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A Preliminary Bacteriological Study of Refrigerated Channel Catfish Sperm

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Abstract.—This study was designed to simulate conditions encountered routinely during refrigerated storage of channel catfish sperm. Sperm samples were stored at 4 C in non-sterile and sterile Hanks' balanced salt solution (HBSS). Non-sterile HBSS was prepared with distilled water stored for 2 wk in a plastic carboy prior to use. Observations were made on the frequency and abundance of bacteria in samples, and on changes in sperm motility and quality. Sperm samples stored in non-sterile HBSS had a complete loss of motility within 72 h. Samples maintained in sterile HBSS showed an initial decrease in motility between 48 and 72 h, and a complete loss of motility within 10 d. Quality of the sperm in each buffer decreased as motility decreased; morphologic changes and reduced motility of sperm were coincident with increased bacterial numbers. Bacteria were cultured on tryptic soy agar and *Pseudomonas* F agar (PFA) by spread-plating 10-μL aliquots from each sample onto bacteriologic media and incubating for 5 d. The dominant bacteria observed were members of the genus *Pseudomonas*, representing 67% of the total bacteria identified. The dominant pseudomonad (*Pseudomonas* sp.) cultured from sperm samples stored in sterile buffer produced caseinase, lecithinase, and was β-hemolytic, whereas the dominant bacteria (*P. putida*) cultured from samples stored in the non-sterile buffers did not. Highly motile pseudomonads, present in two samples stored in sterile buffer, colonized below the surface of the PFA media at 4 C. The attributes of the bacterial contaminants that likely contributed to the decrease in sperm quality were production of extracellular enzymes, consumption of oxygen, and a high level of motility. Potential sources of degradative bacteria were commensal flora of channel catfish and the water used in preparing the storage buffer.

Artificial spawning is used routinely for study and production of fishes. Standard methods include the collection and storage of gametes (Huner and Dupree 1984). Typically, eggs are collected from a ripe female, mixed with a sperm suspension, and fertilized by the addition of water or an activating solution. Storage of gametes prior to fertilization is useful in a number of techniques including polyploidization (Benfey 1989), hybridization (Goudie et al. 1993), and gynogenesis (Ihssen et al. 1990). Effective storage of gametes is also a prerequisite for the establishment of cryopreserved gene banks from improved strains or endangered wild stocks.

Problems with the collection and viability of gametes limit artificial spawning of channel catfish *Ictalurus punctatus*, the most important food fish cultured in the United States. Because the eggs rapidly become inviable after stripping, the time interval for fertilization is narrow. Sperm cannot be stripped from male channel catfish, and the testis must be removed by dissection. The testis is mechanically disrupted to collect sperm which are suspended in buffers for storage (Tiersch et al. 1994). Thus, sperm samples are generally collected and stored in advance to ensure availability when female channel catfish spawn.

Catfish sperm can be stored refrigerated at 4 C (Christensen and Tiersch 1996) or cryopreserved (Guest et al. 1976; Tiersch et al. 1994), although in our experience refrigerated storage appears to be limited by bacterial growth. The presence of bacteria has

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been shown to decrease fertility rates of salmon sperm (Stoss and Refstie 1983) and motility and fertility of carp sperm (Saad et al. 1988); however, microbial contamination of fish sperm has received little attention. A few studies have addressed optimization of refrigerated storage of fish sperm by use of antibiotics but typically without bacteriologic analysis (Scott and Baynes 1980; Stoss 1983).

The present study was designed to simulate conditions encountered during the routine storage of channel catfish sperm. It represents a preliminary attempt to identify dominant bacterial contaminants and to evaluate changes in sperm motility and quality over time. The objectives were to compare sperm motility with the occurrence of bacteria during storage at 4 C and to identify the predominant bacteria found during storage.

