# FLOW CYTOMETRIC EVALUATION OF ANTIBIOTIC EFFECTS ON VIABILITY AND MITOCHONDRIAL FUNCTION OF REFRIGERATED SPERMATOZOA OF NILE TILAPIA

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## ABSTRACT

Improved techniques for storage and evaluation of fish sperm would enhance breeding programs around the world. The goal of this study was to test the effect of antibiotics on refrigerated sperm from Nile tilapia (Oreochromis niloticus) by use of flow cytometry with 2 dual-staining protocols for objective assessment of sperm quality. Concentrations of $1 \times 10^9$ sperm/mL were suspended in Ringer's buffer at 318 mOsmol/kg (pH 8.0). The fluorescent stains Sybr 14 (10 μM), propidium iodide (2.4 mM), and rhodamine 123 (0.13 μM) were used to assess cell viability and mitochondrial function. Three concentrations of ampicillin, gentamicin, and an antibiotic/antimycotic solution were added to fresh spermatozoa. Motility estimates and flow cytometry measurements were made daily during 7 d of refrigerated storage (4 °C). The highest concentrations of gentamicin and antibiotic/antimycotic and all 3 concentrations of ampicillin significantly reduced sperm viability. The highest of each of the 3 antibiotic concentrations significantly reduced mitochondrial function. This study demonstrates that objective sperm quality assessments can be made using flow cytometry and that addition of antibiotics at appropriate concentrations can lengthen refrigerated storage time for tilapia spermatozoa. With minor modifications, these protocols can be adapted for use with sperm from other species and with other tissue types.
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Key words: tilapia, spermatozoa, antibiotics, flow cytometry, motility

## INTRODUCTION

Stored gametes from aquatic species can be used to maintain genetic lines, to aid recovery of depleted populations, and to maximize aquaculture production. However, the presence of microorganisms in gamete samples provides the potential for transfer of disease agents from wild

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and hatchery sources. In addition to the threat of disease transfer, the presence of microorganisms in stored samples can jeopardize valuable germplasm by decreasing fertilizing capability and by lowering cell quality and viability (12).

Although antibiotics are typically added to gametes (human, livestock) to extend storage time and to decrease microbial transfer (3), their application has not been systematically investigated for use with aquatic species (12). Three different methods have been traditionally used to estimate quality and predict fertilizing ability of fish sperm: estimation of motility, evaluation of sperm density, and analysis of seminal plasma (11, 12). Of these methods, motility is the most commonly used parameter to evaluate sperm quality. Sperm motility is the result of cell activation by ion exchange through the cell membrane. However, estimation of motility is typically subjective and is not always a good predictor of fertilization rates (5).

Alternative methods for evaluation of sperm quality using flow cytometry to estimate such characteristics as membrane integrity and mitochondrial function have been developed for sperm of bull (9), turkey (4), and rainbow trout (Oncorhynchus mykiss) (17, 18). This method is only beginning to be applied to fish species and has never been applied to the evaluation of antibiotic effects on fish spermatozoa. The methodology allows for the quantitative evaluation of viability and mitochondrial function of fish spermatozoa with a combination of fluorescent dyes and proper flow cytometry protocols. Our objectives were to 1) develop protocols by generating standard curves with known proportions of viable and nonviable cells for flow cytometric evaluation of sperm viability and mitochondrial function, and 2) evaluate the utility of flow cytometry to monitor the influence of antibiotics on the viability and mitochondrial function of sperm from Nile tilapia (Oreochromis niloticus) during refrigerated storage.

## MATERIALS AND METHODS

Fifteen adult male Nile tilapia (average weight, 400 g; average age, 1 yr) were obtained from Tiltech Aquafarm in Robert, Louisiana, and were maintained at Louisiana State University, Aquaculture Research Station in Baton Rouge. The fish were placed individually in 38-L tanks in a freshwater recirculating system, fed a 32% protein diet for 3 wk prior to sperm collection, and were exposed to a light:dark photoperiod of 10:14 h.

To collect spermatozoa, the genital papillae were dried with a paper towel to avoid activation or contamination with water or feces. Sperm were collected in a 3-mL syringe while gentle pressure was applied to the abdominal area. The samples were transferred to 5-mL tubes, and 5 mL of Ringer's buffer (318 mOsmol/kg, pH 8.0) (19) were added to dilute sperm without sperm activation. Motility was estimated as described by Jenkins and Tiersch (13) before and after the addition of antibiotics and was measured daily for 7 d.

