New Chapter

Simple Extenders for Refrigerated Storage and Cryopreservation of Channel Catfish Sperm

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Introduction

Genetic improvement of channel catfish *Ictalurus punctatus* broodstock is needed to maintain desirable characteristics for aquaculture (e.g., growth rate, feed conversion), to avoid problems associated with inbreeding (e.g., reduction of immune system function), and to ensure conservation of genetic resources. Additionally, hatchery techniques are required to increase the cost effectiveness of aquaculture breeding programs. For example, buffered extender solutions have been the used for refrigerated storage and cryopreservation of channel catfish sperm (Guest et al. 1976, Tiersch et al. 1994, Christensen and Tiersch. 1996). These methods can be used for selective breeding to improve commercially important traits. However, the typical farm does not have the resources necessary for the preparation of extenders used for refrigerated storage and cryopreservation of sperm. Simplification of processes, such as the use of minimal extenders, could advance development of breeding programs and improve production economics. To that end, this study evaluated simplified sperm extenders for use in the refrigerated storage and cryopreservation of channel catfish sperm. Our goal was to identify extenders based on readily available food-grade ingredients useful for artificial spawning that would be accessible to a wide range of farmers and breeders. The objectives were to evaluate the effects of extender composition on refrigerated storage of sperm, cryopreservation of sperm, fertilization percentage, and growth of fingerlings.

Experimental Approach

Extender Preparation

Three extenders were examined (Table 1) made from materials found normally at local grocery stores, and compared these with a widely accepted extender, Hanks' balanced salt solution (HBSS) prepared with eight reagent-grade chemicals (Sigma Chemical Corp., St. Louis, Missouri, USA) (Tiersch et al. 1994). All extenders were adjusted to an osmolality of 290 mOsmol/Kg, although no attempt was made to adjust pH.

Table 1. Composition of extenders used with channel catfish sperm.

Solution (g/L)	$NaHCO_3$	NaCl	pH
Arm & Hammer baking soda®	13.82	--	8.5
Morton salt®	--	9.17	6.0
Combined (baking soda & salt)	6.91	4.59	8.5
Hanks' balanced salt solution	(recipe in Tiersch et al. 1994)		6.5

Sperm Collection

Testes were removed from six mature channel catfish during the normal spawning season in Southern Louisiana (May, 1996). Each testis was cleaned, weighed, and crushed in an

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extender at a ratio of 1 g of testis to 20 mL of extender. The extender/sperm mixtures were filtered through a sieve (Cellector®, E-C Apparatus Corp Milford, MA) to remove pieces of testicular tissue. Samples were stored in 100-mL plastic beakers at 4 °C until use in experiments.

Motility Estimation

Motility was estimated using 200-x dark-field microscopy immediately after the activation of 2-µL of extended sperm with 20-µL of deionized water. Percent motility was defined as the percentage of progressively motile sperm observed during 30 sec within a sample. Sperm that vibrated in place were not considered to be motile.

Refrigerated Storage

To determine the effect of extender composition on the retention of motility over time, 30-mL sperm samples from six channel catfish were stored in 100-mL plastic beakers at 4 °C. The samples were gently swirled and motility estimated daily until it was no longer present.

Cryopreservation Methods

Sperm from five channel catfish were frozen in 0.5-mL straws (IMV International Corp. Paris, France) on aluminum canes with five straws per goblet. Methanol was used as the cryoprotectant at a concentration of 5% (equilibration time, 45 min). Sperm were held in extender for less than 6 h before cryopreservation. We cryopreserved the samples using a computer-controlled freezer (Kryo 10/16, Planer Products Ltd., England) at a rate of –40 °C/min. After reaching –80 °C/min samples were transferred into liquid nitrogen for storage (Christensen and Tiersch 2005). Samples were stored for 24 hr before use in fertilization trials.