Materials and Methods

Preparation of Sperm

Two experiments were conducted. In the first, conducted during July 1993, healthy, mature, 4-yr-old male channel catfish (2–2.5 kg) were killed and their testes removed. Using aseptic technique, adherent tissue was dissected away, and the testes were blotted dry and weighed. Three sperm samples were chosen to represent a range of motility values (i.e., initial motilities of 85%, 60%, and 15%) encountered commonly in sperm used for artificial spawning. Paired samples from the testis of each fish were placed into sterile or non-sterile conditions as follows. One gram of anterior testis (Sneed and Clemens 1963) from each fish was dissociated by crushing, and the spermatozoa suspended in 20 mL of Hanks' balanced salt solution (HBSS) (Tiersch et al. 1994) sterilized by passage through a 0.22-μm filter (Gelman Sciences, Ann Arbor, Michigan, USA). Another gram of testis was dissociated, and the sperm suspended in identical fashion in HBSS prepared with distilled water that had been stored in a plastic carboy for 2 wk prior to use. We chose to use aged, non-sterile water because the distilled water used to prepare buffers in fish hatcheries is often stored in a similar manner. The osmolality of each buffer (290 mOsm/kg) was measured by a vapor pressure osmometer (Model 5500, Wescor Corp., Logan, Utah, USA) to ensure sufficient osmotic pressure to prevent activation of sperm during storage (Bates et al. 1996).

In a second experiment, three sperm samples were collected aseptically and suspended in sterile HBSS as described above. These samples were collected outside of the spawning season (December 1994) and were assessed for sperm motility and the presence of bacteria, which were identified biochemically. The initial motility of the samples was 80%, 80%, and 5%.

Sperm Storage and Motility Estimates

The sperm suspensions in experiments one and two were stored refrigerated at 4 C in sterile 50-mL tissue culture flasks (Corning, Inc., Corning, New York, USA) with loosened caps. Samples were removed aseptically once every 24 h for 10 d to estimate sperm motility and to culture bacteria. To estimate the percentage of motile sperm in each sample, a 1-μL aliquot was added to 40 μL of distilled water on a microscope slide, and the mixtures were viewed immediately for 15 s using darkfield microscopy at 100×. A minimum of three motility estimates were made for each sample.

Bacterial Cultures

Because this was a preliminary bacterial survey, we chose to perform a large number of bacterial cultures (with repeated sampling, various media and two temperatures) and biochemical tests (over 40) on a small number of representative testis samples. We chose this design for two reasons: 1) the patterns of occurrence of bacterial contamination and sperm morphology described in this paper are representative of those observed by us in hundreds of channel catfish

sperm samples studied over the last 5 yr; and 2) future studies can focus on fewer specific bacteria and biochemical tests with larger numbers of catfish. Bacteria from the samples were cultured at 4 C and 24 C by spread-plating 10-μL aliquots onto each of two types of bacteriologic media: 1) tryptic soy agar (TSA) (Difco, Detroit, Michigan, USA), a general nutrient medium; and 2) *Pseudomonas* F agar (PFA) (Difco). The PFA medium enhances production of the pigment fluorescein which differentiates some pseudomonads. The plates were examined at 5 d (4 C incubation) or at 24 h (24 C), after maximal growth had occurred. Viable organisms were counted as colony-forming units (CFU). Selected colonies from TSA and PFA were subcultured on PFA for 24 h at 24 C before transfer to blood agar (TSA supplemented with 5% sheep blood, Carolina Biological Supply Company, Burlington, North Carolina, USA) and incubation for 24 h at 24 C. Bacteria were first identified by Gram staining; all Gram-negative bacteria were identified further by biochemical characterization (Palleroni 1984) using standard tube tests incubated at 24 C. Bacterial flagella were identified with a standard flagellar stain (Carr Scarborough Microbiologicals, Inc., Decatur, Georgia, USA).

Results

A complete loss of motility occurred within 72 h of storage in the samples stored in non-sterile HBSS (Fig. 1). In the samples maintained in sterile HBSS, motility began to decrease between 48 and 72 h, and complete loss of motility occurred within 10 d.

With sterile or non-sterile buffers, the quality of sperm samples decreased as motility decreased. Initially the samples were consistent liquid suspensions and sperm that were not motile appeared structurally intact. At as early as 2 d, the spermatozoa in non-sterile buffer began to clump and could not be collected with a micropipette (Fig. 2A); few motile bacteria could be observed under darkfield microscopy. Morphologic changes included entangled flagella and sperm heads without flagella. Complete loss of motility was associated with degenerated spermatozoa and the presence of a noticeable odor.

