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On-site evaluation of commercial-scale hybrid catfish production using cryopreserved blue catfish sperm

E Hu [a,1], Brian Bosworth [b], Jeff Baxter [c], Terrence R. Tiersch [a,*]

[a] Aquaculture Research Station, Louisiana Agricultural Experiment Station, Louisiana State University Agricultural Center, Baton Rouge, LA 70803, USA
[b] USDA Catfish Genetics Research Unit, Stoneville, MS 38756, USA
[c] Baxter Land Company, 114 E. Speedway, Dermott, AR 71638, USA

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ABSTRACT

Cryopreservation is an effective tool for conservation of genetic resources and is becoming increasingly used worldwide with aquatic species. Broadening the application of this technology to a commercial scale through high-throughput approaches has become essential for use with aquatic species. This study addressed high-throughput sperm cryopreservation of blue catfish at an industrial level. Our objectives were to: 1) optimize the sperm volume used for thawed sperm; 2) evaluate commercial application of high-throughput cryopreserved sperm with standard hatchery techniques, and 3) initiate evaluation of the fertility relationship between individuals and pooled samples. The results showed that a doubling of the previously established volume did not produce significant improvement in fertilization. The working volume of thawed sperm (2 ml at a concentration of 1×10^9/ml for batches of 100–150 ml channel catfish eggs) was practical. There was no significant difference in fry production after artificial fertilization of 2 million eggs with cryopreserved or fresh sperm. Pooled sperm samples and the individual samples used to form the pools produced similar fertilization rates. Blue catfish sperm is valued as a genetic material for hybrid catfish production, and cryopreservation makes genetic material management possible. This study initiates industrialization of this technology for use with aquatic organisms, and because the technology can be generalized, expands the opportunities for application to other species. High-throughput cryopreservation of blue catfish sperm provides new capabilities and can maintain sperm quality sufficiently to support commercial hybrid production.

1. Introduction

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 has grown from less than $1 billion in 1988 to more than $4 billion in 2012 (Newton-Small, 2012). Thus genetic improvement in the dairy industry and assisted reproduction in human medicine each provide an example of genetic resource utilization. The networking of cryopreserved sperm has proven invaluable to human society. Although cryopreservation of fish sperm started at the same time as dairy (Blaxter, 1953), large-scale application of cryopreserved germplasm of aquatic species is not currently utilized. In the past decades, substantial effort and resources have been applied to protocol development in different laboratories with a variety of species. Despite this, cryopreservation remains at a research level in aquatic species. However, there is recognition of the value of this technology. A survey among fish culturists indicated common interest in genetic improvement obtainable by adopting cryopreservation into existing procedures (Boever, 2006), such as among hybrid catfish hatcheries. The expanding demand for genetic improvement in fish culture can be addressed by cryopreservation, and recent development of high-throughput processing (Hu et al., 2011) provides the necessary technical prerequisites.

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 feed conversion (Dunham and Masser, 2012). Although hybrid catfish are currently in high demand by the industry, the production capacity for hybrids is

* Corresponding author at: 2410 Ben Hur Road, Baton Rouge, LA 70820, USA. Tel.: +1 225 765 2848; fax: +1 225 765 2877.
E-mail address: ttiersch@agcenter.lsu.edu (T.R. Tiersch).
[1] Current affiliation: Genetic Resources Management Consulting L.L.C., Baton Rouge, LA 70820, USA.

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constrained primarily due to the lack of natural hybridization between these species and the consequent need for artificial spawning that involves killing of the males for testis collection (Dunham and Masser, 2012). In addition, limited availability of blue catfish males can constrain hybrid production (Avery et al., 2005). 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 also 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 specific 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 damage to 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 sperm can affect the final ratio of motile sperm to eggs (Tiersch et al., 1994) which can in turn affect fertilization rates (Hu et al., 2011; Makeeva and Emel'yanova, 1993; Saksena et al., 1961; Small and Bates, 2001). The use of additional thawed sperm at fertilization to compensate for losses in motility has been used as a potential solution to reduced fertility (Tiersch et al., 1994). 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 increased costs in fry production. In addition, commercial hatchery operators have expressed interest in the utility of increasing the volume of sperm to ensure maximal egg fertilization.

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 (Hu, 2012). Laboratory-scale neurulation testing 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 (Hu et al., 2013). 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, routine hatchery use and genetic improvement applications are feasible and the value of sperm is greatly increased. Proper handling and utilization of sperm also become essential to practice. Because thawed 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 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 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.

