two out-of-control points from the data of 2011, and the rest remained in-control (Figure 5.4). The out-of-control dataset in the CUSUM x chart was one of the lowest post-thaw motility values. In addition, referring to other parameters of the same sample, it also had the lowest initial motility, which reinforced the observations of the quality control chart. The two out-of-control points from the CUSUM x^2 chart were the highest values of post-thaw motility. Therefore, both out-of-control signals were not considered to be quality threats.

Case study: quantitative data different from year to year

Analysis by t-test showed post-thaw membrane integrity was different from 2010 to 2011 (P = 0.000). Therefore, the 2010 data could serve as the normal group for using in Phase 1, and each year data was monitored separately. Because the available data were insufficient to be divided to show Phase 1 and Phase 2 practices, the following strategy was pursued: 1) identify the data distribution using production data early of the year, 2) randomly generate data for the quality control chart (Phase 1), and 3) test the production data with an established control chart (Phase 2). With the use of Minitab 14.12.0 (State College, PA, USA), the 2010 data were fit into a 3-parameter Weibull distribution (P = 0.084) with a shape of 550.73, a scale of 32.31, and a threshold of -31.49. In Phase 1, 30 new datasets were simulated from the same distribution and a CUSUM x chart was generated based on them. The simulated data had a mean μ0 of 0.778, a standard deviation σ0 of 0.074; to detect a 50% change in motility μ1 was set at0.278. After calculation, the lower limit for C_i^{X-} was -0.041. Repeating the same calculation as the last case study, the control limit was h_{cx}=0.55. All simulated data were in-control. In Phase 2, after applying the 2010 data, all data were in-control. For the CUSUM x^2 chart, to detect a large

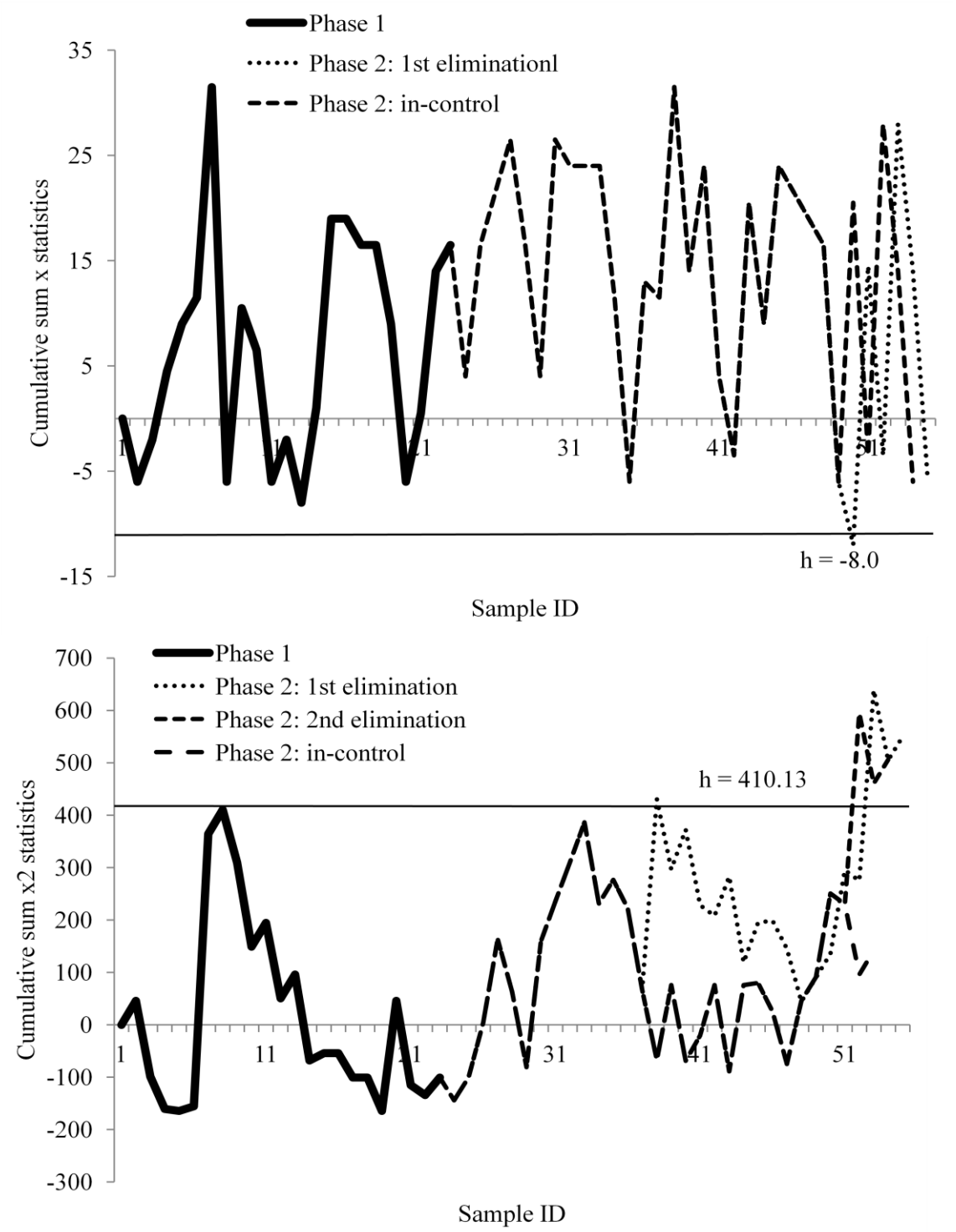

Figure 5.4. Cumulative sum x and x square (x^2) control charts for 2010 and 2011 post-thaw motility data. With 100% inspection, the cumulative sum x chart (above) monitored the mean shifts and the cumulative sum x^2 chart (below) monitored the variation changes. The charts were set up with 2010 data as Phase 1 and the control limit (straight line) kept all Phase 1 in-control. The tested 2011 data were Phase 2. The charts presented the process of eliminating every first out-of-control point utill all Phase 2 data were in-control. h: control limit.

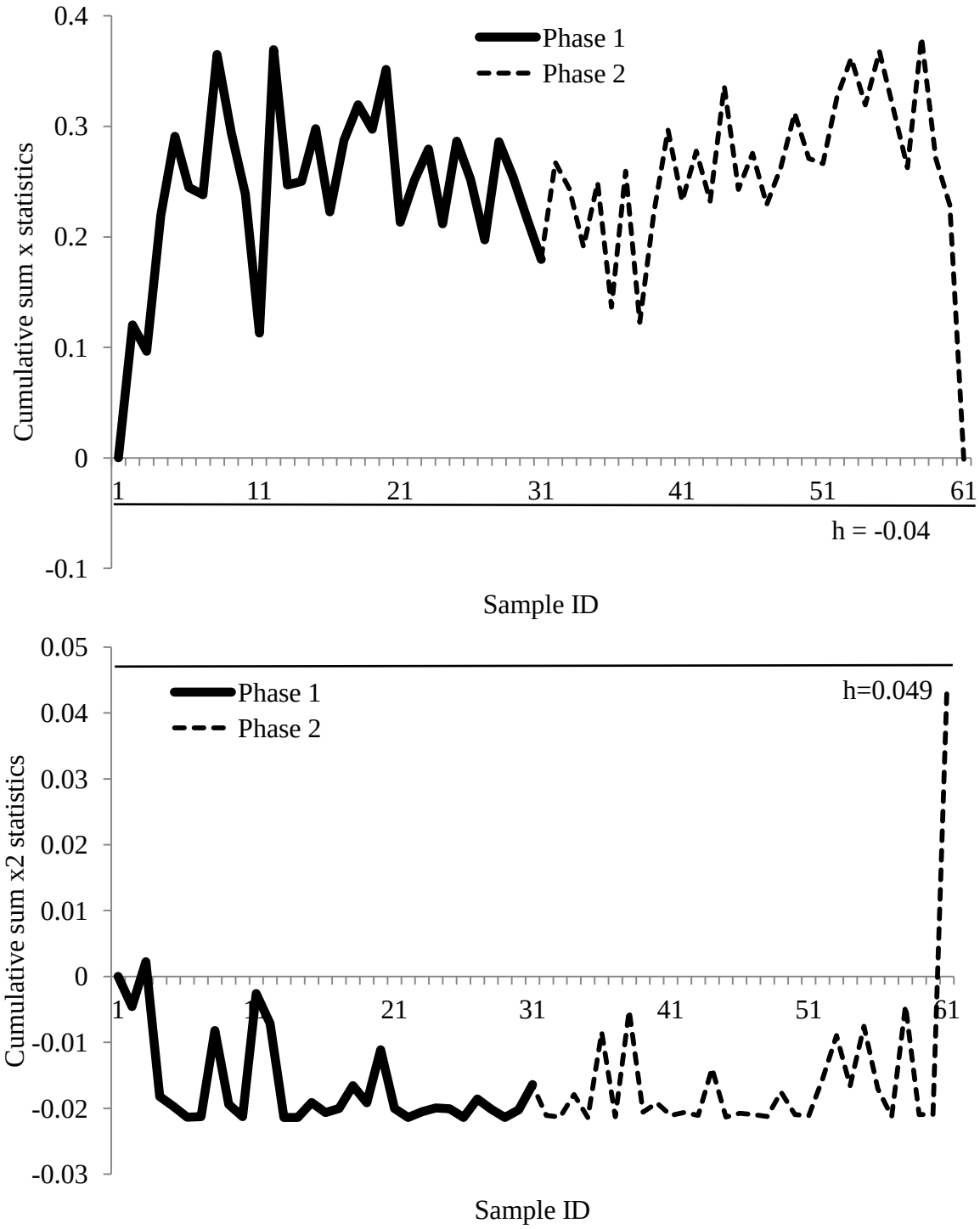

Figure 5.5. Cumulative sum x and x square (x^2) control charts for 2010 post-thaw membrane integrity data. With 100% inspection, the cumulative sum x chart (above) monitored mean shifts and the cumulative sum x^2 chart (below) monitored variation changes. The charts were set up with simulated data from the same distribution of the 2010 data as Phase 1 and the control limit (straight line) kept all Phase 1 data in-control. The 2010 data tested were Phase 2. The cumulative sum x chart and the cumulative sum x^2 chart were all in-control. h: control limit.

standard deviation in the simulated data $\sigma 1$ was set at 0.5, the upper limit for C_i^{X2} was 0.049 and the control limit was h_{cx^2}=8.95. In Phase 2, all data were in-control (Figure 5.5).

The 2011 data was fit into 3-parameter Weibull distribution (P = 0.266) with a shape of 486.46, a scale of 32.39, and a threshold of -31.65. In Phase 1, 50 new datasets were simulated from the same distribution and a CUSUM x chart was generated based on them. The simulated data had a mean μ0 of 0.717, and a standard deviation $\sigma 0$ of 0.078, to detect a 50% change in motility μ1 was set at 0.217. After calculation, the lower limit for C_i^{X-} was -0.033, and the control limit was h_{cx}=0.42. After applying the 2011 data in Phase 2, one data set was out-of-control (Figure 5.6). To detect a large standard deviation in simulated data in the CUSUM x^2 chart $\sigma 1$ was set at 0.5, the calculated upper limit for C_i^{X2} was 0.055, and control limit h_{cx^2} was 9.04. In Phase 2, the same dataset that was out-of-control in the CUSUM x chart also appeared out-of-control in the CUSUM x^2 chart (Figure 5.6). The out-of-control sample had the lowest membrane integrity as well as the lowest initial and post-thaw motility. According to the results from the quality control chart, this sample in 2011 was discarded due to unreliable quality.

Process capability

There were a total of 13 quality characteristics in the current process, and each had specifications based on customer expectation (Creveling, 1997), which was independent to the process operation. Therefore, although the process was in a state of statistical control with the help of quality control (Montgomery, 2008), it still exceeded specification limits occasionally and did not always produce reliable products. The process capability indicated that the operating process was meeting the specifications by comparing the width of the process variation with the width of the specifications. A higher value of process capability meant a better process with

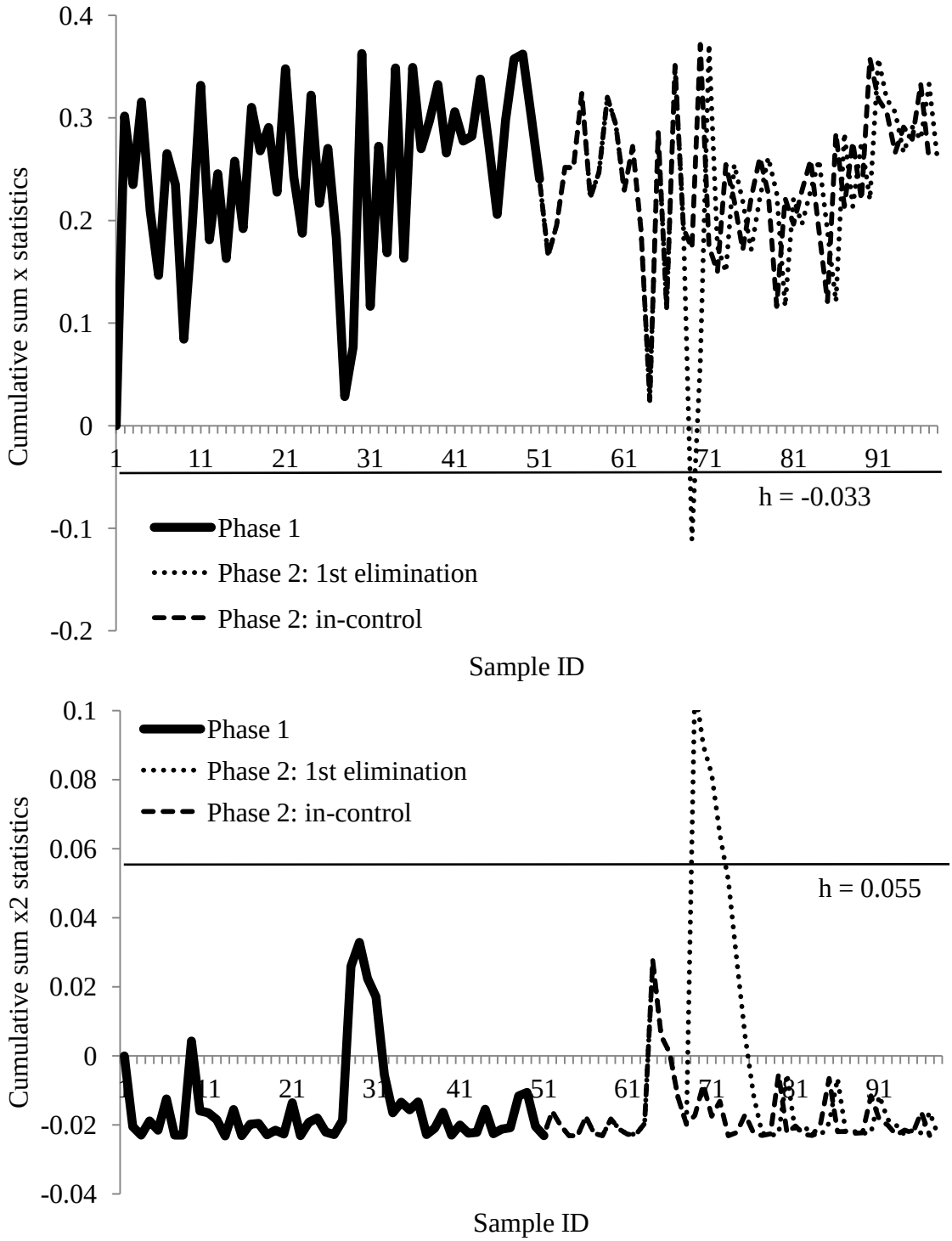

Figure 5.6. Cumulative sum x and x square (x^2) control charts for 2011 post-thaw membrane integrity data. With 100% inspection, the cumulative sum x chart (above) monitored mean shifts and the cumulative sum x^2 chart (below) monitored variation changes. The charts were set up with simulated data from same distribution of 2011 data as Phase 1 and the control limit (straight line) kept all Phase 1 in-control. The tested 2011 data were Phase 2. The charts represented the process of eliminating every first out-of-control point utill all Phase 2 in-control. h: control limit.

respect to the specifications. For example, in a no-mean-shift normal distribution of quality characteristics, when process capability was one, the process was under a three-sigma level of control, which meant only a 0.3% chance of failure (Creveling, 1997).

To carry out process capability analysis, the process must be in-control (Montgomery, 2008). If the process was not in control, it was not stable and cannot be described by one distribution. Without a representative distribution, the process variation cannot be predicted during process capability calculations. After the process was in-control, there were different capability indices for different data distributions. For binomial data, a standard normal distribution for historical defect rates was used to test the average defect rate of in-control data and generated the process Z value. For normally distributed data, the short-term process index of process capability (Cp) represented the ratio of the specification width (USL - LSL) to the process width (6 * estimated standard deviation). For other distributions, long term capability of process (Pp) represented the ratio of the specification width (USL - LSL) to the process width that was the distance between the 99.87th and the 0.13th percentiles in the respective distribution. In general, when the process was under three-sigma level of control, process Z was equal to 3, Cp was equal to 1, and Pp was equal to 1. With the use of Minitab 14.12.0 (State College, PA, USA), the process capabilities of all quality characteristics were calculated when Phase 2 of quality control was in-control (Table 5.7). Some quality characteristics were not listed because they did not have enough data. Only post-thaw motility, equilibration motility, and 2011 initial motility were able to achieve 3-sigma capability, others required improvement by either increasing the width of specifications if appropriate, or by reducing the width of quality variation.

Table 5.7. Process capability of quality characteristics in blue catfish sperm high-throughput cryopreservation. Pp: long term capability of process; Process Z: Z-value using standard normal distribution for average defect rate; Cp: short term process capability.

Quality characteristic	Year*	Data distribution	Index	Value
Post-thaw motility		Smallest Extreme Value	Pp	1.18
Post-thaw membrane integrity	2010	Weibull	Pp	0.60
	2011	Weibull	Pp	0.53
Post-thaw concentration	2010	Weibull	Pp	0.13
	2011	Logistic	Pp	0.08
Sex**	2011	Binomial	Process Z	1.64
Size		Binomial	Process Z	2.10
Color		Binomial	Process Z	1.24
Anterior testis size		Binomial	Process Z	1.40
Initial concentration	2010	Weibull	Pp	0.16
	2011	Normal	Cp	0.71
Initial motility	2010	Smallest Extreme Value	Pp	0.76
	2011	Weibull	Pp	1.01
Equilibration motility		Weibull	Pp	1.46

*When 2010 and 2011 data were statistically different, quality control was performed separately.
** 2010 data showed no defects, therefore, quality control was only performed on 2011 data.

Conclusions

This chapter has presented an initiative taken to formulate a quality assurance plan for a newly developed high-throughput cryopreservation process of fish sperm. Key quality characteristics, for final products and middle products, were identified and their specifications were designed based on data collected during one years of operation (2010, normal group). Different customers could have different quality requirements. For example, genetic evaluation (e.g., in diallel crosses) require lower levels of sperm fertility (only to prodce enough offspring for analysis). In such case, the specifications could be less strict than those presented in this

paper for commercial production scenarios. Even for a particular customer, the requirements could vary on based on specific needs. For example, if all fish were below the specification quality in a certain year (perhaps due to weather conditions), the cryopreservation processer and the customer both face the risk of complete shut down for that year or season. In such a case, the specifications could be redesigned to allow processing of lower quality material to balance the interests of the processer and customers. Overall, specifications played an important role in process standardization. Development of specifications for biological material processing will present new challenges and ideas for research.

It is recommended that the current high-throughput cryopreservation process be monitored by use of a CUSUM chart for 100% inspection. With proper design of control charts, any out-of-control signals will automatically reject the sample. The control chart could provide more information on operational activities. For example, if out-of-control points appeared in equilibration motility, quality personnel could check errors during the previous operation (cryoprotectant or filling preparation). A well-developed quality control system can stabilize variation and increase product quality and competitiveness.

An important feature of this quality assurance plan was the currently intrinsic variation introduced by biological materials. In this study, five of eight key qualitative quality characteristics were used to select proper materials before cryopreservation. Therefore, the quality of incoming materials (live fish) is extremely important. Catfish processing plants inspect the quality of incoming material by sampling before transport (Silva et al., 2001). A similar acceptance-sampling approach is also recommended for high-throughput cryopreservation. If the acceptance sampling can determine the quality of the materials before transport to the

cryopreservation processing facility (Montgomery, 2008), the chance of waste due to the nature of biological materials will be reduced.

The outcome of this study indicates that the basic structure of the quality assurance plan developed in this initiative was functional. With the development of aquatic germplasm cryopreservation, more research on process control will be needed in high-throughput processing. This work can serve as a foundation for the expansion of interests in commercialization of cryopreservation processes in other aquatic species.

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Chapter 6
An Evaluation Method for Artificial Spawning Using Dose-Based Cryopreserved Sperm: Commercial-Scale Sperm Processing of Blue Catfish *Ictalurus furcatus* to Produce Hybrid Catfish

Artificial fertilization technologies are widely used in animal reproduction. These technologies can give better control of propagation, enable crosses not otherwise possible, and provide opportunities for inclusion in genetic improvement programs. Artificial insemination (AI) research on farm animals, especially horses, began in Russia in 1899, and the techniques were well developed for large-scale application in cattle by the 1930s (Foote, 2002). Cryopreservation of bull sperm gained promising and consistent results by the late 1950s (Foote, 2002), and an international market for frozen semen developed quickly afterwards. In comparison to mammals, artificial spawning of fish started even earlier. Trout, salmon, and common carp were artificially spawned in 1767, 1842, and 1860 (Woynarovich and Horvath, 1980, Lichatowich, 2003). Although artificial spawning of fish was applied and generated great economic value (Umali-Maceina, 2007), sperm cryopreservation technology did not attract research and public attention. The first published report of freezing of fish sperm was in 1953 (Blaxter, 1953), but currently, no commercial trade network exists for sperm of aquatic species, even for highly researched biomedical fish such as zebrafish *Danio rerio* (Tiersch et al., 2011).

Because of the genetic benefits brought about by selective breeding and AI technology, cryopreserved livestock sperm is recognized as a product for management of genetic resources. For example, the National Association of Animal Breeders - Certified Semen Services, Inc. (NAAB-CSS, http://www.naab-css.org) is a global network established to provide standards for bull semen. According to this database, direct dairy semen export sales from the US were worth $124,021,998 in 2011 (NAAB-CSS, 2011). The approaches used for livestock semen have been

tested and refined for decades and offer a model available for adaptation to aquatic species. Although cryopreserved fish sperm are not commercialized like bull semen, scientists have recognized the genetic management value of germplasm and was aquatic species were included from the start in the USDA repository of the National Animal Germplasm Program (NAGP, http://www.ars.usda.gov/Main/docs.htm? docid=16979). In addition, with the increasing demand for hybrid catfish, companies and research facilities have cooperated to cryopreserve and use blue catfish *Ictalurus furcatus* sperm at a commercial scale (Hu et al., 2011). Traditional channel catfish culture has experienced depression in the limited states since 2001 (USDA-NASS, 2005). Hybrid catfish (channel catfish female x blue catfish male) have become an alternative to channel catfish for producers due to traits such as high growth rate and disease resistance (Dunham and Masser, 2012). However, culture of blue catfish is limited (Avery et al., 2005), and live fish transportation can be costly and stressful (Wynne and Wurts, 2011). Processing of blue catfish males and cryopreserving sperm for storage and transportation has proven to be a solution to compensate for the problems and uncertainty of using live fish sources.

Cryopreserved fish sperm has been widely researched in different laboratories around the world for more than 3 decades (Tiersch, 2011). In those studies, protocol elements such as cryoprotectants and cooling rates required optimization and standardization to reduce quality variation (e.g., Chapter 3). The use of evaluation methods such as sperm motility or fertilization rate varies considerably among laboratories. Sperm motility has been the most commonly used parameter because tests are straightforward and the results are related to a specific sperm quality feature (Rurangwa et al., 2004). In comparison to the routine accessibility of motility data, fertilization tests are carried out under limitations such as facility (hatchery) availability,

seasonality of spawning of females, and fish availability (e.g., limited by high value or conservation status). Therefore, most fish sperm studies do not provide fertilization results.

Recently, ongoing large-scale production and application of cryopreserved blue catfish sperm has provided the opportunity to generate comprehensive data on fertilization in relation to cryopreserved sperm use. By use of a stand-alone aquatic species cryopreservation facility, high-throughput processing with standardized protocols (Hu et al., 2011) and quality assurance have produced reliable products, and fertilization results were recorded in the hatchery. In addition, routine commercial hatchery practices offer opportunities for further data collection. The number of hybrid catfish fry produced for each hatching trough (Steeby and Avery, 2005) can be estimated before transfer out of the hatchery (Dunham and Masser, 2012). Therefore, the outcome (fry production) can be connected to the specific eggs and sperm used per hatching trough.

With the availability of these data, the goal of this study was to develop a new evaluation method that represented artificial spawning of hybrid catfish when using cryopreserved blue catfish sperm in specified dosages from a high-throughput production line. The objectives were to: 1) identify the similarities and differences between artificial fertilization techniques of cattle and catfish; 2) develop an evaluation method for catfish; 3) assess the utility of the method in a commercial hatchery, and 4) identify factors affecting the new evaluation method.

Comparison between cattle and fish in artificial fertilization

Cattle (beef and dairy) are among the most studied agricultural animals in reproduction. For example, from 2008 to 2011, 50% of funded Phase 1 proposals on animal reproduction in the USDA Small Business Innovation Research program (SBIR, http://www.nifa.usda.gov /funding

/sbir /sbir_abstracts.html) were directly applied to cattle. In the 2013 USDA funding, one third of the health inspection budget for animals will be spent on cattle (UDSA, 2012). Industrial application of cryopreservation is widely accepted for cattle, for example, 86% of cattle breeds have semen stored in Europe (Hiemstra et al., 2011). Catfish aquaculture has begun research to adopt technologies from cattle, such as ultrasound for assessment of gonad development (Guitreau et al., 2012) and large-scale sperm cryopreservation (Lang et al., 2003a). In this study, we looked to adapt the evaluation methods for cattle artificial insemination (AI) to fish artificial spawning by first comparing the similarities and differences between cattle and catfish.

In addition to differences at a genomic level, reproduction varies between cattle and catfish due to different life-history strategies. In broad terms, cattle can be characterized as an example of K-selection which focuses on competing for limited resources, while catfish (like most fishes) represent r-selection which focuses on higher fecundity (Ricklefs and Miller, 2000). Based on the K-selection model, cattle have low fecundity, internal fertilization, and a high survival rate of offspring. The cattle AI industry has been developed based on these reproductive features. In the past decade, 63% of the dairy cattle in the US were bred by AI (O'Connor, 2002). In general, when a cow enters detectable estrus, AI is performed by inserting the contents of a straw (cryopreserved sperm) into the reproductive tract, thereby releasing sperm into the uterine body. Each cow is artificially inseminated with single straw (referred to as a "service") individually for one or multiple times to achieve confirmed pregnancy (Figure 6.1). The "Services per Conception" is defined as the average number of services required to obtain a pregnancy (Cook, 2009). The pregnant cows are under constant monitoring and care. Whether the pregnant cow gives birth or not is recorded as the binary evaluations of "Calving Success" (CS) or "Calving to First Insemination" (CFI) (Donoghue et al., 2004b). The CS describes

whether calving occurred, and CFI describes whether the calving occurred within an acceptable time range after the first insemination (Donoghue et al., 2004a). This time range is also referred to with another term "Days to Calving" which is defined as the time from first insemination to subsequent calving (Buddenberg et al., 1990, Johnston and Bunter, 1996). The time-critical aspect of cattle AI is in determination of the estrus period, which defines the window of conception. Therefore, considerable effort has been invested into regulation and recognition of estrus in cattle (Larson and Ball, 1992).

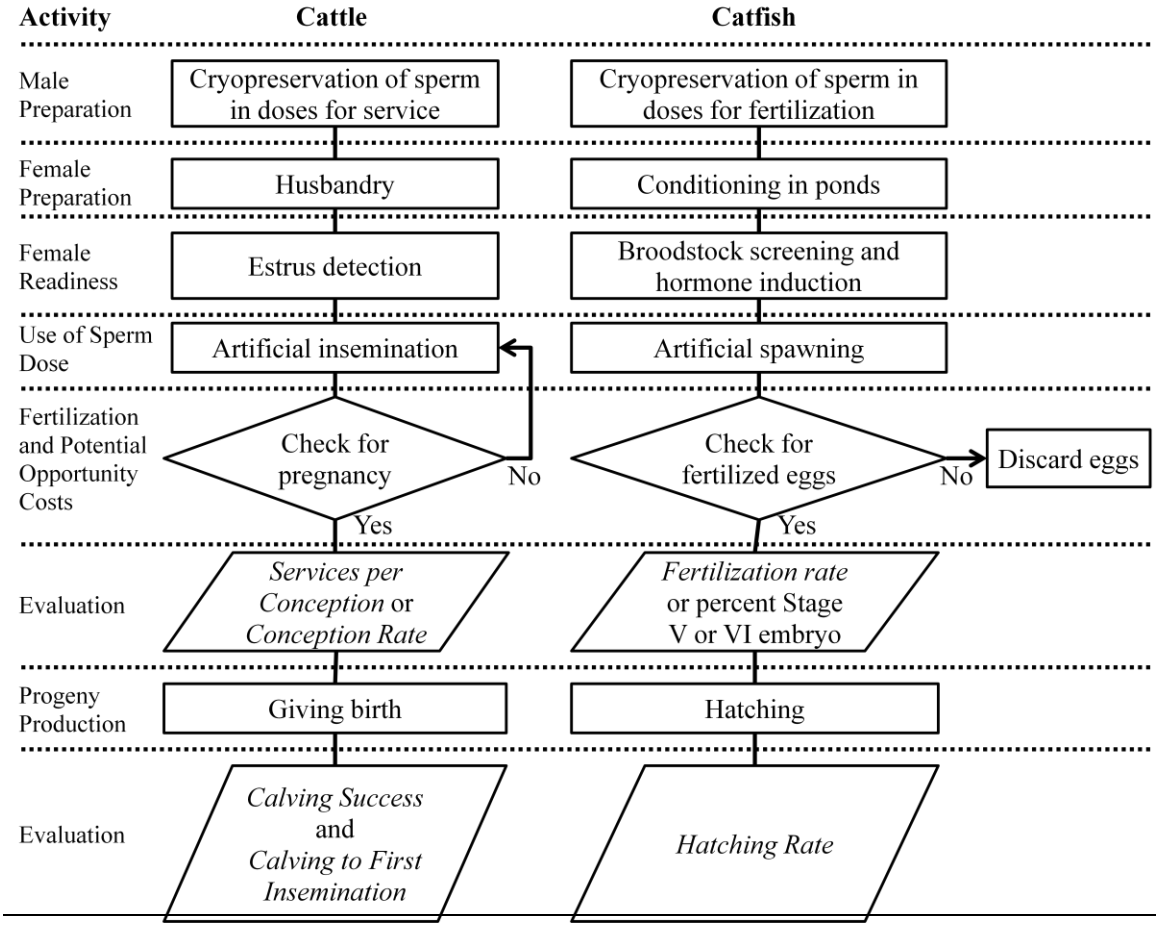

Figure 6.1. Flow charts comparing activities related to cattle artificial insemination and catfish artificial spawning in current commercial application. The shapes of objects in flow charts represent different functions: processes (rectangular), decisions (diamond), or data recording (parallelogram). Specific terms are indicated in italics. Rough equivalency between these two different reproductive processes is indicated by alignments of successive steps.

Methods for artificial spawning vary among fish species. Live-bearing fishes such as the green swordtail *Xiphophorus hellerii* require injection of sperm into the female (Yang et al., 2007). Aquaculture species such as channel catfish *Ictalurus punctatus* (Steeby and Avery, 2005) and striped bass *Morone saxatilis* (Rees and Harrell, 1990) rely on the technique called "hormone-induced strip spawning". In general, females are injected with gonadotropic hormones (e.g., luteinizing-hormone releasing hormone) to induce ovulation. Once the females ovulate, eggs are collected from females by pressing along the belly (i.e., "stripping"). The collected eggs are fertilized with fresh or thawed sperm. In the case of hybrid catfish production, channel catfish eggs are collected and fertilized with sperm from blue catfish (Figure 6.1). Eggs from catfish females only have a single chance within minutes after exposure to water to be fertilized with sperm, and then all eggs, whether fertilized or not, are placed in the hatching system (Avery et al., 2005).

To evaluate fertilization, neurulated embryos (i.e., Stage V, 27 to 30 hr for hybrid catfish at 27 to 29 ℃) or those with initial embryonic mobility (Stage VI, 48 to 50 hr for hybrid catfish at 27 to 29 ℃) are able to be individually identified. The fertilization rate can be assessed as the "Percent Neurulation" (percentage of neurulated embryos) or Stage VI embryo rate (percentage of embryos with initial embryonic mobility). The number of fry can be estimated when they hatch (become free of the chorionic or egg membrane). They are referred to as "sacfry" because of the yolksac they rely on for nutrition. After an additional 5 d, catfish hatcheries estimate the number of swim-up fry (begin feeding and swim freely) in each hatching trough, which contained fertilized egg masses from multiple females (Steeby and Avery, 2005). The ratio between swim-up fry number and the total number of eggs (estimated volumetrically) is termed the "Swim-up Fry Survival Rate". Fertilization (neurulation) rate is not commonly used in

commercial-scale hatcheries as a swim-up fry survival rate (swim-up fry die also referred to as "black fry") and both evaluation methods include the cumulative effects of male variation (e.g., sperm quality), female variation (e.g., egg quality), and hatchery performance (e.g., water quality):

$$\text{Fertilization rate or swim-up fry survival rate} = \mu + \text{sperm}(i) + \text{egg}(j) + \text{water}(k) + \text{error} \quad \text{(Equation 1)}$$

where "μ" is the mean value of fertilization rate or hatching rate, "i" is the number of factors involved in sperm quality, "j" is the number of factors involved in egg quality, "k" is the number of factors involved in water quality. The error represents the residuals (differences between individual rate and mean value) that cannot be explained by the three listed variables (sperm, eggs, and water).

However, with high-throughput cryopreservation processing, quality assurance and dosage control, sperm quality contributes minimum variation to the overall fertility evaluation results:

$$\text{Dose-based fertility evaluation value} = \mu` + \text{egg}(j) + \text{water}(k) + \text{error} \quad \text{(Equation 2)}$$

where "μ`" is the mean value of dose-based fertilization evaluation, "j" is the number of factors involved in egg quality, "k" is the number of factors involved in water quality.

As stated above, differences exist between cattle and catfish in biology and fertilization processes. Because cattle evaluation methods evaluate females individually, they cannot be directly applied to the pooled egg masses used for catfish. However, when using cryopreserved

sperm in hybrid catfish spawning, the dosing (fish sperm in straws) is equivalent to the cattle semen dosing (straws) used in services. In the AI process, cryopreserved cattle semen has been used within the range of 2×10^7 to 10×10^7 sperm/dose (Nadir et al., 1993). The number of sperm is constant for each AI, so that the inseminated cow has sufficient material for conception without waste of valuable semen. Similar to cattle AI, a fish specific evaluation method could therefore integrate standardized sperm doses into existing fish evaluation methods.