Fertilization and Growth Trials

Female channel catfish were induced to spawn by intraperitoneal injection of synthetic luteinizing hormone-releasing hormone (LH-RHa, Sigma Chemical Corp.) at a dose of 100 µg/ Kg body weight. Eggs were collected into greased bowls containing HBSS (Bates et al. 1996). Between 10 and 20 mL of eggs were aliquotted per sample. Samples were thawed in a water bath at 40 °C for 7 sec. Fresh (0.45 mL) or cryopreserved (0.50 mL) sperm were added to eggs and mixed. Water was added to initiate fertilization and water hardening. Fertilization percentage was determined by dissolving the egg matrix with sodium sulfite (15 g/L) for 40-60 sec, placing the eggs on a light box, and counting the number of neurulated embryos at 24-27 hr after fertilization. After hatching, fry were held at equal densities in replicate tanks in an indoor recirculating system for 3.5 months before measurement of length (total and standard) and weight.

Statistical Analysis

All percent motility values were arcsine-square root transformed prior to analysis. Motility data from the refrigerated storage study was compared using a repeated-measures analysis of variance (NCSS 2000, NCSS, Kaysville, Utah, USA). In the cryopreservation study, differences were examined in initial, equilibration, and post-thaw motility using a one-way ANOVA (NCSS 2000) for each analysis. Differences among the treatments in the fresh and cryopreserved sperm fertilization trials were examined with a one-way ANOVA. A randomized block design was used to examine differences in weight, standard length, and total length in the

growth study to address variation in rearing conditions. Means were separated using a Tukey-Kramer multiple comparison test, and were considered significant at the $P < 0.05$.

Results

Refrigerated Storage

During the study, sperm stored in HBSS retained significantly ($P < 0.0001$) higher motility than did sperm stored in other extenders (Figure 1). There were no differences in initial motility ($P = 0.1146$) or equilibration motility ($P = 0.2630$) among the extenders. Sperm cryopreserved in HBSS or the combined extender had significantly higher ($P < 0.0001$) motility than did sperm in baking soda or salt alone.

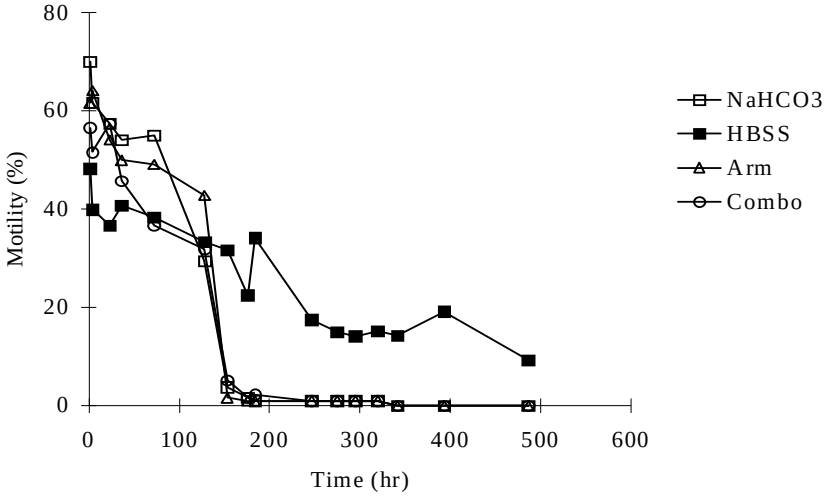

Figure 1. Percent motility of channel catfish sperm stored at 4 °C in four extender solutions. Each point represents the mean of six fish. Sperm stored in HBSS (closed squares) retained motility significantly longer ($P = 0.0001$) than in the other extenders.

Cryopreservation

There were no differences in initial ($P = 0.1146$) and equilibration ($P = 0.2630$) motility among the extenders tested. Sperm cryopreserved in HBSS and the combined extender had higher ($P < 0.0001$) post-thaw motility than did sperm in baking soda or salt (Table 2).

Table 2. Mean (± SD) motility values for sperm frozen in 5% methanol and four extenders. Equilibration was 45 min. Sperm were thawed at 40 °C for 7 sec.

