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Theriogenology
ELSEVIER
Theriogenology 63 (2005) 2103–2112
www.journals.elsevierhealth.com/periodicals/the

Cryopreservation of channel catfish sperm: effects of cryoprotectant exposure time, cooling rate, thawing conditions, and male-to-male variation

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Received 10 November 2003; accepted 5 August 2004

Abstract

The purpose of this study was to extend previous work on the cryopreservation of channel catfish (*Ictalurus punctatus*) sperm. The objectives were to compare the effects of freezing and thawing on motility of sperm for: (1) 1 or 48-h exposure before freezing to 5% methanol and use of 0.5 or 0.25 mL straws; (2) 1 h or 5-day exposure before freezing to 5% methanol; (3) cooling at 45 or 3 °C/min; (4) thawing at 30, 40 or 50 °C using 5 or 10 s duration, and (5) cryopreservation with 5 or 10% methanol of samples from 50 males to analyze male-to-male variation. No differences were found in motility reduction for 1 or 48 h exposure times in 5% methanol, for use of 0.5 or 0.25 mL straws, or for 1 h or 5-day exposures in 5% methanol. A cooling rate of 45 °C/min resulted in lower motility reduction (33 ± 9%) than a rate of 3 °C/min (83 ± 13%) ($P = 0.002$). A thawing temperature of 50 °C resulted in lower motility reduction (25 ± 14%) than 30 °C (51 ± 21%) or 40 °C (59 ± 11%) ($P = 0.001$). A thawing duration of 10 s resulted in lower motility reduction (38 ± 12%) than a duration of 5 s (52 ± 12%) ($P = 0.005$), and there was an interaction between thawing temperature and duration ($P = 0.050$). A concentration of 5% methanol resulted in lower motility reduction (43 ± 17%) than 10% methanol (67 ± 14%) ($P = 0.001$). Regression analysis showed no relationship between motility before freezing and after thawing for 5% methanol ($r^2 = 0.012$) or 10% methanol ($r^2 = 0.011$).

Keywords: Cryopreservation; Channel catfish; Spermatozoa

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0093-691X/$ – see front matter
doi:10.1016/j.theriogenology.2004.08.013

1. Introduction

Gamete cryopreservation and artificial fertilization offer benefits for genetic improvement of commercially produced fishes. Heritability of specific production traits in fish is reported to be higher than that of domestic livestock, such as cattle [1]. The use of artificial breeding and other genetic tools, such as interspecific hybridization, polyploidy, and gynogenesis could allow faster genetic gains in fishes than have been obtained in livestock. Cryopreservation of sperm from genetically superior brood fish can preserve genetic resources and provide greater availability of improved material. The use of cryopreserved bull sperm has provided a database for evaluation of the genetic traits of individual bulls, and their potential for increasing progeny performance [2]. Development of such a database for fishes could benefit commercial aquaculture through increased production capabilities.

Within the order Siluriformes (catfishes), the most important species used worldwide for aquaculture are from three families: Pangasiidae, Clariidae, and Ictaluridae. The pangasiids are cultured throughout Southeast Asia [3], the clariids are cultured predominantly in South Africa [4], and the ictalurids (primarily channel catfish) are cultured in the United States. Cryopreservation of gametes from the pangasiids and clariids has been studied since the 1980s, when problems encountered with spawning and selection programmes generated interest in the use of cryopreservation as a tool to develop stocks with improved culture traits [5].

The farming of channel catfish, *Ictalurus punctatus*, is the largest foodfish aquaculture industry in the United States. Cryopreservation can assist in the genetic improvement of catfish stocks. Initial cryopreservation studies of channel catfish sperm compared various extenders and cryoprotectants [6]. Further studies found methanol to be the best among five tested cryoprotectants for channel catfish sperm, and reported the first use of thawed sperm for production of channel catfish by artificial fertilization [7]. Subsequently it was reported that methanol was a better cryoprotectant than *n*,*n*-dimethyl acetamide and that 0.25 mL straws were superior to 0.5 mL straws for cryopreservation of channel catfish sperm [8].