2. Material and methods

2.1. Fish

Blue catfish males (D&B strain, originally from Crockett, TX) 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 yr old, and ranged from 61 to 97 cm, and 2.8 to 9.8 kg. Prior to transportation, males were selected based on observable secondary sexual characteristics indicative of maturity (e.g. well-muscled head and dark coloration) (Avery et al., 2005). A hauler with a high-pressure oxygen supply was used to transport fish from the 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 placed in aerated outdoor 405-m^2 ponds and fed commercial broodstock diets (Aquaxcel, Cargill™, 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 °C, 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.

2.2. Sperm collection

At the beginning of the channel catfish spawning season (April), 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.

2.3. Determination of sperm concentration

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

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

(Hu et al., 2011).

2.4. Motility estimation

Sperm samples were placed on glass slide and viewed with a 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 h 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% (as such 9% of the individuals were discarded).

2.5. 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 solutions were each prepared in HBSS300 at twice the final concentration, before mixing at 1:1 (v/v) to start equilibration. 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. 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 in the freezer for each cycle ranged from 180 to 240 straws. No additional steps were taken to equalize the thermal mass at each cycle (e.g. using "dummy" straws). After a 30-min equilibration period (defined as starting when cryoprotectant was added to sample and ending at the start of the cooling program), the cooling cycle began from the resting freezer chamber temperature (5 °C) and dropped to −80 °C at a rate of 5 °C/min. At the end of the programmed cooling cycle, the samples were stabilized at −80 °C for another 5 min, and 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.

2.6. 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 used for cryopreservation 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 °C). 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 was used for each egg sample (except different volume treatments in Section 2.7). 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 (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 °C. After 27 to 30 h, normal embryo development would be at the neurula stage (Stage V) (Makeeva and Emel'yanova, 1993; Saksena et al., 1961) 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 to as percent neurulation). After 10 d, the hatched fry had consumed their yolk resources and swam up to the surface to begin exogenous feeding. 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 represented the number of fry for the entire hatching trough. According to existing commercial hatchery requirements, the fertilized egg masses from each day were gradually transferred into two troughs: one for eggs fertilized with thawed sperm; one for those fertilized with freshly prepared sperm. Therefore, after 10 d of hatching, there was only a single number to represent swim-up fry production of either thawed sperm treatment or freshly prepared sperm control. To gain information on fertilization of individual spawns, neurulation was estimated for each egg mass before the transfer.

2.7. Experiment 1: evaluation of double-dosage of thawed sperm

Sperm samples from 8 males were processed individually at the Louisiana State University Agricultural Center, 215 straws representing all 8 males (27 ± 14 straws each) were produced and shipped to Baxter Land Co. Fish Hatchery. All frozen straws were thawed at 40 °C for 20 s, and pooled in a 500-ml Tupperware® containers which are readily available and commonly used in fish hatcheries. 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.

2.8. 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 3000 straws representing all 57 males were shipped to Baxter Land Co. Fish Hatchery. 316 females were used in 7 d of artificial fertilization

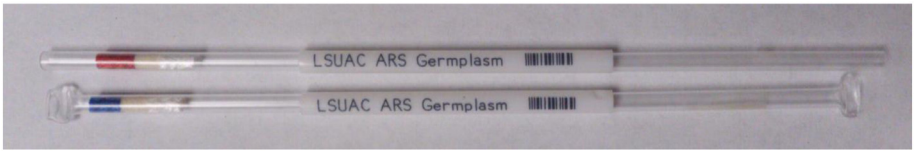

Fig. 1. Example of unsealed (top) and sealed (bottom) 0.5-ml CBS straws (CryoBioSystem, Paris, France) with barcode and alphanumeric label.

at the hatchery. Each day, sperm were thawed at 40 °C for 20 s and pooled in a 500-ml Tupperware container. Fresh sperm were prepared within 24 h before use as 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 the positive control group.

2.9. Experiment 3: fertility relationship between individual and pooled samples

2.9.1. Fertilization by individual males

Straws from 51 of the males from Experiment 2 of 2010 and 2011 were tested in this experiment. The remaining 6 males did not provide sufficient straws for individual post-thaw motility and neurulation testing. Therefore, they were not included in this experiment. All straws were arranged and cataloged at the Aquaculture Research Station before transport to USDA Catfish Genetics Research Unit (Stoneville, MS) for use at a research scale. 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- to 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 (Cuevas-Uribe et al., 2011). During fertilization 0.25 ml of thawed sperm was used for each petri dish, and 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 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, otherwise following routine hatchery procedures (Steeby and Avery, 2005). The incubation temperature was 27 to 29 °C. After 27 to 30 h, 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.