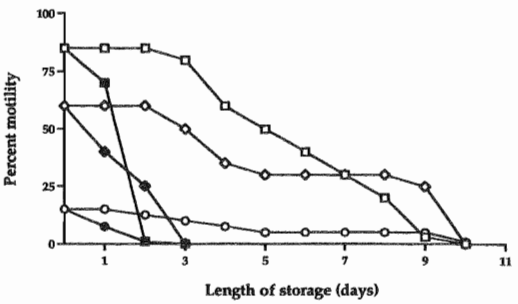

FIGURE 1. *Percent motility of channel catfish sperm stored at 4 C in sterile Hanks' balanced salt solution (HBSS) (open symbols), or in HBSS prepared with aged distilled water (closed symbols). Each point represents the mean value of three fish.*

Motility losses of sperm corresponded to the appearance of bacterial growth on TSA and PFA, and CFU were equivalent on each medium (>50 CFU at day 6). A high level of bacterial motility was indicated by colonies of pseudomonads from two of the samples (with initial motilities of 15% and 80%) that formed below the surface of the PFA when incubated at 4 C. When cultured at 24 C on PFA, these isolates did not colonize below the agar surface. Gram-positive streptococci and bacilli occurred at 0 d and 1 d in all sperm samples stored in non-sterile buffer. Because bacterial results obtained for incubation at 4 C were qualitatively identical to those at 24 C on both media, we chose to report only the biochemical results from the lower temperature which was most relevant to refrigerated storage (Table 1).

The dominant bacteria cultured from the samples in experiment one were members of the genus *Pseudomonas* (Fig. 2B) representing 67% (10 out of 15) of the total bacteria identified. Bacteria (and number of samples in which they occurred) that were isolated from the samples stored in sterile HBSS were: *Pseudomonas* spp. (3/10), *Pseudomonas paucimobilis* (1/10), *Pseu-*

