New Chapter

Development of High-throughput Cryopreservation for Aquatic Species

E Hu and Terrence R. Tiersch

Introduction

High-throughput cryopreservation has been widely applied in livestock industries such as dairy for decades (Pickett and Berndtson 1974), and cryopreserved germplasm constitutes an independent industry encompassing animal breeding, preservation of genetic diversity, and medical research. Fish sperm cryopreservation was demonstrated at about the same time as for humans and livestock species (Blaxter 1953), however, despite the large-scale application in mammals, it remains at a research scale for aquatic species. During the past decades, there have been more than 200 aquatic species reported for sperm cryopreservation (Tiersch and Mazik 2000) and this number is increasing (although an accurate current estimate is not available). 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 improvements of the type that can be provided by cryopreservation (Boever 2006). Therefore, high-throughput cryopreservation for aquatic species comes with high expectations.

As stated above, high-throughput cryopreservation has been widely applied in livestock industries for decades. Its development grew out of advances made in the laboratory, and was scaled up for increased processing speed, capability for mass production, and quality assurance. Available equipment and processes in place for livestock and biomedical applications have been tested for feasibility of use with aquatic species (e.g., Haffray et al. 2008), with extensive work for more than 15 yr at the T. E. Patrick Dairy Improvement Center of the Louisiana State University Agricultural Center. For example, sperm of blue catfish *Ictalurus furcatus* was cryopreserved at this commercial facility using procedures developed for dairy bulls (Lang et al. 2003), as was done for sperm of common carp *Cyprinus carpio*, channel catfish *Ictalurus punctatus*, three species of sturgeon (*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, 2007a). Since then protocols and automated equipment developed for mammals have been specifically adapted for use with aquatic species (described below) including zebrafish *Danio rerio* (Yang et al. 2007), blue catfish (Hu et al. 2010), Eastern oyster *Crassostrea virginica* (Yang et al. unpublished data), channel catfish, and Atlantic salmon *Salmo salar* (Hu 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 straws has been developed for mammalian high-throughput. Adaptation of this equipment has been evaluated for aquatic species to enable commercial-scale use of cryopreserved sperm (e.g., Hu et al. 2010). Briefly, upon mixing with cryoprotectant, sperm samples are placed on the MAPI system, and filling,

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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 °C heat clamps. The straws are 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 follows using standardized procedures. Straws are arrayed on horizontal racks (40 per rack) and placed in a commercial-scale programmable freezer (Micro Digitcool, IMV, France) with a capacity of 280 straws per freezing cycle. If the thermal mass is not controlled at each freezing (e.g. by adding "dummy" straws), the number of straws is regulated within a tight range. The cooling program is initiated at 15-30 min after addition of cryoprotectant. The cooling rate can be programmed from 1 °C /min to 40 °C/min (based on chamber temperature) and various rates are studied (detailed below). When the final target temperature (-80 °C) 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, Cryo Bio System) for long-term storage in liquid nitrogen.