	Motility (%)		
Solution	Initial	Equilibration	Thawed*
Arm & Hammer baking soda®	70 + 6	52 ± 21	3 ± 2^{B}
Morton salt®	53 + 18	63 ± 8	2 ± 2^{B}
Combined (baking soda & salt)	58 + 19	45 ± 11	9 ± 4^{A}
Hanks' balanced salt solution	44 + 17	51 ± 11	13 ± 3^{A}

* Motility estimates sharing letters were not significantly different ($P > 0.05$).

Fertilization and Growth Trials

There were no differences in the fertilization rates among extenders for fresh (P = 0.8002) or cryopreserved (P = 0.3341) sperm (Table 3).

Table 3. Mean (± SD) percent fertilization of refrigerated and cryopreserved sperm. There were no differences in fertilization among extenders for refrigerated (P = 0.8002) or cryopreserved (P = 0.3341) sperm.

	Percent Fertilization	
Treatment	Refrigerated	Cryopreserved
Arm & Hammer baking soda®	80 ± 17	81 ± 12
Morton salt®	82 ± 15	84 ± 3
Combined (baking soda & salt)	83 ± 11	68 ± 26
Hanks' balanced salt solution	84 ± 10	86 ± 13

There were no significant differences in weight (P = 0.9131), standard length (P = 0.7882), or total length (P = 0.8082) of 3.5-month-old juveniles produced from refrigerated sperm (Table 4).

Table 4. Mean (± SD) weight and length of fish produced from refrigerated sperm. There were no differences among the treatments (weight, P = 0.9131; standard length, P = 0.7882; total length, P = 0.8082)

		Length (mm)	
Treatment	Weight (gm)	Standard	Total
Arm & Hammer baking soda®	0.91 ± 0.38	39.7 ± 6.3	48.8 ± 7.2
Morton salt®	0.91 ± 0.31	39.3 ± 4.8	48.9 ± 5.6
Combined (baking soda & salt)	0.92 ± 0.28	39.6 ± 4.4	49.1 ± 5.5
Hanks' balanced salt solution	1.08 ± 0.26	42.4 ± 3.9	52.4 ± 4.6

Potential for Use of Simple Food-Grade Extender Ingredients

This study illustrates that simplified extenders can be used for the refrigerated storage and cryopreservation of channel catfish sperm. Sperm extended in commercially available food-grade preparations of sodium bicarbonate and sodium chloride, or a combination of the two had similar motility and fertilization rates. These rates were similar to those of sperm extended in HBSS, an extender that requires eight ingredients (Tiersch et al. 1994), and has been used extensively for refrigerated storage and cryopreservation of sperm of channel catfish and other species (Guest et al. 1976, Tiersch et al. 1994, Tiersch et al. 1996, Christensen and Tiersch 2005). Previous work has demonstrated the need to maintain the solution osmolality above 290 mOsmol/Kg to preserve sperm quality during storage (e.g., Bates et al. 1996). Additionally, growth and survival of fry produced from cryopreserved sperm were not affected by the extender indicating that sodium bicarbonate and sodium chloride can produce viable offspring. The important distinction is that these simple extenders did not maintain sperm quality as long as HBSS did during refrigerated storage. Based on these results, the use of salt or baking soda as extenders for refrigerated storage prior to freezing should be limited to 3-4 d, and should be evaluated before such use. The HBSS may offer a longer refrigeration period before freezing.

Storage of sperm using these simple extenders offers some advantages. Salt and baking soda are cheaper (considerably so, if cost estimates are made using the calculations provided by Caffey and Tiersch 2000) and are more readily available than the reagent-grade ingredients used to prepare HBSS. In addition, these extenders offer utility when in the field or in conditions with economic or other constraints (such as when working on a tight budget or at non-research hatcheries). Because food-grade salt and baking soda from large global companies were used, reliable quality of such ingredients should be available around the world. In addition, such extenders would likely be useful for a variety of species (e.g., Mongkonpunya et al. 1996, Kwangtong and Bart 2006, Adeyemo et al. 2007), given that preliminary testing of the type done in this study is performed prior to commitment of valuable resources.

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