To generate a standard curve for known ratios of viable and nonviable spermatozoa, a 2-mL sample from each fish of diluted sperm with motilities of 95% at time of collection (final concentration of $1 \times 10^9$ cells/mL) was heated to 70 °C for 5 min to produce nonviable spermatozoa. The exact physiological status of these spermatozoa is difficult to determine, but we will refer to these heat-treated sperm cells as being "nonviable" compared with the untreated fresh sperm cells which will be referred to as "viable." Viable and nonviable spermatozoa were

combined in ratios of 100:0, 75:25, 50:50, 25:75, and 0:100. The mixtures were analyzed with a flow cytometer (FACSCalibur®, Becton Dickinson Immunocytometry Systems (BDIS), San Jose, CA) equipped with an air-cooled, 480-nm argon laser. To calibrate instrument settings, we used FACSComp® software (BDIS, San Jose, CA).

The sperm mixtures were dual-stained with fluorescent dyes (Live/Dead Sperm Viability, Molecular Probes, Eugene, Oregon). Sybr 14 (10 μM) and propidium iodide (PI) (2.4 mM) were used to determine sperm viability; rhodamine 123 (R 123) (0.13 μM) and PI (2.4 mM) were used to evaluate mitochondria functionality. To estimate sperm viability, 1 μL of Sybr 14 was added to a 1-mL sample and incubated for 10 min, and 5 μL of PI were added and the sample was incubated for an additional 10 min prior to analysis. To estimate mitochondrial function, 5 μL of R 123 were added to a 1-mL sample and incubated for 10 min, and 5 μL of PI were added with an additional 10 min incubation prior to analysis. A standard curve was generated for sperm viability, and another was generated for mitochondrial function. A total of 10,000 sperm cells were analyzed per sample daily for 7 d using forward-scatter and side-scatter profiles. For Sybr 14 and PI, density plots using green fluorescence (viable spermatozoa) and red fluorescence (nonviable spermatozoa) were used to assess sperm viability. For R 123 and PI, density plots using green fluorescence (functional mitochondria) and orange fluorescence (nonviable spermatozoa) were used to assess mitochondrial function. Data were analyzed using Cell Quest® Software (BDIS, San Jose, CA).

Three concentrations (Table 1) of ampicillin, gentamicin, and antibiotic/antimycotic solution (Sigma Chemical Company, St. Louis, MO) were used to study the effects of antibiotics on spermatozoa during refrigerated storage. The solutions were added to samples to obtain a final volume of 5 mL with $1x10^9$ cells/mL. Each concentration of the antibiotics plus a control treatment without antibiotic (Ringer's Buffer) was replicated thrice and stored in a

Table 1. Type and concentration of antibiotics used for storage of Nile tilapia spermatozoa for 7 days at 4 °C

| Antibiotic | Concentration | | |
|---|---|---|---|
| | High | Medium | Low |
| Gentamicin (μg/mL) | 750 | 500 | 250 |
| Ampicillin (μg/mL) | 750 | 500 | 250 |
| Antibiotic/Antimycotic | 50% | 20% | 10% |
| Penicillin (units) | 50 | 10 | 5 |
| Streptomycin (mg/mL) | 0.05 | 0.01 | 0.005 |
| Amphotericin (g/mL) | $1.25x10^{-3}$ | $6.25x10^{-3}$ | $1.25x10^{-3}$ |

refrigerator at 4 °C for 7 d. Motility and flow cytometry evaluation of sperm viability and mitochondrial function were performed daily. Data were arcsine-transformed prior to statistical analysis. A simple linear regression (21) was used to determine the correlation between viable and nonviable spermatozoa in the assessment of sperm viability and mitochondrial function, and 2 regression curves were generated. Results from the antibiotic treatments were analyzed by multivariate ANOVA (21), and treatment differences were determined by the Student-Newman-Keul's multiple range test (21). A linear regression (21) was used to determine the correlation between motility and sperm viability, and between motility and mitochondrial function.

## RESULTS

The data obtained by flow cytometric analysis of sperm viability and mitochondrial function were used to generate regression curves (Figure 1). The regression lines generated for each fluorescent dye combination were $r^2 = 0.9439$ for Sybr 14 plus PI, and $r^2 = 0.8908$ for R 123 plus PI. The proportions of viable and nonviable sperm based on mitochondrial function and viability as determined by flow cytometry were within 10% of the values predicted with the exception of the 100:0 mixture which yielded values around 80:20 for viable and nonviable sperm (Table 2).