The sperm dose concept for catfish

The use of cryopreserved sperm in hybrid catfish artificial spawning provides accurate sperm counts and concentrations. Similar to cattle AI services, a single straw can be the basic unit in hybrid catfish artificial spawning. Based on high-throughput processing and quality control, each 0.5-ml straw contains 1×10^9 sperm/ml (i.e., an average 2.5×10^8 sperm/ml motile sperm after thawing) (Chapter 3). The recommended sperm-to-egg ratio for producing hybrid catfish swim-up fry was set at 1.35×10^5 sperm/egg (Chapter 4). In fertilization practice, straws could be thawed and pooled together in the hatchery, and egg count can be standardized as a volumetric estimate such as 40 ± 7 eggs/ml (Chapter 4, N > 300). Therefore, 3704 eggs can be fertilized with each properly handled straw (dose) or 69-142 ml of eggs (egg size variation) can be fertilized with the contents of one straw (using a 95% confidence interval of egg count). This would then provide the basis of the "dose" concept for cryopreserved blue catfish sperm for use in artificial spawning. To fully apply this dose concept, the quality of sperm in straws as well as the transport, handling and utilization of straws should be standardized (Appendix A SOP-12&13, Appendix B).

If the contents of a single straw are placed on more eggs than the recommended dose (i.e., a smaller sperm-to-egg ratio), some fertilizable eggs may not receive sperm. Therefore, the

outcome of this fertilization would be artificially (and avoidably) reduced. Conversely, if a single straw is used to fertilize fewer eggs than the recommended dose (i.e., a larger sperm-to-egg ratio), some motile sperm could be wasted. Therefore, the outcome of this fertilization would use more sperm than needed. However, if a single straw is used to fertilize eggs within the recommended dose, the outcomes can reliably be used in evaluations to make comparisons to standard fertilization outcomes. The outcomes of fertilizations produced within the proper dose range of sperm-to-egg ratio should be distinguished from existing evaluations (e.g., traditional fertilization rate and hatching rate), because these outcomes include the variation inherent to sperm or other male factors (Equation 2). Therefore, with proper use of cryopreserved sperm within the dose, the ratio between eggs fertilized and swim-up fry produced can be defined as the "Swim-up fry Production Efficiency" (SwPE). In previous large-scale application of cryopreserved blue catfish sperm, the SwPE was 59 ± 24% (Chapter 4, N > 300), which can be considered as an initial standard (experimentally derived) SwPE value when using proper dosing. A further application can thus be evaluated by examining the relative difference of the standard SwPE (SwPE Index):

SwPE Index (SwPEI) = SwPE of test trials / standard SwPE

If the SwPEI value was equal to one, the swim-up fry production was not different from standard large-scale hatching results; if the SwPEI values were less than one, variables in the test trial reduced the efficiency of swim-up fry production; if the SwPEI values were more than one, the test trial contained variables that improved production. Specifically, such an evaluation has two assumptions: 1) sperm straws were produced with high quality assurance, and were utilized following proper instructions; 2) fertilization was performed within the dose range (3704 eggs/straw or 69-142 ml eggs/straw). The initial standard SwPE was calculated using

experimental data from one commercial hatchery to initialize the evaluation method. However, with large-scale industrial application of cryopreserved sperm, it would be possible to calculate an industrial standard SwPE each year (or other time period), which could be generated by annual comparison of individual SwPEs across hatcheries. This process could be strengthened by institution of an official testing protocol using standardized sperm samples collected from specific males, pooled and processed for parallel testing at cooperating hatcheries.

Another benefit of this evaluation method is to enable planning with an industrial (or hatchery-specific) standard SwPE and doses. For example, if the target was to produce 1,000,000 swim-up fry, with a standard SwPE of 59%, 1.7 million eggs would be required, and based on the dose of 3704 eggs/straw, at least 459 straws would be required.

Factors that can affect the SwPE evaluation method

Similar to cattle evaluation methods such as Conception Rate (Smith, 1985), the SwPE evaluation method can be affected by various factors. According to the definition of this evaluation method, the reasons for SwPEI of less than one would be low SwPE at the hatchery (Figure 6.2). In general, because with high-throughput and quality assurance the sperm cryopreservation and sperm dose have minimal variation, the reasons for reduced SwPEI would be due to female broodstock management, fertilization methods, or hatchery management.

Broodfish management is the foundation for fry production. Essential aspects of broodstock management include pond water quality (Kelly, 2004, Steeby and Avery, 2005), proper nutrition (Kelly, 2004), temperature effects on gonadal development of female channel catfish (Brauhn and McCraren, 1975, Lang et al., 2003b, Pawiroredjo, 2004), specific traits of breeds or strains (Kelly, 2004), use of gonadotropic hormones (Avery et al., 2005, Dunham and Masser, 2012),

and geographic differences or climate differences and seasonal changes. As such, each hatchery has to create its own schedule of spawning. In addition, disease outbreaks can increase variability in broodstock quality from farm to farm (Wagner et al., 2002). Collection of data based on SwPE can assist development of industry standards depite these large differences in production conditions.

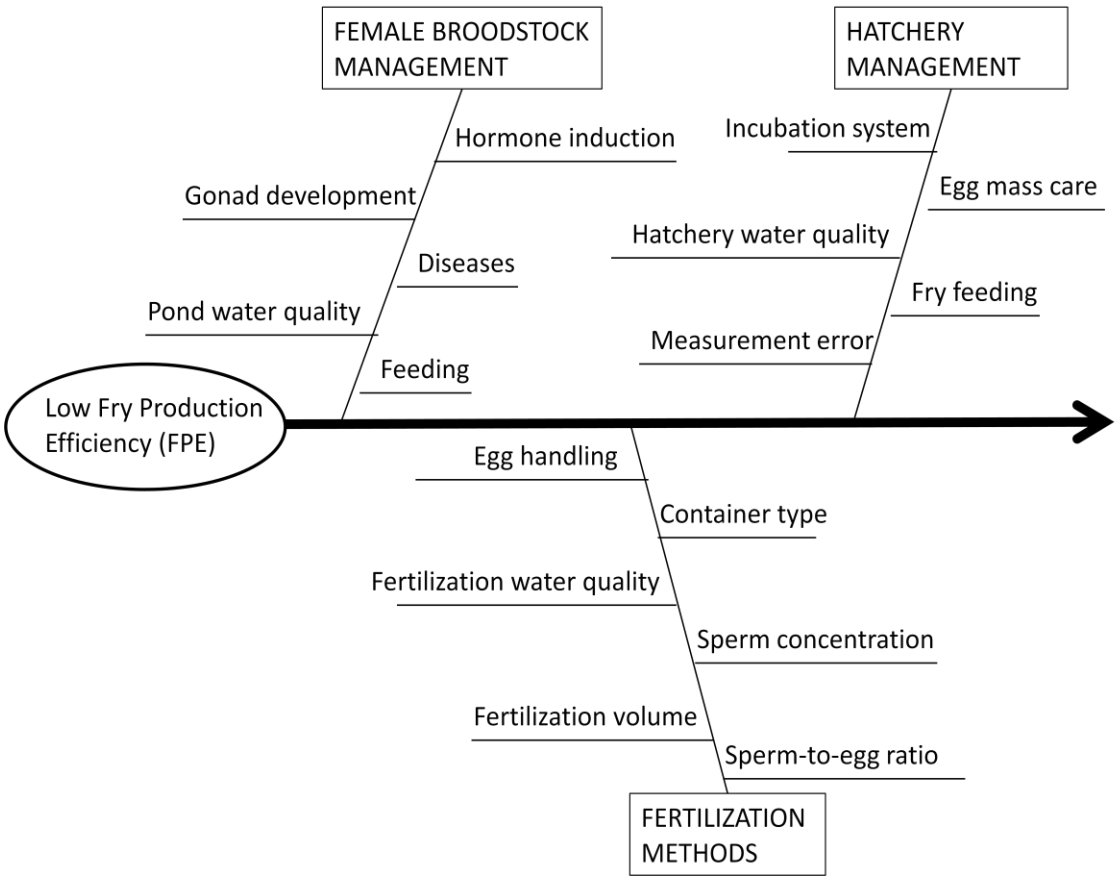

Figure 6.2. Preliminary Cause-Effect Diagram of factors that can potentially affect swim-up fry production efficiency (SwPE) in hybrid catfish production using dose-based cryopreserved sperm. Possible causes for reduced SwPE can be grouped into the three broad categories shown in boxes.

Although the dose defines a sperm-to-egg ratio, there are other important aspects in fertilization. Reporting of the details of fertilizing channel catfish eggs with blue catfish sperm has been variable in previous studies (Table 6.1).

Table 6.1. Representative survey of fertilization details in literature of hybrid catfish fertilization. Five elements were surveyed: container type, sperm concentration (before use in fertilization), sperm volume, activation volume (water added for activation), and number of eggs (or egg volume). These five aspects were defined as fertilization elements. NR: not reported.

Container type	Sperm concentration	Sperm volume	Activation volume	Number of eggs	Literature citation
NR	1×10^{8}/ml	0.5 ml	50 ml	200-300	Tiersch et al., 1994
NR	NR	NR	NR	NR	Bart and Dunham, 1996
210-ml plastic cup	6×10^{9}/ml	0.5 or 1 ml	40 ml	450	Bart et al., 1998
NR	NR	2.5 ml	1000 ml	100 ml	Dunham et al., 2000
NR	NR	1 ml	NR	100	Lang et al., 2003a
NR	NR	2.5 ml	NR	100 g	Phelps et al., 2007
100-ml tri-pour beaker	1×10^{9}/ml	1 or 5 ml	5 or 10 ml	114-167	Cuevas-Uribe, 2011

In general, there are three steps in the artificial spawning methods for hybrid catfish: collection of eggs, mixing and activation of gametes, and egg hardening. The collecting and hardening methods have been well described and widely applied (Tucker, 1991, Avery et al., 2005, Small, 2006). However, the elements of fertilization (size and shape of container, sperm concentration, sperm volume, volume or number of eggs, and activation water volume) are not. These five elements can be considered to be essential elements of fertilization (Figure 6.3) critical to sperm-egg contact.

After eggs have been collected into the container, the number or volume of eggs and the shape of container determines the geometry and potential stacking (layers) of eggs; a suspension of immotile sperm is added (the volume of the suspension is typically smaller than the egg volume and has an uneven distribution of sperm before activation), water is added to activate and mix the gametes, and the total activation volume determines the sperm motility region and potential for sperm-egg contact. Although sperm-to-egg ratios have been reported in the

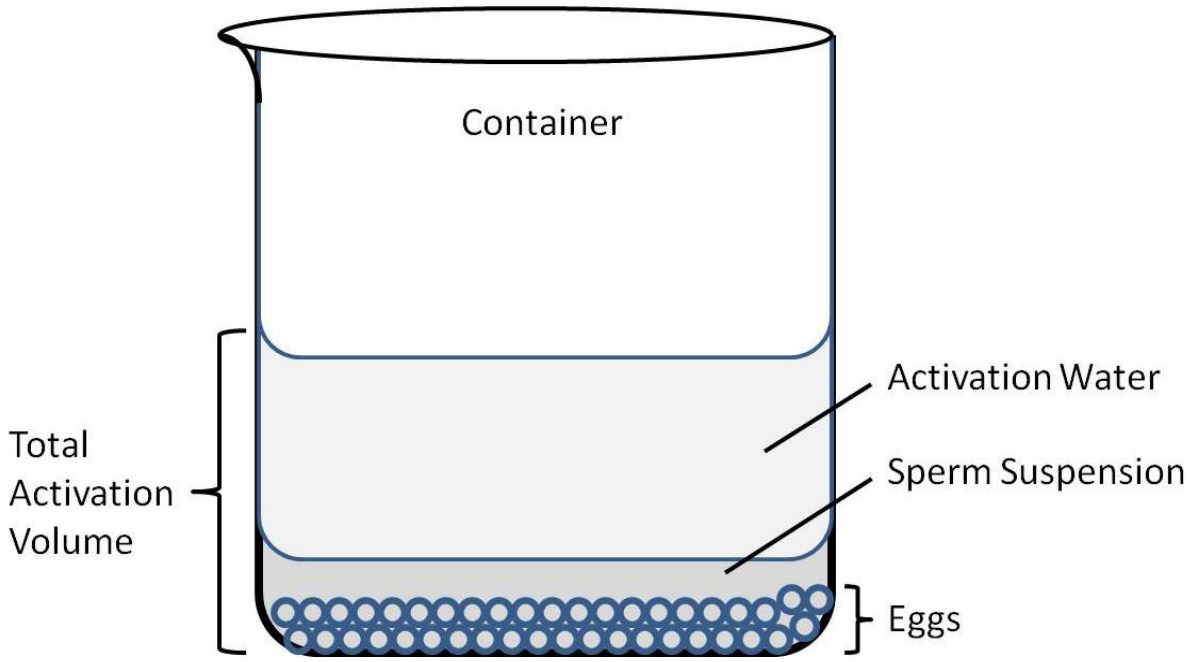

Figure 6.3. The three components of artificial fertilization in a container. Assuming the sperm swim in random directions after activation, the relative volumes of all components (total activation volume) affect the probability of sperm-egg contact and fertilization. The larger the activation volume, the lower the functional sperm concentration (and sperm-to-egg ratio) will be with respect to the available eggs.

literature, the effective relationship between sperm and eggs is dependent on the total fluid volume and cell number in relation to gamete volume and there are few descriptions on how these five elements interact in hybrid catfish production. Of seven hybrid catfish studies that performed fertilization, only two reported all five elements and none of these studies were consistent with each other (Table 6.1). Although experiments conducted in research laboratories serve the purpose of hypothesis testing, differences in fertilization conditions such as these will affect the evaluation methods and the comparability of data among scientific works (Yang et al., 2010). In hatchery production, these fertilization elements would directly affect productivity and SwPE values. As such, part of the process of developing industry standards would be to establish

strict control of these variables. A new potential standard could be "volumetric sperm-to-egg ratio". If both sperm-to-egg ratio and total activation volume were reported, the volumetric sperm-to-egg ratio could be calculated as sperm-to-egg ratio divided by the total activation volume. For example, in two surveyed studies, the volumetric sperm-to-egg ratio were 3.66×10^6 sperm/egg/ml (Bart et al., 1998) and 3.57×10^6 sperm/egg/ml (Cuevas-Uribe, 2011). Control and reporting of these parameters can yield standardized conditions for fertilization.

Many conditions are difficult to standardize among hatcheries due to geographic or management reasons. A practical method of using the new evaluation method is to keep records on identified factors (Fig. 5.2) and fertilization outcomes (SwPE). The standard industrial SwPE could be constantly updated based on broad (perhaps annual) survey of hatcheries using sperm from a standardized source as well as their own proprietary sources. At a minimum, private farms could evaluate the potential for improvement in their own operations, or evaluate performace across years or broodstock sources based on standardized sperm dosing and SwPE values.

As an initial effort to assist industry-wide standardization based on the commercial availability of cryopreserved sperm, a preliminary integrated assessment model has been developed in this dissertation. The concept of "production efficiency" can be applied throughout artificial spawning with assistance of high-throughput cryopreserved sperm (Figure 6.4).

Each "production efficiency" represents measurements at certain phases or steps of the artificial spawning process (Figure 6.4). Male production efficiency (MPE), Female production efficiency (FPE), and Hatchery production efficiency (HPE) are phase production efficiencies, and the others are step production efficiencies. Each phase or step can be assessed qualitatively

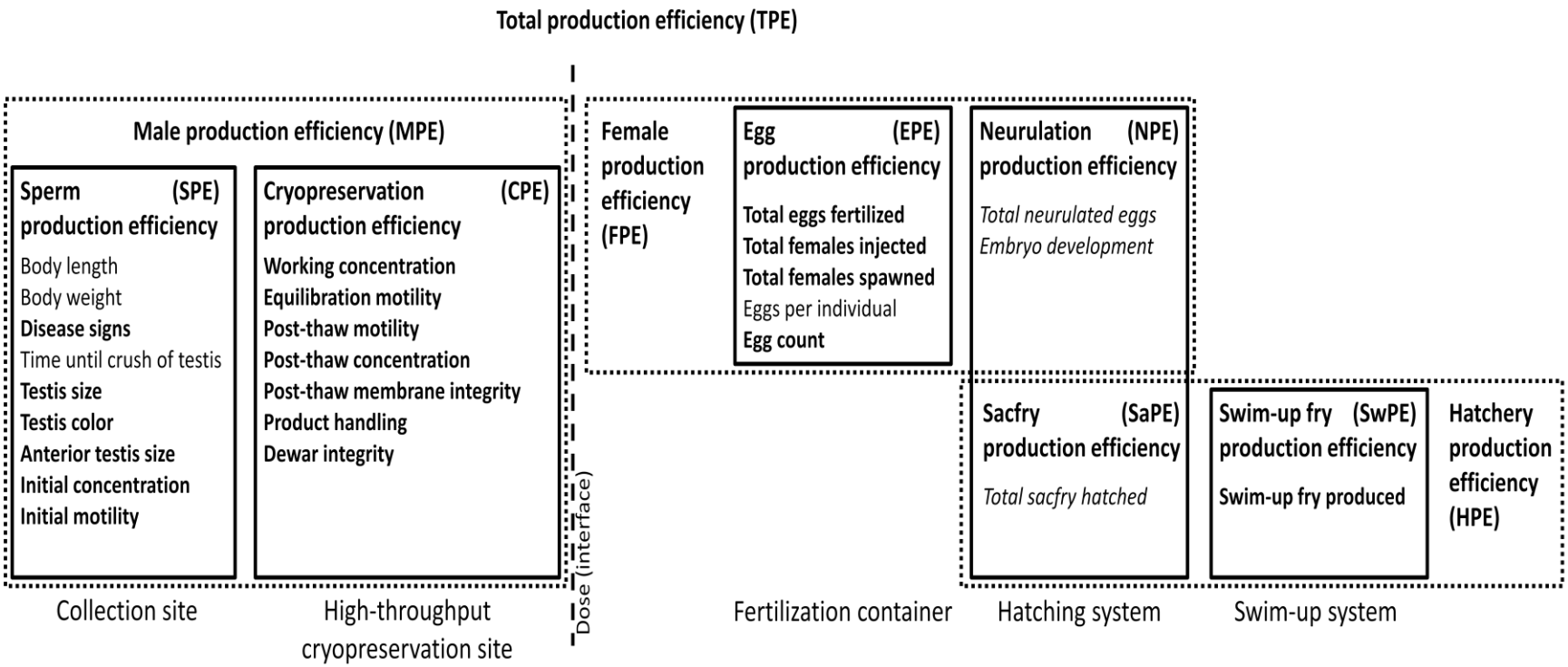

Figure 6.4. Schematic of the production efficiency concept in artificial spawning using high-throughput cryopreserved sperm. Based on the basic commercial and research methods of artificial spawning of channel catfish and hybrids, there are five potential environments (solid boxes): male collection site, high-throughput cryopreservation site, the fertilization container, hatching system, and swim-up fry system. Three main phase production efficiencies (dotted boxes) are used to represent the overall process. The sperm dose (dashed line) links males (sperm) and females (eggs). Under each of the step production efficiencies, there is a list of currently measurable parameters. These or other parameters could be used to assess production efficiency, but the final choices should be standardized to the fullest extent possible. The bold font represents practical parameters used in commercial settings; italicized font represents parameters performed only in the laboratory in current use.

or quantitatively. For example, Egg production efficiency (EPE) could be quantified as the ratio between the number of eggs used in fertilization and the total weight of females injected with spawning hormone; Neurulation production efficiency (NPE) could be quantified as percent neurulation at 30 hr and 27 ℃ (Chapter 3); Sacfry production efficiency (SaPE) could be quantified as percent hatching in terms of the number of sacfry (sometimes referred to as "red fry"). By controlling and monitoring each production efficiency, the artificial spawning process can be standardized. Examples of the conceptual relationships among these terms can also illustrate the relationships that can be used to evaluate quality variation:

TPE = MPE + FPE + HPE

MPE = SPE + CPE

FPE = EPE + NPE

HPE = SaPE + SwPE

In this dissertation, MPE variation was minimized by process standardization and quality assurance developed for SPE and CPE. Therefore, in hatcheries correctly using cryopreserved sperm doses, TPE would predominantly be affected by FPE and HPE. Artificial spawning could then be represented as cumulative effects from FPE to HPE. Although, the SwPE established in this study utilizes the currently available practical data collected from commercial-scale production to evaluate swim-up fry production, EPE, NPE, and SaPE could also be used to evaluate intervening steps and evaluate (or quantify) the sources of variation within the overall process in detail. Collection of these data would require additional efforts in commercial hatcheries that would need to be justified by gains in production efficiency.

Conclusions

This study developed and applied an evaluation method for artificial fertilization to produce hybrid catfish using dose-based cryopreserved blue catfish sperm. The assumptions for this evaluation method were correct handling of sperm cryopreserved with quality assurance and uniform dosing (3704 eggs/straw or 69-142 ml eggs/straw). Different hybrid catfish fry production hatcheries could be evaluated by relative differences among SwPE values and a standard industrial value for SwPE (e.g., 59%) can be used to calculate an SwPE Index. This evaluation method could assist identification of defects or potential improvements in the production system and assist planning for procurement of cryopreserved sperm. The data recording in commercial production would be minimally increased with this evaluation method, and can help systematically identify efficiency variations in catfish production and create opportunities to integrate new technologies or innovations within the industry.

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Chapter 7
Simulation Modeling of High-Throughput Cryopreservation of Aquatic Germplasm: a Comprehensive Study of Commercial-Scale Sperm Processing for Blue Catfish

Computer simulation has been widely used to mimic system behavior (Kelton et al., 2007), and within aquaculture, simulations have been applied to many areas (Table 7.1). These have included structural design, heat transfer, chemical distribution, and farm-level production. Those models assisted aquaculture production by reducing experimentation time and costs, minimizing animal welfare problems, identifying uncontrollable factors, and monitoring of production systems (Lee, 2000, Halachmi, 2006a). With the continued development of computer technology, object-oriented simulation has brought these models ever closer to representing reality (Bolte et al., 2000), and visual interactive simulations can provide straightforward user-friendly interfaces (Porter, 1991). The ARENA software (Rockwell Automation, Inc. Milwaukee, WI) is one of the most popular simulation packages available for discrete event simulation (Halachmi et al., 2005, Halachmi, 2006a), and its visual interaction and fast processing speed have made it a comprehensive tool for use with industrial processes such as automotive, aerospace, and electronics (Takus and Profozich, 1997), as well as aquaculture production processes of interest such as culture of ornamental fish in recirculating systems (Halachmi, 2006a).

As a newly established aquaculture technology, germplasm cryopreservation preserves valuable genetic material (Hu and Tiersch, 2011), and can assist breeding programs and genetic selection. However, compared to the billion dollar global industry that exists for livestock semen (NAAB-CSS, 2011), the industrialization of aquatic germplasm is still at a beginning stage. The only current aquatic application reported in a large-scale commercial farm has been for hybrid catfish production (Hu et al., 2011b). The industrial high-throughput application of aquatic

germplasm will likely be achieved by establishment of facilities capable of year-round processing of multiple species during different spawning seasons (Hu and Tiersch, 2011).

Currently, there is no modeling of cryopreservation processes in any animals, and this dissertation is the initial work for its kind. To model high-throughput cryopreservation, the process pathway needs to be separated into functional components with associated models, which can then be reintegrated (Ernst et al., 2000). The activities in a comprehensive aquatic germplasm facility would share equipment and supplies, but would produce different products from different materials (e.g., various species); different batch sizes (e.g., various packages); different schedules (e.g., various spawning seasons); use slightly different processing (e.g., various collection methods), and as with other agricultural commodities, the incoming sample quality would typically be inconsistent (Parthanadee and Buddhakulsomsiri, 2010). All of these factors provide challenges for process engineering, and to address them all simultaneously has not previously been practical (Lee, 1995).

Based on the first report of commercial-scale application of cryopreserved aquatic sperm (Hu et al., 2011b), the existing high-throughput cryopreservation process for sperm of blue catfish (*Ictalurus furcatus*) can serve as a model for other species, although this species requires dissection of testis (Steeby and Avery, 2005) instead of allowing the stripping of sperm. The same thinking used for knowledge-based control systems (Wu and Joseph, 1992) can be adapted to build a simulation model. Therefore, the goal of this study was to build a simulation model useful for production planning and decision making. The objectives were to: 1) predict the maximum processing output for a typical 8-hr workday; 2) analyze the bottlenecks within the process, and 3) estimate operational costs when the process is run for maximum output.

Table 7.1. Examples of previous uses of computer simulation in aquaculture applications. NR: not reported.

Culture system	Simulation tool	Citation
Cage culture	Self-designed controller	Kim et al., 2011
	commercially available software[1]	DeCew et al., 2010
	NR	Zhao et al., 2010
	NR	Lee et al., 2008
	Self-designed program	Huang et al., 2006
	Self-designed program	Fredriksson et al., 2003
Pond culture	commercially available software[2]	Gutiérrez-Estrada et al., 2012
	commercially available software[3]	Bolte et al., 2000
	NR	Jamu and Piedrahita, 1998
	commercially available software[4]	Hargreaves, 1997
	commercially available software[3]	Gao and Merrick, 1996
	commercially available software[5]	Leung and Shang, 1989
	NR	(Brooks Jr and Kimball, 1981)
Raceway systems	commercially available software[6]	Li et al., 2009
Re-circulating systems	commercially available software[7]	(Halachmi, 2006b)
	commercially available software[7]	Halachmi et al., 2005
	commercially available software[8]	Weatherley et al., 1993

[1] Aqua-FE, University of New Hampshire, Durham, NH, USA.
[2] NUBECILLA 1.0, University of Huelva, Huelva, Spain.
[3] POND, Oregon State University, Corvallis, OR, USA.
[4] STELLA II 3.0.5, ISEE systems, Lebanon, NH, USA.
[5] Microsoft Fortran, Microsoft, Redmond, WA, USA.
[6] MATLAB, MathWorks, Natick, MA, USA.
[7] ARENA, Rockwell Automation, Inc. Milwaukee, WI, USA.
[8] ACSL, The AEgis Technologies Group, Inc., Huntsville, AL, USA.

Methods

The high-throughput cryopreservation of blue catfish sperm can be categorized into six primary steps: 1) dissection, 2) preparation, 3) inspection and organization, 4) equilibration and packaging, 5) freezing and sorting, and 6) storage (Liao et al., 2011). From steps 1 to 3, fish are processed individually. However, from step 4 onward, individual samples can be grouped for maximum utility of system capacity (Liao et al., 2011). The details of each step are described below.

1) Dissection: blue catfish (Hu et al., 2011b) were held in recirculating systems overnight before processing. A single technician brought one male fish from the system to the dissection table. The male was killed by a sharp blow to the head according to IACUC approved procedures. The body cavity was opened by cutting from alongside the anal fin to the pectoral fin, and testes were extracted by carefully separating attached tissues (Appendix A, SOP-2).

2) Preparation: while the dissector continued dissecting fish, another technician cleaned the testes and extracted sperm into extender solution [Hanks' balanced salt solution (Tiersch et al., 1994) at an osmolality of 300 mOsmol/kg, Appendix A, SOP-1]. If the testis weight was below 3 g, the extracted sperm were considered to be insufficient for collection. Therefore, any testes 3 g or smaller were discarded. After extraction, the sperm were filtered through three layers of screens (1-mm mesh, 0.5-mm mesh, and 200-u mesh). Samples sometimes were placed in a queue if labor or tools were not immediately available for filtering. The final filtered sperm suspensions were held in loosely capped 50-ml centrifuge tubes (Appendix A, SOP-3). All testes were prepared within 30 min after dissection.

3) Inspection and organization: technicians gathered all filtered sperm suspensions as a group before analysis. The motilities were tested using darkfield microscopy (Olympus CX41RF, Japan) at 200-× magnification. For each sample, a 1-μl drop of sample was activated by the addition of 20 μl of water on a glass slide on the microscope. Estimation was made based on the percentage of progressively motile sperm in relation to all visible sperm (Appendix A, SOP-4). Concentrations were estimated using a microspectrophotometer (NanoDrop 1000, Thermo scientific, Wilmington, DE). For each sample, 2 μl were used for measurement, and each sample was tested 3 times (Appendix A, SOP-5). To ensure the quality of cryopreserved sperm, two quality specifications were used for inspection: a minimum initial motility of 40%, and a minimum concentration 1×10^9/ml (Chapter 3). The volume of the sample was adjusted in relation to the weight of testis (Appendix A, SOP-7). After rejecting the suspensions that failed inspection, the remaining suspensions were adjusted to the same working concentration 2×10^8 cells/ml (Hu et al., 2011b). A grouping computation was performed for these suspensions (Appendix A, SOP-8). This calculation used to group suspensions into different freezing batches was based on the estimated straw production of each suspension, such that each freezing batch would have minimal waste of freezer space and packaging (Liao et al., 2011).

4) Equilibration & packaging: the operator of the automated packaging system (MAPI, CryoBioSystem, Paris, France) gathered each of the freezing batches, and mixed the suspensions with cryoprotectant to produce a 10% final concentration of methanol (reference number: MX0488-6, EMD, Billerica, MA) to initiate equilibration. The equilibration time was strictly maintained at 30 min. During this time, all mixtures were loaded into straws, sealed at both ends, labeled with alphanumeric and barcode sample information by the MAPI system, and all straws

were placed on horizontal metal racks (reference number: 007119, IMV, Maple Grove, MN) in the freezer (Appendix A, SOP-9).

5) Freezing and sorting: the straws prepared for a single freezing batch were frozen at the same time with the freezing program started at 30 min after cryoprotectant addition. The freezer (Microdigitcool, CryoBioSystem, Paris, France) had a capacity of 280 straws. The chamber was held at 4 ℃ during loading, and after the chamber temperature had stabilized, the freezing program was initiated. The programmed cooling rate was 5 ℃/min from 4 ℃ to -80 ℃ (based on chamber temperature). After the freezer chamber reached -80 ℃, the products were held inside for another 5 min before unloading (Appendix A, SOP-10). The operator oversaw the cooling progress and removed the frozen straws for sorting under liquid nitrogen into daisy goblets (reference number: 015144, Cryo Bio System, Paris, France). Each daisy goblet contained 12 compartments. Each compartment had a capacity of 12 frozen straws. The grouped straws from each sample were held together under liquid nitrogen. During sorting, all straws from each sample were sorted into a single goblet in close proximity to each other (Appendix A, SOP-11). During this time, the freezer chamber was warmed automatically for the next freezing batch.

6) Storage: after freezing of all batches was completed, the daisy goblets were recorded in daily logs and moved into a 370-L storage dewar (MVE 800 series-190, Chart®, Garfield Hts, OH) for long-term storage (Appendix A, SOP-11). The operator managed this process and finished cleaning as needed to complete the day's work.

Timing of the process

During the processing of blue catfish sperm, the elapsed time for each step was recorded 5 to 7 times with digital timers. The recorded times were applied to the ARENA Input Analyzer

to generate distributions, and simulations were performed with process times randomly generated from the respective distributions. Based on data collected, nine distributions were tested and those with lowest square error were selected to represent the data. In general, three types of distributions were applied in the simulation model: normal (NORM) and triangle (TRIA) for dissection and testes process time; and uniform (UNIF) for automated packaging and freezing.

Simulation conditions and parameters

ARENA (Version 13.50.000000) was used for simulation modeling. To mimic real life conditions, the maximum process time preferred was within one 8-hr day. The settings within the simulation model included resources (Table 7.2) and processes (Table 7.3). A simulation run represented a single day of work by 5 people. Each simulation run was performed 50 times with the same settings.

Simulation modeling structure

The simulation model strictly followed the structure of each process step (Figure 7.1). The facility was based on a two-room design: one ("dirty") for fish processing and the other ("clean") for straw processing. There were six components of conceptual logic used to improve model accuracy.

The first component was minimizing the queue on the dissection table. Fish suffered less stress in the holding system than on the floor by the dissection table. In the ARENA model, the Entity Create Model-Object (the basic modeling element) represented the holding system, and each entity that produced by the Entity Create Model-Object represented a single fish. To prevent the Entity Create Model-Object from sending entities constantly regardless of the availability of the next process, a Seize Model-Object was applied. To prevent the dissector from

removing too many fish at once, a logical rule was established that allowed only an available dissector to capture fish to carry to the dissection table (Figure 7.2).

Table 7.2. Settings table for Resources used in the ARENA simulation model. Capacity represents the availability of resources. "$ busy/hour" was the hourly cost during operation. "$ idle/hour" was the hourly cost when the resources were not occupied. "$ per use" was the cost of disposable supplies during operation. Properties define the function of resources. Values in this table were estimated based on actual operation in the Aquatic Germplasm and Genetic Resources Laboratory of the Aquaculture Research Station, Louisiana State University Agricultural Center, Baton Rouge, Louisiana, during 2009-2011.

Resource	Capacity	$ Busy /hour	$ Idle /hour	$ Per use	Properties
Dissector	1	18	0	0	Worker
Dissection table	1	0	0	0.25	Tool (one fish at a time)
Cleaner	3	18	0	0	Worker
Cleaning kit	3	0	0	0.15	Tool (one testis at a time)
Filter	1	0	0	0.15	Tool
Operator	1	18	0	0	Worker (multiple tasks)
Inspection tool	1	0.1	0.1	4	Tool
Grouping calculator	1	0.05	0.05	0	Tool (grouping)
Automated system	1	0.4	0.4	5	Tool (straw packaging)
Freezer	1	2	1	0.25	Tool
Sorting tank	1	0	0	5	Tool (straws sorting)
Inventory computer	1	0.05	0.05	0	Tool

The second logic component was for minimizing labor use. In the ARENA model, labor is considered as a resource. The resource capacity of labor is a constant throughout the process, so it is hard to identify part-time labor. To address this issue, non-paid idle time was applied, such that although the resource capacity remained the same, there was no cost for non-working technicians (Table 7.2). For example, the dissector position would exist until there were no

additional fish in the holding system, and only the time involved with capturing, transporting, and dissection were considered to be paid. In real life, this technician would then be available to assume a new position in the processing pathway.

Table 7.3. Settings table for processes used in the ARENA simulation model. The process names starting with "Lastrun" were part of the control logic to eliminate remainders in the system. Each process employed a worker and tools. The exception was the Dissection process because of the logic for minimizing the queue on the dissection table. The delay values represented process time which was presented as distributions or expressions. All values in this table were measured from the existing process at the Aquatic Germplasm and Genetic Resources Laboratory of the Aquaculture Research Station, Louisiana State University Agricultural Center, Baton Rouge, Louisiana, during 2009-2011. NORM: normal distribution; TRIA: triangle distribution; UNIF: uniform distribution.