The purpose of the present study was to extend this earlier work on cryopreservation and further optimize procedures used in the cryopreservation of channel catfish sperm. Exposure to cryoprotectant prior to freezing is an important factor in the cryopreservation of sperm from many species, and effects will vary depending on the cryoprotectant, time of exposure, and concentration [9]. Cryoprotectants commonly used for fish sperm include dimethyl sulfoxide (DMSO), glycerol, propylene glycol, and methanol. Increased exposure to cryoprotectants such as these can improve the cryoprotective effect, but can also result in increased toxicity to the sperm cells [8,10]. As a part of this study, we analyzed the effect on reduction of sperm motility from before freezing to after thawing of exposure to 5% methanol for periods of 1 h, 48 h, and 5 days. Furthermore, we compared 0.5 and 0.25 mL straws in a factorial treatment arrangement with two exposure times (1 and 48 h).

Cooling rate can affect the rate of osmosis, diffusion, and formation of ice crystals within a cell [9]. In this study, we compared a fast and a slow cooling rate (45 and 3 °C/min, respectively) for cryopreservation. Thawing temperature and duration are also critical factors in the survival of cryopreserved sperm cells [9]. Optimal thawing procedures have not been reported for channel catfish sperm. We compared thawing temperatures of 30, 40, and 50 °C, and thawing durations of 5 and 10 s.

Cryopreservation success and potential fertilizing ability of mammalian sperm are often evaluated by estimation of acrosomal integrity [11,12]. Channel catfish sperm do not possess an acrosome (a condition common to teleost fishes), and motility is often the only technique available for evaluation of sperm after thawing [13,14]. In certain other aquatic species, measurement of percent fertilization provides a useful method of assessment because fertilization can be studied throughout the year. Channel catfish maintained in outdoor ponds spawn naturally only once per year during a 2-month period (May through June in the southern United States), and fertilization trials for assessment of sperm quality are limited to use only during the spawning season. We are aware that motility is not considered to be a viable indicator of fertilization success, although sperm samples exhibiting little to no motility can still result in successful fertilization [15]. Notwithstanding, a comparison of pre-freeze to post-thaw motility is a viable indicator of damage to the cells during the cryopreservation process.

As the final part of this study, we cryopreserved sperm from channel catfish using optimized techniques, with 5 or 10% methanol as the cryoprotectant. This was done to estimate the sperm motility available for commercial-scale application, to determine the level of male-to-male variation in the response to cryopreservation, and to determine if motility after thawing was related to motility before freezing.

2. Materials and methods

Healthy, mature channel catfish (1.5–3 kg and 40–60 cm) from a research population maintained at the Louisiana State University Agricultural Center, Aquaculture Research Station were killed by overdose of tricaine methanesulfonate (a standard fish anesthetic) (Argent Laboratories Inc., Redmond, WA, USA). Because sperm cannot be stripped from channel catfish [6], testes were removed surgically and stored in Hanks' balanced salt solution (HBSS) [7] at 4 °C. All testes were dissociated individually by crushing in HBSS within 6 h after removal. Sperm were rinsed through a 0.5-mm nylon screen (Aquatic Eco-Systems Inc., Apopka, FL, USA) to remove tissue particles, were suspended at 1 g of testis per 20 mL of HBSS, placed in plastic beakers covered with paraffin film, and refrigerated at 4 °C. To ensure that osmotic pressure was sufficient to prevent sperm activation during storage, the final osmolality of extender solutions was adjusted to 295 mOsmol/kg [16] as measured with a vapor pressure osmometer (model 5500, Wescor Corp., Logan, UT, USA).

Motility of each sample was estimated immediately after suspension of sperm and only samples with motility $\geq$50% were used. Motility was estimated by placing 5 μL of each sample on a microscope slide, activating with 50 μL of distilled water, immediately viewing at 100-X magnification with darkfield microscopy, and recording the percentage of visible motile cells. Motility was estimated before freezing (pre-freeze) and within 5 min after thawing (post-thaw).

2.1. Freezing and thawing procedures

Cryoprotectants were diluted 1:1 with HBSS prior to addition to samples, yielding the desired (reported) final concentrations. French straws (IMV International Corp.,

Minneapolis, MN, USA) were plugged with standard PVC powder (solidified by immersion in HBSS), were allowed to equilibrate to the cryoprotectant, and were placed into a controlled-rate freezer (Planer Kryo 10, Middlesex, England). In all studies except the cooling rate study, the temperature was held at 4 °C for 10 min, lowered to −80 °C at 45 °C/min, and held at −80 °C for 10 min. Samples were removed from the freezer and plunged into liquid nitrogen for storage. Straws remained frozen for at least 24 h prior to thawing. Equilibration times were set to yield a cryoprotectant exposure time of 1 h including handling and hold time in the freezer, except in the studies examining the effect of extended exposure.

