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Refrigerated Storage of Channel Catfish Sperm

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Abstract.—Refrigerated storage of sperm is useful for genetic study and artificial breeding of fishes. Due to the potential loss of donor males, storage is important in species such as channel catfish *Ictalurus punctatus* from which sperm cannot be stripped. This study addresses short-term storage (at 4 C) of channel catfish sperm by evaluation of storage methods employed for other species and for cryopreservation of channel catfish sperm. The objectives were to evaluate: 1) storage of intact testes and storage of sperm suspended in an extender solution; 2) use of various storage containers with and without supplemental oxygen; 3) use of extender solution with and without the addition of an antibiotic/antimycotic cocktail; 4) use of extender solution with and without the addition of methanol; and 5) use of extender solution with and without the addition of glucose and methanol. Sperm suspended in extender solution retained motility significantly longer (9 d) than did sperm in intact testis (2 d). Sperm stored in Zip-loc® plastic bags inflated with pure oxygen retained motility significantly longer (12 d) than did sperm stored in Zip-loc® plastic bags without supplemental oxygen (7 d), or sperm stored in plastic beakers (8 d) or test tubes (8 d) without supplemental oxygen. Sperm stored with the addition of antibiotic/antimycotic cocktail or methanol retained motility significantly longer (10–12 d) than did sperm stored without additives (6–8 d). Sperm stored in extender solution without glucose retained motility significantly longer (19–21 d) than did sperm stored in extender with glucose (13–16 d). Motility was retained for as long as 21 d in sperm stored in extender solution with 5% methanol and without glucose. In each experiment, loss of motility was associated with bacterial growth.

Collection and storage of sperm is useful for genetic improvement through artificial breeding, cryopreservation of gametes from valuable strains or individuals, and production of hybrids (Munkittrick and Moccia 1984; Kurokura et al. 1986; Tave 1993). Collection and storage of sperm is possible in many species of fish, including several commercially important species (Morisawa et al. 1983a, 1983b). Sperm can be stored refrigerated for periods ranging from a few hours to several months, depending on the species (Scott and Baynes 1980). Storage of sperm can eliminate the need to keep males available for artificial fertilization and allows faster, easier handling of sperm when eggs are available.

Collection of sperm from some species requires surgical techniques that could involve death of the donor fish. Sperm cannot be stripped from channel catfish *Ictalurus punctatus* (Bart and Dunham 1990) so the testis is removed in whole or in part and dissociated to release sperm into buffered extender solutions (Guest et al. 1976; Tiersch et al. 1994). Methods to optimize sperm storage have been described in species such as rainbow trout *Oncorhynchus mykiss* (Billard 1981), paddlefish (Mims 1991), and Nile tilapia *Oreochromis nilotica* (Harvey and Kelley 1988). However, methods to optimize storage of channel catfish sperm, such as the use of antibiotics, have not been described previously.

The goal of this study was to extend the preliminary observations made for short-term storage of sperm of channel catfish (Guest et al. 1976). In this study, three extender solutions were compared weekly using a qualitative scoring system. For the present study, we quantified motility on a daily basis to evaluate factors found to be important in the storage of sperm of other species and in the cryopreservation of channel catfish sperm (e.g., container type, oxygenation, additives). The objectives were to evaluate: 1) storage of sperm in intact testis and storage of sperm suspended in buffer; 2) use of various sperm storage containers; 3) storage of sperm with supplemental oxygen; 4) storage of sperm with the addition of an antibiotic/antimycotic cock-

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tail; 5) storage of sperm with the addition of methanol; and 6) storage of sperm without a sugar source in the buffer solution. Because bacterial contamination has been associated with reduction of sperm viability (Stoss et al. 1978), we also monitored bacterial presence in sperm samples throughout each experiment.