2.9.2. Hypothesis testing

Individual-weighted proportions (male identification number and number of straws per male) of 6 pooled samples (combinations of the 51 males described in Sections 2.8 and 2.9.1) were recorded and the motility of the pooled sample and resulting neurulation were measured. 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 numbers, 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 measured values were taken directly from pooled samples and statistically independent from theoretical values. The theoretical post-thaw motility and neurulation were compared with the measured values for all 6 pooled samples by paired *T*-test.

2.10. 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 use of the 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.

3. Results

3.1. Experiment 1: evaluation of the double-dose usage of thawed sperm

The post-thaw motility of thawed sperm was 45% (N = 9) and the 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 the different sperm volumes (2 ml and 4 ml) used among the treatments, average outputs on a per-sperm basis were calculated (Table 1). The percent neurulation among all groups was not significantly different ($P = 0.136$).

3.2. 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 that of fresh sperm (43 ± 4%). On a per-day basis, each liter of eggs yielded 23,737 ± 9518 fry using thawed sperm. Using the same volume at a similar concentration for fresh sperm, 26,318 ± 8510 fry were produced per liter of eggs each day. There was no difference ($P = 0.603$) in fry production using similar volumes and concentrations of thawed and fresh sperm (Experiment 1) when fertilizing the same volume of eggs.

3.3. Experiment 3: initiate evaluation of the fertility relationship between individual and pooled samples

The individual post-thaw motility ranged from 0 to 45%. The individual neurulation values were normalized by dividing by the respective egg quality control, and the normalized neurulation values 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%).

4. Discussion

Commercial hatcheries are effective for large-scale production but have limited flexibility for experimental trials. The hatchery where the present commercial-scale trials were performed had the capability of fertilizing eggs collected from more than 40 female channel catfish per day (~450,000 eggs). The traditional catfish egg hatching troughs (Dunham and Masser, 2012) used in the hatchery provided 65 ± 19% neurulation, and a 66% hatching rate (26,318 ± 8510 fry/L eggs divided by 40 ± 7 eggs/ml), which represented appropriate 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

Table 1
Fresh or thawed sperm were used to fertilize batches of channel catfish eggs (100–150 mL, N = 102). 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 (Section 2.6), 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 $\times$ 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
Thawed	4 ml (8 straws)	61 ± 15	2.70	12.15	900

require large-scale fertilization trials over an entire spawning season (e.g. 1–2 months of daily spawning involving millions of fry). For the same reason, the number of treatments was also restricted by the practices within the facility. Estimation of neurulation could serve as an alternative index to gain access to more information during commercial-level fertilization trials, however, hatching results (fry production) remain the most-used (and economically relevant) index for hatchery operators at a commercial facility.

A previous study established a practical total sperm-to-egg ratio of 2.5–3.3 $\times$ 10^5 and derived an optimal equilibration (pre-freeze) 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 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 a range of sperm-to-egg ratios from 1.13 $\times$ 10^4 to 6.90 $\times$ 10^4 in different catfish species (Table 2). The cryopreserved sperm were used at higher ratios than fresh sperm in those studies. In addition, these values reinforce the observation in this study that a doubling of sperm volume in each fertilization pan when using an established sperm-to-egg ratio (2.5–3.3 $\times$ 10^5 sperm/egg) did not improve hybrid catfish fry production because the doubled ratio (5–6 $\times$ 10^5 sperm/egg) was still close to the reported ratio for production of hybrid catfish. 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 scale use warrants further study. However, at this point it cannot be concluded that thawed sperm functions in the same fashion as fresh sperm when using similar volumes and concentrations. More comprehensive experiments will be required to fully refine the performance of cryopreserved sperm. The results that similar volumes of diluted sperm of fresh and thawed sperm yielded similar neurulation and numbers of offspring leads to the conclusion that sperm from a single male, whether cryopreserved or not, can produce consistent numbers of offspring within the current commercial hatchery setting. This equivalence at a per-male basis can reduce the perceived uncertainty of cryopreservation application among farmers. More importantly, the genetic value of sperm can be evaluated, and efforts to improve efficient use of sperm can become a part of hatchery management. This work provides a path for improving efficiency by quantitatively analyzing sperm use and presenting the efficiency on a per-sperm or per-male basis.