In all studies except the thawing study, straws were removed from liquid nitrogen and plunged immediately into a water bath at 40 °C for 5 s for thawing. Straws were dried, and clipped with scissors to release spermatozoa into 1.8 mL microcentrifuge tubes.

2.2. Comparison of exposure times in 5% methanol, and straw sizes

Exposure times of 1 or 48 h, and straw sizes of 0.25 or 0.5 mL were compared in a 2 × 2 factorial arrangement for sperm (from each of five males) suspended in HBSS containing 5% methanol (Fisher Chemical Co., Fair Lawn, NJ, USA). Paired samples were suspended in HBSS or HBSS containing 5% methanol (v/v). After 48 h, sperm suspensions were removed from 4 °C storage and 2 mL samples were distributed into plastic Petri dishes. Methanol was added to those samples that did not initially contain methanol to bring the final concentration to 5%. Samples were drawn into 0.5 or 0.25 mL straws, and allowed to equilibrate for 50 min prior to freezing.

2.3. Comparison of extended exposure times in 5% methanol

The first exposure-time study indicated that exposure to 5% methanol for 48 h did not have a negative effect on cryopreservation. Based on those results and also on previous work indicating an antibacterial effect of 5% methanol during refrigerated storage [17], we chose to examine the effects on cryopreservation of a 5-day exposure to 5% methanol. Exposure times of 1 h or 5 days were compared for sperm (from each of four males) suspended in HBSS containing 5% methanol. Sperm suspensions were removed from storage at 4 °C and methanol was added to each to bring the final concentration to 5%. Samples were drawn into 0.5 mL straws, and allowed to equilibrate for 50 min prior to freezing. The remainder of each sample (now containing 5% methanol) was placed in 4 °C storage for 5 days, after which samples were drawn into 0.5 mL straws prior to freezing.

2.4. Comparison of fast and slow cooling rates

Cooling rates of 45 and 3 °C/min were compared for sperm (from each of four males) suspended in HBSS containing 5% methanol. Sperm suspensions were removed from storage at 4 °C and methanol was added to each to bring the final concentration to 5%. Samples were drawn into 0.5 mL straws, and allowed to equilibrate for 50 min prior to placement in the controlled-rate freezer. Temperature was held at 4 °C for 10 min, lowered

at 45 °C/min (fast) or 3 °C/min (slow) to −80 °C, and held for 10 min until samples were plunged into liquid nitrogen.

2.5. Comparison of thawing temperatures and durations

Thawing temperatures of 30, 40, or 50 °C, and thawing durations of 5 or 10 s were compared for sperm cryopreserved in HBSS containing 5% methanol. Sperm suspensions (from each of four males) were removed from storage at 4 °C, and methanol was added to each to bring the final concentration to 5%. Samples were drawn into 0.5 mL straws, and allowed to equilibrate for 50 min prior to placement in the freezer. For thawing, straws were removed from liquid nitrogen and plunged immediately into a water bath at the respective temperature and duration.

2.6. Variation in cryopreserved sperm of 50 males

Sperm from each of 50 males were suspended in HBSS. Sperm suspensions were removed from storage at 4 °C, and methanol was added to bring the final concentration of paired samples to 5 or 10%. Samples were drawn into 0.5 mL straws, and allowed to equilibrate for 50 min prior to placement in the freezer.

2.7. Statistical analyses

Statistical analyses were performed using SAS Statistical Analysis Software for IBM (SAS Institute, Cary, NC, USA). Values for percent motility were arcsine square-root transformed prior to analysis. Motility reduction was calculated by the following formula:

1 − (post-thaw motility/pre-freeze motility) × 100 = percent reduction.

Exposure time (1 or 48 h) and straw size (0.25 or 0.5 mL) were analyzed by Analysis of Variance (ANOVA) in a 2 × 2 factorial arrangement including all interactions. Thawing temperature (30, 40, or 50 °C) and thawing duration (5 or 10 s) were analyzed by ANOVA in a 3 × 2 factorial arrangement including all interactions. Specific differences among treatment groups were analyzed by the Fisher least significant difference (LSD) test. In the study of male-to-male variation, regression analysis was performed comparing pre-freeze motility and post-thaw motility. Significance was indicated by $P < 0.05$. For regression analysis, an r^2 value > 0.95 was considered significant.