Name	Resources occupied	Delay value (min)
Dissection	Dissection table	NORM(6.12, 2.09)
Testes process	Cleaner, cleaning kit	NORM(12.73, 4.94)
Filtering	Cleaner, filter	TRIA(1.57, 1.78, 2.12)
Inspection	Operator, inspection tool	3.67+1.13 *filtered samples
Organization	Operator, organizing computer	1.5 + 1 *number of acceptance
Straw packaging	Operator, automated system	30
Freezing and sort	Operator, freezer, sorting tank	25
Lastrun.auto system	Operator, automated system	30
Lastrun.Freezing	Operator, freezer	25
Lastrun.sort	Operator, sorting tank	UNIF(15, 25)
Inventory	Operator, inventory computer	0.5+0.75 *number of goblets

The third logic component was inspecting and selecting suspensions. Not all suspensions could pass inspection and all rejected suspensions were discarded to ensure overall product quality. Instead of estimating the suspension population each time after inspection, the simulation model performed random selection on each suspension (Figure 7.3). For example, if the acceptance rate was 90%, each entity that went through the selection process had a 10%

chance of being rejected. This design better represented the real condition during processing than estimation of a selected population because if there were a small number of suspensions in the process, the overall acceptance rate was more likely to be different from 90% due to randomness.

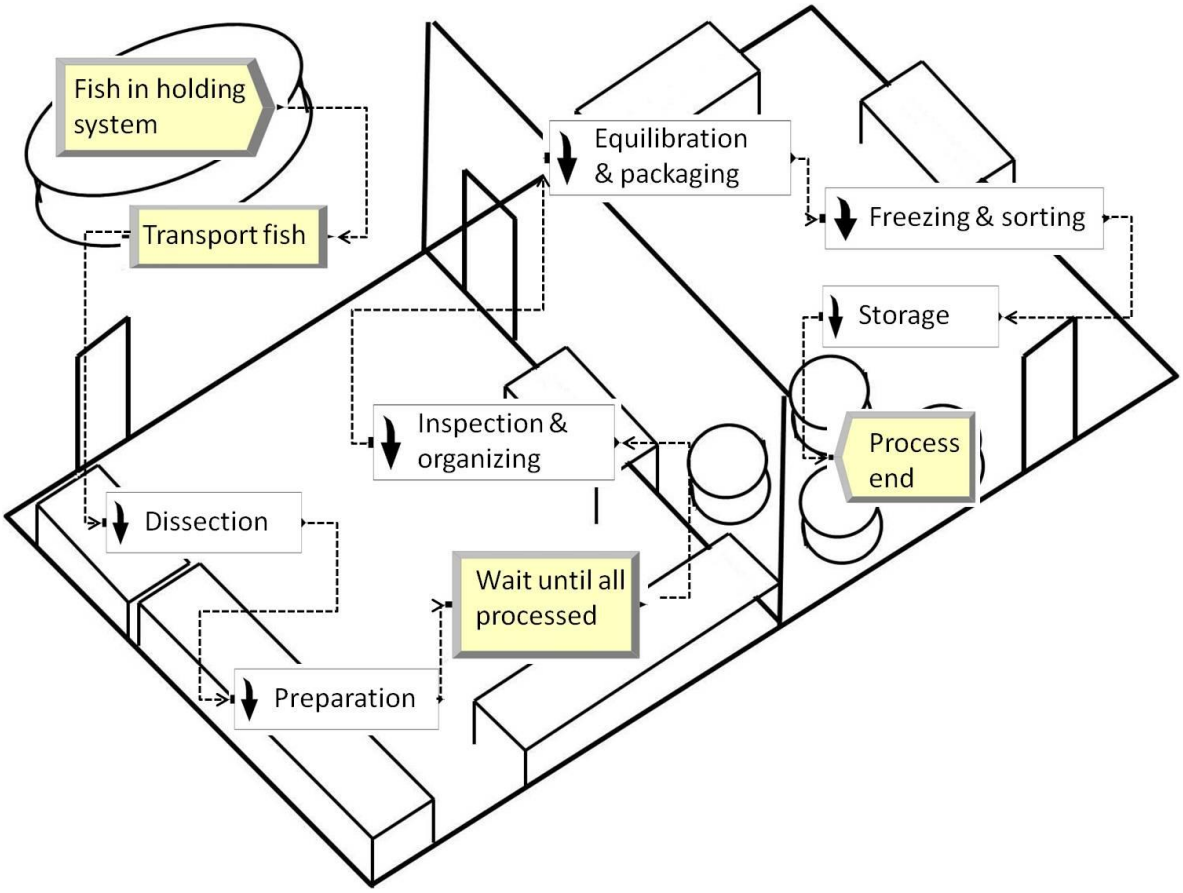

Figure 7.1. Simulation model structure with facility layout. The entities (fish) began from "Fish in holding system", went through the processes (shaded outlines) and submodels (rectangles with black arrows), and finished at "Process end". Each submodel represented one step in the high-throughput cryopreservation process. Transport within the facility was not included in the model.

The fourth logic component was determining freezer loading size by the number of fish. The actual straw productivity of suspensions varied due to biological and technical factors. To simplify the modeling, instead of assigning random straw numbers for each suspension, estimations were made on the average number of suspensions that filled a single freezer chamber

(Figure 7.4). For example, in this study, four suspensions provided sufficient straws to fill the chamber of the MicroDigitcool programmable freezer. The ratio between a full chamber of straws and the number of daisy goblets was one chamber to 2 daisy goblets (maximum capacity of 144 straws each). Therefore, after freezing and sorting, the materials from four sperm suspensions were transferred into two daisy goblets.

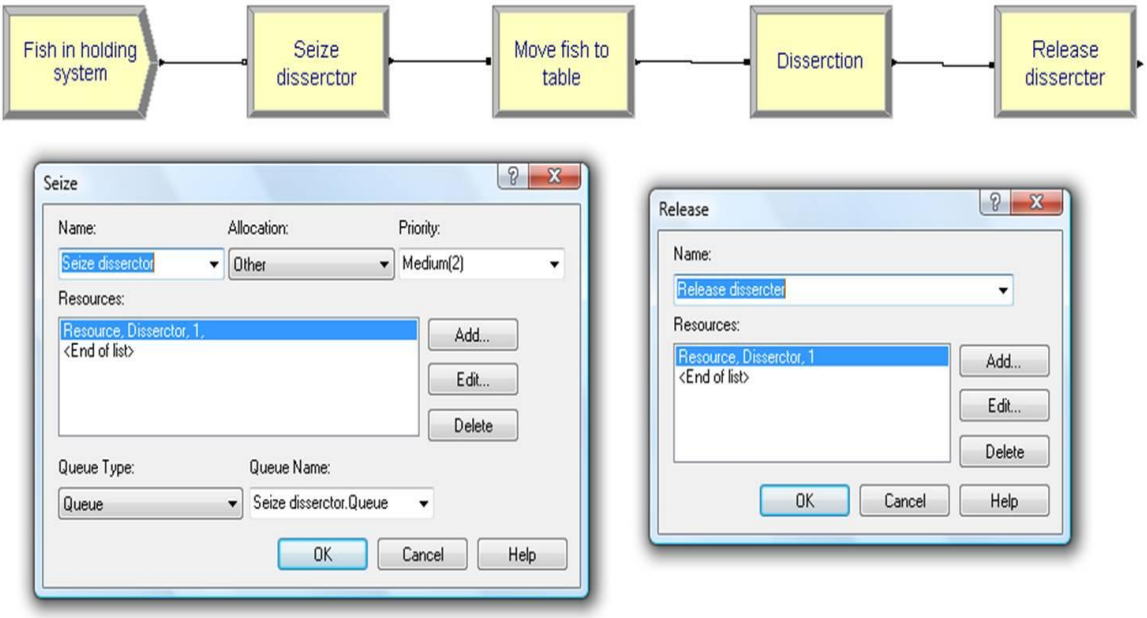

Figure 7.2. The logic used for minimizing the queue on the dissection table. The Seize Model-Object named "seize dissector" forced entity (fish) to associate with the available dissector only (left setting window). As long as the entity was not dissected, the dissector could not capture another fish. Once each fish was processed, the dissector was free again for other entities by "release dissector" (right setting window).

The fifth logic component was to make sure that there were no remainders in the system. Sample remainder was an essential issue for the process simulated. Ideally, this remainder could be held until the next simulation, resulting in continuous processing. However, the blue catfish sperm process did not tolerate a remainder in reality due to the biological characteristics of samples (i.e., quality reduction in sperm cells during storage prior to freezing). If there was not enough suspension for a single freezing batch, the remainder was programmed to be frozen at

once (Figure 7.5). The freezing batch for this remainder would not be performed at full capacity. Therefore, the output from the last freezing batch would change according to the actual batch size.

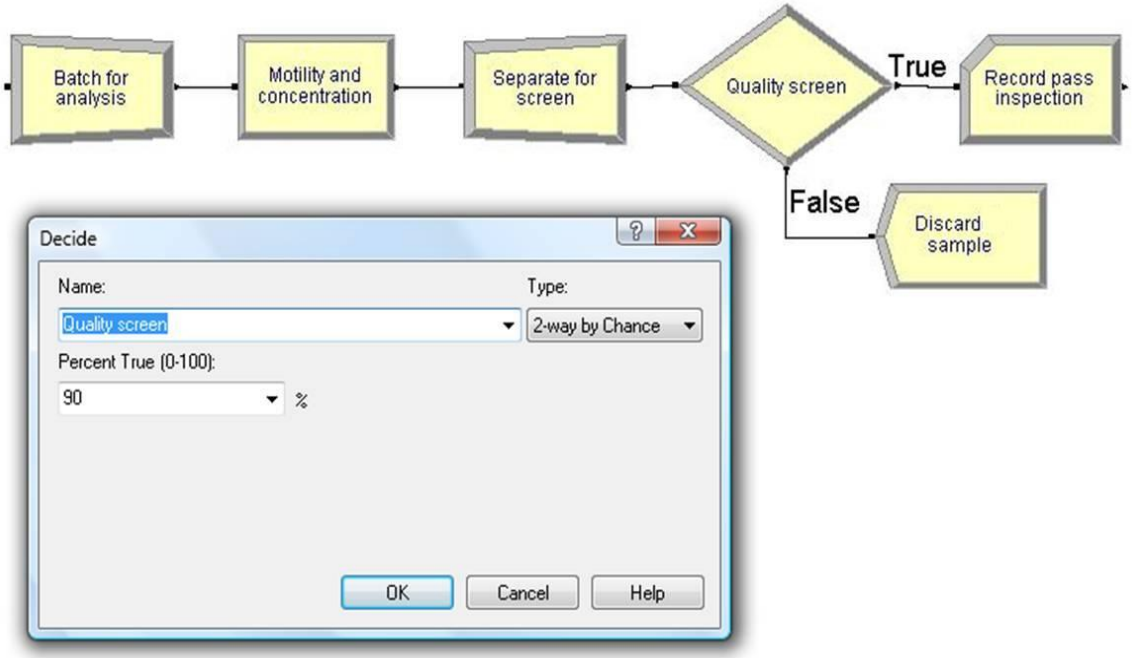

Figure 7.3. The logic used for inspecting and discarding sperm suspensions. All suspensions after filtering were gathered by a Batch Model-Object named “batch for analysis”, and went through inspection (motility and concentration testing) at once. A Separate Model-Object named “separate for screen” broke the batch into original entities (suspensions), and went through a Decision Model-Object named “quality screen”. The number of suspensions that entered next step was determined according to the previously established sample acceptance rate (90% in the setting window).

The sixth logic component was recording of late working hours. Instead of pausing the process at 8 hr and calculating the waste in the system, the efficiency was evaluated by the outputs and consideration of additional working hours because of the quality considerations for biological samples. The timing object in the model would record the current time when a product went through. The time of the final product was considered as the operation time.

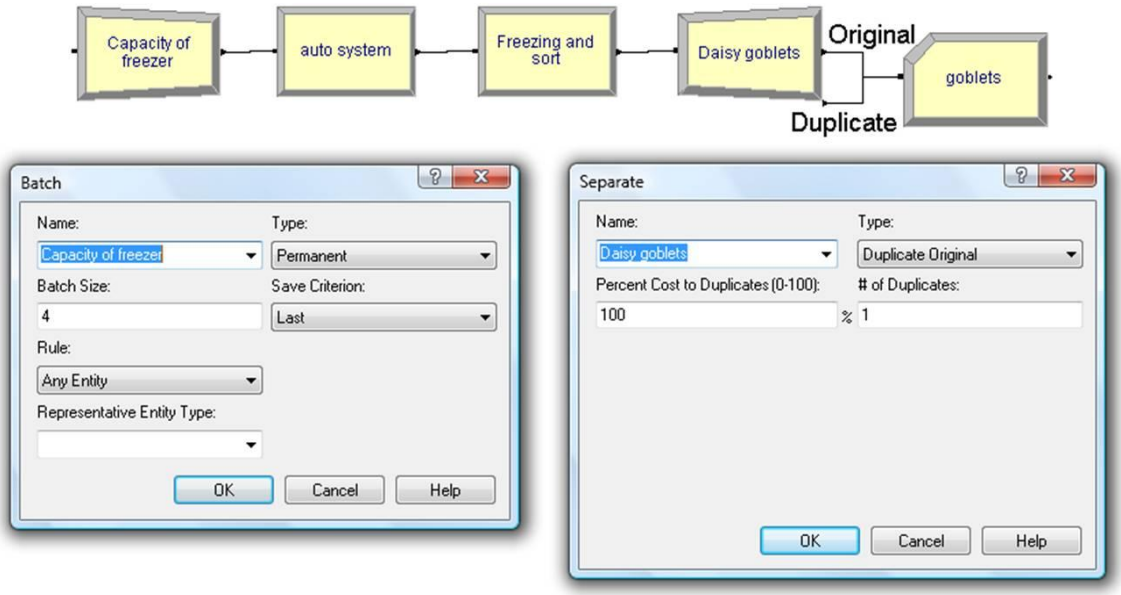

Figure 7.4. The logic used for determining freezer loading size by number of fish. According to the capacities of the automatic packaging system and freezer, the entities (sperm suspensions) were grouped by a Batch Model-Object named "Capacity of freezer" (left setting window). In this case, an average of 4 entities made one batch for freezing. After freezing and sorting, the batched entity was separated by a Separate Model-Object named "daisy goblets", because all straws were sorted into daisy goblets for long-term storage. Each freezing could create two daisy goblets, so in Separate Model-Object, there was 100% chances for each entity (batched freezing) to yield two (daisy goblets) (right setting window).

Model verification and sensitivity analysis

Data used in the model were collected in the Aquatic Germplasm and Genetic Resources Laboratory of the Aquaculture Research Station of the Louisiana State University Agricultural Center during 2009-2011. Steps for model verifications as related to aquaculture have been provided previously (Halachmi, 2005). The first sensitivity analysis was based on daily processed fish as an independent variable and cost per hour (total cost of processing divided by total working hours) as a dependent variable (Figure 7.6). Extreme ranges were applied to the analysis (e.g. as many as 50 blue catfish processed per day). The cost per hour had minimum variation after reduction to $38/hr (at around 15 fish). The existing containment system and process were designed for 10 to 30 fish per day, which would not be affected by hourly cost

variation of the model. The second sensitivity analysis was based on fish processing labor (i.e., dissectors and cleaners) as two dependent variables (Figure 7.7). The target fish to be processed were set as the maximum capacity of the holding system (30 fish). The more labor involved, the higher the cost per hour would be. Specifically, because cleaners and dissectors were paid by hourly wage, when there were more than three cleaners, the testes processing finished sooner and the hourly cost of the entire process stopped increasing; however the cost increased for each new dissector. The setup of the model presented was based on the minimum number of dissectors (one) and optimal number of cleaners (three).

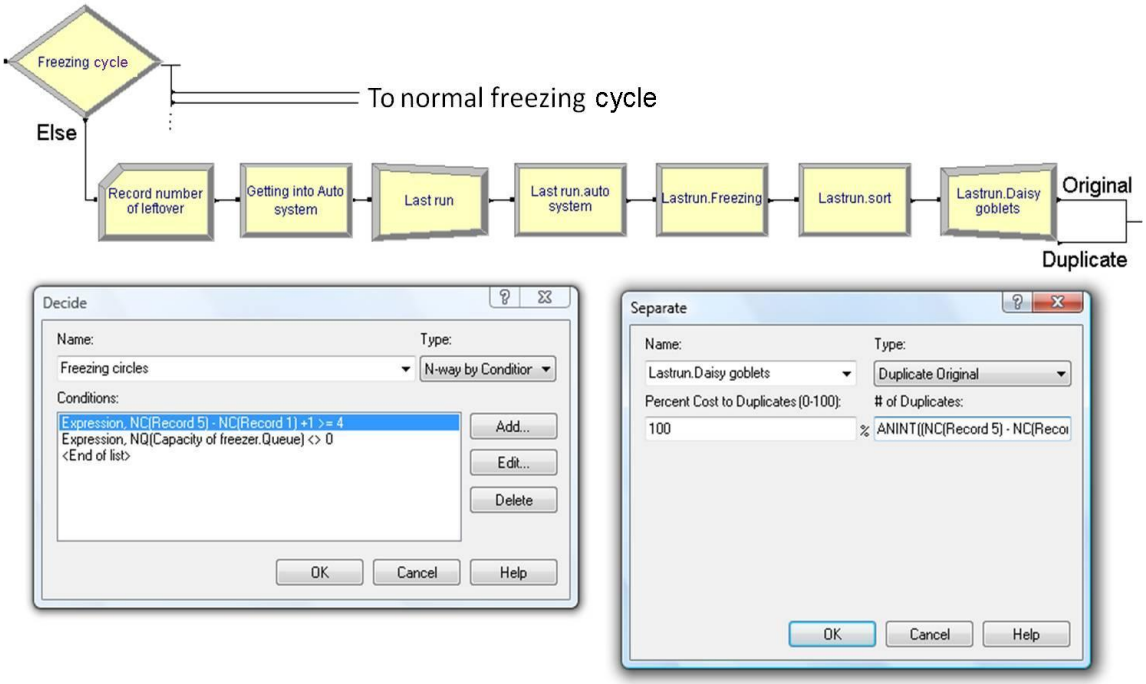

Figure 7.5. The logic used for ensure that no remainders existed in the system. A Decision Model-Object named “freezing batches” was set before entering freezing batches (left setting window). Two conditions were used: remainder in the system was greater than one regular batch size; there were some entities waiting to be batched in normal freezing batches. All entities that failed both conditions went to last run (“Else” pathway). The total number of remainders was recorded by the Record Model-Object named “Record number of remainder”. Therefore, the batch size of Batch Model-Object named “Last run” and the duplication number in the Separate Model-Object named “Lastrun.daisy goblets” (right setting window) were changed accordingly.

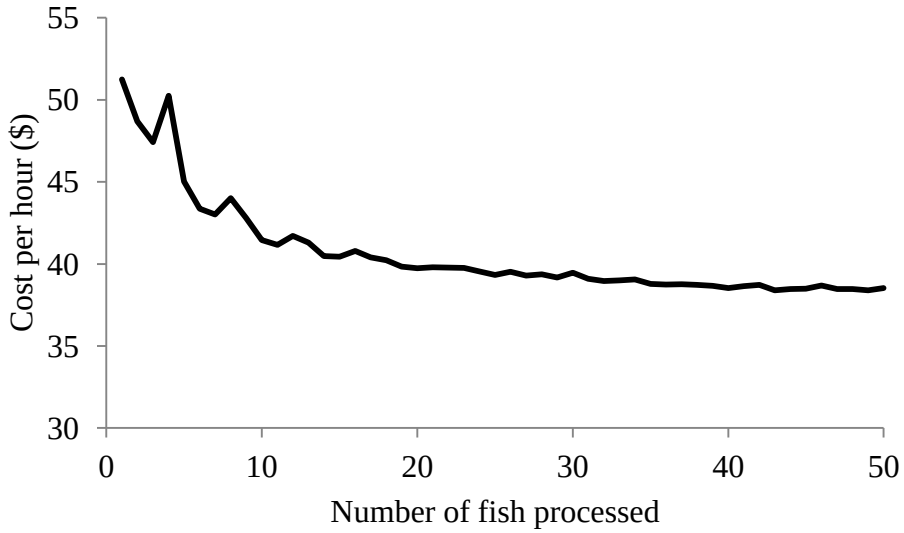

Figure 7.6. Sensitivity analysis of the ARENA simulation model of high-throughput cryopreservation of blue catfish sperm on cost per hour in relation to the number of fish processed.

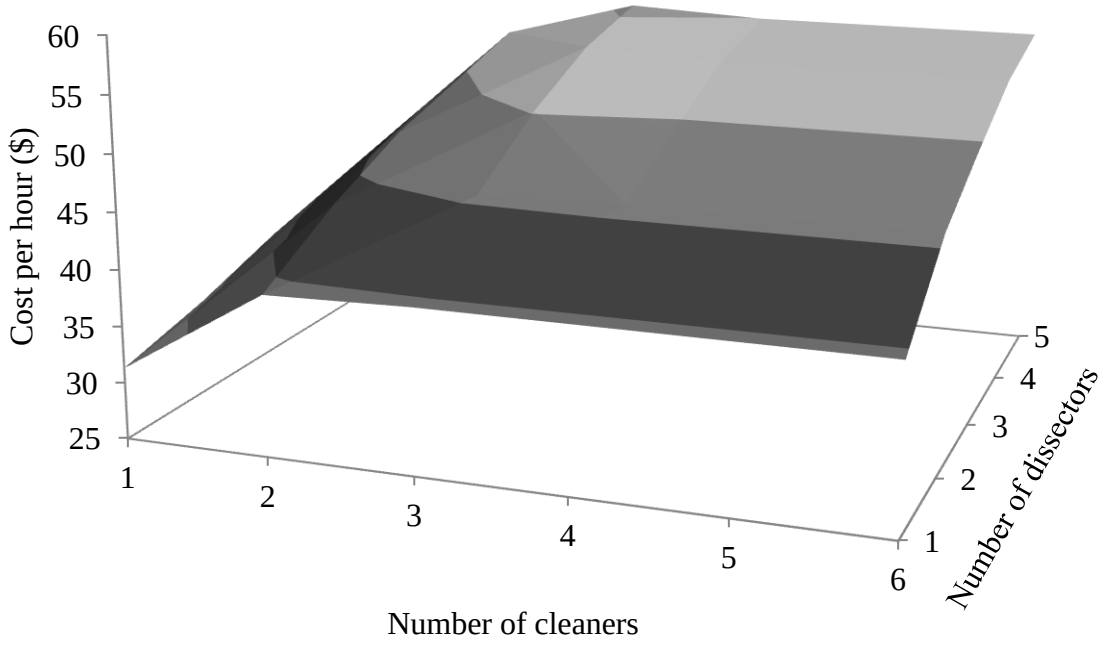

Figure 7.7. Sensitivity analysis of the ARENA simulation model of high-throughput cryopreservation of blue catfish sperm on fish processing labor setup. Two types of labor were involved: dissectors and cleaners. The scenario was set to process 30 fish. The lighter color represented higher cost per hour.

Experiment 1: prediction of maximum output of the process within 8 hr

According to the scale of the existing facility, the containment system could hold a maximum of 30 fish. The simulation model would test for 10 to 30 fish in the containment system and compare the results of 50 model runs.

Experiment 2: analysis of bottlenecks in the process

Based on the selected total entities number from Experiment 1, each process in the model was analyzed for accumulated time (the sum of time spent on this process from each entity) and queue (the entities that waited in line to be processed).

Experiment 3: estimation of the operational cost when the process was run for maximum output

Based on the selected total entities number from Experiment 1, each resource in the model was analyzed for cost according to the values from the settings table (Table 7.2).

Results

Process capability

After testing different total entities from 10 to 30, 18 entities had the process time closest to 8 hr (Table 7.4). Each daisy goblet contained 144 straws, and thus 18 entities produced 1164 ± 33 straws. A linear regression was developed for processing time as follows:

Process time (min) = 0.3671 * Total entities + 1.2045; $R^2 = 0.9975$, $P = 0.00$.

For every increased entity, 0.3671 hr (22 min) was added to the processing time.

Bottlenecks within the operation

The freezing and sorting step consumed the most accumulated total time and accumulated waiting time (Figure 7.8). Other than the freezing and sorting step, testis processing (3.77 ± 0.11

Table 7.4. Process time and output for different inputs of incoming materials (total entities) for high-throughput cryopreservation of blue catfish sperm. The ARENA model simulated each scenario 50 times and presented the mean ± SD for the process time and number of daisy goblets produced.

Total entities	Process time (hr)	daisy goblets produced*
10	4.97 ± 0.15	4.78 ± 0.13
15	6.75 ± 0.15	6.68 ± 0.19
16	6.89 ± 0.13	7.04 ± 0.24
17	7.35 ± 0.17	7.60 ± 0.26
18	7.73 ± 0.16	8.08 ± 0.23
19	8.26 ± 0.16	8.48 ± 0.23
20	8.63 ± 0.13	8.90 ± 0.16
25	10.50 ± 0.10	11.12 ± 0.20
30	12.16 ± 0.18	13.22 ± 0.25

*Because there are 12 compartments in each goblet, fractional use is possible.

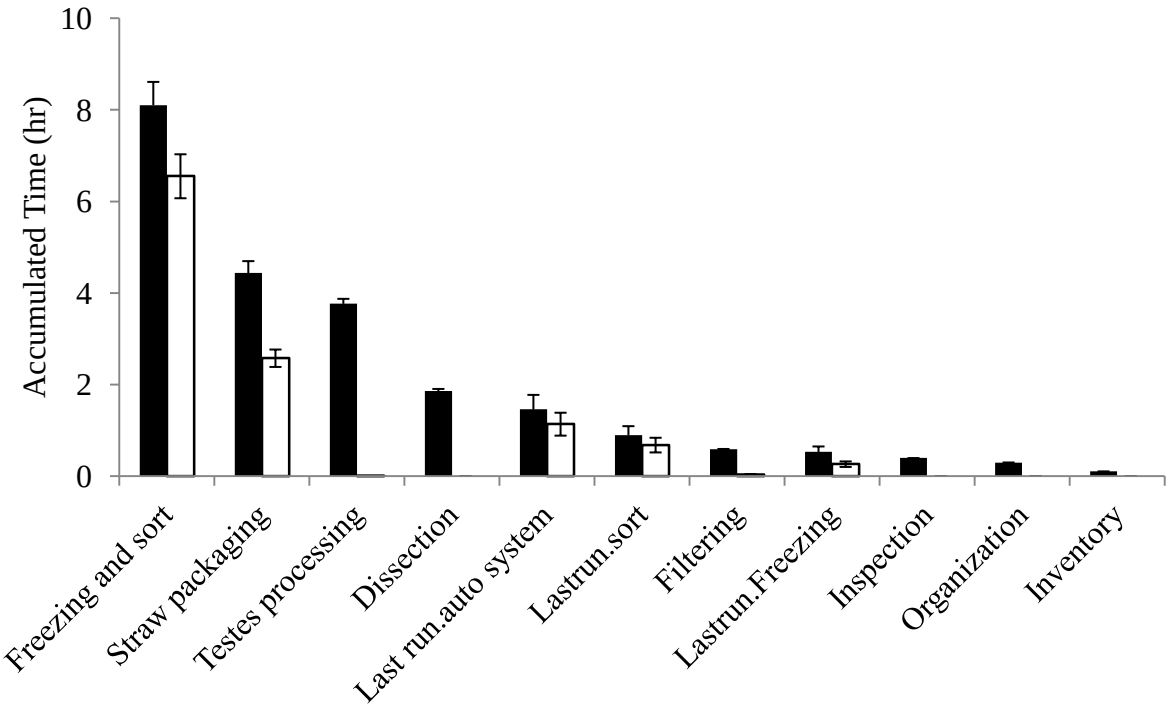

Figure 7.8. Accumulated time of each step (ranked from largest to smallest) for simulations of processing a total of 18 blue catfish in a single 8-hr day. The black bars represent accumulated total time, and the white bars represent accumulated waiting time. The error bars present the standard deviations generated based on data from 50 simulations.

hr) and dissection (1.86 ± 0.05 hr) required the longest accumulated total time. Accumulated waiting time and queue time indicated that the testis processing step (0.0002 hr) and filtering step (0.002 hr) presented only brief delays to the entire process.

Cost of operation

In the case of 18 entities, the operational cost was based on resource usage (Table 7.5). Overall, the single-day processing cost was $310.67, including a 70% working cost (based on efficient use of labor hours and restricting expenses on working hours), 19% usage cost, and 11% idle cost (Figure 7.9). Each daisy goblet cost $38.45, and each straw cost $0.27 (144 straws per daisy goblet).

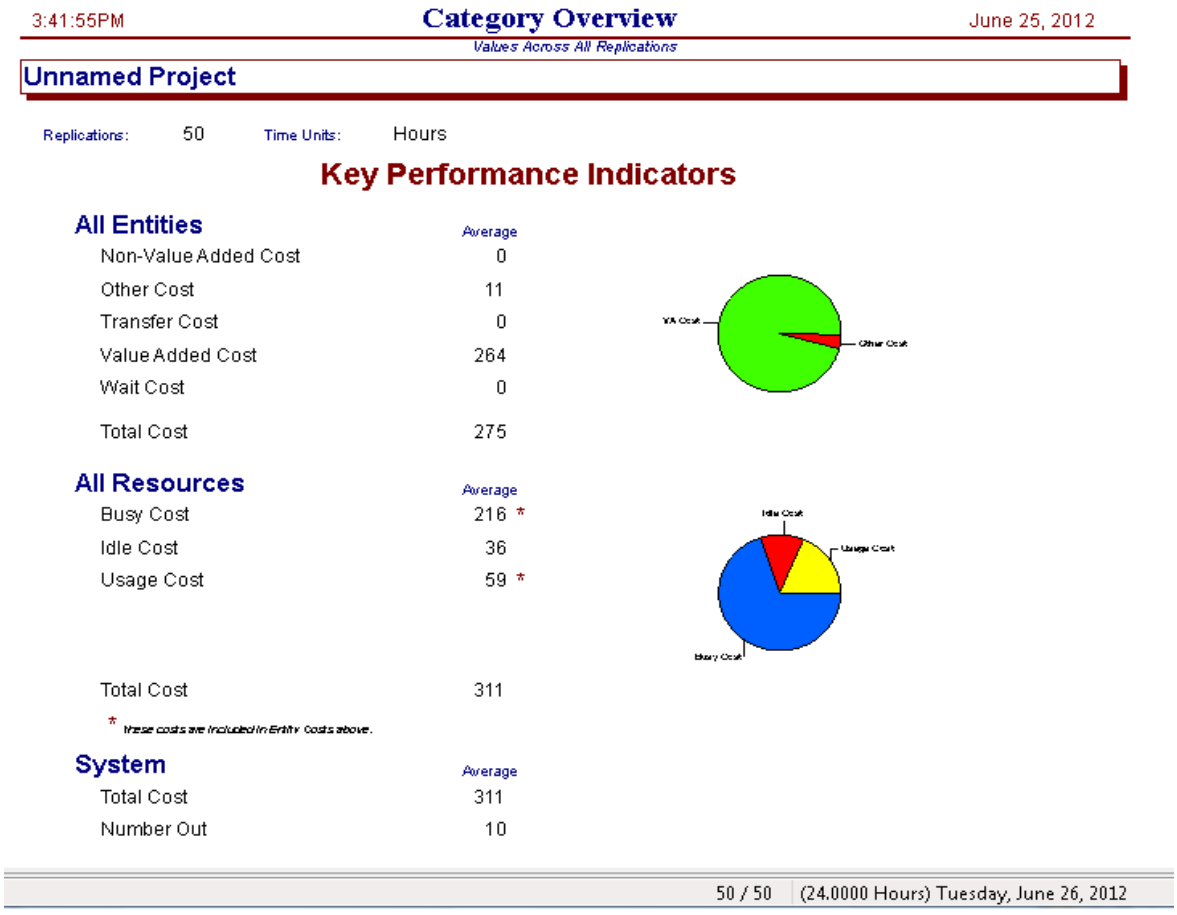

Figure 7.9. Overview page of ARENA summary report for 50 simulations of processing a total of 18 blue catfish in a single 8-hr day.

Discussion

The existing high-throughput pathway could process the testes of as many as 18 blue catfish per day with a single dissector, three cleaners, and one operator (in reality only four people), and could produce around eight full daisy goblets (1,152 straws) of quality product. With the linear relationship between total fish processed and process time, production volume could be increased by adding additional hours or shifts to the work schedule. Based on the fertility of 5×10^8 sperm per cryopreserved straw (Hu et al., 2011a), two straws could produce 1,402 hybrid fry with an egg quality of 66% hatch rate (determined using fresh sperm). Thus, the 1,152 straws produced in a single day could produce 807,552 hybrid fry. Catfish farmers in the US have annually produced 227,681 $\pm$ 18,160 metric tons of channel catfish in the past 5 years (USDA-ERS, 2012) with a market-size fish weight of 0.45 to 0.57 kg (Green and Engle, 2004). By simple calculation, 8 to 10 high-throughput cryopreservation lines could satisfy the overall 0.4 billion fish demand in the US market within one spawning season if all hatchery production switched to artificial spawning for production of channel catfish or complete reliance on hybrids by use of cryopreserved sperm. However, it is not recommended to completely substitute to hybrid catfish for channel catfish production. The ultimate approach should be to focus on breeding and selection of channel catfish, and cryopreservation can greatly assist this process to produce improved fish populations and to transport, maintain and archive germplasm and genetic resources.

To further increase capacity, improvements can be made to increase the working scale. The most time-consuming steps were straw packaging freezing and sorting. However, each step had constant processing times that were determined experimentally (Hu et al., 2011b). To reduce negative effects on time arrangement, the scale could be increased by adapting equipment with

larger capacity or by operating multiple production lines. Therefore, changes in scaling will involve other considerations such as equipment investment, position setup, and operational layout. Software such as ARENA has the capability to test such scenarios and provide evaluations, suitable for decision making.

Table 7.5. Resource costs during the processing of 18 blue catfish males. The ARENA model of high-throughput sperm cryopreservation performed 50 simulations and presented the mean ± SD for three types of costs. "Busy cost" represented the cost when resources were occupied; "idle cost" represented the cost when resources were not in use; "usage cost" represented the cost of supplies that were used each time resources were operated. Unit: USD.

Resource	Busy cost	Idle cost	Usage cost
Dissector	44.04 ± 0.86	0*	0
Dissection table	0	0	4.50 ± 0.00
Cleaner	77.58 ± 1.88	0*	0
Cleaning kit	0	0	2.70 ± 0.00
Filter	0	0	2.70 ± 0.00
Operator	90.24 ± 2.93	0*	0
Inspection tool	0.04 ± 0.00	2.36 ± 0.00	4.00 ± 0.00
Organizing Computer	0.01 ± 0.00	1.19 ± 0.00	0
Automated system	0.87 ± 0.03	8.73 ± 0.03	21.80 ± 0.69
Freezer	3.63 ± 0.12	22.18 ± 0.06	1.09 ± 0.03
Sorting tank	0	0	21.80 ± 0.69
Inventory Computer	0.01 ± 0.00	1.19 ± 0.00	0

* Assumptions included that labor was paid with working hours and there was no cost for existing equipments.