Healthy, mature catfish (1.5–3 kg, 40–60 cm) were killed by overdose of MS-222 anaesthetic (Argent Laboratories, Inc., Redmond, Washington, USA). Whole testes were removed and placed in appropriate extender solutions until dissociation. Sperm from the anterior portion of the testis (Sneed and Clemens 1963) were used for analysis. Intact testes were refrigerated at 4 C in Hanks balanced salt solution (HBSS) (Tiersch et al. 1994) for comparison of sperm from intact testes to sperm suspension samples. Otherwise, all testes were dissociated within 6 h, and sperm were suspended at 1 g testis/20 mL HBSS or modified HBSS, placed in storage containers, and refrigerated at 4 C. All open-top storage containers (plastic beakers) were covered with parafin film. To insure that osmotic pressure was high enough to prevent activation of sperm during storage (Bates et al. 1996), the final osmolality of all solutions was adjusted to ~295 mOsm/kg as measured with a vapor pressure osmometer (Wescor model 5500, Logan, Utah, USA).

Evaluation of storage methods was done by comparison of sperm motility (the percentage of actively swimming sperm upon activation with water) throughout each experiment. Use of motility values provided a rapid, simple estimation of the quality of samples and allowed for experimentation to proceed outside of the spawning season.

Motility was estimated by placing 5 μL of sperm sample onto a microscope slide, activating with 50 μL of deionized water (18 mOsm/kg) and viewing at 100-× magnification with dark-field microscopy. Motility of each sample was estimated immediately after initial sperm preparation, and once daily until motility was no longer detected upon activation. Only sperm exhibiting vigorous forward movement were considered motile; sperm that were vibrating in place were not considered as motile. Qualitative scoring systems such as that used by Guest et al. (1976) emphasize the presence or absence of movement of any kind, whereas a system such as we used quantifies the relative proportion of actively swimming sperm.

Bacteria were identified as any microscopic organisms without tails that were observed moving actively in sperm samples, although no attempts were made to taxonomically classify bacteria in the sperm samples. Bacterial presence was considered to be significant when bacterial number in the sample was estimated to be equal to or larger than the number of sperm cells.

Storage of sperm in intact testes was compared to storage of sperm that was released from testes and suspended in HBSS. Testes were removed from each of ten fish and placed into two treatment groups (five testes each) in a randomized fashion. Testes from the first group were placed in HBSS in 50-mL polypropylene centrifuge tubes and refrigerated. Testes from the second group were dissociated to release sperm, which was suspended in HBSS, placed in 100-mL plastic beakers, and refrigerated. Each day, small pieces were removed from three locations on the intact testes, pooled, and dissociated for comparison of motility to that of sperm suspended in HBSS.

Three types of containers were chosen for comparison of sperm storage. Ten-mL samples of sperm suspensions from each of four fish were placed into 15-mL centrifuge tubes, 100-mL plastic beakers, quart-sized Zip-loc® plastic bags (Dow Chemical Co., Inc., Indianapolis, Indiana, USA), and Zip-loc® plastic bags inflated with pure oxygen. Samples were refrigerated and motility was monitored daily. The plastic bags containing supplemental oxygen were inflated each day immediately after motility estimates.

Because the use of antibiotics for storage of channel catfish sperm has not been de-

scribed previously, a multipurpose commercially prepared solution was selected that contained two antibiotics and an antimycotic (Product No. A-7292; Sigma Chemical Co., St. Louis, Missouri, USA). Concentrations were selected based on manufacturer's recommendation for cell culture. The antibiotic/antimycotic cocktail (A/AC) was formulated to contain 10,000 units penicillin, 10 mg streptomycin, and 25 μg amphotericin per mL in 0.9% sodium chloride solution when reconstituted with sterile water. Sperm samples stored with the addition of A/AC were compared to samples stored without addition of the A/AC. Twenty-mL samples of sperm suspensions from each of four fish were placed into quart-sized Zip-loc® plastic bags without A/AC, plastic bags containing 0.1% or 1% A/AC (v/v), plastic beakers without A/AC, or plastic beakers containing 1% A/AC. Samples were refrigerated and motility was monitored daily. All plastic bags were inflated with pure oxygen immediately after motility estimates.