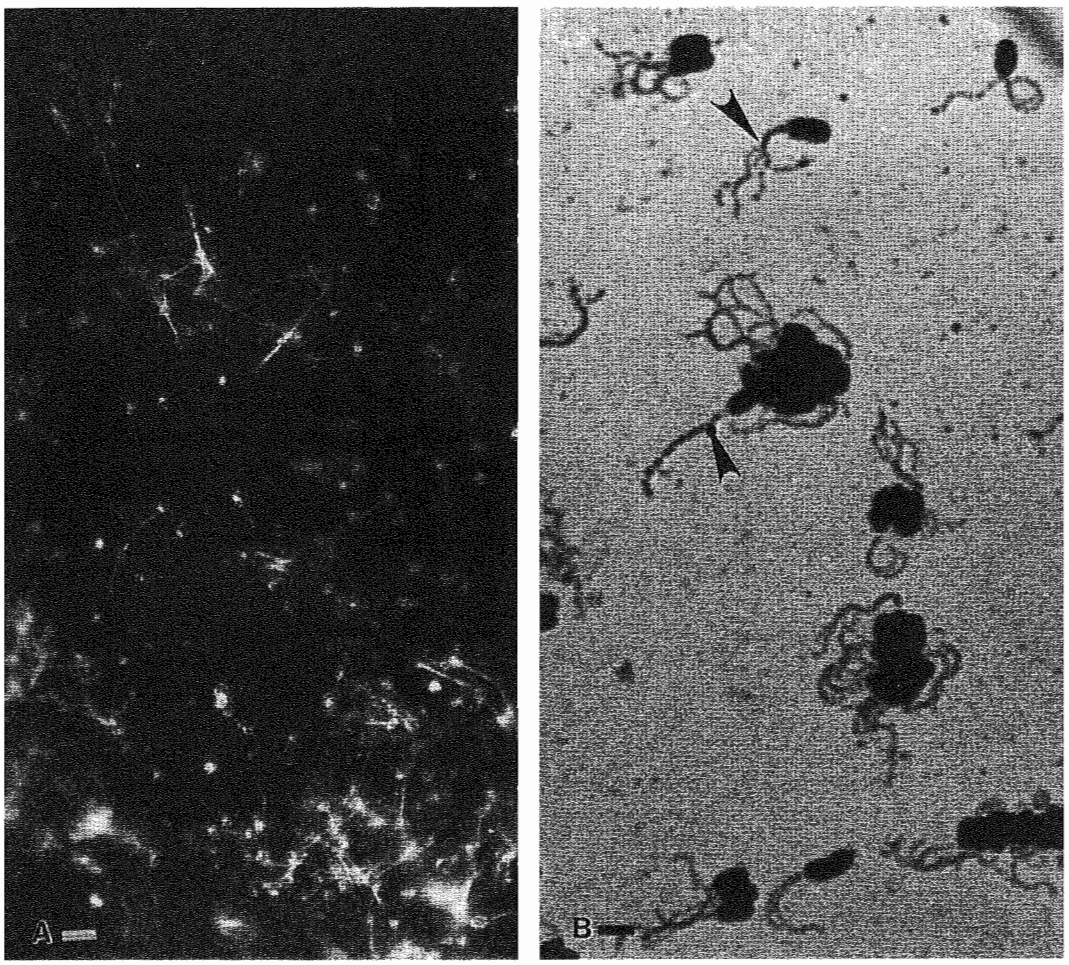

FIGURE 2. *Channel catfish sperm diluted in Hanks' balanced salt solution and stored for 2 d (A) viewed with darkfield microscopy (bar = 5 μm; total magnification = 576×). Members of the genus Pseudomonas (B) were the dominant bacteria found in refrigerated sperm samples viewed with brightfield microscopy (bar = 2 μm; total magnification = 1440×). Note the presence of bacterial flagella (arrowheads).*

domonas putida (1/10), *Pantoea agglomerans* (1/10), *Aeromonas hydrophila* (1/10), and *Klebsiella oxytoca* (1/10). The most frequently encountered pseudomonad cultured from these sperm samples produced proteases (Table 1). Bacteria (and number of samples in which they occurred) isolated from sperm samples stored in non-sterile HBSS were: *P. putida* (3/10) and *P. fluorescens* (1/10). In all cultures, pseudomonads were the most numerous microorganism present. *Pseudomonas* was the sole genus observed at day 10 on either medium.

Bacteria in experiment two (and number of samples in which they occurred) were: *Pseudomonas paucimobilis* (2/3) and *Pseudomonas maltophilia* (1/3). Two gram-positive, non-spore-forming rods and one strain of streptococci were present in 2 of 3 samples. No bacteria were cultured from the third sperm sample. By day 6, four times more CFU were present in the sample with 5% initial motility than in the samples with 80% initial motility (data not shown).

Discussion

Studies addressing sperm quality during storage have been conducted extensively in

TABLE 1. *Nutritional and physiological characteristics[a] of the dominant pseudomonads cultured from samples of channel catfish sperm stored at 4 C in sterile (S) and non-sterile (N) Hanks' balanced salt solution.*

Characteristic	S	N	Characteristic	S	N
Enzyme function			Carbohydrate utilization		
Arginine decarboxylase	+	+	xylose	−	+
Lysine decarboxylase	−	−	malonate	+	−
Ornithine decarboxylase	−	−	adonitol	−	−
Urease	+	+	arabinose	−	+
Nitrate reductase	+	+	cellibiose	−	−
Nitrite reductase	−	−	dextrose	+	+
β-galactosidase	−	−	dextrose (anaerobic)	−	−
Cytochrome oxidase	+	+	dulcitol	−	−
Lecithinase	+	−	galactose	−	+
Gelatinase	−	−	glycerol	−	−
Phenylalanine deamidase	−	−	inositol	−	−
Utilization of citrate	+	+	lactose	−	−
Esculin hydrolysis	−	−	levulose	−	−
Blood agar hemolysis	β	γ	maltose	−	−
Starch hydrolysis	−	−	mannitol	−	−
Casein hydrolysis	+	−	mannose	+	+
Other characteristics			melibiose	−	+
Motility	+	+	raffinose	−	−
Gram reaction	−	−	rhamnose	−	−
Fluorescein production	+	+	salicin	−	−
Hydrogen sulfide	−	+	sorbitol	−	−
Vogues-Proskauer	−	−	sucrose	+	+
Indole production	−	−	trehalose	−	−

[a] Results of biochemical tests: "+", positive or present; "−", negative or absent.

domesticated farm animals (Bartlett et al. 1976), but few are available for fish sperm (e.g., Sneed and Clemens 1956; Scott and Baynes 1980; Saad et al. 1988). No data are available on the bacteriology of stored catfish sperm. Bacterial contamination can alter the success of artificial spawning of fishes. Decreases in motility have been related directly to decreased fertilizing capacity (Stoss and Refstie 1983; Saad et al. 1988). In our study, the decreased motility of sperm stored at 4 C was associated directly with an increased prevalence of bacteria. Morphologic changes of catfish spermatozoa were observed during storage, similar to those identified for carp sperm stored at 4 C (Saad et al. 1988).