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, although they did not control sperm concentration. Others have examined incorporation of industrial-scale protocols from dairy semen approaches to aquatic species. (Dong et al., 2005a,b; Lang et al., 2003). 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 use of excess sperm has been a standard practice in the production of hybrid catfish. Farmers have routinely fertilized eggs with a larger volume of sperm solution (2.7 $\times$ 10^5 sperm/egg, if one male is used to fertilize eight females) (Avery et al., 2005) than would be optimal based on the findings of recent studies (Hu et al., 2011). In the present study, freshly prepared sperm had the same concentration as thawed sperm ($P > 0.05$) when used at same volume. The results from this study indicate that under current commercial hatchery conditions the functional utility of cryopreserved sperm was the same as that of fresh sperm despite differences in sperm numbers.

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

Table 2
Survey of sperm-to-egg ratios used in cultured catfish species. Only one study (indicated below) specified the ratio as being for motile sperm.

Species	Sperm type	Sperm-to-egg ratio (sperm/egg)	Citation
African catfish	Cryopreserved	4.90 $\times$ 10^4	Steyn and Van Vuren (1987)
Clarias gariepinus	Fresh	1.50 $\times$ 10^4	Rurangwa et al. (1998)
	Cryopreserved	1.13 $\times$ 10^4	(Viveiros et al. (2000)
Striped catfish	Cryopreserved	6.90 $\times$ 10^7	Kwantong and Bart (2003)
Pangasius hypophthalmus	Fresh	1.89 $\times$ 10^6	Kwantong and Bart (2009)
	Cryopreserved	6.94 $\times$ 10^6	Kwantong and Bart (2009)
Channel catfish	Cryopreserved	2.00 $\times$ 10^5[a]	Tiersch et al. (1994)
Hybrid catfish (channel catfish female $\times$ blue catfish male)	Fresh	1.25 $\times$ 10^5	Bart and Dunham (1996)
	Cryopreserved	1.33 $\times$ 10^7	Bart et al. (1998)
	Cryopreserved	2.50 $\times$ 10^5	Hu et al. (2011)

[a] Motile sperm concentration.

sperm to fertilize small research-size batches of catfish eggs (e.g., 10–12 ml) suitable for use in diallel crosses or other genetic evaluations to characterize the relative genetic merit of male broodstock.

Differences between measured and predicted post-thaw motility values indicated that the pooled samples were not characterized by their extreme values. Motility differences 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 evaluating sperm quality before pooling. 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 in fertilization by certain males in pooled samples 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 (swimming behavior of sperm) (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 require further study that combines hatchery management and parental identification with genetic analysis. In addition, the capability of dividing the straws for commercial production and testing of the same males for fertility also demonstrated the advantages of cryopreserved sperm in terms of genetic resource storage and reuse.

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 required. 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. The 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. The 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. The use of cryopreserved sperm would enable tracking and repeated use of males (i.e., across years) and would be 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 that was contained in 1300 straws. With high-throughput process reported previously (Hu et al., 2011), we demonstrated that 1300 straws can be produced from 20 blue catfish males in about 8.5 h (Hu, 2012).

In practice, the fertilizations in this study were performed with a small excess of sperm (i.e., standard industry practice). Cryopreserved sperm from the standardized processing contained 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 (indicated by neurulation) 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 (obtained from preliminary fertilization trials to characterize large batches). In the long term, the 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).

4.1. Genetic material 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 into future hybrid catfish production. The use of hybrid catfish within the industry should be viewed as a temporary measure used because comparable genetic improvement in channel catfish has not yet been made. Hybrids involve additional costs of production and can present environmental hazards if they escape, potentially causing cross-species introgression of genes into wild populations. Cryopreserved sperm have been the primary means used to produce the tremendous genetic improvement in the dairy industry. Such use in channel catfish can greatly accelerate production of improved lines for aquaculture and avoid or reduce the need to produce hybrids. The results of this and other recent studies (Hu et al., 2011) lead directly to large-scale or commercial application of cryopreservation technology in management of genetic resources in aquatic species. This genetic resource management concept is based on the traditional phases of production (Fig. 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 freed 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 allow banking 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 the “savings account”.