3. Results

3.1. Comparison of exposure times in 5% methanol, and straw sizes

Post-thaw motility was estimated for channel catfish sperm cryopreserved in 0.5 and 0.25 mL straws after 1 or 48 h exposure to 5% methanol. No difference was found between the motility reduction of samples exposed for the two times ($P = 0.766$), and no difference was found in motility reduction between 0.5 and 0.25 mL straws ($P = 0.892$). There was no interaction between exposure time and straw size ($P = 0.809$) (Table 1).

Table 1
Post-thaw motility[a] (mean ± S.D.) for channel catfish sperm cryopreserved after exposure for 1 or 48 h in HBSS containing 5% methanol

Straw size (mL)	Exposure time (h)	
	1	48
0.25	21 ± 12%	22 ± 19%
0.5	24 ± 18%	19 ± 16%

Exposure time and straw size (0.25 or 0.5 mL) were analyzed in a 2 × 2 factorial arrangement. No differences were found in any treatment group. Five males were used for replication.
[a] Pre-freeze motility was >70% in all samples.

3.2. Comparison of extended exposure times in 5% methanol

Post-thaw motility was estimated for channel catfish sperm cryopreserved in 0.5 mL straws after 1 h or 5-day exposure to 5% methanol. The motility reduction (mean ± S.D.) for cryopreserved sperm exposed to 5% methanol for 1 h was 33 ± 9% and for 5 days was 52 ± 15%. Although the mean for 1 h was lower than the mean for 5 days, there was no difference in motility reduction for 1 h or 5-day exposure ($P = 0.137$).

3.3. Comparison of cooling rates

Post-thaw motility was estimated for channel catfish sperm cryopreserved in 5% methanol at two cooling rates. The motility reduction (mean ± S.D.) for sperm cryopreserved at a cooling rate of 45 °C/min was 33 ± 9% and at a cooling rate of 3 °C/min was 83 ± 13%. A cooling rate of 45 °C/min resulted in lower motility reduction than did 3 °C/min ($P = 0.002$).

3.4. Comparison of thawing temperatures and durations

Post-thaw motility was estimated for channel catfish sperm cryopreserved in 5% methanol and thawed using temperatures of 30, 40, or 50 °C and durations of 5 or 10 s (Table 2). A thawing temperature of 50 °C resulted in lower motility reduction (25 ± 14%) than did 30 °C

Table 2
Post-thaw motility[*] (mean ± S.D.) for channel catfish sperm thawed at temperatures of 30, 40, or 50 °C, and durations of 5 or 10 s

Thawing temperature (°C)	Thawing duration (s)		Pooled (by temperature)
	5	10	
30	20 ± 4%[d]	31 ± 6%[bc]	26 ± 8%
40	21 ± 5%[cd]	39 ± 13%[ab]	30 ± 13%
50	46 ± 5%[a]	45 ± 6%[a]	46 ± 5%
Pooled (by duration)	29 ± 13%	38 ± 10%	–

Thawing temperature and duration were analyzed by ANOVA with a Duncan's multiple range test. A temperature and duration of 50 °C for 5 s, 50 °C for 10 s, and 40 °C for 10 s resulted in lower motility reduction ($P = 0.001$) than did all other combinations. Four males were used for replication. Values sharing superscript letters were not significantly different.
[*] Pre-freeze motility was >70% in all samples.

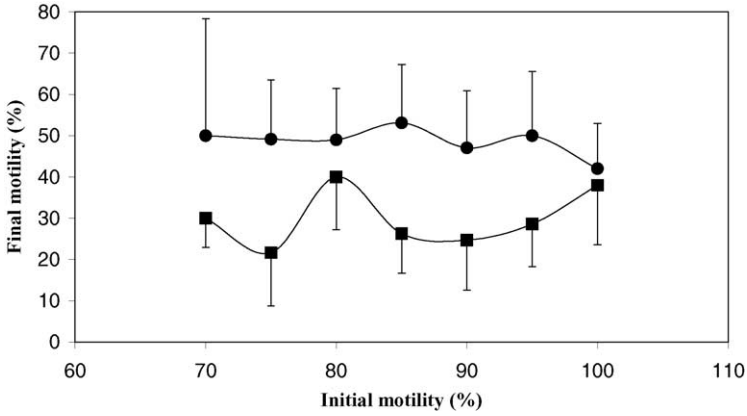

Fig. 1. Pre-freeze motility versus post-thaw motility of channel catfish sperm cryopreserved in HBSS containing 5% (circles) or 10% (squares) methanol. The use of 5% methanol resulted in lower motility reduction ($P = 0.001$) than did the use of 10% methanol. Each point represents the mean ± S.D.