Meanwhile, improvements can also be made in the existing procedures. The cumulative time for the process could be shortened by optimizing the number of resources available. With additional workers and tools for each step, more entities could be processed within a given amount of time. Therefore, overall time could be shortened. In this study, testis processing and dissection required the second-highest amount of time. If there were two dissectors and two sets

of dissection supplies available, fish processing would be doubled. Furthermore, the time required by the process could be reduced by improving the quality of resources. An experienced technician could prepare one testis in 10 min, compared to 12.7 min of cleaning time required for a novice cleaner. In real-life production, any improvements in technique could provide significant positive effects. Future modeling will evaluate these changes with a focus on optimization of resources to maximize production. This is necessary, for example, to avoid existing or emergent bottlenecks that would negate rate improvements in earlier steps.

In addition to bottleneck identification, cost analysis also provided important information. There was an 11% idle cost that provided no value to the final products. Although some idle cost is unavoidable, such as during freezing and automated system operation, redundant equipment such as separate computers for organizing and storage could be combined. In addition, within the 70% busy cost, more than 90% came from labor costs (Lee, 1995). Therefore, increases or decreases in labor costs would affect overall cost more significantly than changes in other parameters. It should be noted that labor costs in this analysis were strictly based on working hours to enable focus on other components. Future modeling of specific scenarios could include more full-time positions if workers with more experience or higher education levels were employed.

Overall, these results clearly demonstrate the value and efficiency of simulation modeling to optimize a process such as high-throughput cryopreservation (Leung, 1986), and provide options and planning tools for further improvements of the existing process in the following areas: 1) increased production output with additional automated systems and freezers; 2) increased production speed with optimized processing of fish and testis , and 3) reduced costs when developing new facilities, or choosing equipment. In addition, to further evaluate the

changes (Wu and Joseph, 1992), an expert system approach which could interpret process data and predict out-of-control situations (Alexander, 1987) could be adapted for further analysis.

Cryopreservation protocols have been developed in several hundred aquatic species (Tiersch, 2011a). Essentially all of this work has been performed at a laboratory scale that is not applicable for high-throughput commercial-scale application. For example, almost all laboratory studies involve results based entirely from study less than 100 straws (or other containers) during one to several days of effort. This usually involved hand filling of containers and freezing in small batches not relevant to commercial-scale activities. With increasing demand, adoption of high-throughput cryopreservation techniques will play a more important role in aquaculture. The simulation model in this study was designed to provide a template that can be easily adapted to other species or applications. In fact, although there were multiple aspects directly involving cryopreservation (Leibo, 2011), from the process level, only three factors will cause major changes in the model (Table 7.6): refrigerated storage, shipment, and sperm quantity from each animal. This would likely hold true for other species.

Refrigerated storage is essential to cryopreservation, because a suitable extender solution will protect sperm cells and prolong the handling time (Billard et al., 2004, Christensen and Tiersch, 2007, Cloud, 2011, Wayman et al., 2011). If fish processing is geographically distant from the cryopreservation facility, shipping would be required. The receiving facility would not perform the sample collection, but an additional quality inspection step would be required upon receipt. Because, much like refrigerated storage, shipping conditions directly affect sample quality (Huszar et al., 2004, Tiersch, 2011b), shipping with sub-optimal conditions can cause quality reduction or loss of entire collections (Dong et al., 2005).

Table 7.6. Binary identification table for adapting the simulation model from this study to other species.

Condition	Type of change	Consideration during changes
1.Capable of refrigerated storage	Logic	Hold samples until cryopreservation
a. Processing on site	Structural	Add processer and steps
b. Processing off site (shipping)	Structural	Add inspection on arrival
2.Not capable of refrigerated storage	Logic	Continuous processing
a.Processing on site	Structural	Add processer and steps
i.Sufficient sperm available	Logic	Freeze as individuals
ii.Insufficient sperm available	Logic	Freeze as pooled samples
b. Processing off site (shipping)	N/A	Contingency plan for low quality samples

The definition of "sufficient sperm" will vary according to the capacity of equipment and packaging type, but generally, large-bodied fishes would be more likely to provide sufficient sperm at the individual level. For small-bodied fishes such as biomedical research models, (e.g., zebrafish *Danio rerio*), pooling of samples would be necessary for high-throughput processing based on existing automated equipment (Tiersch et al., 2011). Other species-dependent details such as equilibration time, loading concentration and type of cryoprotectant, can be modified by changing values in the settings tables without other logic or structural modifications.

Conclusions

This study represents the first simulation modeling of high-throughput cryopreservation. The results from this model provided valuable information on existing processes and can support future decision making. The model provides the capability for planning and expanding the scale

of high-throughput cryopreservation production. As such, future research on related economic and engineering topics can be performed in this "virtual facility". The significant necessary investments in construction and management can be examined rationally, and hypothesis testing can be accelerated. With the development of tools such as simulation models, the use of aquatic germplasm can more quickly become a valuable tool for aquaculture, conservation biology, fisheries management, and genetic research.

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Chapter 8[†]
Summary and Conclusions

As described in the previous chapters, high-throughput cryopreservation has been widely applied for decades in livestock industries such as dairy (Pickett and Berndtson, 1974). Consequently, cryopreserved germplasm constitutes an independent industry encompassing animal breeding, preservation of genetic diversity, and medical research. Fish sperm cryopreservation was first demonstrated at about the same time as for humans and livestock species (Polge and Rowson, 1952, Blaxter, 1953), however, despite the large-scale application in mammals, it remains at a research scale for aquatic species. As such, there is currently no system for large-scale production of cryopreserved germplasm of aquatic species. Recently, a survey of fish culturists revealed a high demand for genetic improvements of the type that can be provided by cryopreservation (Boever, 2006). Therefore, high-throughput cryopreservation for aquatic species comes with high expectations.

The development of high-throughput cryopreservation in livestock grew out of advances made in the laboratory that were subsequently scaled up for increased processing speed, capability for mass production, and quality assurance. Prior to this dissertation project, available equipment and processes in place for livestock and biomedical applications had been tested for feasibility of use with aquatic species (Haffray et al., 2008), with extensive work for more than 15 yr at the T. E. Patrick Dairy Improvement Center of the LSUAC. For example, sperm of blue catfish *Ictalurus furcatus* were cryopreserved at this commercial facility using procedures developed for dairy bulls (Lang et al., 2003a), as was done for sperm of common carp *Cyprinus carpio*, channel catfish *Ictalurus punctatus*, three species of sturgeon (of the genera

[†] A portion of this chapter was 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). Reprinted by permission.

Scaphirhynchus and *Acipenser*), striped bass *Morone saxitilis*, white bass *M. chrysops*, yellow bass *M. mississippiensis*, and marine species such as spotted seatrout *Cynoscion nebulosus*, red snapper *Lutjanus campechanus* (Roppolo, 2000), and diploid and tetraploid Pacific oysters *Crassostrea gigas* (Dong et al., 2005, Dong et al., 2007). Since then protocols and automated equipment developed for mammals have been specifically adapted in this dissertation for use with aquatic species (described below) including zebrafish *Danio rerio* (Yang et al., 2007), blue catfish (Hu et al., 2011), Eastern oyster *Crassostrea virginica* (Yang et al., 2012), channel catfish, and Atlantic salmon *Salmo salar* (Barbosa et al. unpublished data).

Automated equipment

High-throughput can be achieved in different ways, and adoption of automated systems is one of the most efficient methods. An automated system named MAPI (CryoBioSystem Inc. Paris, France) for loading, sealing, labeling and reading of plastic straws has been developed for mammalian high-throughput processing with high biosecurity (e.g., protection from transfer of pathogens). Adaptation of this equipment has been evaluated for aquatic species to enable commercial-scale use of cryopreserved sperm (Hu et al., 2011). In brief, upon mixing with cryoprotectant, sperm samples are placed on the MAPI system (Figure 1), and filling, sealing and labeling of the straws are controlled by a proprietary computer program (SIDE, CryoBioSystem, Inc.). Samples are drawn into plastic 0.5-mL (CBS) straws by vacuum, and are continuously transferred to the sealing platform where both ends are sealed by use of 158 ℃ heat clamps. The straws are permanently labeled with alphanumeric information and bar-coding on the identification jacket with an ink printer (A400, Domino, IL, USA) before transfer for label verification and quality control evaluation. This system can routinely produce 11 straws per min.

Freezing is performed using standardized procedures. Straws are arrayed on horizontal racks (40 per rack) and placed in a commercial-scale programmable freezer (Figure 8.1, Micro Digitcool, IMV, France) with a capacity of 240 straws per freezing cycle. If the thermal mass is not controlled exactly at each freezing (e.g. by adding "dummy" straws), the number of straws is regulated within a tight range (±8 %). The cooling program is initiated at 15-30 min (set exactly for a particular species) after addition of cryoprotectant. The cooling rate can be programmed from 1 ℃ /min to 40 ℃/min (based on chamber temperature) and various rates are studied (detailed below). When the final target temperature (-80 ℃) is reached and held for 5 min, the samples are removed and sorted under liquid nitrogen into 12-compartment storage containers (Daisy goblets, reference number: 015144, CryoBioSystem) for long-term storage in liquid nitrogen.

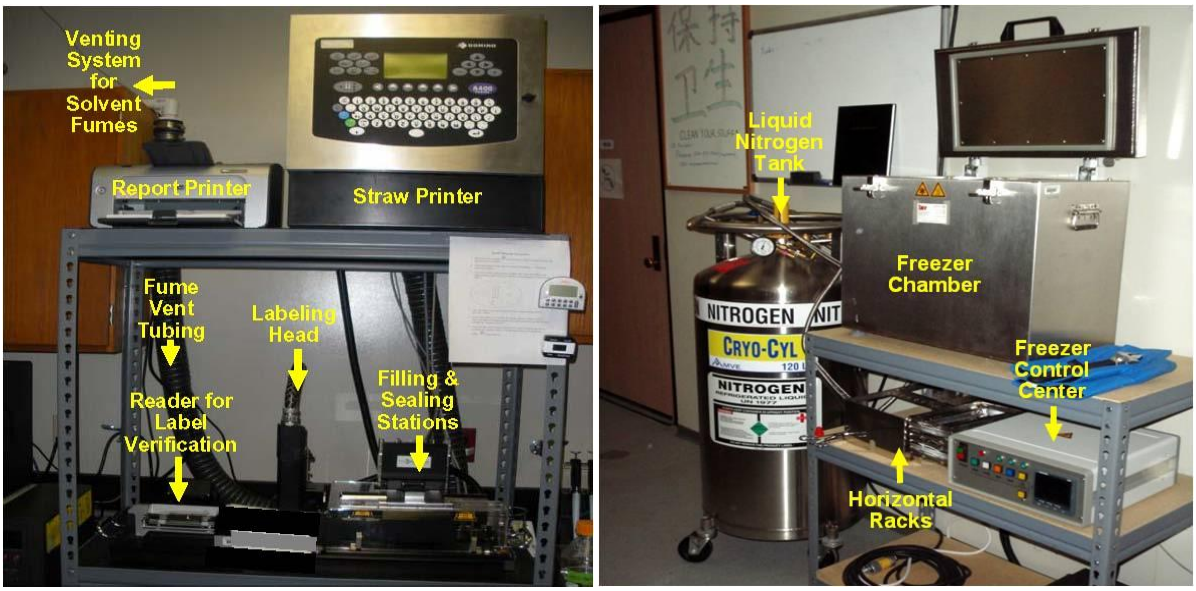

Figure 8.1. In the MAPI system (left), sperm suspensions are loaded and packaged into straws at filling and sealing stations, labeled under the labeling head driven by the straw printer, and pass a barcode reader for label verification. Next, the straws are laid on horizontal racks and placed in a programmable freezer (right) controlled by regulating liquid nitrogen flow from a pressurized storage tank.

Protocol development with automated system

Catfish sperm cryopreservation has been relatively well studied for aquatic species. A systematically developed methodology has analyzed refrigerated storage (Christensen and Tiersch, 2007), cryoprotectant toxicity (Christensen and Tiersch, 1997), cooling rate (Christensen and Tiersch, 2005), and fertilization (Tiersch et al., 1994). The involvement of automated systems did not directly affect the previous methodology, but did define constraints during processing (Figure 8.2). These constraints included container (straw size), production scale (number of straws packaged per minute), and freezer capacity (number of straws frozen each time).

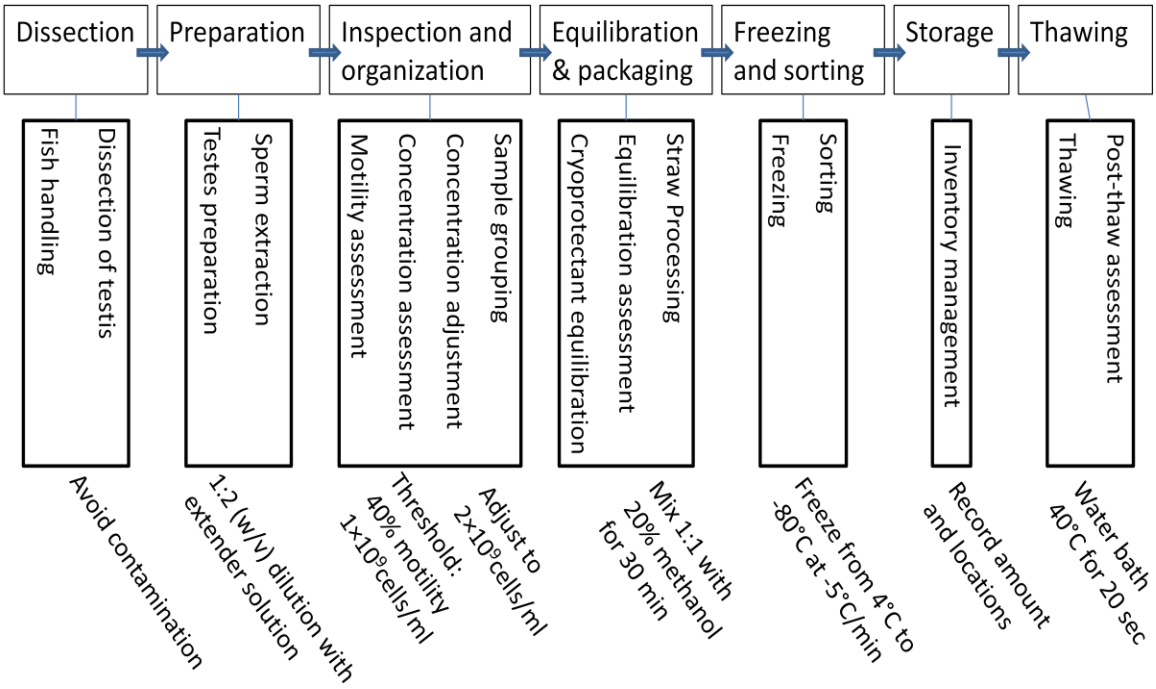

Figure 8.2. Schematic overview of cryopreservation of catfish sperm using automated processing. The central process elements were divided into seven steps (top, connected by arrows), each step entailed specific activities (upright rectangles) that were optimized individually by research experiments. The optimized steps were integrated into standardized protocols (specific examples provided at bottom in angled text).

In this dissertation, individual samples retained their identity throughout processing (i.e. there was no pooling of sample material from different males), which increased the complexity

of arranging process order (Liao et al., 2012). The constraints and complexity accumulated, as the process evolved from laboratory-oriented to production-oriented viewpoints. After details of the process were determined during experiments, cryopreserved sperm from individuals were used to test fertility with eggs collected from 5-8 individual female channel catfish. Sperm characteristics were tested for correlations with the characteristics related to fertility, which led to development of quality assurance criteria for the overall process and large-scale application of cryopreserved sperm.

High-throughput pathways

High-throughput pathway development is essential to future production (Hu and Tiersch, 2011). Critical aspects of high throughput are to arrange the sequencing of procedures and to balance the inputs and outputs between connected steps. With development of pathway sequences, industrial designers and economists can further evaluate the utility of resources and processing costs. In general, processing analysis has two elements: time and resources (Figure 8.3).

An industrial engineering approach would focus on reducing the time and cost at each step to increase the overall efficiency, and on reducing the possibility of bottlenecks between steps. In this dissertation, the basic strategies were to: 1) manage resources to reduce the waiting time of materials (queue formation) and overall working hours of resources (accumulated busy time for resources), and 2) use batches to maximize the usage of equipment. The simulation model that was developed from the process pathway can test strategies based on time and resource utilization data collected from real life (Chapter 7). From the simulation results, the existing process could process 18 blue catfish males in an 8-hr working day, produce 1440 straws of cryopreserved sperm, at a cost of $ 0.27 per straw.

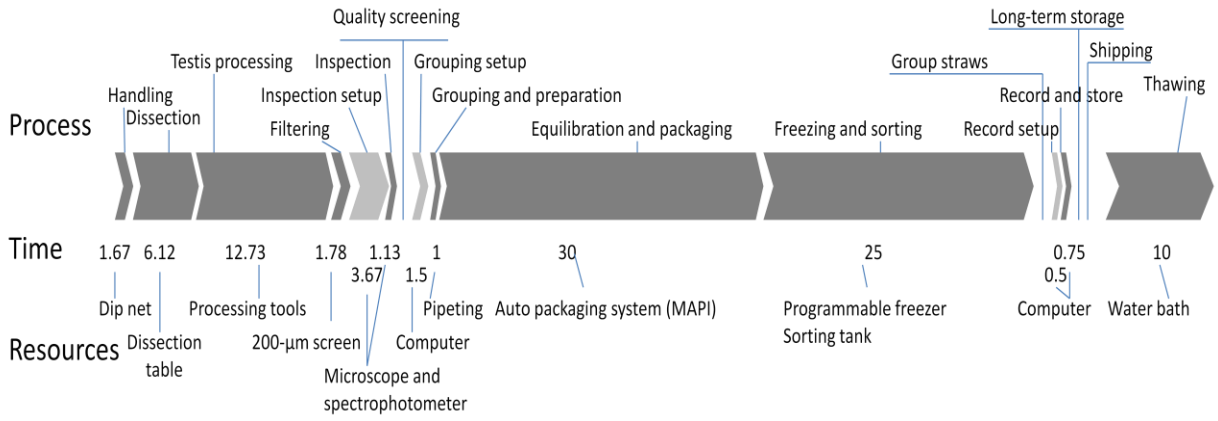

Figure 8.3. Schematic representation of a cryopreservation pathway established for sperm collection by dissection of testis from blue catfish. In its basic form, the process comprises 13 standardized, integrated steps from sample collection through thawing. The steps are presented as arrows sized in proportion to the total time (min) required for processing and cryopreservation of a sample from a single male. The light arrows are setup steps, and dark arrows are operation steps. Resources included the tools used during respective steps.

Process quality assurance

Quality assurance of the sperm cryopreservation process was mainly focused on standardization of protocols for product quality (cryopreserved sperm with fertility). In application, quality of the final product would be essential to customer satisfaction. Therefore, post-thaw quality characteristics (motility, membrane integrity, and sperm concentration) were identified (Figure 8.4). However, during processing, multiple internal quality characteristics can have influences on final product quality. Establishing specifications for all quality characteristics can standardize the process, reduce the potential of rejection after processing, and avoid waste in using materials that cannot meet acceptable product standards (Creveling, 1997). With specifications, the final product will meet the basic quality requirements (e.g. fertilize eggs and produce fry), but further improvements of quality and operation consistency will rely on the existence of a quality control plan.

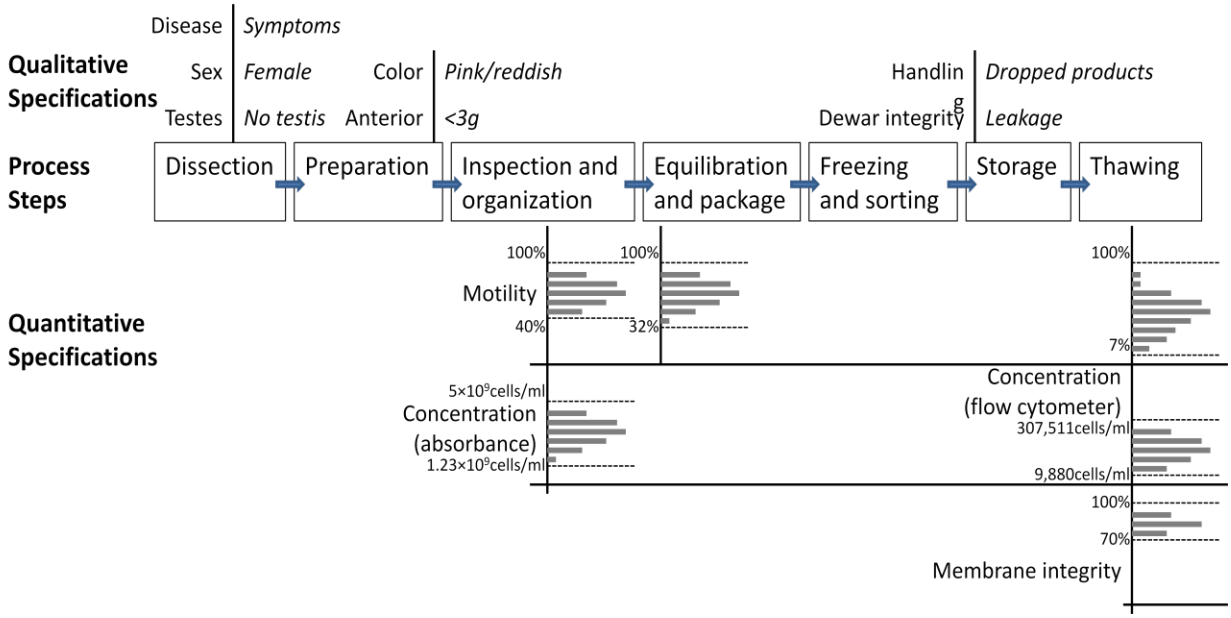

Figure 8.4. Key quality characteristics (Qch) and specifications for the catfish sperm cryopreservation process developed in this project. Along the process pathway, several Qch were associated with each step. For the qualitative specifications (listed above the process), the defect conditions were listed beside their Qch (italics); for the quantitative specifications (listed below the process), the upper and lower limits are presented (dotted lines) along with example population distributions.

This quality control plan was specifically designed for processing of individual (non-pooled) samples using the 3-sigma rule which defined the statistical quality control limits as being within three standard deviations of the sample mean (Montgomery, 2008). The process in this study was structured as a continuous process (only producing cryopreserved sperm using blue catfish sperm) with 100% inspection (measurements were taken from every male). To match this specific structure, the quality control plan was based on the cumulative sum (CUSUM) method (Bourke, 2001, Montgomery, 2008, Reynolds and Stoumbos, 2010). Qualitative and quantitative records of quality characteristics were both analyzed in the CUSUM chart. The quality control chart was used to test if individual samples exceeded control limits of quality characteristics (Figure 8.5). Those samples exceeding the limits (out-of-control limits) were defined as fail-quality points. The information on fail-quality points can be used to provide

explanations for quality variation, alerts for process errors, and improvement suggestions to management. Quality assurance would be the milestone of process transformation, because it would reflect the change of perspective from research of comprehensive scenarios to a streamlined focus on production of uniform products.

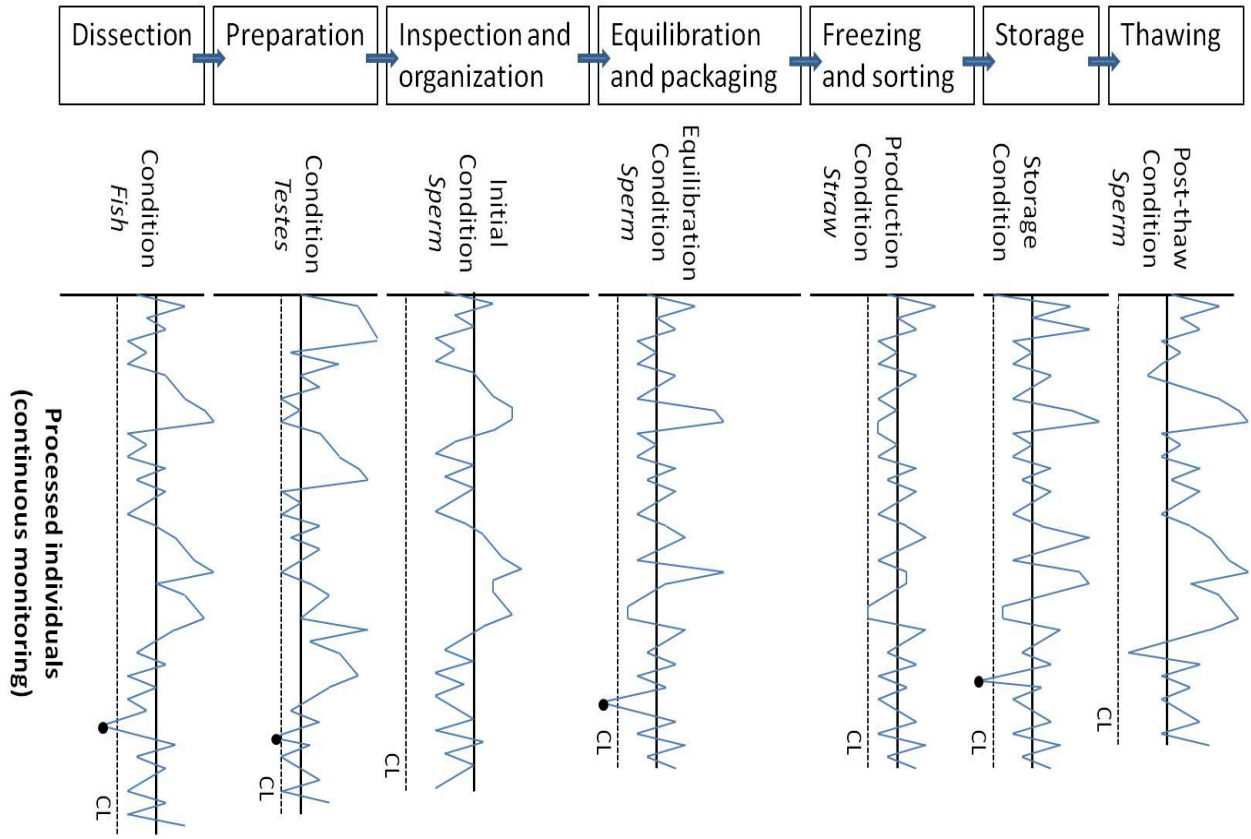

Figure 8.5. Demonstration of sperm cryopreservation processing under quality control monitoring. At each step, the quality condition of individual samples was described by quality characteristics (Qch). If the Qch failed quality screening, this sample would be eliminated from the process, therefore, the number of samples that entered next step decreased (i.e. yielding a shortened axis). If the Qch passed screening, the value of the Qch would be calculated by quality control equations and input into a 2-dimensional coordinate system as a single data point. The Y axis (horizontal solid lines) represents calculated values, and the X axis (vertical solid lines) represents sequential sample identification numbers. All data points are connected (coordinate graphs) to form the quality control charts for that Qch (e.g., the CUSUMx chart shown here for blue catfish sperm) to compare with existing control limits (CL, dotted lines). If the respective data points in the control chart exceeded the control limit, the quality of this sample was not acceptable (black dots).

The complete production line is presented in the materials organized in the Appendices. The facility component list (Appendix C) provides supply information sufficient to operate one production line. Inventory spreadsheets (Appendix D) provide all quality related records throughout the process, so quality assurance can be executed. Standard Operation Sheets (Appendix E) are formatted to serve as personnel training materials and work sign-up sheets. The manual of straw thawing (Appendix B) was designed as a production manual and public communication material. In reality, with investment, the production line can be assembled by following the facility component list and process structure (Chapters 3 and 7) and personnel can be trained with the Standard Operation Sheets. After materials arrive, the production line is operated and records information is captured in inventory spreadsheets for the quality assurance plan (Chapter 5). The frozen products would be sent to customers with educational materials attached with the product manual. Thus, within this dissertation, the entire system is covered from planning to operation at the production line, to terminal use of products by customers.

Commercial-scale evaluation of high-throughput processing

Aquaculture is a profit-driven labor-intensive endeavor. Therefore, cryopreserved sperm has to be tested on a commercial scale with practical labor scenarios. Close cooperation with a commercial hybrid catfish hatchery from 2009 to 2012 confirmed the potential of adopting cryopreserved sperm. First, there were no technical barriers to adaptation of cryopreserved sperm in the hatchery. The thawing method was straightforward (Appendix B). Thawed sperm could be handled in the same way as freshly prepared sperm suspensions and maintained motility after thawing for at least one day under refrigerated conditions (Hu et al., 2011). Secondly, there was no fertility loss associated with use of cryopreserved sperm. As indicated in Chapter 4, there were no significant differences between fresh and thawed sperm in terms of fertilizing the same

volume of eggs. This result was based on fertilization of more than one million eggs from 300 females which was larger than the previous large-scale fertilization trials performed in rainbow trout *Oncorhynchus mykiss* (Cabrita et al., 2001), turbot *Scophthalmus maximus* (Chen et al., 2004), yamu *Brycon amazonicus* (Velasco-Santamar á et al., 2006), striped bass *Morone saxatilis* (Kerby et al., 1985) and Atlantic salmon *Salmo salar* (Alderson and Macneil, 1984) and thus represents the largest fertilization study done with cryopreserved fish sperm. With the capability of processing large numbers of males and fertilization of eggs from large numbers of females, the data collected from application trials expanded geometrically. Statistical analysis of these data could reveal aspects that might be hidden in smaller-sized experiments. For example, after individual fertilization tests, statistics showed that the neurulation of pooled sperm could be predicted from the neurulation of composite individuals (Chapter 4). Therefore, cryopreserved sperm would not simply be a replacement of freshly prepared sperm in the hatchery, but creates new opportunities for aquaculture such as genetic resources management (see below).

Large-scale application of cryopreserved sperm can also bring a change of view to traditional artificial spawning in hybrid catfish production. The use of standardized cryopreservation sperm as a dose in artificial spawning provides an opportunity to exclude sperm or male variability from fertilization outcomes. With automated processing and quality assurance, cryopreserved sperm could provide standard genetic input throughout hybrid catfish hatcheries. The availability of a corresponding product, dairy semen, created a global billion-dollar industry (NAAB-CSS, 2011), and now similar opportunities have been revealed for aquaculture. To evaluate artificial spawning in test trials, two assumptions should be met: 1) cryopreserved sperm straws were produced under quality assurance and used in fertilization following proper instructions; 2) the number of eggs fertilized by one straw was within the dose (3704 eggs/straw

or 69-142 ml eggs/straw). Once the test trial meets the assumptions swim-up fry production efficiency (SwPE) can be calculated as the number of swim-up fry divided by the number of egg used for fertilization. The relative difference can also be calculated between the SwPE of test trials and a standard SwPE derived from large-scale hybrid catfish production using cryopreserved sperm (SwPE Index). The initial SwPE derived in this dissertation (59%) was used in this way for spawning evaluation across test trials.

Similar to the conception rate concept used for livestock (Smith, 1985, Cook, 2009), this evaluation method and SwPE value can be affected by extraneous factors (Figure 6.1) such as female broodfish management (e.g. deficient feeding of female broodfish), fertilization methods (e.g. insufficient water for activation), and hatchery management (e.g. low dissolved oxygen or poor water quality in hatching systems). Previous studies on channel catfish ovarian development (Dorman and Torrans, 1987, Patiño et al., 1996), temperature-induced out-of-season spawning (Lang et al., 2003b), and gonadotropic hormone effects on ovulation (Habibi et al., 1989, MacKenzie et al., 1989) have targeted such broodstock factors. The use of the SwPE evaluation method can further assist identification of variations in different activities in hybrid catfish fry production. The use of dose and standard SwPE for evaluation in hybrid catfish production also provides quantitative information that can be used to plan fry production and purchases of cryopreserved sperm.

Another challenge for the process developed in this dissertation was the actual throughput scale. There are no specific quantitative definitions of "high-throughput", however, if the throughput of processing cannot meet the production scale of potential customers, the public will not accept the products due to the risk of interruption in the supply chain. Currently, in-field evaluations and simulation modeling have both indicated that daily production from 16-18 males

is more than 1,000 straws, which will produce close to one million hybrid fry from about 40 L of catfish eggs. Presently this level of production could sustain production at a single commercial-scale hatchery that produces 500,000-1,000,000 fry per day. During the two-month spawning period each year, this production would be equivalent to 27-54% of the entire hybrid catfish fry production in 2011 (about 111 million) (Chatakondi, 2012) or 4-9 % of the entire cultured catfish fry and fingerling production (about 700 million) (USDA-NASS, 2011).Thus this prototype facility can provide commercially relevant throughput and could be expanded as needed, or be used as a model to develop additional facilities to respond to increase in demand for catfish. The other 10 months in the year would e available for work with other species (see below).

Integration of genetic resource management into aquaculture

This dissertation collected sufficient data to show the capability of cryopreserved sperm in assisting aquaculture activities. With high-throughput production and quality assurance, cryopreserved sperm can lead to predictable output (e.g., hybrid catfish fry). With this output, broad applications would be enabled: 1) valuable fish genetic features can be recovered or improved if the sperm of interest remain available, and 2) less significant features can be maintained in the frozen state rather than in live fish. These applications emphasize the importance of genetic resource management.

This concept of genetic resource management in aquaculture activities can be applied to other related activities for aquatic animals (Figure 8.6). For example, conservation programs for aquatic species can cryopreserve sperm from wild populations and develop repositories. The inventory of those repositories can be used for restoring local populations or maintaining population diversity in other areas. Biomedical model fish research could bank sperm from multiple genotypes and reduce or enhance live fish maintenence (Tiersch et al., 2011).

Cryopreservation technology is the key to genetic resource management, which can provide a change in the patterning of interactions of aquatic species with human society. A central facility equipped with high-throughput cryopreservation production could plan activities throughout the year according to the different natural spawning seasons of aquatic species (Figure 8.7) or at other times that gametes can be made available. Year-round activity in aquatic species will increase the utility of the facility, and maximally support genetic resource management.