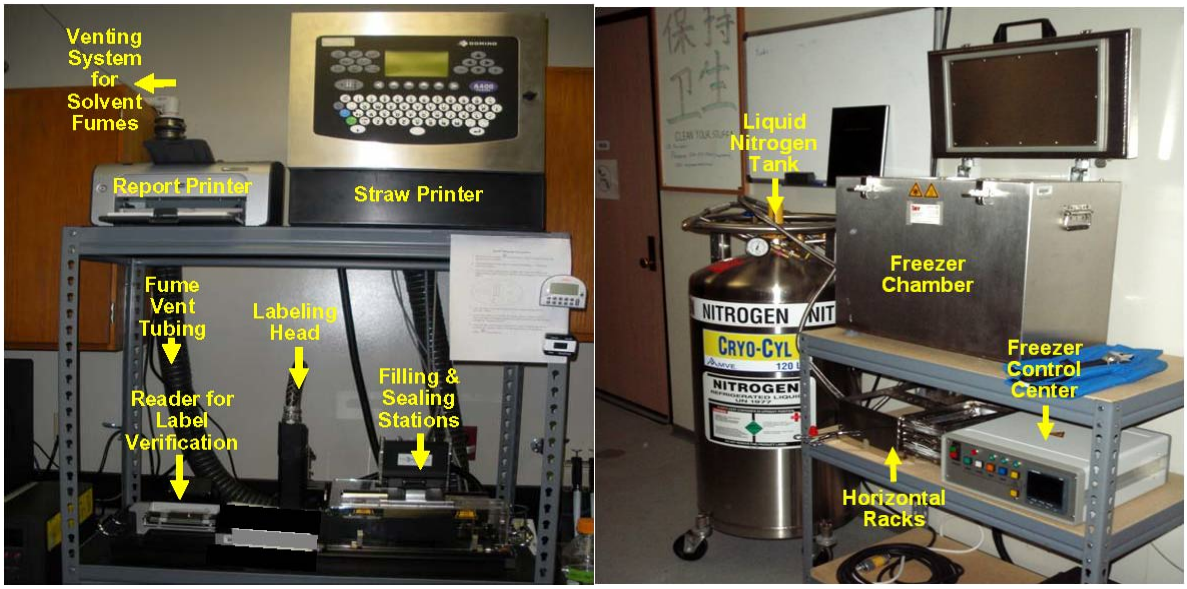

Figure 1. In the MAPI system (left), sperm suspensions are loaded and packaged into straws at filling and sealing stations, labeled under the labeling head driven by the straw printer, and pass a barcode reader for label verification. Next, the straws are laid on horizontal racks and placed in a programmable freezer (right) controlled by regulating liquid nitrogen flow from a pressurized storage tank. To see MAPI in action, *click here*.

High-throughput Pathways

Critical aspects of high throughput are arranging the sequence of procedures and balancing the inputs and outputs between connected steps. For any sequence developed, industrial designers and economists can further evaluate production resource utility and processing costs. In general for fish that require dissection for sperm collection, we have identified 5 major steps: dissection, sample processing, freezing and sorting, storage, and

utilization (Figure 2). An industrial engineering approach would focus on reducing the time and cost at each step to increase the efficiency of the production cycle, and reduce the possibility of bottlenecks between steps. In preliminary economic analyses of blue catfish, the estimated cost per straw was US$1.50 without labor, and each month there was an additive storage cost of US$0.02 per straw for supplies such as liquid nitrogen (our unpublished data).

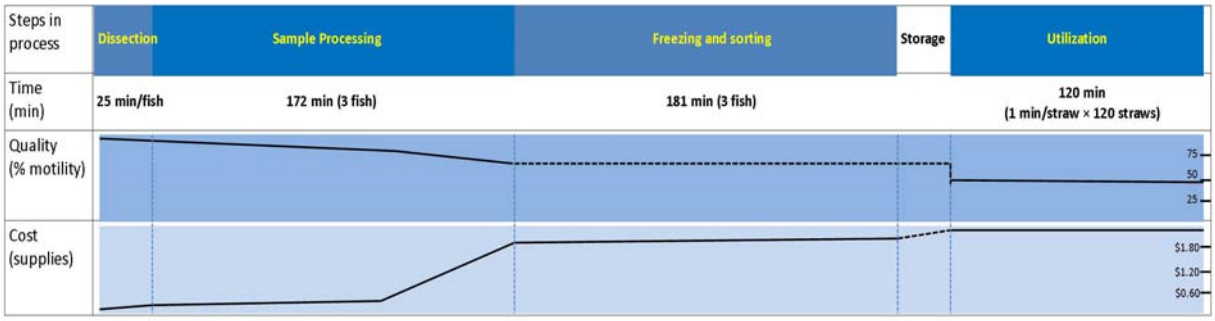

Figure 2. A simplified (5-step) schematic of the cryopreservation process for blue catfish. Each step is displayed horizontally in proportion to the amount of time required (in min) in relation to the sperm quality observed (expressed as percent motility) and supply costs (US$) for the number of fish or straws handled at each step. The long time intervals in the sample processing, freezing and sorting, and utilization steps were recognized as bottlenecks to be addressed in future research.

Commercial-scale Evaluation of High-throughput Processing

High-throughput cryopreservation is only beginning in aquatic species. Currently, marine species such as southern flounder *Paralichthys lethostigma* and Pacific oyster have been tested with laboratory-scale fertilization, anadromous species such as Atlantic salmon have been tested with hatchery-scale fertilization, and blue catfish was tested at a commercial scale with more than 1,400 straws in a single trial with 242 channel catfish females (Table 1). With a specified sperm-to-egg ratio (5×10^5 sperm per egg), thawed sperm had the same fertilization capability as fresh sperm on a per-male basis, and thawed sperm yielded production of 200,000 fry per day.

Table 1. Commercial-scale production parameters for use of high-throughput cryopreservation of blue catfish sperm. Cryopreseved sperm from 17 males and fresh sperm from 16 males were cross-tested during 4 d using eggs from 242 females. (P-values obtained from T-test).