Storage times of sperm suspended in HBSS were compared to storage times of sperm suspended in HBSS containing 5% methanol (a cryoprotectant used for freezing of catfish sperm). Fifty-mL samples of sperm suspensions from each of five fish were distributed into two treatment groups. Group one was suspended in normal HBSS and group two was suspended in HBSS containing 5% methanol. A diluted solution of 50% HBSS and 50% methanol was used for the addition of methanol to reduce acute toxicity. Samples were refrigerated and motility was monitored daily.

A factorial treatment arrangement was used to compare storage times of sperm suspended in HBSS, HBSS without glucose, HBSS with 5% methanol, and HBSS without glucose with 5% methanol. Testes were removed from each of five fish and cut into two sections. One section of each testis was suspended in normal HBSS, and the other section was suspended in HBSS without glucose. Methanol was added (5% by volume) to one half of each suspension yielding four treatment groups. Samples were refrigerated and motility was monitored every 2 d.

Statistical analysis was performed using SAS statistical analysis software for IBM® (SAS Institute, Cary, North Carolina, USA). Percent motilities were arcsine square-root transformed before analysis. Regression lines for each treatment group were calculated for motility versus time. Treatment groups were compared by multisource regression which compared the slopes and intercepts of each regression line to determine if a difference was present. Specific differences between treatment groups were identified using Fisher LSD test by comparison of the total number of days motility was retained for each treatment group. The level of significance was set at $P < 0.05$.

Sperm dissociated from the testes and suspended in HBSS retained motility significantly longer ($P = 0.001$) than did sperm left in intact testes. Sperm from intact testes retained motility for 2 d, while sperm dissociated from testes and suspended in HBSS retained motility for 9 d. Bacterial presence was evident after 2 d in intact testes and evident after 9 d in sperm suspensions.

Sperm suspensions in plastic bags inflated with pure oxygen retained motility significantly longer ($P = 0.001$) than did suspensions in other storage treatments (Fig. 1). Plastic beakers provided approximately the same storage times as plastic bags without supplemental oxygen, but plastic beakers provided easier handling. Bacterial presence was evident after 8 d in samples without supplemental oxygen and evident after 12 d in samples with supplemental oxygen.

Sperm suspensions in plastic bags and plastic beakers containing 1% A/AC retained motility significantly longer ($P = 0.001$) than did sperm suspensions in other treatments (Fig. 2). However, motility in samples containing 1% A/AC was significantly lower ($P = 0.009$) at day one com-

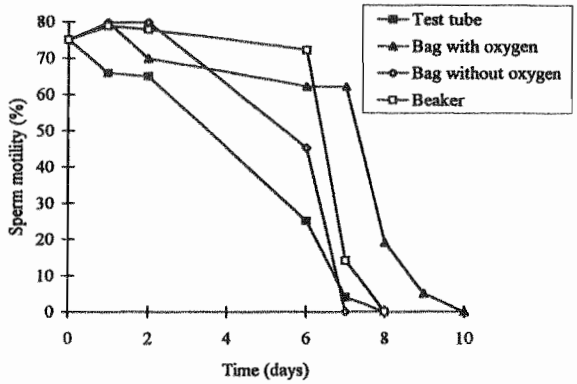

FIGURE 1. *Motility of channel catfish sperm suspensions during refrigerated storage in various containers, including storage with supplemental oxygen. Each point represents the mean of four fish.*

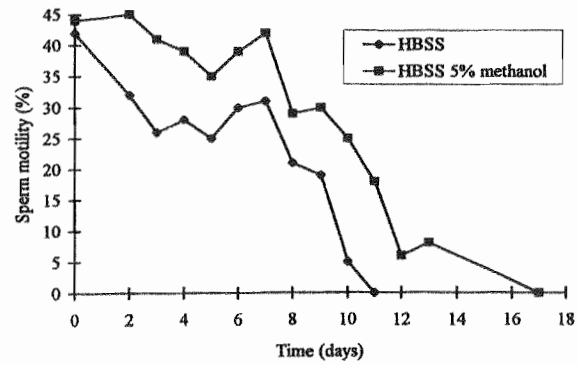

FIGURE 3. *Motility of channel catfish sperm suspensions with and without the addition of 5% methanol during refrigerated storage. Each point represents the mean of five fish.*

pared to other samples. Bacterial presence was evident after 6 d in samples without A/AC, evident after 6–8 d in samples with 0.1% A/AC, and evident after 10 d in samples with 1% A/AC.