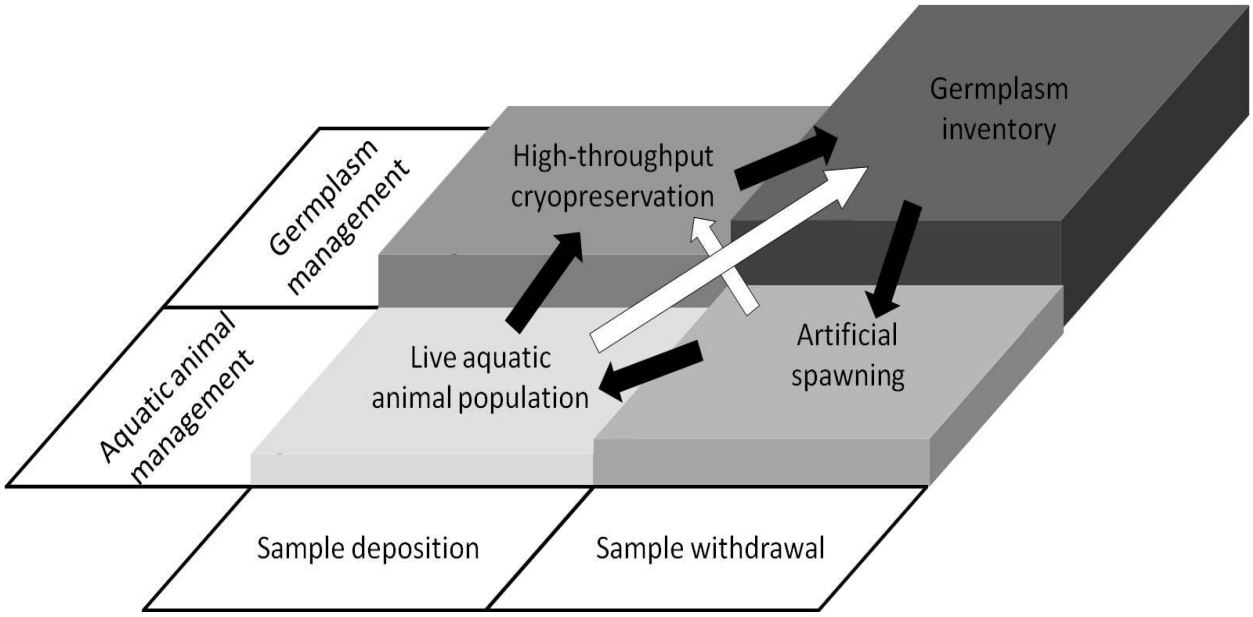

Figure 8.6. Model of integration of genetic resource (GR) management and aquatic animal activities. There are four elements of this model: Live aquatic animal populations, high-throughput cryopreservation, germplasm inventory, and artificial spawning. The black arrows represent GR flows: GR as a form of live animals in populations is an input into the cryopreservation process (sample deposition); GR accumulates into inventory (germplasm management); artificial spawning withdraws GR from inventory (sample withdrawal); GR are used to improve fry grow out (aquatic animal management). The white arrows represent information feedback: spawning results can help improve cryopreservation process; broodfish genetic features can help inventory management. The height of each element block represents the concentration of the GR in terms of storage space.

Conclusions

This dissertation demonstrates a roadmap for transforming laboratory protocols to the production line. More importantly, when rising above the details contained within the chapters,

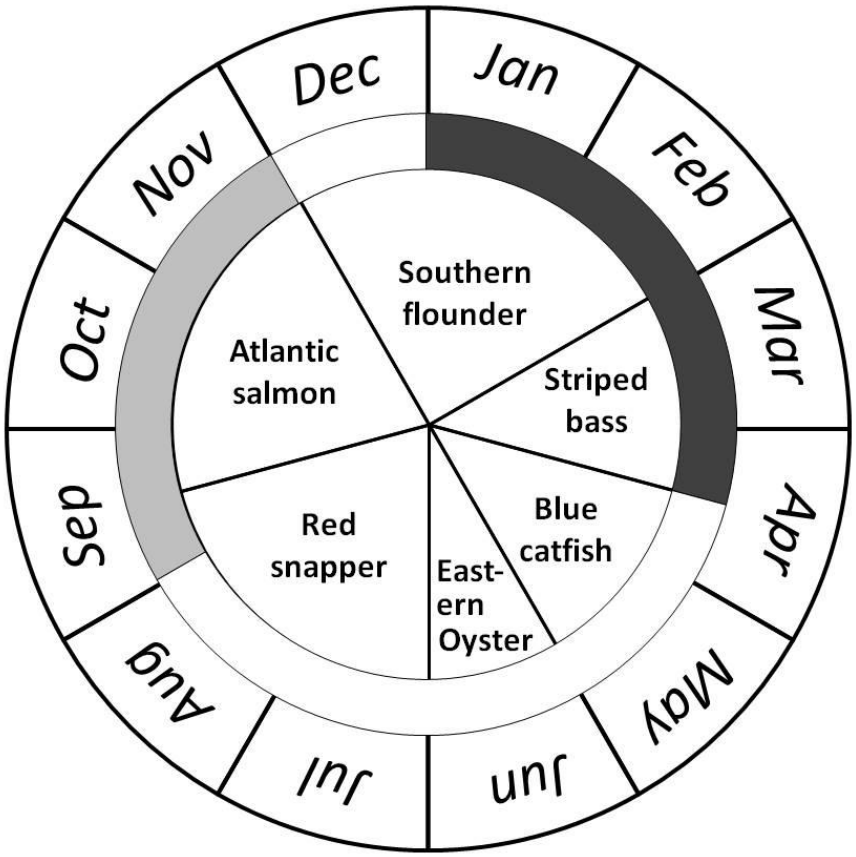

Figure 8.7. Example of an annual production cycle for a cryopreservation facility based on aquatic species that have been studied. A facility performing high-throughput cryopreservation could be operational year round if activities included work with a variety of species during their natural spawning seasons (interior segments). Work could also be done during out-of-season spawning by temperature-photoperiod manipulation (Daniels et al., 2010) in tanks (e.g., southern flounder, light gray ring), or temperature-control (Lang et al., 2003b) in ponds (e.g., blue catfish, dark gray ring).

this dissertation is a call for behavior changes within potential users of sperm cryopreservation products: changes in production scale; changes from research-orientation to uniform commercially relevant production; changes in emphasizing the genetic value of male characteristics, and changes of view about aquatic genetic resources. The content presented arose from a 5-year PhD program which was built upon decades of previous aquatic species research and industrial application in livestock industries. Eventually, this dissertation can serve

additional scientific research by providing ideas that can accelerate the upcoming progress of aquatic genetic resource management. Ultimately, increases in food production can extend the time for human societies to avoid stress from hunger, provide opportunities for economic development through aquaculture, and reduce pressure on wild aquatic populations or assist in their management.

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Appendix A. Standard Operating Procedures

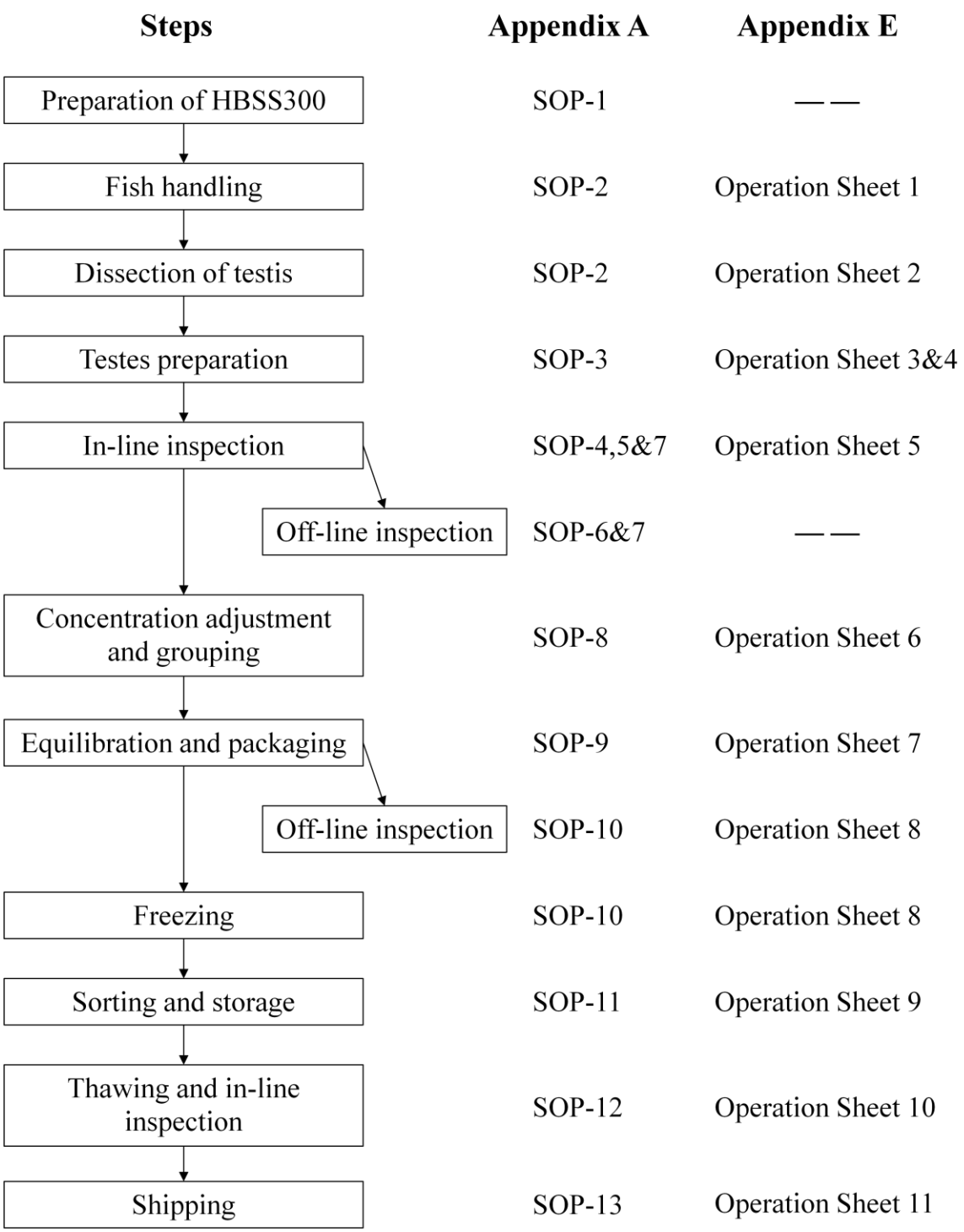

Figure A.1. Overview of relationships among process steps, standard operation procedures (Appendix A), and standard operation sheets (Appendix E).

SOP-1. Preparation of 300 mOsmol/kg Hanks' Balanced Salt Solution (HBSS300) as Blue Catfish Sperm Extender Solution

Materials and supplies needed:

Latex gloves	NaCl
Balance	KCl
Stirring hot plates	$CaCl_2 \cdot 2H_2O$
4-L flasks	$MgSO_4 \cdot 7H_2O$
Label tapes	Na_2HPO_4
Metal spoon	KH_2PO_4
Permanent marking pen	$NaHCO_3$
Weigh boats	$C_6H_{12}O_6$ (glucose)
Magnetic stirrers	Water purification
1-L bottles	Refrigerator

Procedure:

1. Combine the ingredients (32 g NaCl, 1.6 g KCl, 0.64 g $CaCl_2 \cdot 2H_2O$, 0.8 g $MgSO_4 \cdot 7H_2O$, 0.24 g $NaHPO_4$, 0.24 g KH_2PO_4, 1.4 g $NaHCO_3$, 4 g glucose) with distilled water and bring total volume to ~ 3.9 L
2. Stir the solution until all solutes dissolved
3. Verify the osmolality of mixture using osmometer; adjust to 300 mOsmol/kg by adding water
4. Distribute solution into 1-L bottles and label with date and name of operator.
5. Store bottles in refrigerator

References:

Tiersch, T. R., Goudie, C. A. & Carmichael, G. J. 1994. Cryopreservation of channel catfish sperm: storage in cryoprotectants, fertilization trials, and growth of channel catfish produced with cryopreserved sperm. Transactions of the American Fisheries Society, 123: 580-586.

SOP-2. Fish handling and Dissection

Materials and supplies needed:

Dip net	Paper towels
Basket	Ethanol 95%
Electronic balance (60 kg capacity)	Scalpels
Dissection table	Weighing boats
Hammer	Trash can
HBSS300 (SOP-1)	

Procedure:

1. Check fish repository records and locate blue catfish males; fish identification numbers were assigned sequentially
2. Scoop fish from holding system to processing area
3. Kill fish by sharp blow to the head
4. Weigh the fish to nearest gram and record
5. Move fish to dissection table and remove fluids using paper towels and HBSS300
6. Measure the length from head to the end of the tail (total length) and record
7. Open fish body cavity without damage to the intestine, expose and separate testes from connecting tissues with minimum bleeding, and record visual characteristics of fish and testes
8. Place testes in weighing boat and cover with HBSS300

References:

Hu, E., Yang, H. & Tiersch, T. R. 2011. High-throughput cryopreservation of spermatozoa of blue catfish (Ictalurus furcatus): Establishment of an approach for commercial-scale processing. Cryobiology, 62: 74-82.

SOP-3. Sperm Extraction

Materials and supplies needed:

Weighing boats	0.5-ml snap-cap tube
Tweezers	200-µ filters
Scissors	25-ml pipet tips
Electronic balance (1 kg capacity)	50-ml vials
Ziplog bags (qt.)	Cooler (10 gal.) with ice
HBSS300 (SOP-1)	Paper towels
Rolling pin	1-mm strainer
25-ml pipetor	0.5-mm strainer

Procedure:

1. Remove remaining blood and connective tissue without breaking testes
2. Dry the testes on paper towel, separate and weigh the anterior and posterior portions
3. Discard testes that are less than 3 g
4. Place the anterior testes into Ziploc bag and add 1ml of HBSS300 for each g of testes. Extract air in the Ziploc bag and seal it
5. Place Ziploc bag on flat smooth surface and crush the testes using rolling pin without breaking the bag
6. Cut a corner of Ziploc bag to release the contents through double strainers into 50-ml vials and record volume
7. Transfer contents from 50-ml vial through 200-ul filter to a clean 50-ml vial
8. Place filtered sperm suspensions (in 50-ml vials) into refrigerator or on ice without direct contact with ice

References:

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SOP-4. Motility Estimation with Computer Assisted Sperm Analysis (CASA,CEROS®, Hamilton Thorne, Inc., Beverly, MA)

Materials and supplies needed:

CASA unit (CEROS®, Hamilton Thorne, Inc., Beverly, MA)

Glass slides

Deionized water

10-ul pipet

100-µl pipet

10-µl pipet tips

200-µl pipet tips

KimWipes

Procedure:

1. Turn on computer, microscope, and software program
2. Determine the file folder for image storage
3. Select the analysis setting as "Catfish"
4. Input animal name as "species + date" (ITFddmmyy)
5. Input study number following fish identification number assigned to fish in SOP-2
6. Place 1 ul of sperm on glass slide, and activate with 10 ul of fresh water, without cover slide place under microscope for immediate motility check and write down naked-eye estimation
7. Step on foot paddle 3-5 times to record CASA imagines in different areas of sample within 15 sec

References:

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

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SOP-5. Measurement of sperm concentration with the NanoDrop® microspectrophotometer (Thermo Scientific, Wilmington, DE)

Materials and supplies needed:

Eppendof tubes	10-µl pipet tips
HBSS300 (SOP-1)	200-µl pipet tips
Deionized water	KimWipes
10-ul pipet	Eppendorf tube rack
100-ul pipet	Vortex mixer

Procedure:

1. Add 90 ul of HBSS300 into an empty Eppendof tube
2. Add 10 ul of sperm suspension into the eppendof tube with 90 ul of HBSS300
3. Mix well with vortex mixer
4. Turn on computer and NanoDrop
5. Select program "cell culture"
6. Blank the NanoDrop with 2 ul deionized water during program initialization
7. Reblank NanoDrop with 2 ul HBSS300
8. Load 2 ul of mixed sample and click "measure"

Note: if the reading from NanoDrop is higher than 1.00, the sample needs to be further diluted with HBSS300 (SOP-1). The dilution ratio in concentration calculation will need to be adjusted accordingly

9. Click "measure" 2 more times to collect triplicate measurements
10. Average all 3 measurements and input into the equation $Y=(5.12\times10^{8}X-4.07\times10^{7})\times20$ as "X" value
11. Calculation concentration as "Y" times dilution ratio and record

References:

Cuevas-Uribe, R. & Tiersch, T. R. 2011. Estimation of fish sperm concentration by use of spectrophotometry. In: TIERSCH, T. R. & GREEN, C. C. (eds.) Cryopreservation in Aquatic Species, 2nd edition. Baton Rouge, LA: World Aquaculture Society. Pp. 162-200.

SOP-6. Measurment of Membrane Integrity by Accuri™ Flow cytometry (BD Biosciences, San Jose, CA)

Materials and supplies needed:

Eppendof tubes

10-ul pipet

1000-ul pipet

10-µl pipet tips

1000-µl pipet tips

Filter screen 20 ul

Live/Dead Cell Double Staining Kit, 04511, Sigma-Aldrich

HBSS300 (SOP-1)

Accuri™ flow cytometry (BD Biosciences, San Jose, CA)

Procedure:

1. Turn on computer, flow cytometry, and software program
2. Load 6-peak and 8-peak beads sample to validate program
3. Determine the file save folder and file name
4. Set program at 10 ul volume, speed at medium
5. Add 998 of ul HBSS300 into an empty Eppendof tube
6. Add 2 ul of sperm suspension into tube 998 ul of HBSS300
7. Mix well
8. Filter mixture through 20-µm screen
9. Transfer 250 µl of filtered suspension into new Eppendof tube
10. Add 1.25 µl of SYBR-14 and 1.25 µl of PI into the 250-µl sample and incubate in dark for 10 min
11. Load sample on flow cytometry and analyze

References:

BD BIOSCIENCES. BD Accuri C6 Flow cytometry Instrument Manual. Available: http://www.bdbiosciences.com/documents/BD_Accuri_C6Flow_Cyto_Instrument_Manual.pdf.

SOP-7. Off-Line Quality Screening of Samples

Procedure:

1. Perform motility check on filtered sperm suspensions
2. Discard if less than 40% motility
3. Perform spectrophotometer measurement on samples
4. Discard if less than 1×10^9/ml
5. (optional) Take 2 ul sample for flow cytometry analysis (SOP-6)

SOP-8. Sample Grouping and Preparation for Cryoprotectant Equilibration

Materials and supplies needed:

Microsoft® Excel
50-ml vials
25-ml pipetor
25-ml pipet tips
10-ml pipet tips
Methanol
HBSS300 (SOP-1)

Procedure:

1. Estimate the volume of each suspension and record
2. Calculate and dilute all suspension to 2×10^9 cells/ml and record the HBSS300 volume added
3. Prepare 20% methanol individually with the same volume as each 2×10^9/ml sample and record volume

Note: For concentrations between 1×10^9 and 2×10^9 cells/ml, do not dilute sperm suspensions, adjust cryoprotectant concentration instead

4. Calculate theoretical straw number produced from each sample and record
5. Group samples for maximum efficiency of using freezer capacity (240 straws) and record

References:

Liao, T. W., Hu, E. & Tiersch, T. R. 2012. Metaheuristic approaches to grouping problems in high-throughput cryopreservation operations for fish sperm. Applied Soft Computing, 12: 2040-2052.

SOP-9. Equilibration and Packaging Using the MAPI® System (CryoBioSystem, Paris, France)

Materials and supplies needed:

Timer
Injection nozzle
CBS straws
Straw racks
MAPI (CryoBioSystem, Paris, France)

Procedure:

1. Turn on computer, MAPI printer, MAPI control box, and initialize software
2. Perform "ink check" function of the program with previously used straws
3. Input labels for individual samples into program, formatting as "fish ID + species + year + strain" (##ITFyyyyB&D)
4. Connect two injection nozzles by inserting head to tail for filling sperm suspension
5. Load the straw holding box with CBS straws
6. Mix sperm suspension with respective methanol solution for all individuals in one group, start 30 min count down (equilibration) with timer
7. Equip MAPI with extended injection nozzle and load mixture to be distributed into straws with the estimated straw number
8. Record wasted straws due to package failure and straws produced from each sample
9. Change injection nozzle and label between samples
10. Place filled straws on racks

Note: any straws from different samples that share one rack have to be placed towards opposite direction, recording of straw location (rack number) is recommended

References:

Hu, E., Yang, H. & Tiersch, T. R. 2011. High-throughput cryopreservation of spermatozoa of blue catfish (Ictalurus furcatus): Establishment of an approach for commercial-scale processing. Cryobiology, 62: 74-82.

SOP-10. Freezing Using Microdigitcool® Programmable Freezer (CryoBioSystem, Paris, France) and Inspection

Materials and supplies needed:

10-ul pipet

100-ul pipet

10-ul pipet tips

200-ul pipet tips

Microscope

Glass slides

KimWipes

Microdigitcool freezer (CryoBioSystem, Paris, France)

Procedure:

1. Turn on computer, freezer control box, liquid nitrogen tank, and software program
2. Select freezing program "blue catfish" and run until sample loading instruction shows on the computer
3. Place racks with desired order into freezer, wait for timer countdown
4. When timer beeps, start freezing cycle
5. Perform motility test on equilibrated samples and record
6. (optional) Take 2 ul of equilibrated samples for flow cytometry
7. Continue freezing process for another 5 min after the chamber reaches -80C and record freezing status

References:

Hu, E., Yang, H. & Tiersch, T. R. 2011. High-throughput cryopreservation of spermatozoa of blue catfish (Ictalurus furcatus): Establishment of an approach for commercial-scale processing. Cryobiology, 62: 74-82.

SOP-11. Sorting and Storage of Cryopreserved straws

Materials and supplies needed:

Styrofoam box

Plastic holding cups

Latex gloves

Cryogloves

Daisy goblets, CryoBioSystem, Paris, France

Long tweezers

Markers

Inventory canister of inventory dewar

Procedure:

1. Fill Styrofoam with liquid nitrogen, submerge plastic holding cups into liquid nitrogen holder
2. Remove straws and place into cups under liquid nitrogen in the positions they were on the rack. Warm up freezer for next freezing
3. Sort all straws into 12-compartments of Daisy goblets starting from the black compartment (12:00 position) with clockwise direction (Figure B.2)
4. Record sorted straws for each sample and location (compartments, Daisy goblets)

Note: different sperm sources should not share the same compartment

5. Label the Daisy goblet as soon as it is filled with the format "species + year + fish IDs" (ITFyyyy ##, ##, ##, …)
6. Leave filled Daisy goblet submerged in liquid nitrogen until all straws are sorted
7. Label the inventory canister holder with format "species + year + canister ID" (ITFyyyy#)
8. Put 5 Daisy goblets into one canister and put all canisters into inventory dewar immediately

SOP-12. Thawing and In-Line Inspection

Materials and supplies needed:

Items listed in Appendix B

Procedure:

1. Fill liquid nitrogen holder with liquid nitrogen, and submerge an empty Daisy goblet
2. Sort one straw from each sample into the empty Daisy goblet
3. Thaw the straws following Appendix B but release straw contents individually into Eppendorf tubes
4. Perform motility testing on thawed samples
5. Perform flow cytometry on thawed samples

SOP-13. Shipping Preparation

Materials and supplies needed:

Shipping dewar Cryogloves

Procedure:

1. Fill shipping dewar two days before with liquid nitrogen
2. Fill shipping dewar again one day before
3. Transfer Daisy goblets into shipping dewar on the day of shipping
4. Close and lock the cap of shipping dewar
5. Ship with priority shipping, make sure the sample can arrive within 3 d maximum

Appendix B. Manual for Using Cryopreserved Sperm Stored in 0.5-ml Straws

Introduction to Cryopreserved Sperm

This alternative sperm source can be available on demand.

Artificial spawning has been used for nearly 70 years in aquatic species, and provides enhanced control in every step of fish reproduction. With well-developed methods and materials, artificial spawning can achieve higher production than natural spawning. By extracting sperm and eggs researchers and farmers can systematically select or hybridize species of interest for genetic improvement. However, the application of such genetic improvement remains limited in aquaculture. One of the critical limitations is that accessing large amounts of sperm on a commercial scale is difficult. For example, the availability of sperm is influenced by the quantity and quality of males. Male broodstock management often takes extra labor and resources. In our facility, we provide services that gather males from reliable sources, extract sperm with standard methods, and provide quality control following industrially engineered designs. The final product of cryopreserved sperm can be stored for decades or centuries and transported to any location. Once the cryopreserved sperm are thawed and used at the recommended dosage, they function similarly to normal sperm and can be easily adapted to large-scale production. This manual addresses the use of cryopreserved sperm step by step, so that the thawing procedure will be effective and simple.

Product Features

Cryopreservation is a technology that preserves cells under cryogenic temperatures (e.g., < -100 ℃) while retaining their biological function. During cryopreservation, the cells are treated with chemicals to induce dehydration and minimize freezing damage. After the cells are frozen, they are transferred to liquid nitrogen (LN2) for storage. Therefore, there are two safety issues involved:

- All products contain **chemicals** that may be harmful.
- Products stored in **LN2** can cause injury if mishandled.

> Note
>
> The temperature of Liquid Nitrogen is -196 ℃ (-321 ℉).

CryoBioSystem™ (CBS™, Paris, France) straws and French straws (IMV, Paris, France) are the containers used for frozen sperm (Figure B.1). The CBS™ straws have

higher biosecurity features than the more common French straws. This straw has a unique “jacket” design that prevents rapid heat exchange. The “jacket” makes the straw less sensitive to environmental changes and makes field handling more feasible.

Figure B.1. Plastic 0.5-ml straws showing cotton end (left) and identification label jacket. The top two are CBS^{TM} straws, and the bottom two are French straws.

During the cryopreservation process, the jacket of each straw is labeled with product information and barcode. During transportation, CBS^{TM} straws are positioned in 12-compartment goblets (Daisy goblets). To identify the location of straws, the compartments are color coded. There are 12 colors: black, grey, yellow, powder blue, royal blue, chartreuse, orange, red, purple, brown, pink, and white. Beginning with the black compartment at position No. 1 (12:00), the numbering of each compartment increases in a clockwise manner to compartment No. 11, with compartment No. 12 (round) in the middle (Figure B.2). Be aware of mishandling of compartments during shipping. If there is any doubt about order or color, color is the default indicator. The labels on the straws should be used for final validation of sample before thawing. Each compartment has the capability to hold a small amount of LN2, which provides a little more buffer time for handling.

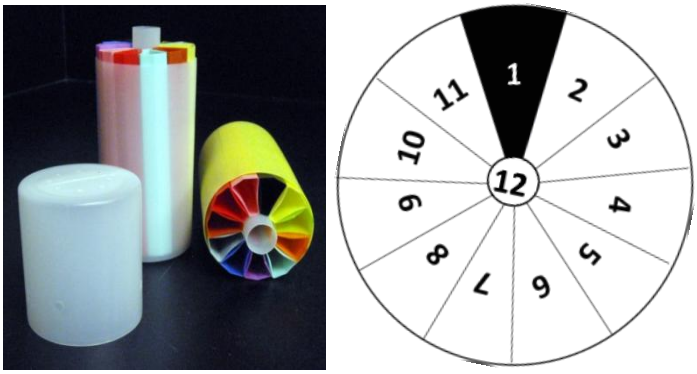


Figure B.2. Color-coded compartments of a daisy goblet (left). Identification of the Daisy goblet compartments illustrated by the diagram (right) begins with the black compartment and progresses in a clockwise direction.

Note

It can sometimes be difficult to remove the top of a fully-loaded Daisy goblet (e.g., every compartment contains 11-13 straws) coming out of LN2. The cover can be sealed by ice from water vapor in the air. Safety aspects of opening Daisy goblets are described in the following chapters.

Safety Precautions

Personnel should be required to wear long pants, gloves, goggles and closed-toe shoes. Please be aware that Cryo-gloves are different from laboratory gloves. They are multi-layer insulated gloves and can be placed directly in contact with LN2 for a short time, whereas laboratory gloves are thinner and made of nitrile rubber. Caution: other types of gloves (e.g. leather or cotton) can trap LN2 and cause burns.

To ensure safety

- Safety goggles
- Laboratory gloves
- Cryo-gloves
- Closed-toe shoes

During the performance of procedures listed in this manual, several icons are used to emphasize the safety equipment required for each step.

Tools and Supplies

The method was designed for use in any hatchery with common tools and supplies.

The tools and supplies required for the procedures in this manual are listed alphabetically below. This manual can also be used as a checklist.

Cryo-gloves

Because of the low temperature of LN2, cryo-gloves are required when handling cold objects. *Caution: cryo-gloves cannot fully protect hands from freezing.*

Goggles

Goggles are necessary when performing all procedures. Nitrogen is in gaseous form at room temperature. When subjected to high pressure, the temperature decreases and nitrogen transforms into a liquid at -196 ℃ (-321 ℉), which is suitable for cryogenic preservation. During thawing, liquid nitrogen will expand dramatically in volume and can cause ejection of objects. The use of goggles is the most effective way to protect your eyes. CBS™ straws are heat-sealed on both ends, which minimizes the chance of LN2 entering the straw.

Hot water

Any reasonable water sources should suffice. Examples are tap water, hatchery water, or well water. The optimal temperature is 40 ℃ (104 ℉) and usually is prepared by mixing hot water with water at room or tap temperature. The lowest acceptable temperature is 35 ℃ (95 ℉). The volume of water depends on the size of the thawing container. *Temperature is critical to thawing procedure.*

Laboratory gloves

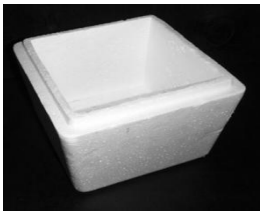
Laboratory gloves are necessary during all procedures. Gloves can protect the skin from chemicals in sperm suspension and prevent sample contamination. When dealing with LN2, laboratory gloves will not protect hands from freezing. *Do not use cloth gloves when handling LN2.*

Paper towels

Before releasing sperm from straws, drying is essential. Any remaining water on the straw will potentially activate sperm and reduce the thawed sperm quality. A typical roll of paper towels has individual segments that can cover the whole straw. Use of good quality paper towels will help avoid contaminating samples.

Plasticware

Disposable plasticware (e.g., tupperware™) can be used for holding of sperm suspensions. The volume of the plasticware depends on the volume of sperm being thawed. When all sperm has been released, the container should remain half-empty. This space in the plasticware is used to avoid spillage during movement and to provide oxygen for the sperm. The tops of plasticware should not be sealed so that oxygen can continue to move over the surface.



Polystyrene foam box

As one of the most commonly used tools for maintaining temperature, a polystyrene foam box provides good space for mixing water and thawing straws. Although there is no strict requirement for size, the opening of the box should be wide enough for both hands. Other equipment such as an electronic water bath can be used for the same purpose.

Scissors

Scissors are used to remove the ends of the plastic straws. Short-blade kitchen scissors work more efficiently and give more control than long-blade scissors.

Thermometer

A thermometer (digital or glass) should be used to make sure the temperature of the water is within the required range of 40 ℃ (104 ℉). If a thermometer is not available, a dissolved oxygen meter or pH meter with a thermometer function can serve the same purpose.

Timer

The thawing procedure is time sensitive. A timing device is necessary for accuracy during submersion of the straws in hot water. Digital timer or a watch will each work as long as the seconds are clearly visible. Recording of the timing of the entire process and at each step is also recommended as a quality assurance check.

Thawing Procedure

The general steps are narrated as a single-person task, followed by suggestions for inclusion of additional workers if desired.

Step 1: Prepare 40 ℃ (104 ℉) water

A typical tap water source should provide hot water at approximately 50 ℃ (122 ℉) and cold water at approximately 20 ℃ (68 ℉) although this can vary by location and time of the year. Starting with hot water in the polystyrene foam box, slowly add in cool water and keep stirring. Once the mixture temperature is slightly above 40 ℃ (104 ℉), the water is ready to use. When a hot water source is not available, a stove or coffee maker can be used to heat water.

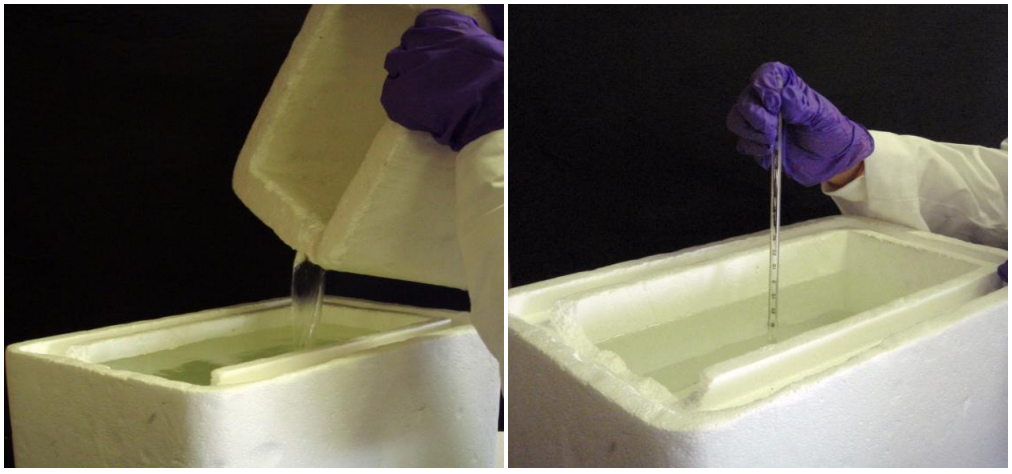


Figure B.3. Step 1 illustration. Mixing of hot and cool water to yield 40 ℃ (104 ℉) water (left) and measuring the temperature using a thermometer. (right).

Step 2: Preparation of paper towels

A sheet of paper towel (15.5 cm × 28.8 cm or 6.1 in ×11.3 in) will allow CBS™ straws (13 cm or 5.1 in length) to lay across and be covered by the width of the paper towel. To prepare paper towels for drying, fold them in half lengthwise. All pieces are laid on the table before use. Thawing 6 goblets (about 864 straws) should consume one roll of paper towel.

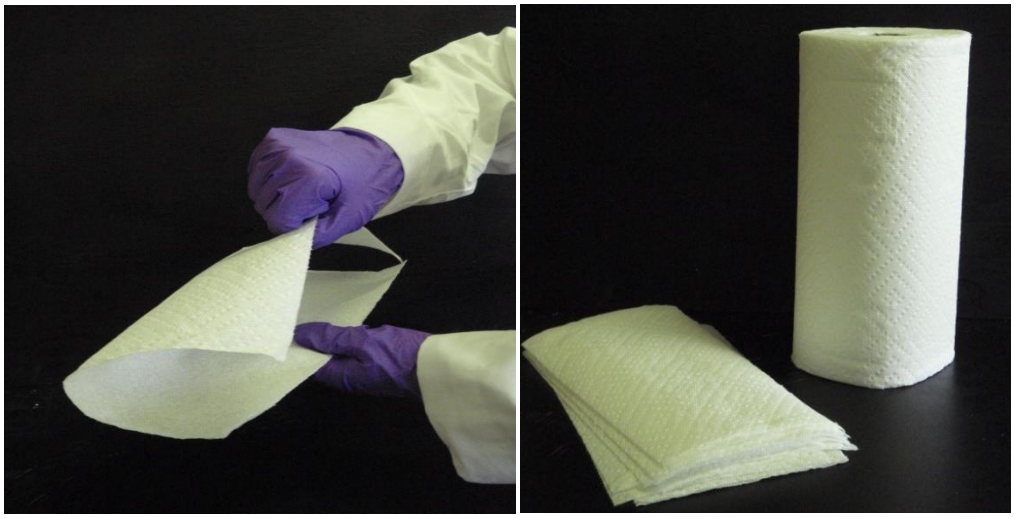


Figure B.4. Step 2 illustration. Folding two layers of paper towel (left) and stocking for later use (right).