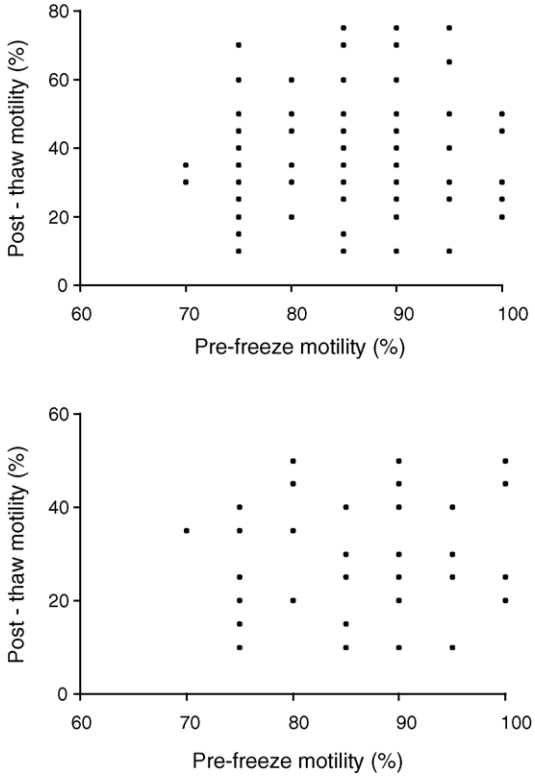

Fig. 2. Post-thaw motility versus pre-freeze motility of channel catfish sperm samples ($N = 50$) cryopreserved in HBSS containing 5% methanol (top chart) or 10% methanol (bottom chart). Regression analysis indicated no relationship between pre-freeze and post-thaw motility values for 5% methanol ($r^2 = 0.012$) or 10% methanol ($r^2 = 0.011$).

Table 3
Pre-freeze and post-thaw motility statistics for channel catfish sperm samples ($N = 50$) frozen in 5 or 10% methanol (MeOH)

Statistical parameter	Pre-freeze motility (%)	Post-thaw motility	
		5% MeOH	10% MeOH
Mean	87	48%	28%
Mode	90	50%	30%
Minimum–maximum	70–100	20–75%	10–50%
S.D.	8	13%	12%
r^2	–	0.0124	0.0105

Values for r^2 are presented from regression analysis of post-thaw versus pre-freeze motility.

(51 ± 21%) or 40 °C (59 ± 11%) ($P = 0.001$). A lower motility reduction was found missing a 10 s duration than with a 5 s duration ($P = 0.005$). An interaction was found between thawing temperature and duration ($P = 0.050$). Because an interaction was found, a non-factorial ANOVA with a Duncan's multiple range test was performed on six treatment groups. Thawing temperatures and durations of 50 °C for 5 s, 50 °C for 10 s, and 40 °C for 10 s resulted in lower motility reduction than did all other treatment groups ($P = 0.001$).

3.5. Variation in cryopreserved sperm of 50 males

A concentration of 5% methanol resulted in lower motility reduction than did 10% methanol ($P = 0.001$; Fig. 1). Post-thaw motility values ranged from 10 to 75% for 5% methanol and from 10 to 50% for 10% methanol (Table 3). Mean motility reduction values were 43 ± 17% for 5% methanol and 67 ± 14% for 10% methanol. Regression analysis showed no relationship between pre-freeze motility and post-thaw motility for 5% methanol ($r^2 = 0.012$; Fig. 2) or 10% methanol ($r^2 = 0.011$; Fig. 2).