Parameter	Thawed sperm	Fresh sperm	P-value
Sperm use per day (cells)	$17 \pm 6 \times 10^{10}$	$24 \pm 12 \times 10^{10}$	0.173
Hatching rate	43 ± 15%	52 ± 4%	0.158
Sacfry produced (per day)	$17 \pm 7 \times 10^4$	$28 \pm 7 \times 10^4$	0.034

Development of quality control and assurance are as important as development of proper protocols, and are likewise only just beginning for high throughput application in aquatic species. Male-to-male variation in the quality of cryopreserved sperm is commonly observed in species including mammals, fishes, and invertebrates (Mazur et al. 2008, Yang et al. 2009), although the underlying mechanisms for the variability remain unresolved (Dong et al. 2007b,

Tiersch et al. 2007). Unlike comparable previous reports in aquatic species, no significant variation was observed in post-thaw motility and fertility among ten blue catfish males (Figure 3) used in our commercial-scale study (Hu et al. 2011). This is desirable for high-throughput technology because it can provide products with consistent and predictable output. Possible explanations for the lack of variability include: 1) a small sample size of 10 males (although comparable to previous studies); 2) unknown attributes of the reproductive biology of blue catfish; 3) tight standardization of sperm concentration 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 (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 majority 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.

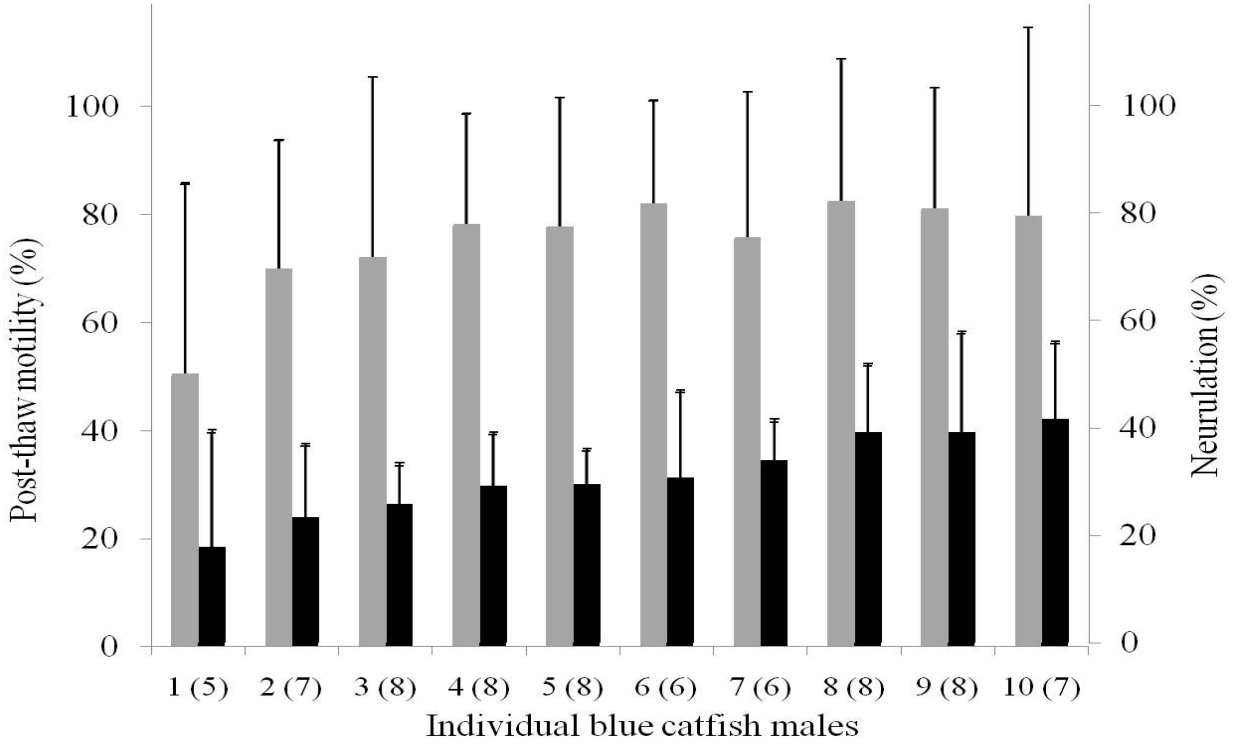

Figure 3. Sperm from 10 blue catfish males was cryopreserved using 1×10^9 cells/ml, 10% methanol, and a 5 °C/min cooling rate. Sperm were thawed and used to fertilize eggs from 5-8 channel catfish females (numbers of females indicated in parentheses). Post-thaw motility (dark bars) and neurulation (light bars) were used to evaluate male-to-male variation. Post-thaw motility and fertility of thawed sperm were not significantly different among the 10 individuals (Hu et al. 2011).