Step 3: Verifying inventory

Before accessing the shipping dewar, be familiar with the location of samples. The inventory list includes description of samples and the storage location. Read the list carefully and make plans for the amount to be thawed each day.

Step 4: Removal of goblets 

Note

Make sure workers are familiar with all steps from this point forward prior to proceeding, because from this step forward, timing will be critical. If a problem occurs, placing the Daisy goblets back into the shipping dewar will keep samples safe. Although the Daisy goblets and CBS™ straws have resistance to heat transfer, the maximum exposure time to ambient air for Daisy goblets is less than 2 min before samples will be affected.

The single-canister and multiple-canister shipping dewars from MVE have a hard case protecting the outside. To open the case, all three butterfly-shaped metal tabs on the side of dewar need to be lifted and rotated counter-clockwise 180 °to open the latches (there are other dewars with different locks). After opening the case, the sample neck can be seen, which is filled by a foam cap. After removing the cap, the numeric labels of each canister will be visible. The goblet canisters are “L” shaped facing out. Choose the desired canister according to the inventory. Move the canister horizontally until it is directly under the entrance/exit of the shipping dewar, and move the canister vertically until the goblets inside can be recognized and accessed easily. Each canister contains two Daisy goblets and a metal lifter which helps lift the goblets out. If the desired goblet is at the bottom, it should be rearranged to the top for easy access. To rearrange the goblets, both should be taken out and placed on a flat surface, and their order reversed when placed back in the canister. The whole action should be performed within 30 seconds. Now, raise the lifter again and remove desired goblet which should be placed vertical (standing) on a flat surface. After the goblet is removed, lower the canister and rest it in the middle of shipping dewar.

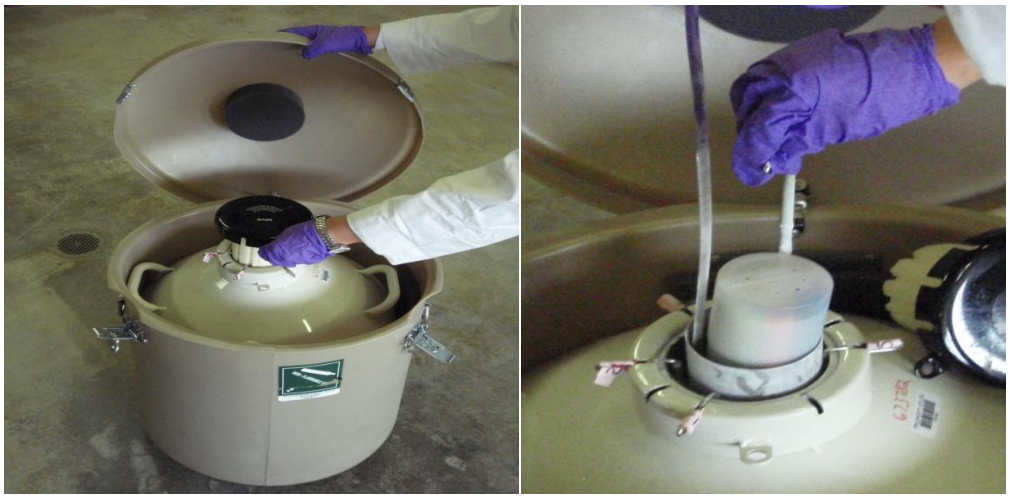


Figure B.5. Step 4 illustration. Open the case of the shipping dewar and lift the cover (left), then raise the goblets by lifting the canister (right).

Step 5: Opening of Daisy goblets

After the goblet is out of the shipping dewar, it will remain at a safe low temperature for 3 min. Each compartment contains a small volume of LN2, thus the goblet should be positioned vertically to aid in keeping the liquid nitrogen in the individual compartments. Because of the low temperature, the goblet will have ice crystal formation outside in humid environments. Use dry paper towels to clean visible white ice, and remove the top with cryo-gloves. Place the top on a flat surface.

> Note
>
> External ice can reduce gripping force and may cause difficulty in opening the Daisy goblet. Therefore, the ice should be removed before trying to open.

Figure B.6. Step 5 illustration. Daisy goblet covered by ice crystals (left), and removed of top (right).

Step 6: Returning opened goblet to canister

Once the top is removed and straws are visible, return the goblet to the canister and place it in the middle of shipping dewar. By doing this, exposure of the straws to room temperature will be kept at a minimum to prevent premature thawing.

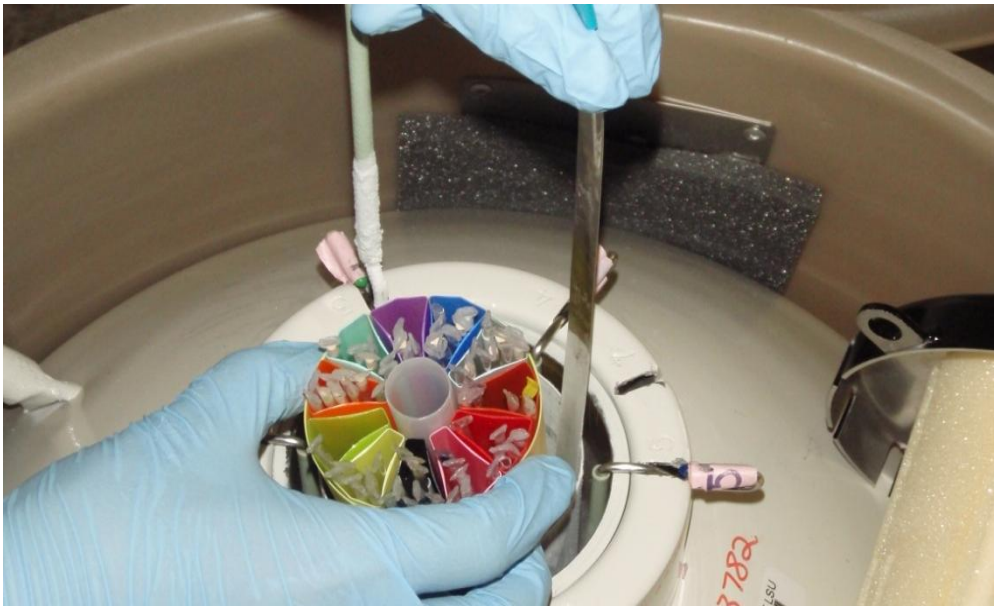


Figure B.7. Step 6 illustration. Place the opened daisy goblet back into the cold environment (shipping dewar).

Step 7: Removing straws for thawing

If a small number of samples need to be thawed, the safest way is to raise the canister and remove the desired straws individually while the goblet is still in the canister. However, for a large production, remove the goblet from the canister and place it on the table and remove two or three compartments simultaneously with the straws inside. In this step, cryo-gloves are usually too large to handle small straws, so laboratory gloves are recommended.

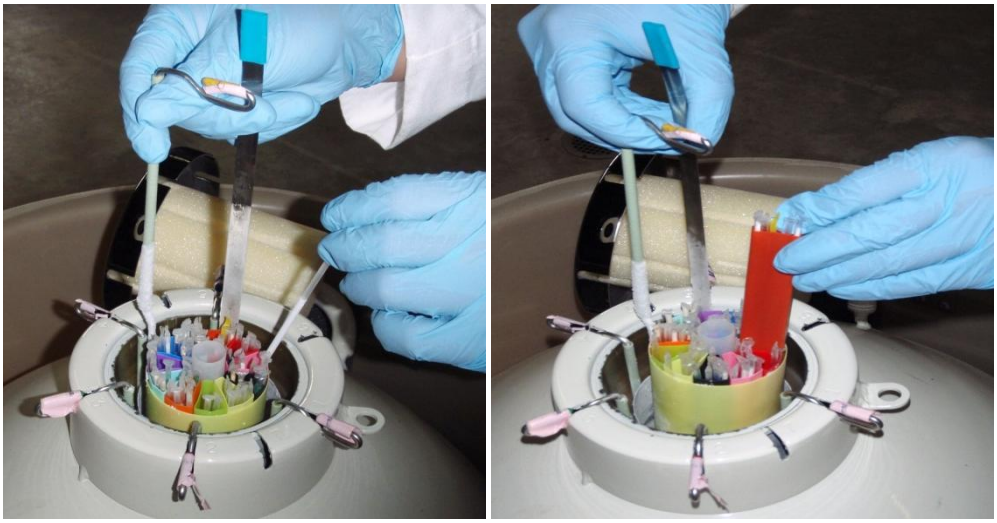


Figure B.8. Step 7 illustration. For thawing, straws can be removed individually (left) or in compartments (right).

Step 8: Thawing

Cryo-gloves cannot get wet. Use two pairs of laboratory gloves only for this step. Drop the straws into the 40 ℃ water immediately. Once the straws are fully submerged, it takes 20 sec for the CBS™ straws and 12 sec for the French straws to be thawed. When thawing an entire compartment, pour the contents of the compartment into the water. If there are any straws remaining in the compartment, manually move them and drop into the water. The small amount LN2 inside the compartment may immediately vaporize and form a thick, harmless fog on top of the water. Once the straws are in the water, move them to prevent the straws from sticking together, and to ensure consistent thawing.

> Note
>
> The temperature of the thawing water should be rechecked after repeated thawing or passage of time. Hot water can be added to return the temperature to 40 ℃ (104 ℉) before thawing of subsequent goblets.

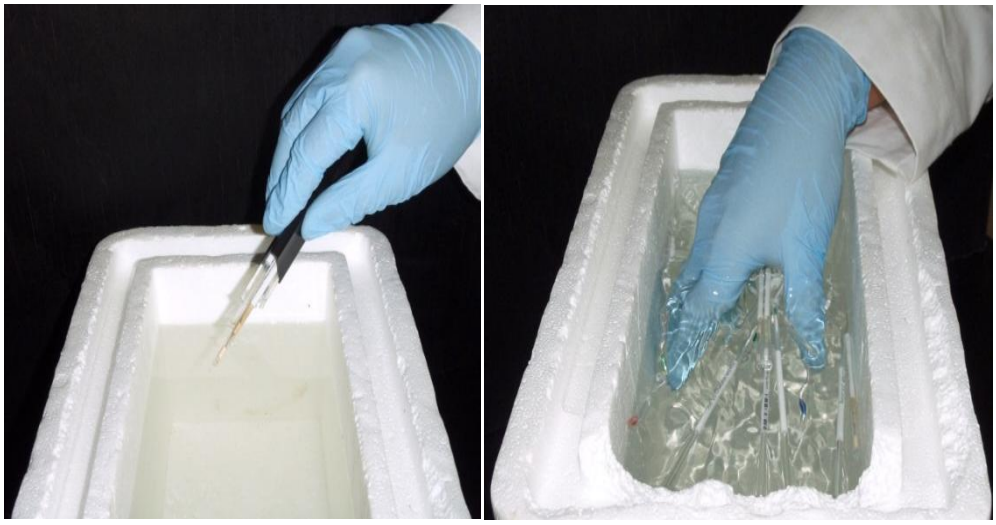


Figure B.9. Step 8 illustration. Placement of straws in 40 ℃ water (left), and stirring for 20 sec (right).

Step 9: Drying and arrangement of straws

After thawing, remove the straws and place them on the prepared paper towels. All remaining water should be carefully dried. Arrange all the straws in the same orientation (i.e., align the cotton plugs on one side). Final verification of labels can be performed if desired.

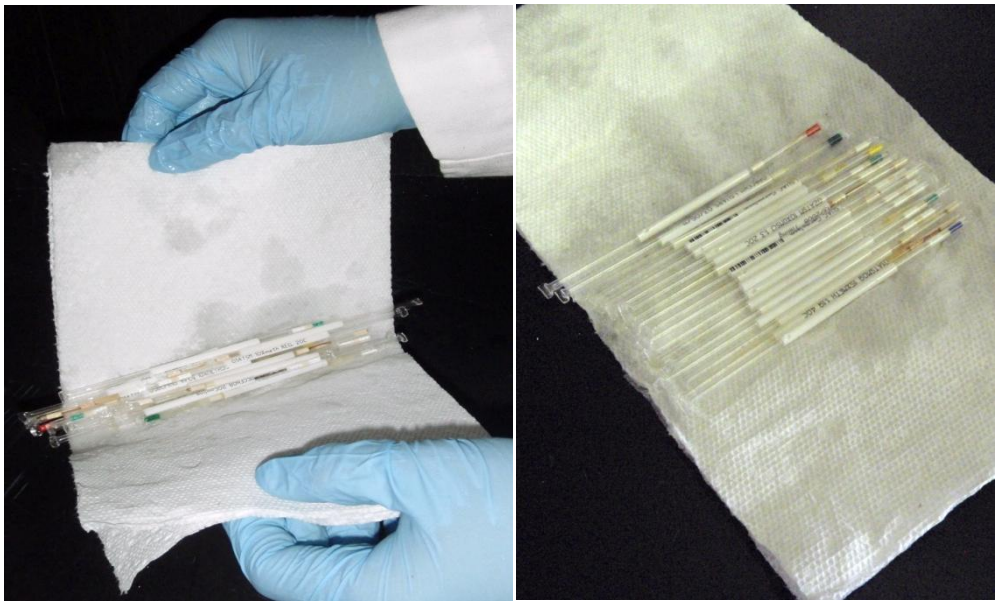


Figure B.10. Step 9 illustration. Thawed straws are dried by paper towel (left) then arranged in the same orientation (right).

Step 10: Release of sperm

The plasticware should be cleaned and dried before use. Hands should also be cleaned and dried. Holding 10~15 straws, adjust them to the same level with the cotton side facing up. Hold the jacket of the straws and shake once to make the samples move towards the cotton end. Cut the empty (air space) ends. Do not worry about sperm release, because the other sealed end holds the sample in by creating negative pressure at the top of the straw. Turn the straws over and with one hand still holding the jacket, place the cut ends into the plasticware and cut the below the cotton plug to release the sperm. If straws are accidentally dropped into the plasticware, they can be removed carefully with dry clean tweezers.

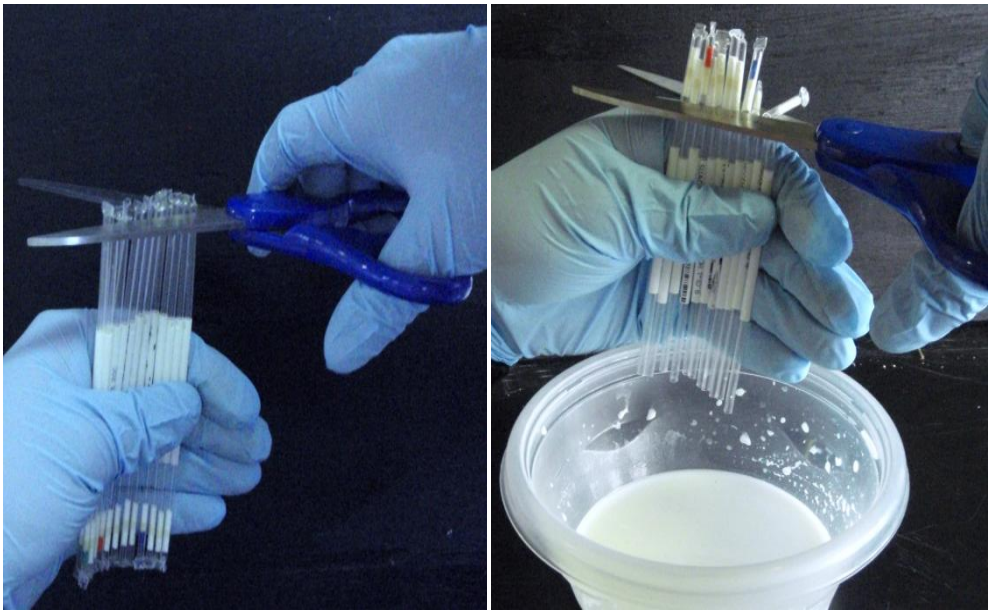


Figure B.11. Step 10 illustration. To release sperm from straws, the empty ends are cut first (left), then pointed the plasticware and the cotton ends are removed (right).

Assembly line layout

The thawing procedure can be managed by one person, however, if more than one person is available, an assembly line may be used to improve efficiency and speed. The thawing procedure can be divided into two parts: thawing and cutting. Two parts can be done separately without affecting sample quality. If there are two persons, the polystyrene foam box can be placed next to the paper towels (Figure 3.10). One person thaws all straws and arranges them on paper towel; the other dries them and cuts the straw to release the sperm. Furthermore, if there are three persons thawing, one can remove samples, one can thaw, and the third does the cutting based on specific working environments, there might be differences in layout. *In any event, the thawing procedure is time sensitive until samples are thawed. After thawing, the samples are safe in straws if held at 4 ℃ used within 2 hr.*

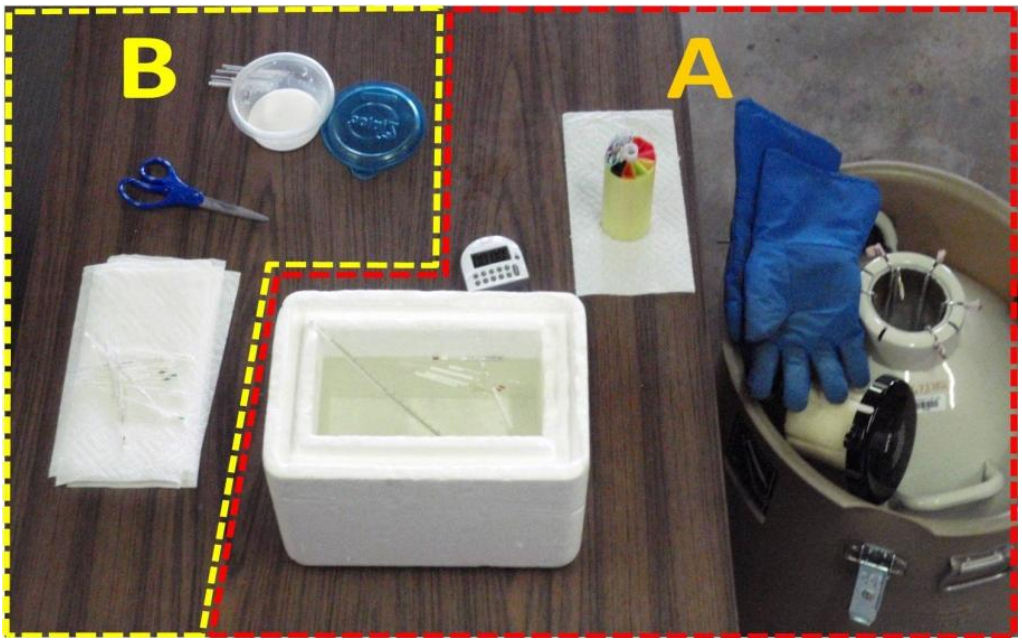


Figure B.12. General layout for thawing of straws. In practice, one person can operate Area A for straws and thawing; another person can operates Area B for drying straws and cutting. Two people can coordinate and work at the same time.

Recommended Usage during Fertilization

Thawed sperm can be used in fertilization similar as fresh prepared sperm. Keep sperm suspension on ice or in refrigerator all the time and avoid any contamination from outside environment or cross contamination from other sperm suspensions. The recommended usage is 3704 egg/straw or 69-142 ml eggs/straw (i.e., 7408 egg/ml or 138-284 ml eggs/ml).

Appendix C. Component List for a High-throughput Cryopreservation Facility

According to the modeled structure of high-throughput cryopreservation in Chapter 7, there are seven steps: Transportation of fish (T), Dissection (D), Preparation (P), Inspection and organization (I), Equilibration and packaging (E), Freezing and sorting (F), and Storage (S). The chemical solution preparation (C) and general cleaning (G) should be routine activities of the facility. This Appendix lists all the supplies and equipment (>$1,000) required for each step except for the automated packaging and freezing systems and large-scale storage dewar. The automated system and dewar each cost more than $20,000 and require quotes from sales companies. The items in this list were sorted alphabetically.

Item	Step	Source	Stock Id	Price($)	Quantity	Total($)
Aprons/lab clothes	D	University store[1]	SS00930	10.72	3	32.16
Centrifuge tubes (15-ml)	P	University store	SS17880	30.88	1	30.88
Clorox wipes	G	fishersci.com	34133	155.43	5	777.15
Daisy goblets (bundle of 5)	F	CryoBioSystem	015152	7.50	20	150.00
Desktop computer	I	bestbuy	bundle [2]	449.98	2	899.96
Dip net	T	fishnetco.com	DN-DH2	73.95	2	147.90
Eppendof tubes (1.5-ml)	I	University store	SS17867-07D	10.93	5	54.65
Exam gloves (small)	D	University store	SS40277-B	12.56	2	25.12
Exam gloves (large)	D	University store	SS40277-D	12.45	2	24.90
Exam gloves (medium)	D	University store	SS40277-C	12.55	2	25.10
Fillet knives	D	walmart.com	047527	24.97	2	49.94
Filters screen (200-micron)	P	aquaticeco.com	M200	17.43	1	17.43
First aid kit	D	walmart.com	AOZ3522	41.99	1	41.99
Fish basket	T	fishnetco.com	FB-2	21.95	2	43.90
Flasks (4000-ml)	C	University store	SS34105-P	44.99	2	89.98
Forceps	D	fishersci.com	10-316A	24.46	4	97.84
Funnels	P	University store	SS35619-90C	6.19	3	18.57
Garbage bags (10-gal)	D	walmart.com	E8-5060	12.47	2	24.94
Glass beakers (1000-ml)	C	University store	SS05681-10P	19.30	2	38.60
Glass beakers (50-ml)	C	University store	SS04675-G	2.05	2	4.10
Glass beakers (600-ml)	C	University store	SS04675-M	3.13	2	6.26
Glass bottles (1000-ml)	C	University store	SS08240-40F	7.84	5	39.20
Glass bottles (250-ml)	C	University store	SS08240-40D	4.26	5	21.30
Glass bottles (400-ml)	C	University store	SS08240-40E	6.20	5	31.00
Glass flasks (1000-ml)	C	University store	SS34105-K	4.86	2	9.72
Glass flasks (100-ml)	C	University store	SS34105-D	3.25	2	6.50
Glass flasks (250-ml)	C	University store	SS34105-F	2.46	2	4.92
Glass flasks (500-ml)	C	University store	SS34105-H	4.39	2	8.78

(table cont.)

Item	Step	Source	Stock Id	Price($)	Quantity	Total($)
Glass tube holders	P	University store	SS79929	10.13	4	40.52
Glassware washers	G	walmart.com	QTY1025	11.99	2	23.98
Graduated cylinder (1000-ml)	C	University store	SS24665-J	62.24	1	62.24
Graduated cylinder (100-ml)	C	University store	SS24665-E	20.83	1	20.83
Graduated cylinder (250-ml)	C	University store	SS24665-G	28.81	1	28.81
Graduated cylinder (500-ml)	C	University store	SS24665-H	30.84	1	30.84
Ice chest (40-qt)	D	walmart.com	44576	80.00	1	80.00
Injection nozzle	E	CryoBioSystem	014623	91.80	2	183.60
Intercom	C	walmart.com	KX-TS105B	26.74	1	26.74
Macroman	P	Pipetman	F110752	426.00	2	852.00
Magnetic stirrer (large)	C	fishersci.com	50093335	105.96	1	105.96
Magnetic stirrer (medium)	C	fishersci.com	50093334	71.41	1	71.41
Metal canes	C	fishersci.com	378441	67.40	4	269.60
Micro tubes (5-ml)	P	fishersci.com	03-391-168	14.27	7	99.89
Microscope	I	fishersci.com	12-562-1	1,500.16	1	1,500.16
Microscope slides	I	University store	SS68775	10.86	10	108.60
Nature wire ties (zip ties)	S	walmart.com	76180	11.91	1	11.91
Paper towel	G	walmart.com	3700081438	13.97	2	27.94
PCR tube (0.5ml)	P	University store	SS17867-44B	19.32	1	19.32
PH meter	C	University store	SS29976	407.10	1	407.10
pipet (1000-ul)	I	fishersci.com	02-681-171	98.67	1	98.67
Pipet (100-ul)	I	fishersci.com	21-197-2F	77.90	1	77.90
Pipet (10-ul)	I	fishersci.com	13-676-10J	87.23	1	87.23
pipet (200-ul)	I	fishersci.com	02-681-152	97.08	1	97.08
Pipet aid charge stand	P	Pipetman	F1077501	85.00	1	85.00
Pipet tips (1000-ul)	I	University store	SS69722-12C	13.23	10	132.30
Pipet tips (10-ml)	P	University store	SS69719-51B	20.13	5	100.65
Pipet tips (10-ul)	I	University store	SS69722-26AB	8.50	10	85.00
Pipet tips (200-ul)	I	University store	SS69722-22B	8.98	10	89.80
Pipet tips (25-ml)	P	labdepotinc.com	P-0500-S01	83.78	1	83.78
Refrigerator	P	bestbuy	GTH18GBDWW	599.99	1	599.99
Safety glasses	D	fishersci.com	SB1810S	5.79	4	23.16
Scale (100-g)	P	fishersci.com	CS200-001	122.02	1	122.02
Scale (100-mg)	P	fishersci.com	14-557-555	418.20	1	418.20
Scale (60-kg)	D	fishersci.com	02-113-31	507.66	1	507.66
Scalpel blades	D	fishersci.com	12-640	34.02	10	340.20
Scalpel holder	D	fishersci.com	371070	25.92	2	51.84

(table cont.)

Item	Step	Source	Stock Id	Price($)	Quantity	Total($)
Scissors (4” blades)	D	walmart.com	308	12.97	2	25.94
Scissors (3” blades)	D	fishersci.com	08-945	20.05	4	80.20
Steel wool	G	walmart.com	898018	2.98	1	2.98
Stirring hot plates	C	University store	SS41059	445.91	2	891.82
Straws (0.5-ml)	E	CryoBioSystem	014627	153.00	5	765.00
Syringes (1-ml)	D	University store	SS79401-21AA	14.23	10	142.30
Syringes (3-ml)	D	University store	SS79401-25A	10.40	10	104.00
Tape colored	C	walmart.com	W549	4.87	1	4.87
Test tube racks	P	University store	SS79930-06	10.79	5	53.95
Tissue grinder	P	fishersci.com	885451-0020	43.30	2	86.60
Trash can	D	walmart.com	RMC3417	29.99	2	59.98
Tube holder (5-ml)	P	rpicorp.com	146145	35.00	4	140.00
Vaccum tube (100 pack)	D	Medexsupply.com	BD-366450	33.75	1	33.75
Vials (15-ml)	P	University store	SS83244	84.40	1	84.40
Vials (50-ml)	P	fishersci.com	4558	196.49	1	196.49
Volumetric flask (100-ml)	C	University store	SS34855-G	23.30	1	23.30
Volumetric flask (250-ml)	C	University store	SS34855-J	33.71	1	33.71
Volumetric flask (500-ml)	C	University store	SS34855-K	41.80	1	41.80
Vortex mixer	I	University store	SS76609-21C	357.44	1	357.44
Wash bottles	D	University store	SS09486-60E	6.06	4	24.24
Water bath	C	fishersci.com	15-462-2SQ	884.35	1	884.35
Water purification	C	labwater.com	D11901	5,067.30	1	5,067.30
Weighing boats (10-cm)	P	fishersci.com	08-732-113	102.54	5	512.70
Ziploc bags (qt & gal)	P	walmart.com	bundle [3]	7.16	5	35.80
					Total of all supplies	$19,347.64

[1] items from University store are generally cheaper than those purchased directly from producers.
[2] bundled items: K410-11681KU and L2262
[3] bundled items: 0002570000320 and 0002570000310;

Appendix D. Data Recording Spreadsheets

Table D.1. sample preparation data log for recording sample conditions from fish to sperm suspension.

Sample preparation log									
Date[1] ____/____/_____					Operator(s) _____				
Species[2] _____					FAO species code[3] __ __ __ _____				
Fish source _____					Genetic strain _____				
Fish ID[4]	Fish status		Weight (kg)	Length (cm)	Testis status		Anterior testis (g)	Concentration ($\times 10^9$ cells/ml)	Motility (%)
	Sick	Female			No testis	reddish			
	☐	☐			☐	☐			
	☐	☐			☐	☐			
	☐	☐			☐	☐			
	☐	☐			☐	☐			
	☐	☐			☐	☐			
	☐	☐			☐	☐			
	☐	☐			☐	☐			
	☐	☐			☐	☐			
	☐	☐			☐	☐			
	☐	☐			☐	☐			
	☐	☐			☐	☐			
	☐	☐			☐	☐			
	☐	☐			☐	☐			
Every killed fish should be recorded in this log. Check the boxes if observed respective conditions. 1. Date format: "MMDDYYYY" 2. Species format: English common name 3. FAO species code follows FAO ASFIS list: 3A_ code then taxocode (e.g. blue catfish is ITF 1411000204) 4. Fish ID format: numerical continuous throughout a fiscal year (do not restart every log sheet)									

Table D.2. Sample grouping data log for calculating dilution methods to make sperm suspension ready for equilibration and packaging.

Grouping log

Date[1] ____/____/_____ Operator(s) _____

Target CPA concentration[2] _____ FAO species code[3] __ __ __ _____

Equilibration time _____

Fish ID[4]	Volume (ml)	Add extender solution (ml)	Double dose CPA (ml)	Estimated straws	Group[5]	Equilibration motility (%)

Only samples passed quality screen enter this log.

1. Date format: "MMDDYYYY"
2. The cryoprotectant type and concentration during equilibration (e.g. DMSO 10%)
3. FAO species code follows FAO ASFIS list: 3A_ code then taxocode (e.g. blue catfish is ITF 1411000204)
4. Fish ID of samples corresponds to the same as Sample preparation log
5. Group ID restart every day

Table D.3. Packaging and freezing data log for recording automated system operation and straw produced.

Packaging and freezing log

Date[1] ____/____/_____ Operator(s) _____

Straw label format[2] _____ FAO species code[3] __ __ __ _____

Freezing procedure From ____ to ___ at ____ ℃/min

From ____ to ___ at ____ ℃/min

From ____ to ___ at ____ ℃/min

Group[4]	Straw produced	Filling failure	Sealing failure	Labeling failure	Freezing status
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					

1. Date format: "MMDDYYYY"
2. The recommended format: "fish ID + FAO 3A_code + Date + genetic strain + protocol changes (if applicable)"
3. FAO species code follows FAO ASFIS list: 3A_ code then taxocode (e.g. blue catfish is ITF 1411000204)
4. Group ID corresponds to the same as Grouping log

Table D.4. Sorting and storage data log for recording product location.

Sorting and storage log				
Date[1] ____/____/_____			Operator(s) _____	
Daisy goblet format[2] _____			FAO species code[3] __ __ __ _____	
Group[4]	Straw sorted	Fish ID included with compartment number[5]	Daisy goblet number	Canister label[6]
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				

1. Date format: "MMDDYYYY"
2. The recommended format: "FAO 3A_code + Date + fish ID"
3. FAO species code follows FAO ASFIS list: 3A_ code then taxocode (e.g. blue catfish is ITF 1411000204)
4. Group ID remain the same as Packaging and freezing log
5. Entry format: "Fish ID (number of compartments)" (e.g. 07(2))
6. The recommended format: " FAO 3A_code + Year + numerical ID"

Appendix E. Standard Operation Sheets for sperm high-throughput cryopreservation

Table E.1. Standard Operation Sheet for Operation 1: fish handling.

OPERATION SHEET

Operation No.	1	**Material**	Blue catfish males
Operation Name	Fish handling	**Data log**	Sample preparation log
Original	_____	**Changes**	_____
Checked	_____	**Approved**	_____

No.	Operation Description	Machine/Equipment	Setup Description	Operate Hr/Unit
1	Locate blue catfish males		Check fish repository records	
2	Catch fish from holding system	Dip nets (18'' above)	Dip nets were treated with bleach and properly dried	
3	Transfer fish to processing area	Dip nets (18'' above) or fish baskets (Ø17'')	Dip nets and fish baskets were treated with bleach and properly dried	

Table E.2. Standard Operation Sheet for Operation 2: fish dissection.

OPERATION SHEET

Operation No.	2	**Material**	Blue catfish males
Operation Name	Fish dissection	**Data log**	Sample preparation log
Original	_____	**Changes**	_____
Checked	_____	**Approved**	_____

No.	Operation Description	Machine/Equipment	Setup Description	Operate Hr/Unit
1	Kill fish by sharp blow to the head	Hammer (4 lb. above)	Hammer was bleached and cleaned	
2	Weigh the fish	Weight scale (max. 60 kg)	Set a fish basket on the weight scale and zero it, then put fish in	
3	Move fish body to dissection table and clean the blood using paper towel and HBSS300		Dissection table was cleaned using paper towel and 95% ethanol	
4	Measure the length from fish head to the end of the tail	Ruler		
5	Open fish body cavity by not break intestine , expose testes, separate testes from connecting tissues with minimum bleeding	Scalpels	Scalpels were decontaminated	
6	Place fish testes in weight bowl submerging under HBSS300		The HBSS was warmed up to room temperature	

Table E.3. Standard Operation Sheet for Operation 3: testes processing.

OPERATION SHEET

Operation No.	3	**Material**	Blue catfish testes
Operation Name	Testes processing	**Data log**	Sample preparation log
Original	_____	**Changes**	_____
Checked	_____	**Approved**	_____

No.	Operation Description	Machine/Equipment	Setup Description	Operate Hr/Unit
1	Detach remaining blood and extra connecting tissue from testes without breaking testes	Tweezers and scissors	Tweezers and scissors were decontaminated	
2	Dry the testes on paper towel, separate anterior and posterior testes, then weigh the anterior portion and the overall	Weight scale (max. 3 kg)	Set a weight bowl on the weight scale and zero it, then put testes in	
3	Discard testes that were less than 3 g			
4	Place the anterior testes into Ziploc bag then add in HBSS300 following the rule as 1ml HBSS300 for 1g testes, then extract air in the Ziploc bag and seal it	Liquid transfer (25 ml)	Use new Ziploc bag	
5	Place Ziploc bag on flat smooth surface and crush the testes into pieces using hard objects without breaking the bag	Testes crusher such as bend ion ruler	Dry the surface then lay the Ziploc bag	

Table E.4. Standard Operation Sheet for Operation 4: filtering sperm suspension.

OPERATION SHEET

Operation No.	4	**Material**	Crushed testes
Operation Name	Filtering sperm suspension	**Data log**	Sample preparation log
Original	_____	**Changes**	_____
Checked	_____	**Approved**	_____

No.	Operation Description	Machine/Equipment	Setup Description	Operate Hr/Unit
1	Cut a corner of Ziploc bag to release all content through double strainers into 50-ml centrifuge tubes	Strainers (1-mm and 0.5-mm mesh)	Strainers were washed then rinsed in HBSS300 and dried by paper towel	
2	Transfer all content from first 50-ml centrifuge tube through 200-ul filter to another clean 50-ml centrifuge tube	200-ul mesh filter	Filters were washed, rinsed in HBSS300 and dried by paper towel	
3	Place filtered sperm suspensions (in 50-ml centrifuge tubes) into refrigerator or on ice without direct contacting with ice	Ice chest or refrigerator	Fresh made ice with minimum melting	

Table E.5. Standard Operation Sheet for Operation 5: inspection.