Table 2. Proportions of viable sperm (mean ± SD) estimated by mitochondrial function or sperm viability analyzed by flow cytometry using rhodamine 123 and propidium iodide or Sybr 14 and PI dual-staining combinations

| Viable : Nonviable[a] | Sybr 14 (% viable) | rhodamine 123 (% viable) |
|---|---|---|
| 100:0 | 82 ± 10 | 78 ± 14 |
| 75:25 | 68 ± 13 | 63 ± 15 |
| 50:50 | 46 ± 4 | 46 ± 11 |
| 25:75 | 27 ± 5 | 28 ± 4 |
| 0:100 | 3 ± 1 | 1 ± 1 |

[a] Known proportions of viable and nonviable spermatozoa were produced by mixing fresh sperm with sperm cells heated at 70 °C for 5 minutes from the same fish.

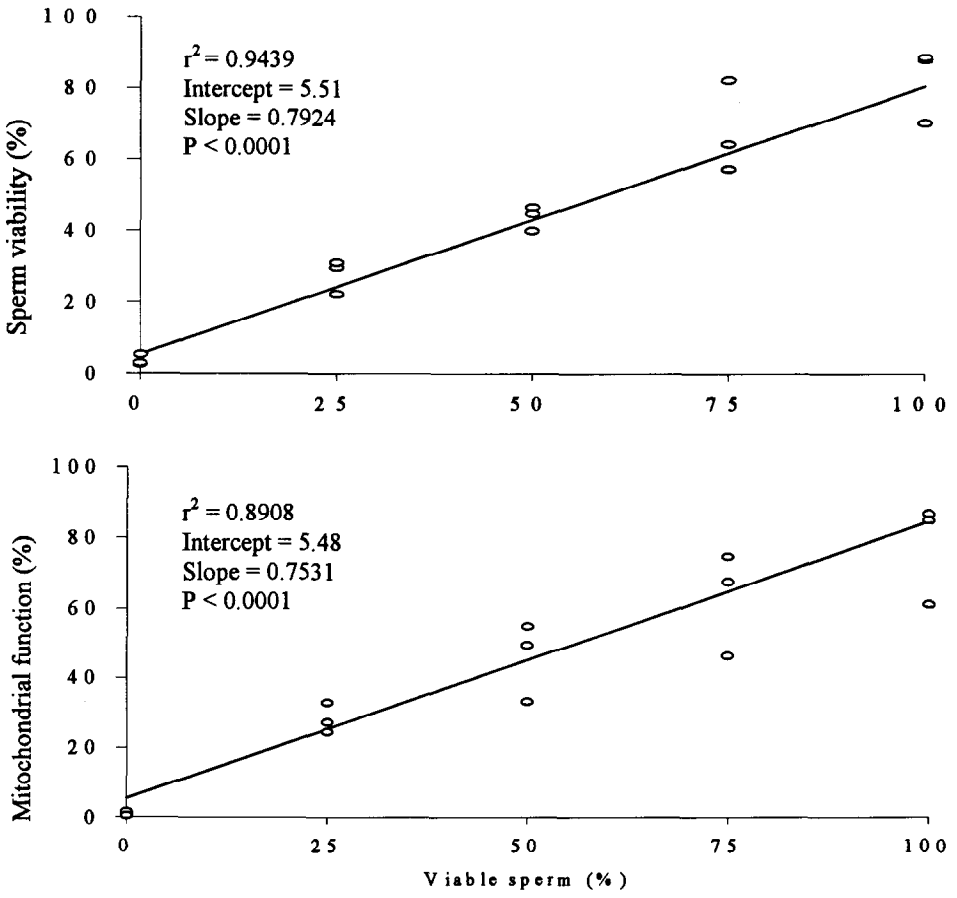


Figure 1. Linear regression of sperm viability estimated with Sybr14 plus propidium iodide for different percentages of viable Nile tilapia spermatozoa (top panel). Linear regression of mitochondrial function estimated by staining with rhodamine 123 plus PI for different percentages of viable tilapia spermatozoa (bottom panel).

During the 7 d of refrigerated storage there were no significant differences (P = 0.8500) in sperm motility among the 3 antibiotic treatments (Figure 2). There were significant differences in motility among the three different concentrations of each of the antibiotics (P = 0.0001), where the highest concentration of each antibiotic significantly reduced motility.