Sperm suspended in buffer containing methanol retained motility significantly longer ($P = 0.027$) than did sperm in buffer without methanol (Fig. 3). Bacterial presence was evident after about 7 d in sperm without methanol and evident after 10 d in sperm containing methanol.

Sperm suspended in buffer containing no glucose retained motility significantly longer ($P = 0.039$) than did sperm in buffer containing glucose (Fig. 4). Storage time of sperm suspensions containing 5% methanol was not significantly different ($P = 0.080$) from that of suspensions without methanol. There was no significant interaction ($P = 0.808$) between the glucose and methanol.

This work extends findings made in an earlier study of the storage of channel catfish sperm (Guest et al. 1976). According to the scoring system used in that study, the sperm used in each of our experiments would have been scored initially at values of 3 to 5 (with 5 as the highest quality). The initial values for sperm quality in the study by Guest et al. (1976) were all below 1.5 (most sperm vibrating without forward movement). They continued observations on vibrating sperm (classified as non-motile

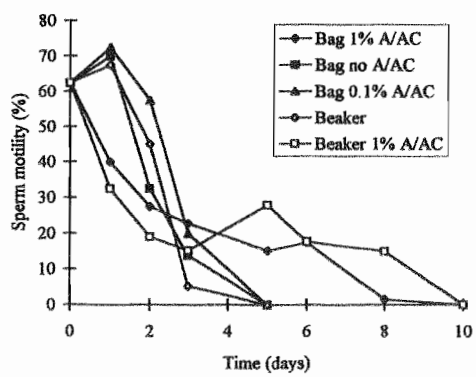

FIGURE 2. *Motility of channel catfish sperm suspensions with and without the addition of antibiotic/antimycotic cocktail during refrigerated storage. Concentrations of antibiotic/antimycotic cocktail were 1% and 0.1% in plastic bags and 1% in plastic beakers. Each point represents the mean of five fish.*

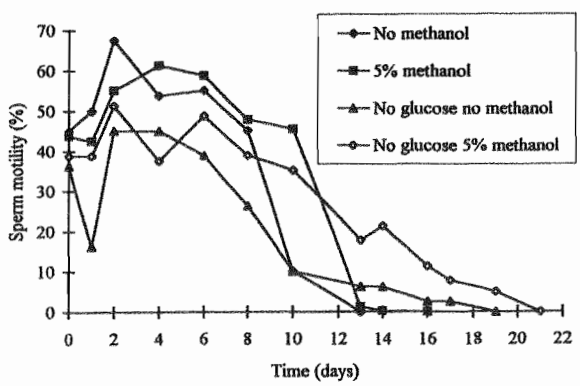

FIGURE 4. *Motility of channel catfish sperm suspended in Hanks' balanced salt solution (HBSS) prepared with and without glucose, and with and without 5% methanol. Each point represents the mean of four fish.*

in our system) for up to 9 wk (we discarded samples upon reaching a score of 1). While the fertilizing ability of sperm capable only of weak vibrating motion (without forward movement) remains undetermined, shorter storage time of more vigorous sperm samples would seem most useful in the hatchery.