OPERATION SHEET

Operation No.	5	**Material**	Sperm suspensions
Operation Name	Inspection	**Data log**	Sample preparation log
Original	_____	**Changes**	_____
Checked	_____	**Approved**	_____

No.	Operation Description	Machine/Equipment	Setup Description	Operate Hr/Unit
1	Place a drop of 1 ul sperm on glass slide, activate with 10 ul fresh water, without cover slide place under microscope for immediate motility check and CASA recording	CASA, Hamilton Thorne, Inc., Beverly, MA	Select storage directory beforehand and name each CASA file, use "Catfish" setting	
2	Discard if less than 40% motility			
3	Dilute 10 ul sperm to 200 ul using HBSS300, then place 2 ul diluted sperm on NanoDrop for absorbance measurement 3 times	NanoDrop, Thermo Scientific, Wilmington, DE	Use cell culture function, make baseline with DiH2O and secondary baseline with HBSS300	
4	Calculate concentration of suspension using $Y=(5.12\times10^{8}X-4.07\times10^{7})\times20$	Computer by NanoDrop	Setup Excel table for calculation	
5	Discard if less than 1×10^{9}/ml			
6	Take 2 ul suspension to dilute to 1×10^{6}/ml for flow cytometer membrane integrity test	Accuri™ Flow cytometer, BD Biosciences	Follow flow cytometer standard protocol	

Table E.6. Standard Operation Sheet for Operation 6: preparing and planning for freezing.

OPERATION SHEET

Operation No.	6	**Material**	Sperm suspensions
Operation Name	Preparing and planning for freezing	**Data log**	Grouping log
Original	_____	**Changes**	_____
Checked	_____	**Approved**	_____

No.	Operation Description	Machine/Equipment	Setup Description	Operate Hr/Unit
1	Estimate the volume of each suspension			
2	Calculate and dilute all suspension to 2×10^9 cells/ml	Liquid transfer		
3	Prepare 20% methanol individually with same volume as each 2×10^9/ml sample			
4 (optional)	For concentration between 1×10^9/ml and 2×10^9/ml, do not dilute sperm suspension, but make higher concentration methanol to achieve final 10% methanol and 1×10^9/ml sperm mixture	Computer	Setup Excel table for calculation	
5	Calculate theoretic straw number produced from each sample	Computer	Setup Excel table for calculation	
6	Group samples for maximum efficiency of using freezer capacity	Computer	Follow Liao et al. 2012 *Applied Soft Computing*, 12: 2040-2052	

Table E.7. Standard Operation Sheet for Operation 7: packaging.

OPERATION SHEET

Operation No.	7	**Material**	Sperm suspensions
Operation Name	Packaging	**Data log**	Packaging and freezing log
Original	_____	**Changes**	_____
Checked	_____	**Approved**	_____

No.	Operation Description	Machine/Equipment	Setup Description	Operate Hr/Unit
1	Mix sperm suspension with respective methanol solution, start 30 min count down with timer	Timer	Set 30 min on timer	
2	Load mixture on MAPI to distribute mixture into straws	MAPI, CryoBioSystem, Paris, France	Start up system, design labels, type all formats for current freezing batch, fully fill the straw holder, made enough injection nozzles; before each sample, set the target straw number according to previous estimation	
3	Place straws on racks in designated position, any straws from different samples that share one rack have to be in opposite position	racks		

Table E.8. Standard Operation Sheet for Operation 8: freezing and off-line inspection.

OPERATION SHEET

Operation No.	8	**Material**	Unfrozen straws
Operation Name	Freezing and off-line inspection	**Data log**	Packaging and freezing log
Original		**Changes**	
Checked		**Approved**	

No.	Operation Description	Machine/Equipment	Setup Description	Operate Hr/Unit
1	Place racks into freezer, wait for timer beeping	Microdigitcool, CryoBioSystem, Paris, France	Start up system, run profile with 5 ℃/min cooling rate, hold on loading temperature as 4 ℃	
2	When timer beeps, start freezing			
3	Place a drop of 1 ul sperm from remaining mixtures on glass slide, then activate with 10 ul fresh water, without cover slide place under microscope for motility check and CASA recording	CASA, Hamilton Thorne, Inc., Beverly, MA	Select storage directory beforehand and name each CASA file, use "Catfish" setting	
4 (optional)	Take 3 ul mixture to dilute to 1×10^6/ml for flow cytometer membrane integrity	Accuri™ Flow cytometer, BD Biosciences, San Jose, CA	Follow flow cytometer standard protocol	
5	When freezing stops at -80C, take out straws and place into cups under liquid nitrogen following the positions as they were on the rack, warm up freezer for next freezing	Freezer and liquid nitrogen sorting tank/container	Fill liquid nitrogen sorting tank/container with enough liquid nitrogen that can just cover the plastic cups, submerge 4-5 cups	

Table E.9. Standard Operation Sheet for Operation 9: Sorting and storage.

OPERATION SHEET

Operation No.	9	**Material**	Frozen straws
Operation Name	Sorting and storage	**Data log**	Sorting and storage log
Original	_____	**Changes**	_____
Checked	_____	**Approved**	_____

No.	Operation Description	Machine/Equipment	Setup Description	Operate Hr/Unit
1	All straws sorted into 12-compartment Daisy goblets sequentially by compartment, different sperm sources did not share same compartment	Liquid nitrogen sorting tank/container	Label the Daisy goblets with straw number and related sperm sources	
2	Transfer Daisy goblets into canisters in inventory dewar	Inventory dewar	Canisters were labeled with production time and species	
3	Report inventory list			

Table E.10. Standard Operation Sheet for Operation 10: in-line quality check.

OPERATION SHEET

Operation No. 10 **Material** Frozen straws

Operation Name In-line quality check

Original _____ **Changes** _____

Checked _____ **Approved** _____

No.	Operation Description	Machine/Equipment	Setup Description	Operate Hr/Unit
1	Take one straw from each sperm source (male) to combine in one Daisy goblet	Liquid nitrogen sorting tank/container	Fill liquid nitrogen sorting tank/container with enough liquid nitrogen that can just cover the Daisy goblet	
2	Thaw no more than 12 straws at once under 40 ℃ for 20 sec	Water bath	Heat the water bath to 40 ℃	
3	Dry the straws, cut the ends individually, release sperm into 1.5 ml centrifuge tubes	Scissors	Label all 1.5 ml centrifuge tubes with sperm sources and arrange on tube rack	
4	Place a drop of 1 ul of sperm from a tube on glass slide, then activate with 10 ul fresh water, without cover slide place under microscope for immediate motility check and CASA recording	CASA, Hamilton Thorne, Inc., Beverly, MA	Select storage directory beforehand and name each CASA file, use "Catfish" setting	
5	Take 3 ul of sperm to dilute to 1×10^6 cells/ml for flow cytometer membrane integrity test	Accuri™ Flow cytometer, BD Biosciences	Follow flow cytometer standard protocol	

Table E.11. Standard Operation Sheet for Operation 11: transportation preparation

OPERATION SHEET

Operation No. 11 **Material** Daisy goblets

Operation Name Transportation preparation

Original _____ **Changes** _____

Checked _____ **Approved** _____

No.	Operation Description	Machine/Equipment	Setup Description	Operate Hr/Unit
1	Fill shipping dewar two days before	Shipping dewar		
2	Fill shipping dewar again one day before	Shipping dewar		
3	Transfer Daisy goblets into shipping dewar on the day of shipping	Shipping dewar	Check the inventory for locations of samples	
4	Close and lock the cap of shipping dewar	Shipping dewar		
5	Ship with priority shipping, make sure the sample can arrive within 3 days maximum		Check shipping companies with their service and fill documents properly	

Appendix F. Original Data in Research Chapters

Chapter 3

Table F.1. Methanol concentration, sperm density and cooling rate combined effect.

Id	Concentration %	Density sperm/ml	Cooling rate ℃/min	Fresh motility %	Equilibrium motility %	Post-thawing motility %		
1	5	1.00E+08	5	57.5	57.5	17.5	20	17.5
1	5	1.00E+09	5	57.5	60	17.5	22.5	22.5
3	5	1.00E+08	5	60	60	10	10	15
3	5	1.00E+09	5	60	60	25	20	15
4	5	1.00E+08	5	55	55	6.5	10	10
4	5	1.00E+09	5	55	60	12.5	15	15
1	5	1.00E+08	40	57.5	60	0	0	0
1	5	1.00E+09	40	57.5	57.5	2	0.5	2
3	5	1.00E+08	40	60	55	0	0	0
3	5	1.00E+09	40	60	60	2	2	3
4	5	1.00E+08	40	55	55	0	0	0
4	5	1.00E+09	40	55	60	2	0.5	2
1	10	1.00E+08	5	57.5	57.5	27.5	27.5	27.5
1	10	1.00E+09	5	57.5	60	27.5	20	40
3	10	1.00E+08	5	60	55	22.5	27.5	22.5
3	10	1.00E+09	5	60	60	27.5	25	37.5
4	10	1.00E+08	5	55	57.5	10	15	17.5
4	10	1.00E+09	5	55	60	15	12.5	-
1	10	1.00E+08	40	57.5	57.5	2	2	0.5
1	10	1.00E+09	40	57.5	60	2	3	4
3	10	1.00E+08	40	60	57.5	4	4	3
3	10	1.00E+09	40	60	60	7.5	7.5	7.5
4	10	1.00E+08	40	55	55	4	4	3
4	10	1.00E+09	40	55	60	7.5	2	2

Table F.2. Effect of methanol concentrations.

Id	Concentration %	Density	cooling rate	equilibrium motility %		post-thawing motility %		12hr motility after thawing %
6	5	1E9/ml	5C/min	70	30	-	-	25
8	5	1E9/ml	5C/min	65	17.5	20	20	12.5
9	5	1E9/ml	5C/min	57.5	12.5	12.5	-	25
10	5	1E9/ml	5C/min	60	12.5	12.5	12.5	10
11	5	1E9/ml	5C/min	65	12.5	12.5	-	12.5
12	5	1E9/ml	5C/min	57.5	10	10	-	5
6	10	1E9/ml	5C/min	60	37.5	35	37.5	27.5
8	10	1E9/ml	5C/min	57.5	35	-	-	27.5
9	10	1E9/ml	5C/min	57.5	37.5	37.5	-	37.5
10	10	1E9/ml	5C/min	57.5	25	25	-	25
11	10	1E9/ml	5C/min	62.5	40	-	-	40
12	10	1E9/ml	5C/min	60	-	-	-	-

Table F.3. Loading density and thawing condition effects.

Id	Density sperm/ml	CPA	thawing condition	fresh motility %	equilibrium motility %		post-thawing motility %	
20	1.00E+09	10%Meth	20C40s	45	42.5	32.5	37.5	32.5
21	1.00E+09	10%Meth	20C40s	50	42.5	10	15	15
22	1.00E+09	10%Meth	20C40s	57.5	52.5	40	30	35
20	1.00E+09	10%Meth	40C20s	45	40	32.5	32.5	37.5
21	1.00E+09	10%Meth	40C20s	50	7.5	25	27.5	12.5
22	1.00E+09	10%Meth	40C20s	57.5	52.5	35	42.5	40
20	1.70E+09	10%Meth	20C40s	45	42.5	2.5	7.5	0.5
21	1.70E+09	10%Meth	20C40s	50	42.5	0	2.5	0
22	1.70E+09	10%Meth	20C40s	57.5	52.5	0.5	5	2.5
20	1.70E+09	10%Meth	40C20s	45	40	0.5	5	15
21	1.70E+09	10%Meth	40C20s	50	7.5	5	0.5	0.5
22	1.70E+09	10%Meth	40C20s	57.5	52.5	2.5	15	5

Table F.4. Individual male fertilization.

Date	Female	Male	Thawed fertilization %	Control fertilization%
14-May	5	20	41.67	53.33
14-May	16	20	25.00	46.67
14-May	32	20	16.67	23.33
15-May	1	20	51.67	46.67
15-May	12	20	68.33	53.33
15-May	22	20	21.67	63.33
15-May	31	20	61.67	60.00
15-May	40	20	50.00	60.00
14-May	6	22	35.00	80.00
14-May	17	22	31.67	60.00
14-May	33	22	15.00	26.67
15-May	2	22	50.00	36.67
15-May	13	22	46.67	46.67
15-May	23	22	38.33	30.00
14-May	7	23	26.67	56.67
14-May	18	23	30.00	53.33
14-May	34	23	28.33	60.00
15-May	4	23	33.33	23.33
15-May	14	23	0.00	16.67
14-May	8	24	38.33	63.33
14-May	19	24	25.00	33.33
14-May	35	24	30.00	60.00
15-May	5	24	28.33	26.67
15-May	15	24	48.33	33.33
15-May	24	24	38.33	66.67
15-May	32	24	35.00	43.33
15-May	41	24	50.00	33.33
14-May	9	25	36.67	63.33
14-May	23	25	38.33	46.67
14-May	36	25	16.67	60.00
15-May	6	25	75.00	60.00
15-May	16	25	51.67	46.67
15-May	25	25	33.33	36.67
15-May	34	25	30.00	26.67
15-May	42	25	75.00	36.67
14-May	11	26	36.67	66.67
14-May	37	26	3.33	36.67
15-May	7	26	75.00	60.00
15-May	17	26	61.67	46.67

(table cont.)

Date	Female	Male	Thawed fertilization %	Control fertilization%
15-May	26	26	60.00	13.33
15-May	35	26	40.00	43.33
15-May	43	26	45.00	33.33
14-May	12	27	25.00	56.67
14-May	27	27	50.00	50.00
14-May	38	27	26.67	50.00
15-May	8	27	63.33	30.00
15-May	18	27	5.00	40.00
15-May	27	27	28.33	43.33
15-May	36	27	45.00	23.33
15-May	44	27	63.33	50.00
14-May	13	28	40.00	83.33
14-May	28	28	41.67	76.67
14-May	39	28	26.67	43.33
15-May	9	28	56.67	46.67
15-May	19	28	65.00	66.67
15-May	28	28	20.00	23.33
15-May	37	28	46.67	43.33
15-May	45	28	56.67	43.33
14-May	14	29	38.33	63.33
14-May	29	29	43.33	53.33
14-May	40	29	3.33	6.67
15-May	10	29	25.00	46.67
15-May	20	29	46.67	33.33
15-May	29	29	38.33	33.33
15-May	38	29	20.00	46.67
14-May	15	30	33.33	56.67
14-May	30	30	25.00	30.00
15-May	11	30	41.67	40.00
15-May	21	30	23.33	40.00
15-May	30	30	33.33	36.67
15-May	39	30	45.00	33.33

Table F.5. Biological information of males in individual fertilization.

Male	Weight (kg)	Length (in)	Anterior testis weight(g)	Posterior testis weight (g)	Body factor	GSI	Dilution	Motility %	Density sperm/ml	equilibrium motility %	post-thaw motility %
23	3.8	27.5	5.70	1.769	0.011	0.196	1:2	40	9.13E+08	40	17.92
29	3.9	28	8.11	1.310	0.011	0.243	1:2	47.5	2.26E+09	37.5	23.33
27	5.6	32	7.94	2.850	0.011	0.191	1:2	50	1.93E+09	45	25.83
24	4.2	28	7.46	1.387	0.012	0.213	1:2	45	3.73E+09	45	29.17
20	6.4	32	16.77	1.695	0.012	0.288	1:2	42.5	1.68E+09	42.5	29.50
30	3.8	28	12.13	0.930	0.011	0.340	1:2	67.5	1.15E+09	57.5	30.83
22	4.3	28.5	8.16	1.533	0.011	0.226	1:2	55	1.72E+09	57.5	34.00
25	3.76	29	5.29	2.416	0.009	0.205	1:2	52.5	3.10E+09	42.5	39.17
28	7.0	34	5.20	3.000	0.011	0.117	1:2	55	2.42E+09	45	39.17
26	3.7	27.5	12.50	1.540	0.011	0.377	1:2	62.5	2.15E+09	45	41.67

Table F.6. Genetic influence between blue catfish and channel catfish.

Female	Male	Sperm /monolayer	post-thaw motility %	Neurulation %				Embryo %	
B12	bc35	1.00E+08	30	45	56	52	92	85	76
B24	bc35	1.00E+08	30	5	6	3	0	1	-
B65	bc35	1.00E+08	30	16	16	11	-	42	16
B12	bc36	1.00E+08	27.5	-	21	45	-	19	73
B24	bc36	1.00E+08	27.5	4	11	4	1	2	2
B65	bc36	1.00E+08	27.5	20	13	28	65	57	52
B12	bc38	1.00E+08	27.5	-	45	45	-	77	69
B24	bc38	1.00E+08	27.5	5	1	2	3	0	0
B65	bc38	1.00E+08	27.5	13	18	11	45	53	5
B12	bc40©	1.00E+08	50	42	50	-	126	122	99
B24	bc40©	1.00E+08	50	15	6	13	10	3	8
B65	bc40©	1.00E+08	50	24	20	22	111	99	92
B12	cc5	1.00E+08	25	30	66	60	96	82	53
B24	cc5	1.00E+08	25	3	3	4	0	2	0
B65	cc5	1.00E+08	25	24	14	17	28	60	-
B12	cc5©	1.00E+08	50	65	67	-	125	119	63
B24	cc5©	1.00E+08	50	16	11	10	1	13	7
B65	cc5©	1.00E+08	50	20	19	16	52	55	71
B12	cc6	1.00E+08	10	59	45	62	112	-	
B24	cc6	1.00E+08	10	3	2	1	0	0	1
B65	cc6	1.00E+08	10	25	14	16	25	39	50
B12	cc6&7©	1.00E+08	7.5	48	60	74	107	108	141
B24	cc6&7©	1.00E+08	7.5	3	8	11	4	9	6
B65	cc6&7©	1.00E+08	7.5	17	19	25	43	79	50
B12	cc7	1.00E+08	1	38	38	41	65	52	84
B24	cc7	1.00E+08	1	1	3	4	-	-	3
B65	cc7	1.00E+08	1	10	9	8	-	30	22

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Table F.7. Neurulation data of treatments with 4 ml thawed sperm and 2 ml thawed sperm

	ID	%	ID	%	ID	%	ID	%
Control	12	23.68	4	58.54	10	69.31	9	76.92
	16	27.78	2	61.22	5	69.77	28	78.05
	33	36.84	9	62.50	8	70.94	11	78.49
	17	38.24	27	63.10	42	72.84	15	79.69
	27	43.48	14	63.49	7	73.08	-	81.33
	13	44.07	2	64.58	-	74.32	7	82.35
	37	46.32	7	67.53	-	74.51	6	85.00
	21	50.00	13	67.53	-	74.55	26	85.45
	24	50.98	41	68.12	2	74.71	19	85.48
	46	55.56	23	68.42	2	76.06	16	86.75
	6	57.32	31	69.23	14	76.12	19	90.63
	24	90.22						
2 ml thawed sperm	33	32.18	42	61.70	14	68.85	4	80.00
	2	34.33	23	61.90	18	70.00	6	80.00
	3	46.00	6	62.50	13	70.97	12	80.56
	27	48.31	6	65.15	14	71.13	2	84.21
	37	53.10	-	65.96	28	74.36	19	77.91
	41	59.26	19	66.18	16	77.78	24	67.00
	2	60.00						
4 ml thawed sperm	12	12.77	27	55.07	42	61.80	2	71.54
	24	39.06	13	55.26	9	62.11	6	74.63
	21	39.78	31	57.41	24	63.41	19	75.00
	17	41.10	14	58.93	46	64.65	27	77.78
	13	46.77	6	60.00	7	64.77	28	80.88
	37	48.94	14	60.00	15	68.52	2	81.25
	5	51.16	26	60.19	23	70.13	19	82.46
	33	52.56	10	61.76	41	71.43	16	88.06

Table F.8. Large scale application neurulation.

Date	Date ID	Treatment	Percentage
5/24/2010	d1	Cryo	33.9
5/24/2010	d1	Cryo	28.6
5/24/2010	d1	Cryo	32.1
5/24/2010	d1	Cryo	48.2
5/24/2010	d1	Cryo	35.7
5/24/2010	d1	Cryo	41.1
5/24/2010	d1	Cryo	35.7
5/24/2010	d1	Cryo	46.4
5/24/2010	d1	Cryo	28.6
5/24/2010	d1	Cryo	55.4
5/24/2010	d1	Cryo	37.5
5/24/2010	d1	Cryo	44.6
5/24/2010	d1	Cryo	33.9
5/24/2010	d1	Cryo	58.9
5/24/2010	d1	Cryo	30.4
5/24/2010	d1	Cryo	37.5
5/24/2010	d1	Cryo	37.5
5/24/2010	d1	Cryo	41.1
5/24/2010	d1	Cryo	66.1
5/24/2010	d1	Cryo	37.5
5/24/2010	d1	Cryo	60.7
5/24/2010	d1	Cryo	50.0
5/24/2010	d1	Cryo	35.7
5/24/2010	d1	Cryo	73.2
5/24/2010	d1	Fresh	35.7
5/24/2010	d1	Fresh	55.4
5/24/2010	d1	Fresh	32.1
5/24/2010	d1	Fresh	44.6
5/24/2010	d1	Fresh	42.9
5/24/2010	d1	Fresh	51.8
5/24/2010	d1	Fresh	62.5
5/24/2010	d1	Fresh	44.6
5/24/2010	d1	Fresh	76.8
5/24/2010	d1	Fresh	32.1
5/24/2010	d1	Fresh	55.4
5/24/2010	d1	Fresh	50.0
5/24/2010	d1	Fresh	46.4
5/24/2010	d1	Fresh	32.1
5/24/2010	d1	Fresh	53.6
5/24/2010	d1	Fresh	17.9

(table cont.)

Date	Date ID	Treatment	Percentage
5/24/2010	d1	Fresh	21.4
5/24/2010	d1	Fresh	10.7
5/24/2010	d1	Fresh	82.1
5/24/2010	d1	Fresh	76.8
5/24/2010	d1	Fresh	71.4
5/24/2010	d1	Fresh	48.2
5/24/2010	d1	Fresh	67.9
5/24/2010	d1	Fresh	50.0
5/25/2010	d2	Cryo	42.3
5/25/2010	d2	Cryo	44.2
5/25/2010	d2	Cryo	67.3
5/25/2010	d2	Cryo	28.8
5/25/2010	d2	Cryo	23.1
5/25/2010	d2	Cryo	30.8
5/25/2010	d2	Cryo	44.2
5/25/2010	d2	Cryo	25.0
5/25/2010	d2	Cryo	59.6
5/25/2010	d2	Cryo	23.1
5/25/2010	d2	Cryo	7.7
5/25/2010	d2	Cryo	9.6
5/25/2010	d2	Cryo	30.8
5/25/2010	d2	Cryo	9.6
5/25/2010	d2	Cryo	51.9
5/25/2010	d2	Cryo	65.4
5/25/2010	d2	Cryo	17.9
5/25/2010	d2	Cryo	50.0
5/25/2010	d2	Cryo	66.1
5/25/2010	d2	Cryo	23.2
5/25/2010	d2	Cryo	35.7
5/25/2010	d2	Cryo	33.9
5/25/2010	d2	Cryo	35.7
5/25/2010	d2	Cryo	14.3
5/25/2010	d2	Fresh	38.5
5/25/2010	d2	Fresh	46.2
5/25/2010	d2	Fresh	46.2
5/25/2010	d2	Fresh	51.9
5/25/2010	d2	Fresh	46.2
5/25/2010	d2	Fresh	42.3
5/25/2010	d2	Fresh	48.1
5/25/2010	d2	Fresh	26.9

(table cont.)

Date	Date ID	Treatment	Percentage
5/25/2010	d2	Fresh	63.5
5/25/2010	d2	Fresh	23.1
5/25/2010	d2	Fresh	36.5
5/25/2010	d2	Fresh	25.0
5/25/2010	d2	Fresh	36.5
5/25/2010	d2	Fresh	26.9
5/25/2010	d2	Fresh	44.2
5/25/2010	d2	Fresh	48.1
5/25/2010	d2	Fresh	32.1
5/25/2010	d2	Fresh	66.1
5/25/2010	d2	Fresh	57.1
5/25/2010	d2	Fresh	75.0
5/25/2010	d2	Fresh	57.1
5/25/2010	d2	Fresh	51.8
5/25/2010	d2	Fresh	53.6
5/25/2010	d2	Fresh	53.6
5/26/2010	d3	Cryo	63.5
5/26/2010	d3	Cryo	50.0
5/26/2010	d3	Cryo	36.5
5/26/2010	d3	Cryo	42.3
5/26/2010	d3	Cryo	46.2
5/26/2010	d3	Cryo	48.1
5/26/2010	d3	Cryo	36.5
5/26/2010	d3	Cryo	53.8
5/26/2010	d3	Cryo	25.0
5/26/2010	d3	Cryo	32.7
5/26/2010	d3	Cryo	73.1
5/26/2010	d3	Cryo	55.8
5/26/2010	d3	Cryo	51.9
5/26/2010	d3	Cryo	57.7
5/26/2010	d3	Cryo	44.2
5/26/2010	d3	Cryo	38.5
5/26/2010	d3	Cryo	61.5
5/26/2010	d3	Cryo	69.2
5/26/2010	d3	Cryo	55.8
5/26/2010	d3	Cryo	34.6
5/26/2010	d3	Cryo	40.4
5/26/2010	d3	Cryo	75.0
5/26/2010	d3	Cryo	50.0
5/26/2010	d3	Cryo	40.4

(table cont.)

Date	Date ID	Treatment	Percentage
5/26/2010	d3	Fresh	48.1
5/26/2010	d3	Fresh	53.8
5/26/2010	d3	Fresh	48.1
5/26/2010	d3	Fresh	42.3
5/26/2010	d3	Fresh	46.2
5/26/2010	d3	Fresh	46.2
5/26/2010	d3	Fresh	55.8
5/26/2010	d3	Fresh	50.0
5/26/2010	d3	Fresh	34.6
5/26/2010	d3	Fresh	65.4
5/26/2010	d3	Fresh	44.2
5/26/2010	d3	Fresh	65.4
5/26/2010	d3	Fresh	53.8
5/26/2010	d3	Fresh	55.8
5/26/2010	d3	Fresh	71.2
5/26/2010	d3	Fresh	55.8
5/26/2010	d3	Fresh	59.6
5/26/2010	d3	Fresh	65.4
5/26/2010	d3	Fresh	42.3
5/26/2010	d3	Fresh	65.4
5/26/2010	d3	Fresh	50.0
5/26/2010	d3	Fresh	63.5
5/26/2010	d3	Fresh	67.3
5/26/2010	d3	Fresh	42.3
5/27/2010	d4	Cryo	69.6
5/27/2010	d4	Cryo	42.9
5/27/2010	d4	Cryo	42.9
5/27/2010	d4	Cryo	42.9
5/27/2010	d4	Cryo	57.1
5/27/2010	d4	Cryo	64.3
5/27/2010	d4	Cryo	67.9
5/27/2010	d4	Cryo	41.1
5/27/2010	d4	Cryo	39.3
5/27/2010	d4	Cryo	60.7
5/27/2010	d4	Cryo	44.6
5/27/2010	d4	Cryo	51.8
5/27/2010	d4	Cryo	57.1
5/27/2010	d4	Cryo	35.7
5/27/2010	d4	Cryo	33.9
5/27/2010	d4	Cryo	55.4

(table cont.)

Date	Date ID	Treatment	Percentage
5/27/2010	d4	Cryo	67.9
5/27/2010	d4	Cryo	46.4
5/27/2010	d4	Cryo	48.2
5/27/2010	d4	Cryo	44.6
5/27/2010	d4	Cryo	69.6
5/27/2010	d4	Cryo	53.6
5/27/2010	d4	Cryo	71.4
5/27/2010	d4	Cryo	71.4
5/27/2010	d4	Fresh	51.7
5/27/2010	d4	Fresh	51.7
5/27/2010	d4	Fresh	50.0
5/27/2010	d4	Fresh	65.0
5/27/2010	d4	Fresh	53.3
5/27/2010	d4	Fresh	63.3
5/27/2010	d4	Fresh	56.7
5/27/2010	d4	Fresh	55.0
5/27/2010	d4	Fresh	33.3
5/27/2010	d4	Fresh	46.7
5/27/2010	d4	Fresh	56.7
5/27/2010	d4	Fresh	51.7
5/27/2010	d4	Fresh	43.3
5/27/2010	d4	Fresh	70.0
5/27/2010	d4	Fresh	36.7
5/27/2010	d4	Fresh	25.0
5/27/2010	d4	Fresh	61.7
5/27/2010	d4	Fresh	45.0
5/27/2010	d4	Fresh	76.7
5/27/2010	d4	Fresh	75.0
5/27/2010	d4	Fresh	46.7
5/27/2010	d4	Fresh	78.3
5/27/2010	d4	Fresh	53.3
5/27/2010	d4	Fresh	80.0
6/1/2011	d5	Cryo	63.3
6/1/2011	d5	Cryo	3.3
6/1/2011	d5	Cryo	63.3
6/1/2011	d5	Cryo	40.0
6/1/2011	d5	Cryo	60.0
6/1/2011	d5	Cryo	40.0
6/1/2011	d5	Cryo	50.0
6/1/2011	d5	Cryo	36.7

(table cont.)

Date	Date ID	Treatment	Percentage
6/1/2011	d5	Cryo	66.7
6/1/2011	d5	Cryo	53.3
6/1/2011	d5	Cryo	46.7
6/1/2011	d5	Cryo	76.7
6/1/2011	d5	Cryo	50.0
6/1/2011	d5	Cryo	33.3
6/1/2011	d5	Cryo	50.0
6/1/2011	d5	Cryo	86.7
6/1/2011	d5	Cryo	83.3
6/1/2011	d5	Cryo	80.0
6/1/2011	d5	Cryo	43.3
6/1/2011	d5	Cryo	83.3
6/1/2011	d5	Cryo	50.0
6/1/2011	d5	Cryo	40.0
6/1/2011	d5	Cryo	46.7
6/1/2011	d5	Cryo	80.0
6/1/2011	d5	Cryo	63.3
6/1/2011	d5	Cryo	53.3
6/1/2011	d5	Cryo	96.7
6/1/2011	d5	Cryo	66.7
6/1/2011	d5	Cryo	70.0
6/1/2011	d5	Cryo	90.0
6/1/2011	d5	Fresh	83.3
6/1/2011	d5	Fresh	73.3
6/1/2011	d5	Fresh	70.0
6/1/2011	d5	Fresh	50.0
6/1/2011	d5	Fresh	100.0
6/1/2011	d5	Fresh	63.3
6/1/2011	d5	Fresh	73.3
6/1/2011	d5	Fresh	76.7
6/1/2011	d5	Fresh	63.3
6/1/2011	d5	Fresh	76.7
6/1/2011	d5	Fresh	53.3
6/1/2011	d5	Fresh	66.7
6/1/2011	d5	Fresh	93.3
6/1/2011	d5	Fresh	80.0
6/1/2011	d5	Fresh	80.0
6/1/2011	d5	Fresh	90.0
6/1/2011	d5	Fresh	76.7
6/1/2011	d5	Fresh	70.0

(table cont.)

Date	Date ID	Treatment	Percentage
6/1/2011	d5	Fresh	70.0
6/1/2011	d5	Fresh	46.7
6/1/2011	d5	Fresh	50.0
6/1/2011	d5	Fresh	76.7
6/1/2011	d5	Fresh	63.3
6/1/2011	d5	Fresh	96.7
6/1/2011	d5	Fresh	76.7
6/1/2011	d5	Fresh	60.0
6/1/2011	d5	Fresh	96.7
6/1/2011	d5	Fresh	43.3
6/1/2011	d5	Fresh	80.0
6/1/2011	d5	Fresh	76.7
6/2/2011	d6	Cryo	80.0
6/2/2011	d6	Cryo	73.3
6/2/2011	d6	Cryo	46.7
6/2/2011	d6	Cryo	66.7
6/2/2011	d6	Cryo	50.0
6/2/2011	d6	Cryo	73.3
6/2/2011	d6	Cryo	70.0
6/2/2011	d6	Cryo	43.3
6/2/2011	d6	Cryo	43.3
6/2/2011	d6	Cryo	83.3
6/2/2011	d6	Cryo	60.0
6/2/2011	d6	Cryo	70.0
6/2/2011	d6	Cryo	83.3
6/2/2011	d6	Cryo	93.3
6/2/2011	d6	Cryo	90.0
6/2/2011	d6	Cryo	83.3
6/2/2011	d6	Cryo	83.3
6/2/2011	d6	Cryo	56.7
6/2/2011	d6	Cryo	90.0
6/2/2011	d6	Cryo	90.0
6/2/2011	d6	Cryo	63.3
6/2/2011	d6	Cryo	83.3
6/2/2011	d6	Cryo	93.3
6/2/2011	d6	Cryo	56.7
6/2/2011	d6	Cryo	50.0
6/2/2011	d6	Fresh	56.7
6/2/2011	d6	Fresh	86.7
6/2/2011	d6	Fresh	93.3

(table cont.)

Date	Date ID	Treatment	Percentage
6/2/2011	d6	Fresh	86.7
6/2/2011	d6	Fresh	90.0
6/2/2011	d6	Fresh	66.7
6/2/2011	d6	Fresh	83.3
6/2/2011	d6	Fresh	86.7
6/2/2011	d6	Fresh	86.7
6/2/2011	d6	Fresh	90.0
6/2/2011	d6	Fresh	93.3
6/2/2011	d6	Fresh	86.7
6/2/2011	d6	Fresh	100.0
6/2/2011	d6	Fresh	83.3
6/2/2011	d6	Fresh	60.0
6/2/2011	d6	Fresh	56.7
6/2/2011	d6	Fresh	40.0
6/2/2011	d6	Fresh	73.3
6/2/2011	d6	Fresh	76.7
6/2/2011	d6	Fresh	56.7
6/2/2011	d6	Fresh	70.0
6/2/2011	d6	Fresh	40.0
6/2/2011	d6	Fresh	86.7
6/2/2011	d6	Fresh	63.3
6/2/2011	d6	Fresh	86.7
6/12/2011	d7	Cryo	56.7
6/12/2011	d7	Cryo	63.3
6/12/2011	d7	Cryo	73.3
6/12/2011	d7	Cryo	66.7
6/12/2011	d7	Cryo	46.7
6/12/2011	d7	Cryo	60.0
6/12/2011	d7	Cryo	70.0
6/12/2011	d7	Cryo	56.7
6/12/2011	d7	Cryo	46.7
6/12/2011	d7	Cryo	73.3
6/12/2011	d7	Cryo	36.7
6/12/2011	d7	Cryo	63.3
6/12/2011	d7	Cryo	23.3
6/12/2011	d7	Cryo	33.3
6/12/2011	d7	Cryo	73.3
6/12/2011	d7	Cryo	63.3
6/12/2011	d7	Cryo	83.3
6/12/2011	d7	Cryo	70.0

(table cont.)