4. Discussion

A previous study [17] found that 5% methanol increased the storage time of channel catfish sperm at 4 °C. In the present study, there was no difference in motility reduction of samples stored for as long as 5 days in 5% methanol prior to freezing, indicating that storage of sperm in 5% methanol was surprisingly non-toxic. Other research has shown that 10% or greater methanol can cause motility reduction in sperm of blue catfish, *Ictalurus furcatus*, within 30 min [15]. The use of 5% methanol as a standard ingredient in extender solutions (such as HBSS) for channel catfish sperm could prove beneficial by increasing refrigerated storage time while simultaneously preparing sperm for cryopreservation.

Cooling rates can affect success of cryopreservation of fish sperm. Best results for the cryopreservation of sperm of African sharptooth catfish, *Clarias gariepinus*, were obtained using a two-step cooling regime, including a cooling rate of 5 °C/min [5]. In the present study, a cooling rate of 45 °C/min yielded higher post-thaw motility than did 3 °C/min. The dairy bull industry uses a cooling rate of 16 °C/min [2,11], which shows

promise for use with catfish sperm [15]. The present research indicates a faster cooling rate results in less motility reduction, but there may be a plateau at which increasing the cooling rate further does not improve the motility reduction. If the plateau is reached at a cooling rate lower than 16 °C/min, the standardized dairy bull method of cryopreservation could prove to be the best method for large-scale cryopreservation of channel catfish sperm.

As with cooling rates, thawing rates are critical in the cryopreservation of sperm cells. We found that a thawing temperature of 50 °C with durations of 5 or 10 s or a temperature of 40 °C with a duration of 10 s performed best for channel catfish sperm. Successful production of hybrid catfish (channel catfish × blue catfish) was reported using sperm cryopreserved with dairy bull protocols in 0.5 mL straws and thawed at 40 °C for 7 s [15]. Thawing times and temperatures are dependent on a number of variables including sample volume and containers. For example, sperm of Mekong giant catfish, *Pangasianodon gigas*, cryopreserved in 1 mL ampoules resulted in a fertilization rate of only 12%, whereas sperm cryopreserved in artificial-insemination catheters produced a fertilization rate of 67% [18].

Earlier studies of channel catfish sperm reported no reduction in pre-freeze motility of sperm held in 5 or 10% methanol for <30 min [17]. A concentration of 10% methanol could be expected to perform better as a cryoprotectant than 5% methanol, although this was not true in the present study. One explanation would be that 10% methanol is toxic to channel catfish sperm. If so, minimizing the exposure time before freezing might result in higher observed motility.

Variation in the success rate of cryopreservation can be caused by variation in the response of sperm of individual males to the cryopreservation process. The final part of this study was to establish a basic understanding of male-to-male variation in channel catfish sperm. We cryopreserved sperm from 50 males in 5 or 10% methanol to study these concentrations with increased replication. We found on average an ∼60% retention of initial motility for cryopreservation of channel catfish sperm with 5% methanol. We found no relationship between pre-freeze motility and post-thaw motility of high quality (pre-freeze motility of 70–100%) sperm samples, indicating variation in the response of individual males, and disallowing prediction of post-thaw motility of sperm for individual males. Given the high initial sperm motility in channel catfish (often 90% or better), there is the potential for increasing the post-thaw motility as techniques are optimized further. This is especially relevant given that selective breeding has not been applied to improvement of male reproductive performance of catfish. Use of selective breeding could improve factors in male catfish such as motility, percent fertilization (dependent on egg quality), or possibly even cryopreservation performance.

Research is now being conducted on the use of commercial-scale dairy techniques for cryopreservation of fish sperm [14,15]. The use of dairy bull semen freezing techniques (involving cooling at 16 °C/min to −140 °C before plunging into liquid nitrogen) provides a consistent methodology for research and commercial storage of fish sperm. This technique has been employed for blue catfish sperm, but remains un-optimized for channel catfish sperm. Commercial-scale efforts for cryopreservation of catfish sperm will create greater demand for standardization of labeling, record-keeping, and sample transfer agreements.

Acknowledgements

This research was supported in part by funding from the Louisiana Catfish Promotion and Research Board and the United States Department of Agriculture. We thank Tony's Seafood of Baton Rouge for providing catfish, and M. Bates, M. Mayeaux, L. Pittman-Cooley, and W. Wayman for assistance in collection of samples. Approved by the Director of the Louisiana Agricultural Experiment Station as manuscript 03-11-1502.

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