High levels of reliability are necessary for any technology to become commercially relevant. For high-throughput cryopreservation of aquatic sperm to move from the laboratory into commercial application, the process can no longer be viewed as simple combinations of cryobiological factors. Instead, from a manufacturing point of view, the process needs to be recognized as a sequence of operations relying on different equipment and resources (Figure 4).

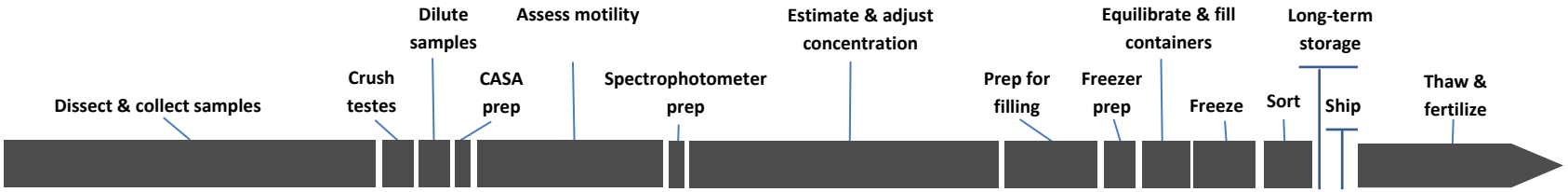

Figure 4. Schematic representation of a cryopreservation pathway established for sperm collection by dissection of the testis (e.g., for blue catfish or zebrafish *Danio rerio*). In its basic form, the process comprises 15 standardized, integrated steps from sample collection through thawing and use for fertilization. The steps are presented as rectangles sized in proportion to the total time required for processing and cryopreservation of samples from 20 individual males. CASA: computer-assisted sperm analysis.

With this perspective, further studies can focus on the optimization of overall system efficiency, while balancing biological and other factors, leading to a robust commercial model.

Thus, establishment of process pathways will assist in ensuring efficiency and quality of high-throughput products. In addition, these process pathways can be standardized or harmonized to provide central pathways for movement of material into comprehensive germplasm repositories achieving high throughput in two ways: intrinsically by themselves, and extrinsically by acting as a funnel for supplemental sources of cryopreserved material (Figure 5). New equipment, supplies, and reagents will eventually be necessary to

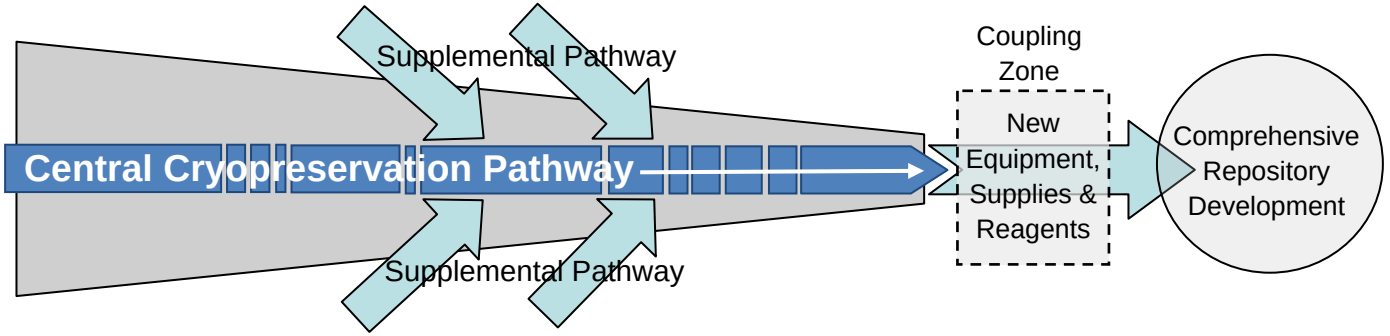

Figure 5. Conceptual approach for reinforcing the existing cryopreservation pathway and using it as a central route to funnel materials from other laboratories through a coupling zone that will integrate future commercial advances in high-throughput cryopreservation technology specific for aquatic species to support development of large repositories capable of handling hundreds of thousands of samples.

fully address the specific needs of aquatic species, although such a system could begin operation by adaptation of commercially available products developed for livestock and biomedical application.