Date	Date ID	Treatment	Percentage
6/12/2011	d7	Cryo	66.7
6/12/2011	d7	Cryo	56.7
6/12/2011	d7	Cryo	60.0
6/12/2011	d7	Cryo	70.0
6/12/2011	d7	Cryo	53.3
6/12/2011	d7	Cryo	46.7
6/12/2011	d7	Cryo	43.3
6/12/2011	d7	Cryo	60.0
6/12/2011	d7	Cryo	23.3
6/12/2011	d7	Cryo	76.7
6/12/2011	d7	Cryo	53.3
6/12/2011	d7	Cryo	30.0
6/12/2011	d7	Fresh	53.3
6/12/2011	d7	Fresh	56.7
6/12/2011	d7	Fresh	66.7
6/12/2011	d7	Fresh	63.3
6/12/2011	d7	Fresh	40.0
6/12/2011	d7	Fresh	60.0
6/12/2011	d7	Fresh	66.7
6/12/2011	d7	Fresh	96.7
6/12/2011	d7	Fresh	73.3
6/12/2011	d7	Fresh	66.7
6/12/2011	d7	Fresh	90.0
6/12/2011	d7	Fresh	66.7
6/12/2011	d7	Fresh	13.3
6/12/2011	d7	Fresh	20.0
6/12/2011	d7	Fresh	23.3
6/12/2011	d7	Fresh	70.0
6/12/2011	d7	Fresh	43.3
6/12/2011	d7	Fresh	43.3
6/12/2011	d7	Fresh	83.3
6/12/2011	d7	Fresh	90.0
6/12/2011	d7	Fresh	70.0
6/12/2011	d7	Fresh	26.7
6/12/2011	d7	Fresh	83.3
6/12/2011	d7	Fresh	53.3
6/12/2011	d7	Fresh	56.7
6/12/2011	d7	Fresh	83.3
6/12/2011	d7	Fresh	66.7
6/12/2011	d7	Fresh	53.3

(table cont.)

Date	Date ID	Treatment	Percentage
6/12/2011	d7	Fresh	93.3
6/12/2011	d7	Fresh	93.3

Table F.9. Large scale application in daily swim-up fry production.

	Thawed sperm					Fresh sperm			
	Fry	egg volume (L)	Female number	sperm volume ml	Fry /1E9 sperm	Fry	egg volume (L)	sperm volume ml	Fry /1E9 sperm
5/23/2010	156000	9.55	62	127	965	216000	10.65	142	1757
5/24/2010	102222	9.1	64	121	663	216000	9.1	121	2056
5/25/2010	258000	12.55	70	167	1214	360000	16.15	215	1931
5/26/2010	156000	8.2	46	109	1123	312000	18.85	251	1433
5/31/2011	168000	5.1	29	68	1945	156000	5.1	68	2649
6/1/2011	96000	2.55	18	34	2223	108000	2.55	34	3668
6/12/2011	108000	3.8	27	51	1678	108000	3.8	51	2461

Table F.10. Individual male information for predict pooled sample characteristics.

Date	Male ID	Straw number	Initial motility (%)	Post-thaw motility (%)	Post-thaw membrane integrity (%)	Normalized neurulation
31-May	5	60	57.5	40	0.281807	1.01701
31-May	10	84	72.5	45	0.212792	0.879479
31-May	4	37	52.5	35	0.281189	0.914767
31-May	3	48	62.5	22.5	0.337755	1.013844
31-May	2	17	57.5	7.5	0.367431	0.944625
31-May	6	12	65	45	0.208734	1.047774
1-Jun	6	98	65	45	0.208734	1.047774
1-Jun	5	9	57.5	40	0.281807	1.01701
1-Jun	7	16	52.5	35	0.310453	0.941911
1-Jun	13	33	57.5	42.5	0.260679	1.053203
1-Jun	12	61	65	42.5	0.303999	1.245024
1-Jun	10	57	72.5	45	0.212792	0.879479
1-Jun	8	42	52.5	22.5	0.286257	1.036012
1-Jun	11	14	52.5	42.5	0.239488	0.803474
1-Jun	13	12	57.5	42.5	0.260679	1.053203
12-Jun	44	38	50	42.5	0.273949	0.811618
12-Jun	38	50	52.5	7.5	0.416877	1.031488
12-Jun	40	36	52.5	10	0.336597	1.028773
12-Jun	25	8	35	1.5	0.644013	1.278502
12-Jun	29	25	50	32.5	0.383271	1.039631
12-Jun	26	14	55	30	0.360091	0.877669
12-Jun	15	3	45	0.5	0.508483	1.001629
23-May	2	92	65	22.5	0.228659	0.327684
23-May	6	29	55	25	0.22293	0.319474
23-May	9	7	42.5	0	0.212766	0.304909
23-May	10	62	42.5	0.5	0.34947	0.500816
23-May	11	53	62.5	7.5	0.250097	0.358407
23-May	18	37	52.5	35	0.239836	0.343702
23-May	23	57	67.5	22.5	0.135818	0.194636
23-May	25	1	57.5	12.5	0.196429	0.281496
23-May	22	1	67.5	12.5	0.23985	0.343723
24-May	1	27	65	12.5	0.204678	0.293319
24-May	5	36	45	7.5	0.281022	0.402724
24-May	7	46	52.5	0	0.335802	0.481229
24-May	11	144	62.5	7.5	0.250097	0.358407
24-May	22	12	67.5	12.5	0.23985	0.343723
24-May	19	84	47.5	25	0.194638	0.278931
24-May	25	12	57.5	12.5	0.196429	0.281496
24-May	17	39	45	12.5	0.175214	0.251094
24-May	24	78	42.5	4	0.229167	0.328412
25-May	48	144	70	*45*	0.182692	*0.543*
25-May	22	144	67.5	12.5	0.23985	0.343723

(table cont.)

Date	Male ID	Straw number	Initial motility (%)	Post-thaw motility (%)	Post-thaw membrane integrity (%)	Normalized neurulation
25-May	1	11	65	12.5	0.204678	0.293319
25-May	5	14	45	7.5	0.281022	0.402724
25-May	7	18	52.5	0	0.335802	0.481229
25-May	11	57	62.5	7.5	0.250097	0.358407
25-May	22	5	67.5	12.5	0.23985	0.343723
25-May	19	33	47.5	25	0.194638	0.278931
25-May	25	5	57.5	12.5	0.196429	0.281496
25-May	17	15	45	12.5	0.175214	0.251094
25-May	24	31	42.5	4	0.229167	0.328412

Table F.11. 2010-2011 process data: concentration and motility.

ID	Date	Dilution	Concentration (sperm/ml)	Initial motility (%)	Equilibration motility (%)	Post-thaw motility (%)
1	4/14/2010	0.50	3.16E+09	65	45	12.5
2	4/14/2010	0.50	2.64E+09	65	57.5	22.5
3	4/14/2010	0.50	1.61E+09	35		
4	4/14/2010	0.50	1.15E+09	30		
5	4/14/2010	0.50	2.29E+09	45	45	7.5
6	4/14/2010	0.50	2.78E+09	55	47.5	25
7	4/15/2010	0.50	1.28E+09	52.5	52.5	0
8	4/15/2010	0.50	8.05E+08	32.5		
9	4/15/2010	0.50	1.26E+09	42.5	45	0
10	4/20/2010	0.50	1.03E+09	42.5	45	0.5
11	4/20/2010	0.50	1.74E+09	62.5	62.5	7.5
12	4/20/2010	0.25	3.58E+08	42.5		
13	4/22/2010	1.00	4.47E+09	35		
14	4/22/2010	1.00	2.51E+09	45	37.5	27.5
15	4/22/2010	1.00	4.13E+09	65	52.5	30
16	4/22/2010	1.00	2.63E+09	70	62.5	50
17	4/27/2010	1.00	1.58E+08	45	37.5	12.5
18	4/27/2010	1.00	8.30E+07	52.5	55	35
19	4/27/2010	1.00	2.36E+08	47.5	52.5	25
20	4/27/2010	1.00	1.26E+08	55	52.5	
21	4/27/2010	1.00	1.83E+08	55	42.5	
22	4/27/2010	1.00	1.44E+08	67.5	45	12.5
23	4/27/2010	1.00	3.99E+08	67.5	57.5	22.5
24	4/27/2010	1.00	2.10E+08	42.5	37.5	4
25	4/27/2010	1.00	1.02E+08	57.5	42.5	12.5
26	4/29/2010	1.00	1.09E+08	55	35	27.5
27	4/29/2010	1.00	2.17E+08	65	65	37.5
28	4/29/2010	1.00	3.64E+08	55	60	37.5
29	4/29/2010	1.00	1.79E+08	55	45	35
30	4/29/2010	1.00	1.93E+08	65	65	35
31	4/29/2010	1.00	1.35E+08	55	40	7.5
32	4/29/2010	1.00	1.60E+08	50	30	27.5
33	4/29/2010	1.00	2.22E+08	55	35	12.5
34	4/29/2010	1.00	1.64E+08	42.5	30	10
35	4/29/2010	1.00	2.13E+08	55	55	25
36	4/29/2010	1.00	2.34E+08	60	55	32.5
37(28C5)	4/29/2010	1.00	1.95E+08	55	40	35
41	5/14/2010	1.00	3.48E+09	62.5	42.5	
43	5/14/2010	1.00	2.78E+09	62.5	52.5	
44	5/14/2010	1.00	1.22E+09	52.5	45	

(table cont.)

ID	Date	Dilution	Concentration (sperm/ml)	Initial motility (%)	Equilibration motility (%)	Post-thaw motility (%)
45	5/19/2010	1.00	2.36E+09			
46	5/19/2010	1.00	7.06E+09	67.5		
47	5/19/2010	1.00	2.19E+09	55		
48	5/19/2010	1.00	1.15E+09	70	62.5	
49	5/19/2010	1.00	8.76E+09			
50	5/19/2010	1.00	1.45E+09			
52	5/19/2010	1.00	4.10E+09			
53	5/19/2010	1.00	2.80E+09			
54	5/19/2010	1.00	2.46E+09	67.5		
55	5/19/2010	1.00	4.67E+09	65		
1	4/29/2011	1.00	6.42E+08	57.5		
2	4/29/2011	1.00	1.45E+09	57.5	32.5	7.5
3	4/29/2011	1.00	1.66E+09	62.5	42.5	22.5
4	4/29/2011	1.00	1.96E+09	52.5	52.5	35
5	4/29/2011	1.00	2.53E+09	57.5	45	40
6	4/29/2011	1.00	3.53E+09	65	55	45
7	4/29/2011	1.00	2.13E+09	52.5	60	35
8	4/29/2011	1.00	2.38E+09	52.5	42.5	22.5
10	4/29/2011	1.00	3.32E+09	72.5	65	45
11	4/29/2011	1.00	2.85E+09	52.5	42.5	42.5
12	4/29/2011	1.00	2.33E+09	65	52.5	42.5
13	4/29/2011	1.00	2.97E+09	57.5	55	42.5
14	5/13/2011	1.00	1.82E+09	65	55	30
15	5/13/2011	1.00	1.52E+09	45	45	0.5
16	5/13/2011	1.00	6.84E+08	40		
17	5/13/2011	1.00	4.26E+08	40		
18	5/13/2011	1.00	7.19E+08	65	37.5	
19	5/13/2011	1.00	3.20E+09	50	45	7.5
20	5/13/2011	1.00	9.98E+08	60	35	7.5
21	5/13/2011	1.00	1.66E+09	40	45	12.5
22	5/13/2011	1.00	2.79E+09	40	45	37.5
23	5/13/2011	1.00	1.91E+09	55	45	
25	5/13/2011	1.00	8.76E+08	35	32.5	1.5
26	5/13/2011	1.00	9.00E+08	55	50	30
27	5/13/2011	1.00	3.27E+09	40	40	50
28	5/13/2011	1.00	1.49E+09	60	42.5	0.5
29	5/13/2011	1.00	1.91E+09	50	42.5	32.5
30	5/16/2011	1.00	1.91E+09	50	42.5	0.5
31	5/16/2011	1.00	1.49E+09	40	37.5	
32	5/16/2011	1.00	1.60E+09	45	52.5	42.5
33	5/16/2011	1.00	4.35E+09	35	35	7.5
34	5/16/2011	1.00	2.42E+09	40	40	10

(table cont.)

ID	Date	Dilution	Concentration (sperm/ml)	Initial motility (%)	Equilibration motility (%)	Post-thaw motility (%)
35	5/16/2011	1.00	3.72E+09	40	35	0.5
36	5/16/2011	1.00				
37	5/16/2011	1.00	1.08E+09	45	45	22.5
38	5/16/2011	1.00	2.93E+09	52.5	40	7.5
39	5/16/2011	1.00	2.76E+09	37.5	45	10
40	5/16/2011	1.00	2.60E+09	52.5	42.5	10
41	5/16/2011	1.00	2.54E+09	22.5		
42	5/16/2011	1.00				
43	5/16/2011	1.00	2.78E+09	42.5	52.5	15
44	5/16/2011	1.00	2.35E+09	50	52.5	42.5
45	5/16/2011	1.00	2.14E+09	42.5	50	27.5
46	5/16/2011	1.00	3.24E+09	40	40	0.5
47	5/16/2011	1.00	4.00E+08	42.5		
48	5/16/2011	1.00	3.04E+09	42.5	42.5	42.5
49	5/19/2011	1.00	3.05E+09	40	37.5	7.5
50	5/19/2011	1.00	2.29E+09	52.5	40	40
51	5/19/2011	1.00	3.27E+09	62.5	60	37.5
52	5/19/2011	1.00	2.56E+09	55	50	35
53	5/19/2011	1.00	2.14E+09	45	37.5	12.5
54	5/19/2011	1.00	2.72E+09	40	37.5	12.5
55	5/19/2011	1.00	2.95E+09	62.5	55	45
56	5/19/2011	1.00	3.25E+09	42.5	45	15
57	5/19/2011	1.00	1.71E+09	62.5	55	50
58	5/19/2011	1.00	2.64E+09	52.5	40	32.5
59	5/19/2011	1.00	3.27E+09	62.5	52.5	12.5

Table F.12. 2010-2011 process data: flow cytometer analysis during processing.

		Pure		Crushed		Equilibrium	
ID	Date	Membrane integrity	Concentration	Membrane integrity	Concentration	Membrane integrity	Concentration
1	4/14/2010	0.90996	1.65E+06	0.850974	1.45E+07	0.606059	6.02E+06
2	4/14/2010	0.91313	2.55E+06	0.874219	1.35E+07	0.68839	8.22E+06
3	4/14/2010	0.902363	1.12E+06	0.872508	6.49E+06		
4	4/14/2010	0.851809	8.06E+05	0.861451	3.23E+06		
5	4/14/2010	0.904887	1.26E+06	0.860264	1.47E+07	0.500194	6.77E+06
6	4/14/2010	0.911843	1.35E+06	0.914437	8.95E+06	0.65739	4.03E+06
7	4/15/2010					0.41632	9.48E+06
8	4/15/2010					0.492	8.03E+06
9	4/15/2010					0.498	6.64E+06
14	4/22/2010			0.795116	8.07E+06	0.792449	6.60E+06
15	4/22/2010			0.833101	6.24E+06	0.827928	5.45E+06
16	4/22/2010			0.760634	7.87E+06	0.75437	6.17E+06
17	4/27/2010	0.77069	8.18E+06	0.869143	3.85E+06	0.860154	3.57E+06
18	4/27/2010	0.86006	7.21E+06	0.832415	9.56E+06	0.832364	7.29E+06
19	4/27/2010	0.865482	8.12E+06	0.845888	2.66E+06	0.846085	2.30E+06
20	4/27/2010	0.820289	1.38E+07	0.855571	6.96E+06	0.856341	5.92E+06
21	4/27/2010	0.837092	1.08E+07	0.864814	5.58E+06	0.861307	5.05E+06
22	4/27/2010	0.867481	9.87E+06	0.867861	7.17E+06	0.86808	5.82E+06
23	4/27/2010	0.920089	1.24E+07	0.892309	3.73E+06	0.89621	2.74E+06
24	4/27/2010	0.831801	7.14E+06	0.798506	2.29E+06	0.776903	1.99E+06
25	4/27/2010	0.825503	1.35E+07	0.855516	7.14E+06	0.844059	6.10E+06
26	4/29/2010	0.843165	1.21E+07	0.8758	7.06E+06	0.875091	5.10E+06
27	4/29/2010	0.913515	1.08E+07	0.861324	4.38E+06	0.859834	4.10E+06
28	4/29/2010	0.820783	1.20E+07	0.842109	9.61E+06	0.903437	7.22E+06
29	4/29/2010	0.832844	1.36E+07	0.890075	9.35E+06	0.886116	7.48E+06
30	4/29/2010	0.913373	1.12E+07	0.855691	1.18E+07	0.869167	8.78E+06

(table cont.)

		Pure		Crushed		Equilibrium	
ID	Date	Membrane integrity	Concentration	Membrane integrity	Concentration	Membrane integrity	Concentration
31	4/29/2010	0.813344	1.07E+07	0.8628	5.77E+06	0.852063	4.85E+06
32	4/29/2010	0.870106	1.34E+07	0.906626	4.94E+06	0.902114	4.18E+06
33	4/29/2010	0.833077	1.40E+07	0.85579	1.13E+07	0.856928	9.32E+06
34	4/29/2010	0.837849	1.29E+07	0.892146	2.57E+06	0.899099	2.46E+06
35	4/29/2010	0.872808	1.34E+07	0.885898	4.80E+06	0.879464	4.42E+06
36	4/29/2010	0.864788	1.42E+07	0.854817	8.78E+06	0.893588	7.28E+06
37(28C5)	4/29/2010	0.886679	1.41E+07	0.899325	4.99E+06	0.902062	4.45E+06
41	5/14/2010	0.86266	1.45E+07	0.833394	1.46E+07	0.814014	1.25E+07
43	5/14/2010	0.872143	1.26E+07	0.815359	1.39E+07	0.806371	1.36E+07
44	5/14/2010	0.626302	1.11E+06	0.736342	1.29E+07	0.746551	1.04E+07
46	5/19/2010	0.834298	1.38E+07	0.886865	5.04E+06	0.889708	5.04E+06
47	5/19/2010	0.835679	1.44E+07	0.878549	1.16E+07	0.866038	1.32E+07
48	5/19/2010	0.792823	1.30E+07	0.906613	2.86E+06	0.895782	3.03E+06
54	5/19/2010	0.765243	1.29E+07	0.894254	1.45E+07	0.879793	1.44E+07
55	5/19/2010	0.833831	1.38E+07	0.900344	8.73E+06	0.898992	8.43E+06

Table F.13. 2010-2011 process data: flow cytometer analysis on thawed samples and fertilization tests.

Male	Date	Membrane integrity	Concentration	Neurulation	Normalized neurulation
1	4/14/2010	0.795322	8507	0.470588235	0.674386481
2	4/14/2010	0.771341	16400	0.583333333	0.835958242
5	4/14/2010	0.718978	13632	0.630769231	0.903937264
6	4/14/2010	0.77707	7850	0.545454545	0.781675239
7	4/15/2010	0.664198	20050	0.433333333	0.620997551
9	4/15/2010	0.787234	9400	0.447761194	0.641673704
10	4/20/2010	0.65053	401881	0.509433962	0.730055176
11	4/20/2010	0.749903	386450	0.536231884	0.768458508
17	4/27/2010	0.824786	11642	0.453125	0.64936042
18	4/27/2010	0.760164	703632	0.391891892	0.561609012
19	4/27/2010	0.805362	135473	0.491803279	0.70478915
22	4/27/2010	0.76015	374400	0.517241379	0.741243762
23	4/27/2010	0.864182	201931	0.293103448	0.420038132
24	4/27/2010	0.770833	4752	0.465517241	0.667119385
25	4/27/2010	0.803571	11144	0.621212121	0.890241245
26	4/29/2010	0.756923	16089	0.634920635	0.909886522
27	4/29/2010	0.79085	7612	0.476190476	0.682414892
28	4/29/2010	0.84	8663	0.369230769	0.529134008
29	4/29/2010	0.798913	9109	0.493333333	0.706981828
30	4/29/2010	0.794118	10200	0.535211268	0.766995892
31	4/29/2010	0.854602	318905	0.333333333	0.477690424
32	4/29/2010	0.889501	280050	0.582089552	0.834175815
33	4/29/2010	0.847222	190800	0.521126761	0.74681179
34	4/29/2010	0.895699	201294	0.52238806	0.748619321
35	4/29/2010	0.843896	200743	0.433333333	0.620997551
36	4/29/2010	0.79052	195224	0.407407407	0.583843852
37(28C5)	4/29/2010	0.908257	10846	0.306451613	0.439167003
41	5/14/2010	0.799065	31782		
43	5/14/2010	0.756438	30861		
44	5/14/2010	0.52381	20792		
48	5/19/2010	0.817308	10348		
2	4/29/2011	0.632569	1245572	0.435	0.944625407
3	4/29/2011	0.662245	986468	0.466875	1.013843648
4	4/29/2011	0.718811	1072178	0.42125	0.914766558
5	4/29/2011	0.718193	977426	0.468333333	1.017010496
6	4/29/2011	0.791266	1050891	0.4825	1.047774159
7	4/29/2011	0.689547	1117178	0.43375	0.941910966
8	4/29/2011	0.713743	812687	0.477083333	1.036011582

(table cont.)

Male	Date	Membrane integrity	Concentration	Neurulation	Normalized neurulation
10	4/29/2011	0.787208	1258557	0.405	0.879478827
11	4/29/2011	0.760512	986634	0.37	0.803474484
12	4/29/2011	0.696001	784752	0.573333333	1.245023525
13	4/29/2011	0.739321	955025	0.485	1.05320304
14	5/13/2011	0.657011	75572	0.51	1.107491857
15	5/13/2011	0.491517	1683284	0.46125	1.001628664
19	5/13/2011	0.752877	873333	0.45375	0.98534202
20	5/13/2011	0.581723	95274	0.43375	0.941910966
21	5/13/2011	0.818086	568856	0.505	1.096634093
22	5/13/2011	0.659315	85721	0.4	0.868621064
25	5/13/2011	0.355987	76866	0.58875	1.278501629
26	5/13/2011	0.639909	2255373	0.404166667	0.8776692
27	5/13/2011	0.83871	81741	0.4675	1.015200869
28	5/13/2011	0.641658	1701592	0.49	1.064060803
29	5/13/2011	0.616729	133831	0.47875	1.039630836
30	5/16/2011	0.721568	1090750	0.48875	1.061346363
32	5/16/2011	0.685942	1141692	0.488	1.059717698
33	5/16/2011	0.638428	1676070	0.5475	1.188925081
34	5/16/2011	0.688955	605700	0.45125	0.979913138
35	5/16/2011	0.728364	795622	0.5425	1.178067318
37	5/16/2011	0.693701	708507	0.47875	1.039630836
38	5/16/2011	0.583123	39502	0.475	1.031487514
39	5/16/2011	0.688733	887562	0.37375	0.811617807
40	5/16/2011	0.663403	1356866	0.47375	1.028773073
43	5/16/2011	0.696158	890100	0.4	0.868621064
44	5/16/2011	0.726051	940547	0.37375	0.811617807
45	5/16/2011	0.65619	930600	0.51275	1.113463626
46	5/16/2011	0.587856	1068069	0.482916667	1.048678972
48	5/16/2011	0.752187	34300	0.54375	1.180781759
49	5/19/2011	0.680704	1069204	0.48875	1.061346363
50	5/19/2011	0.743437	938159	0.50125	1.088490771
51	5/19/2011	0.687057	77264	0.561666667	1.219688744
52	5/19/2011	0.82479	839850		
53	5/19/2011	0.785232	802438	0.47	1.02062975
54	5/19/2011	0.77275	597015	0.47125	1.023344191
55	5/19/2011	0.733242	1306800	0.469583333	1.019724937
56	5/19/2011	0.757953	1326169	0.4575	0.993485342
57	5/19/2011	0.746099	942327	0.46875	1.017915309
58	5/19/2011	0.8	27000	0.41875	0.909337676

(table cont.)

Male	Date	Membrane integrity	Concentration	Neurulation	Normalized neurulation
59	5/19/2011	0.72556	1058700	0.500833333	1.087585957

Table F.14. 2009-2011 process data: material defects and testis weight. “0” means not observed; “1” means observed.

Date	ID	Sick	Female	No testis	Reddish	Anterior testis weight (g)
4/5/2009	1	0	0	0	0	12.04
4/5/2009	2	0	0	0	0	6.35
4/5/2009	3	0	0	0	0	13.33
4/5/2009	4	0	0	0	0	5.04
4/5/2009	5	0	0	0	0	7.56
4/17/2009	6	0	0	0	0	13.57
4/17/2009	7	0	0	0	0	2.68
4/17/2009	8	0	0	0	0	8.16
4/17/2009	9	0	0	0	0	4.54
4/17/2009	10	0	0	0	0	9.99
4/17/2009	11	0	0	0	0	12.23
4/17/2009	12	0	0	0	0	4.84
4/25/2009	13	0	0	0	0	9.79
4/25/2009	14	0	0	0	0	10.39
4/25/2009	15	0	0	0	0	7.29
4/25/2009	16	0	0	0	0	10.91
4/27/2009	17	0	0	0	0	12.69
4/27/2009	18	0	0	0	0	8.75
4/27/2009	19	0	0	0	0	8.18
5/8/2009	20	0	0	0	0	16.769
5/8/2009	21	0	0	0	0	2.93
5/8/2009	22	0	0	0	0	8.16
5/9/2009	23	0	0	0	0	5.696
5/9/2009	24	0	0	0	0	7.4553
5/9/2009	25	0	0	0	0	5.2911
5/9/2009	26	0	0	0	0	12.5
5/9/2009	27	0	0	0	0	7.94
5/9/2009	28	0	0	0	0	5.2
5/9/2009	29	0	0	0	0	8.11
5/9/2009	30	0	0	0	0	12.13
5/12/2009	31	0	0	0	0	6.45
5/20/2009	32	0	0	0	0	4.937
5/20/2009	33	0	0	0	0	6.63
5/20/2009	34	0	0	0	0	6.94
5/21/2009	35	0	0	0	0	7.61
5/21/2009	36	0	0	0	0	6.28
5/21/2009	37	0	0	0	0	6.33
5/21/2009	38	0	0	0	0	8.92
4/14/2010	1	0	0	0	0	4.365
4/14/2010	2	0	0	0	0	23.84
4/14/2010	3	0	0	0	0	5.73
4/14/2010	4	0	0	0	0	9.34

(table cont.)

Date	ID	Sick	Female	No testis	Reddish	Anterior testis weight (g)
4/14/2010	5	0	0	0	0	10.86
4/14/2010	6	0	0	0	0	9.49
4/15/2010	7	0	0	0	1	*3.0001*
4/15/2010	8	0	0	0	0	7.62
4/15/2010	9	0	0	0	0	7.85
4/20/2010	10	0	0	0	0	14.11
4/20/2010	11	0	0	0	0	26.27
4/20/2010	12	0	0	0	0	14.24
4/22/2010	13	0	0	0	0	8.321
4/22/2010	14	0	0	0	0	12.41
4/22/2010	15	0	0	0	0	14.21
4/22/2010	16	0	0	0	0	27.16
4/27/2010	17	0	0	0	0	12.8
4/27/2010	18	0	0	0	0	18.71
4/27/2010	19	0	0	0	0	8.74
4/27/2010	20	0	0	0	0	13.98
4/27/2010	21	0	0	0	0	8.22
4/27/2010	22	0	0	0	0	4.42
4/27/2010	23	0	0	0	0	5.66
4/27/2010	24	0	0	0	0	25.36
4/27/2010	25	0	0	0	0	8.77
4/28/2010	26	0	0	0	0	5.39
4/28/2010	27	0	0	0	0	19.05
4/28/2010	28	0	0	0	0	5.92
4/28/2010	29	0	0	0	0	5.23
4/28/2010	30	0	0	0	0	18.84
4/28/2010	31	0	0	0	0	3.87
4/28/2010	32	0	0	0	0	3.9
4/28/2010	33	0	0	0	0	5.65
4/28/2010	34	0	0	0	1	0
4/28/2010	35	0	0	0	0	5.32
4/28/2010	36	0	0	0	0	5.83
4/28/2010	37	0	0	0	0	3.07
4/28/2010	38	0	0	0	1	0
5/7/2010	39	0	0	0	0	9.44
5/7/2010	40	0	0	0	0	7.32
5/7/2010	41	0	0	0	0	11.18
5/14/2010	42	0	0	0	0	6.9
5/14/2010	43	0	0	0	1	1.735
5/14/2010	44	0	0	0	0	9.54
5/14/2010	45	0	0	0	0	20.33
5/19/2010	46	0	0	0	0	7.4
5/19/2010	47	0	0	0	0	14.87

(table cont.)

Date	ID	Sick	Female		No testis		Reddish		Anterior testis weight (g)
5/19/2010			48	0	0	0	0		16.065
5/19/2010			49	0	0	0	0		16.345
5/19/2010			50	0	0	0	0		10.136
5/19/2010			51	0	0	0	0		10.257
5/19/2010			52	0	0	1	0		0
5/19/2010			53	0	0	0	1		3.168
5/19/2010			54	0	0	0	1		2.623
5/19/2010			55	0	0	0	0		12.5
5/19/2010			56	0	0	0	0		11.63
4/29/2011			1	1	0	0	0		0
4/29/2011			2	0	0	0	0		11.3
4/29/2011			3	0	0	0	0		6.12
4/29/2011			4	1	0	0	0		0
4/29/2011			5	0	0	0	0		9.83
4/29/2011			6	0	0	0	0		10.09
4/29/2011			7	0	0	0	0		9.27
4/29/2011			8	0	0	0	0		13.55
4/29/2011			9	0	0	0	0		5.67
4/29/2011			10	0	0	0	0		7.9
4/29/2011			11	0	0	0	1		1.95
4/29/2011			12	0	0	0	0		18.32
4/29/2011			13	0	0	0	0		3.68
4/29/2011			14	0	0	0	0		12.3
4/29/2011			15	0	0	0	0		8.03
5/13/2011			16	0	0	0	0		8.28
5/13/2011			17	0	0	0	0		3.78
5/13/2011			18	0	0	0	0		10.08
5/13/2011			19	0	0	0	0		10.18
5/13/2011			20	0	0	0	0		4.67
5/13/2011			21	0	0	0	0		18.12
5/13/2011			22	0	0	0	0		14.76
5/13/2011			23	0	0	0	0		12.61
5/13/2011			24	0	0	0	0		5.85
5/13/2011			25	0	0	0	0		14.6
5/13/2011			26	0	0	1	0		0
5/13/2011			27	0	0	0	0		6.17
5/13/2011			28	0	0	0	0		8.13
5/13/2011			29	0	0	0	0		7.67
5/13/2011			30	0	0	0	0		11.72
5/13/2011			31	0	0	0	0		9.1
5/16/2011			32	0	0	0	0		13.73
5/16/2011			33	0	0	0	0		4.97
5/16/2011			34	0	0	0	0		7.71

(table cont.)

Date	ID	Sick	Female	No testis	Reddish			Anterior testis weight (g)
		5/16/2011	35	0	0	0	0	5.89
		5/16/2011	36	0	0	0	0	9.44
		5/16/2011	37	0	0	0	0	3.51
		5/16/2011	38	0	0	0	0	14.41
		5/16/2011	39	0	0	0	0	11.15
		5/16/2011	40	0	0	0	0	5
		5/16/2011	41	0	0	0	0	9.37
		5/16/2011	42	0	0	0	0	3.37
		5/16/2011	43	0	0	0	1	2.23
		5/16/2011	44	0	0	0	0	9.83
		5/16/2011	45	0	0	0	0	7.77
		5/16/2011	46	0	0	0	0	7.52
		5/16/2011	47	0	0	0	0	4.92
		5/16/2011	48	0	0	0	0	4.39
		5/16/2011	49	0	0	0	0	5.33
		5/19/2011	50	0	0	0	0	8.28
		5/19/2011	51	0	0	0	0	6.72
		5/19/2011	52	0	0	0	0	8.01
		5/19/2011	53	0	0	0	0	3.75
		5/19/2011	54	0	0	0	0	3.28
		5/19/2011	55	0	0	0	1	2.35
		5/19/2011	56	0	0	0	0	5.34
		5/19/2011	57	0	0	0	0	5.51
		5/19/2011	58	0	0	0	0	7.36
		5/19/2011	59	0	0	0	0	9.07
		5/19/2011	60	0	0	0	0	7.32

Table F.15. Timing process operations of preparing sperm suspensions.

Male	5	6	7	8	1	2	3	4	5	6	7	8
Dissection	-	4'31"	3'51"	3'59"	10'	5'	10'	6'	6'	6'	6'	6'
Clean	4'48"	6'49"	-	5'9"	8'42"	2'12"	3'	-	4'40"	-	8'	7'20"
Weight testis	1'13"	-	-	-	15'	11'49"	-	-	-	-	7'33"	-
Crush	8'32"	4'04"	5'34"	4'41"								
Filter	-	-	-	54"	-	-	-	-	-	-	-	-
Motility	-	-	-	2'	-	-	-	-	-	-	-	-
Adjust concentration	-	-	-	12'30"	-	-	-	-	-	-	-	-
Count cells	-	-	-		28'40"	26'40"	27'	-	27'	-	27'	-

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Chapter 3

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Vita

E Hu was born in 1984 in Shaoyang City, Hunan Province, China. He spent 6 years in Zijiang River Elementary School and 6 years in No. 2 Middle School. In 2003, he graduated with a high school diploma and enrolled in Zhejiang University, majoring in Labor and Social Security in the College of Economy. In 2004, he changed his major to Biotechnology in the College of Life Sciences. In 2007, he completed the Zhejiang University Student Research Training Program with the research topic "Change of Fatty Acids Composition and Distribution of Germ Cells during Spermatogenesis" supervised by Dr. Wanxi Yang from Spermlab (www.spermlab.org) in the College of Life Sciences. In the same year, E Hu graduated with a Bachelor of Science degree and enrolled as a Master's degree student in the School of Renewable Natural Resources, in the Agricultural Center Aquaculture Research Station of Louisiana State University with Dr. Terrence Tiersch as his supervisor. In 2009, he transferred to the current PhD program while continuing research under Dr. Tiersch. During this time, E Hu twice won the Best Abstract Awards (1st place) at the Annual Meeting of the Louisiana Chapter of the American Fisheries Society (2010 and 2011), and in 2011, he won the United States Aquaculture Society Best Abstract/Travel Award at the Aquaculture America meeting, the Presentation Award of the Fish Culture Section of the American Fisheries Society, and the United States Aquaculture Society, and Aquaculture America 2011 spotlight presentation participation award. He is a member of the Louisiana Chapter of the American Fisheries Society, World Aquaculture Society, Louisiana State University Aquaculture and Fisheries Club, and Honor Society of Phi Kappa Phi. In 2010, E Hu served as the sports coordinator of the Louisiana State University Chinese Scholar and Student Association, and in 2012, he was selected and sponsored to serve in the Farm Animal Integrated Research (FAIR) 2012 conference as graduate

recorder. Currently, E Hu is a PhD candidate for the degree of Doctor of Philosophy in Wildlife and Fisheries Science in December 2012.