The initial differences in motility of sperm samples could be attributed to male-to-male variability. Alternatively, initial motility could be influenced by microorganisms derived from commensal flora. Our results indicate that the flora of the testes of channel catfish is composed of organisms representing the genera *Pseudomonas*, *Pantoea*, *Aeromonas*, and *Klebsiella*. Some gram-positive rods and cocci were also present. Pseudomonads were indigenous to five of the six channel catfish samples examined, and they were the most numerous bacteria cultured from the samples. The *Pseudomonas* sp. derived from sperm samples stored in sterile HBSS produced proteolytic enzymes not produced by the *Pseudomonas putida* obtained from the samples stored in non-sterile HBSS. Organisms producing proteolytic enzymes are more likely to invade tissues (Gilardi 1985). Upon death and rupture of spermatozoa, the proteins released could provide nutrients for bacteria, and proteases could degrade other spermatozoa.

The use of the non-sterile aged distilled water in HBSS could have introduced *Pseu-*

domonas putida as a buffer contaminant. Members of the genus *Pseudomonas* can originate from the natural environment because they are motile, free-living bacteria ubiquitous in aquatic habitats (Thune et al. 1993). Pseudomonads are non-fermentative, obligate aerobes that can multiply rapidly in nutrient-rich and confined environments (Gilardi 1985). They can affect spermatozoa directly or compete for substrate in storage media. Oxygen consumption by these bacteria was implicated in reduced survival of carp sperm samples (Saad et al. 1988).

As opportunistic pathogens pseudomonads can employ high levels of motility as a virulence mechanism. McManus et al. (1980) found that mutants of *Pseudomonas aeruginosa* displaying a loss of spreading in soft agar had diminished virulence in sepsis of burn wounds. In our study, the migration of *Pseudomonas* spp. below the surface of PFA indicated a high level of motility at 4 C. The motility displayed at this temperature likely contributed to bacterial invasiveness and subsequent colonization of refrigerated sperm samples. It should also be noted that given the high level of motility of *Pseudomonas* and their similarity in size to catfish sperm (Fig. 2) inexperienced hatchery personnel could mistake bacterial motility for sperm motility. Use of poor quality sperm samples for fertilization could result in the loss of valuable eggs.

Multiplication of microorganisms may be suppressed by refrigeration, but it is not stopped, and microorganisms are not necessarily killed by freezing. For example, *Pseudomonas aeruginosa* which produces exotoxin A has been associated with low fertility in bulls and can be transmitted in frozen semen (Getty and Ellis 1967). To control infectious agents that might be transmissible by sperm, the addition of antibiotics is common in breeding practices with farm animals (Hafez 1993). With regard to fishes, this practice has been studied most in salmonids (Stoss 1983) and has increased storage time of refrigerated channel catfish sperm (Christensen and Tiersch 1996). Cryopreservation of sperm has become an accepted technique for selective breeding and genetic improvement in livestock industries. This technology is being developed rapidly for use in aquaculture species and would enable widespread distribution and exchange of sperm samples and, potentially, of disease organisms. Cryopreservation of sperm is especially valuable for channel catfish because sperm cannot be stripped, and it is necessary to remove the testis or kill valuable broodstock to collect sperm. In addition, gene banks are needed for protecting endangered fish species and for producing improved stocks for aquaculture. Therefore, research is required to identify non-spermicidal antibiotics that will control the microorganisms that occur in fish sperm.

This study represents the first bacteriological evaluation of catfish sperm samples and one of the few for fish sperm in general. With increased numbers of bacteria, the motility, quality, and viability of the channel catfish sperm decreased. Morphologic changes and reduced motility of sperm were coincident with increased bacterial numbers. The characteristics of the bacteria that likely contributed to reduced sperm quality were production of proteolytic enzymes and metabolic by-products, consumption of oxygen, and a high level of motility. Protocols for the collection and storage of sperm free from bacteria would minimize vertical and horizontal transmission of microorganisms, and improve fertilization success in the intensive culture conditions used in hatcheries. By defining microbial populations, antibiotics can be selected to test against target species of bacteria. Because incubation at 4 C and 24 C yielded identical bacterial results in this study, it would seem most useful to incubate cultures at 24 C to speed diagnosis of contaminants in refrigerated sperm samples. The availability of these baseline data allows development of improved gamete stor-

age techniques, such as cryopreservation, and benefits artificial breeding programs.

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