Future Needs for Commercial Application of Cryopreservation in Aquatic Species

For high-throughput cryopreservation to assume a functioning role in assisting aquaculture production and aquatic species conservation it has to proceed beyond development of the initial technical requirements and requires changes in conventional thinking. For example, high throughput by definition involves large numbers of samples. Inherent in this is the need to minimize waste and inefficiency because small mistakes or inefficiencies are multiplied and can become costly. For example, use of a sperm concentration of 10^8 cells/mL was not biologically different from the use of 10^9 cells/mL (Hu et al. 2011), and at a typical laboratory scale of 100 straws or fewer, not economically different as well. However, in a high-throughput setting for the same total number of sperm cells, the use of 10^8 cells/mL would require 20,000 straws compared to only 2,000 straws for 10^9 cells/mL – a difference of US$27,000 (US$30,000 vs. US$3,000) for the numbers of straws used in our commercial blue catfish trial.

A series of activities are required to be in place for full-scale application. These activities have not yet been fully implemented anywhere for aquatic species. There are problems and barriers at each step, but getting started is usually the hardest part. The entry-level requirements for equipment, facilities, and training are high and force potential users to focus on technology development and technical problems (e.g., Caffey and Tiersch 2000a,b). However after these hurdles are passed, the focus can shift to coordination of activities and realization of the substantial opportunities provided by cryopreservation. This would include establishment of high-throughput capabilities, which has recently been recognized as a focus for necessary research by the US National Institutes of Health for biomedical models including fishes (workshop summary available at: *www.ncrr.nih.gov/publications/#reports*). A final phase of maturation in application would see cooperation and connections forming among governmental agencies, non-governmental organizations, academic institutions, and private companies. The major activities in this potential pathway for application would include (Tiersch 2008):

1) Development of necessary technical capabilities and facilities at well-funded and secure locations sited near aquatic species activities, or easily accessed by overnight shipping services.
2) Establishment of training programs for procedural efficiency, and education of personnel.
3) Development of appropriate biosecurity safeguards to control movement in and out of facilities of pathogens and other adverse biological effects (e.g., Tiersch and Jenkins 2003).
4) Development of functioning storage repositories, with rules for use and disposal of samples, and with appropriate security for basic services (e.g., electricity, liquid nitrogen, refrigeration capabilities, and aeration for aquaria and holding tanks).
5) Implementation of archival-quality labeling of samples and the creation of robust databases capable of handling biological information concerning samples (including geographical information system (GIS) data on collections), and maintaining correct inventory and identification of sample locations.
6) Further development of capabilities computing and information transfer including the ability to interact and exchange information with other databases such as the Genetic Resources Information Network of the USDA (*www.ars-grin.gov*).

7) Increasing of sample processing capabilities to enable high throughput of samples. This would include installation and use of automated or semi-automated equipment for labeling, filling, and sealing of straws, and the procurement of commercial-scale freezing and storage capabilities.

8) After central facilities have developed strong operational capabilities, a sustained effort should be made to develop cooperation with other organizations and facilities. These relationships can include sharing of samples, capabilities and expertise. Efforts should be made to link cryopreservation with existing or planned activities such as fish sampling programs or cooperation with specialized hatcheries during spawning seasons.

9) To assist interactions among organizations, basic arrangements should be discussed and put forth as formal transfer agreements that can be negotiated and put in place to describe things such as responsibilities, rights, and ownership of samples.

10) Because different facilities will have different approaches there should be establishment of quality control protocols and standardization or harmonization of protocols, labeling, terminology, reporting of results, and databases. Development of Best Practices Manuals or other guidelines would assist these efforts.

11) Essentially, individual repositories can at this point be linked by establishment of a full repository system, and end users of cryopreserved sperm, such as hatcheries or farms could interact with this system.

12) Coordinated regional activities could take place in individual countries or be administered across borders by treaties or other agreements to encompass river systems or ecosystems.

13) Establishment of pricing structures, marketing, and business practices will be necessary for initial and continued commercial investment.

Conclusions

High-throughput technology has wide potential application. In biomedical science, it can preserve valuable strains for research purposes; in fisheries management, it can assist hatcheries in recovery efforts, and in aquaculture, it can enable and accelerate selective breeding and genetic improvement. High-throughput processing will serve as a fundamental technology for aquatic germplasm banking. These efforts will proceed more easily with large-bodied fishes or species that can provide copious volumes of sperm such as salmon, but can also proceed with small biomedical fishes such as zebrafish (Yang et al. 2007) or *Xiphophorus* (Yang et al. 2007, 2009) by pooling of samples to represent lines and increase the volume available for automated processing. Eventual development of processing equipment and containers that can rapidly handle small volumes (e.g., 10-50 uL) in a standard format or by use of microfluidic technologies would allow high-throughput of even individual small fishes or samples.

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