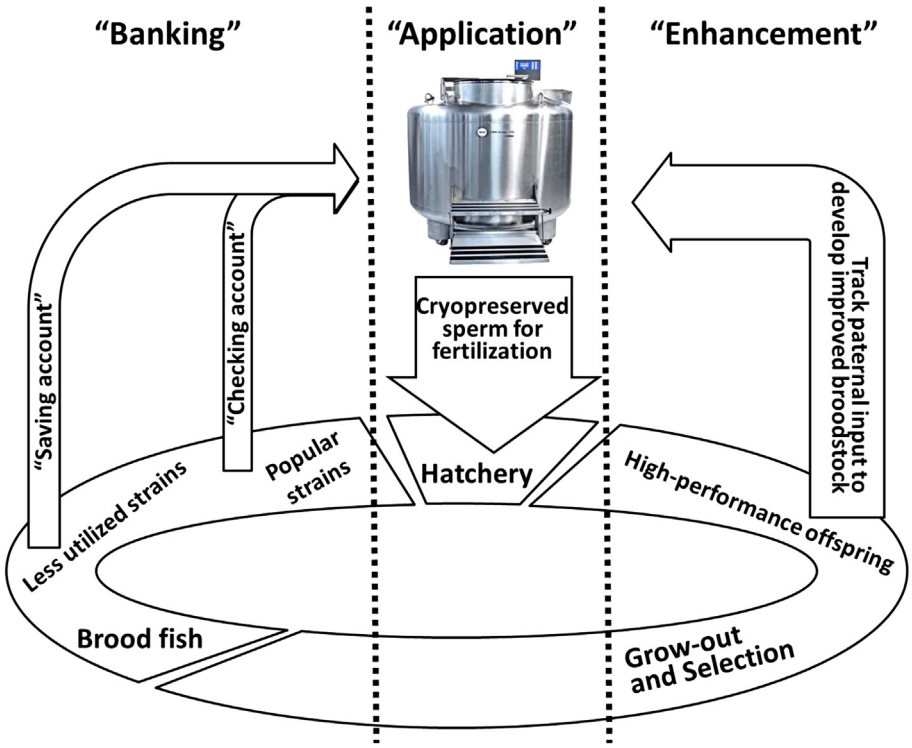

Fig. 2. A summary of genetic resource management concepts as potentially integrated into hybrid catfish production. During hybrid catfish production, all activities can be grouped into three broad categories: broodfish, hatchery, and grow-out and selection. Genetic resource management can be grouped into three aspects (separated by dotted lines): banking, application, and enhancement. In broodfish management, Banking can be used to store the less-utilized strains to liberate farm resources as a long-term archival collection ("savings account"), and store popular strains to increase their accessibility as a working repository ("checking account"). 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 involves collection of information about hybrid offspring performance and links it back to the sperm donors. Live broodfish and cryopreserved sperm are each considered as genetic resources in this conceptual framework.

Application: With the banking repository model, all hatcheries can access their own archival ("savings") and working repository ("checking") accounts regardless of distance and time. For example, if a farm decided to start a new hatchery at a remote site in 2 years, there would be no need to transport populations of live blue catfish to the new site, or to wait 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 could 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 sires (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).

The genetic resource management concept is achievable with recently developed and described high-throughput processing methods. For example, the banking of five blue catfish strains in an archival collection with 100 straws for each individual (http://nrrc.ars.usda.gov) can preserve 30 individuals from each strain with 15,000 straws. A popular strain of blue catfish such as D&B could be held in a working repository collection. To support a commercial-level hatchery with daily consumption of 1300 straws during the 50 d spawning season, a total 65,000 straws of D&B sperm would be required. Thus a total of 80,000 straws would be needed to form the initial repository collection for blue catfish sperm. During application, straws in the working collection could be used to produce hybrid catfish at a commercial scale, which provides the opportunity for genetic enhancement. The parental identification of high-performance offspring could be linked back to the repository. The remaining straws of identified individuals could be used for production of additional high-performance offspring or be transferred into the archival collection for future use. With existing production capacity (Hu, 2012), 69 d of processing would be sufficient to produce the 80,000 straws necessary to establish this system of genetic resource management that would benefit hybrid catfish production in the short- and long-term.

5. 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 on-demand killing of 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 resource management for genetic improvement. The use of cryopreserved sperm could increase the profitability of catfish aquaculture in the long term, and offer opportunities for development of new markets and industries in aquatic genetic resources and germplasm.

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