Rapid dissociation of testis and suspension of sperm in extender increased the storage time of channel catfish sperm. Removal of the testis interrupts circulation of blood which impedes delivery of dissolved oxygen and nutrients and the removal of waste products. In turn, tissues surrounding the sperm cells break down, releasing waste products that can accumulate and cause destruction of sperm (Stoss et al. 1978). Suspension of sperm removes most of the testicular tissue, reducing the amount of waste being generated, and supplies sperm with nutrients and oxygen for metabolism, increasing storage time. Suspension of sperm should occur as soon as possible after surgical removal of testis to minimize loss of motility. Placing testes directly into chilled HBSS increases the safe storage time prior to sperm preparation (data not shown).

Containers used for storage of sperm in previous studies have included plastic beakers and plastic bags (Stoss et al. 1987; Mims 1991). These containers are disposable, easily labeled, and clean (although not sterile) before use. In our study, these containers were easy to use, and minimized contamination of samples. Plastic beakers provided better handling throughout all experiments, but they were not airtight and therefore prevented maintenance of high levels of oxygen. Accordingly, if increased storage time is desirable, plastic bags inflated with pure oxygen would seem the container of choice. If improved handling is desirable, plastic beakers would seem most useful.

In each experiment, a decrease in motility was associated with an increase in bacterial numbers. The addition of 1% A/AC extended the time before decreased motility was observed, but reduced motility on day 1, suggesting a toxic effect from the A/AC. Stoss et al. (1978) found a toxic effect with the use of penicillin and streptomycin in rainbow trout sperm. An antimycotic (amphotericin) was not included in that study, and antibiotic toxicity was observed at ~100 times greater dosages than those we used. This suggests that the toxic effect we observed could be a result of an interaction among the compounds in the A/AC, or from the amphotericin. Another explanation would be a higher susceptibility to toxicity from antibiotics or antimycotics in channel catfish sperm than in rainbow trout sperm. Further work is necessary to determine optimal conditions to limit growth of microorganisms in refrigerated channel catfish sperm.

In channel catfish (Tiersch et al. 1994) and in other species, such as zebrafish *Brachidanio rerio* (Harvey et al. 1982) methanol has been shown to be a more effective cryoprotectant for sperm than DMSO or glycerol. As a part of the study by Tiersch et al. (1994), refrigerated storage time was evaluated for channel catfish sperm suspended in HBSS containing methanol, although the use of methanol as a regular additive to extender solutions was not evaluated. Addition of methanol to extender solutions prior to suspension of sperm allows simultaneous storage and preparation for cryopreservation. In the two methanol experiments in the present study, one experiment showed a significant increase in storage time of samples prepared with methanol. The second experiment did not show a significant difference ($P = 0.080$), but did suggest a weak effect. Methanol did not affect the storage time negatively in either experiment. It was observed that increase in bacterial numbers was faster in samples without methanol, but the bactericidal action of methanol requires a concentration of 95% (Kolb et al. 1952). This suggests that methanol is acting either as a bacteriostatic agent or is in some way protecting the sperm cells from waste products or enzymes produced by bacteria. Further

study is necessary to determine the effects of methanol on sperm and on bacterial growth in sperm suspensions.

The factorial experiment testing extender solutions with and without glucose showed increased storage time in samples without glucose. This was associated with increased time before bacterial growth was evident. Sperm were not adversely affected by lack of glucose, but bacterial growth could have been slowed by lack of a carbohydrate. Samples without glucose retained motility the longest of any group in this study (up to 21 d).

Suggested procedures for sperm preparation and storage at 4 C include the aseptic removal and dissociation of testes, exposure of sperm to adequate oxygen, and use of fresh, sterile HBSS prepared without glucose and containing 5% methanol. Sterile buffers can be prepared by filter-sterilization. The osmotic pressure of the solution should be verified to ensure an osmolality of >270 mOsm/kg to prevent the activation of stored sperm cells (Bates et al. 1996). Stored sperm samples should be monitored daily and discarded when contaminated to lessen the possibility of transferring bacteria to other samples. It should be noted that the bacterial contaminants we observed were highly motile and similar in size to catfish sperm. Training of inexperienced personnel is suggested for estimation of sperm quality prior to use for fertilization.

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