Sperm viability was not different among the 3 antibiotic treatments (P = 0.0668 ; Figure 3). Viability of tilapia sperm declined significantly during storage (P = 0.0001). The viability values of spermatozoa in the highest concentration of each antibiotic, all 3 concentrations of ampicillin and the control (no antibiotic), were significantly different from viability values from the medium and low concentrations of gentamicin and antibiotic/antimycotic (P = 0.0166). A weak correlation was found between sperm viability and motility (r = 0.3969).

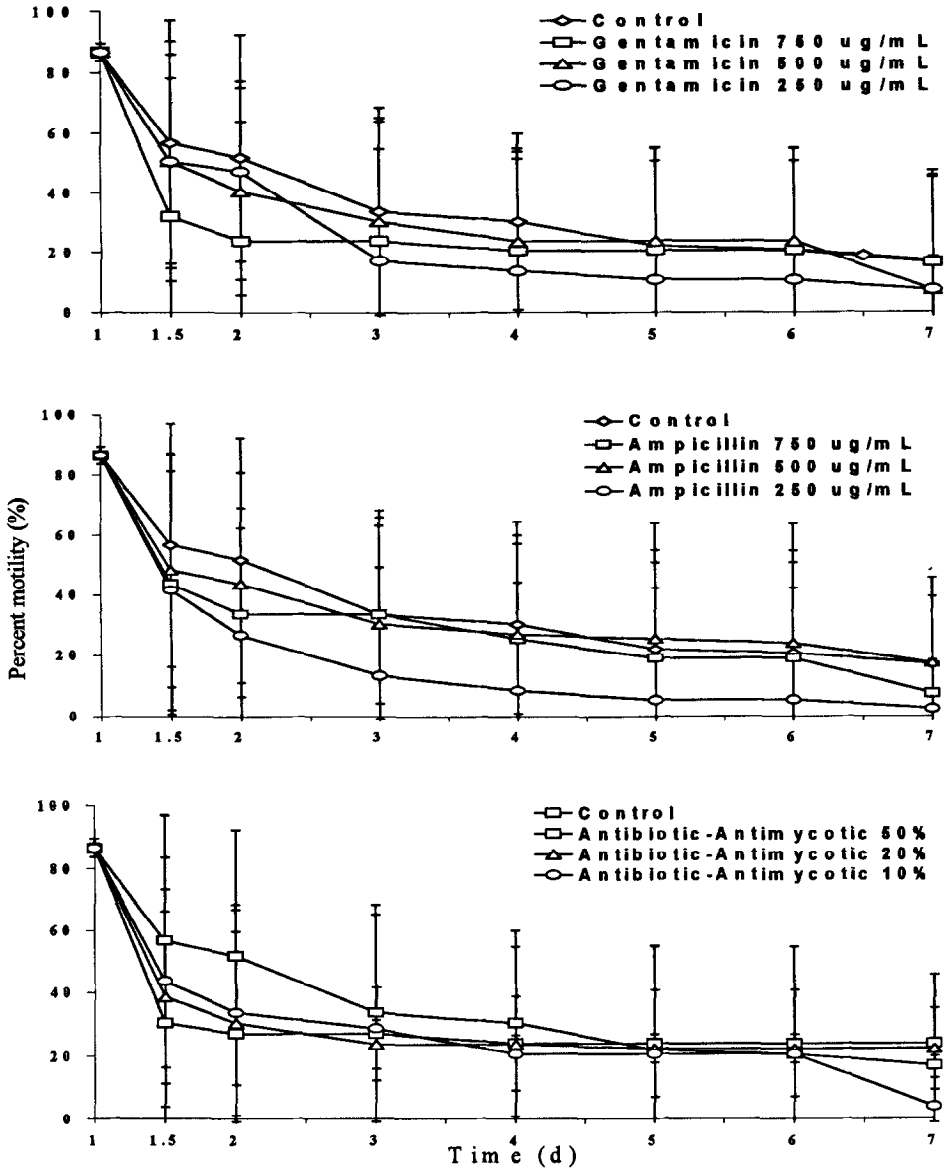


Figure 2. Estimation of percent motility (means ± SD) of Nile tilapia sperm stored in Ringer's buffer, without antibiotics (control), or with gentamicin (top panel), ampicillin (middle), or antibiotic/antimycotic (bottom) solution.

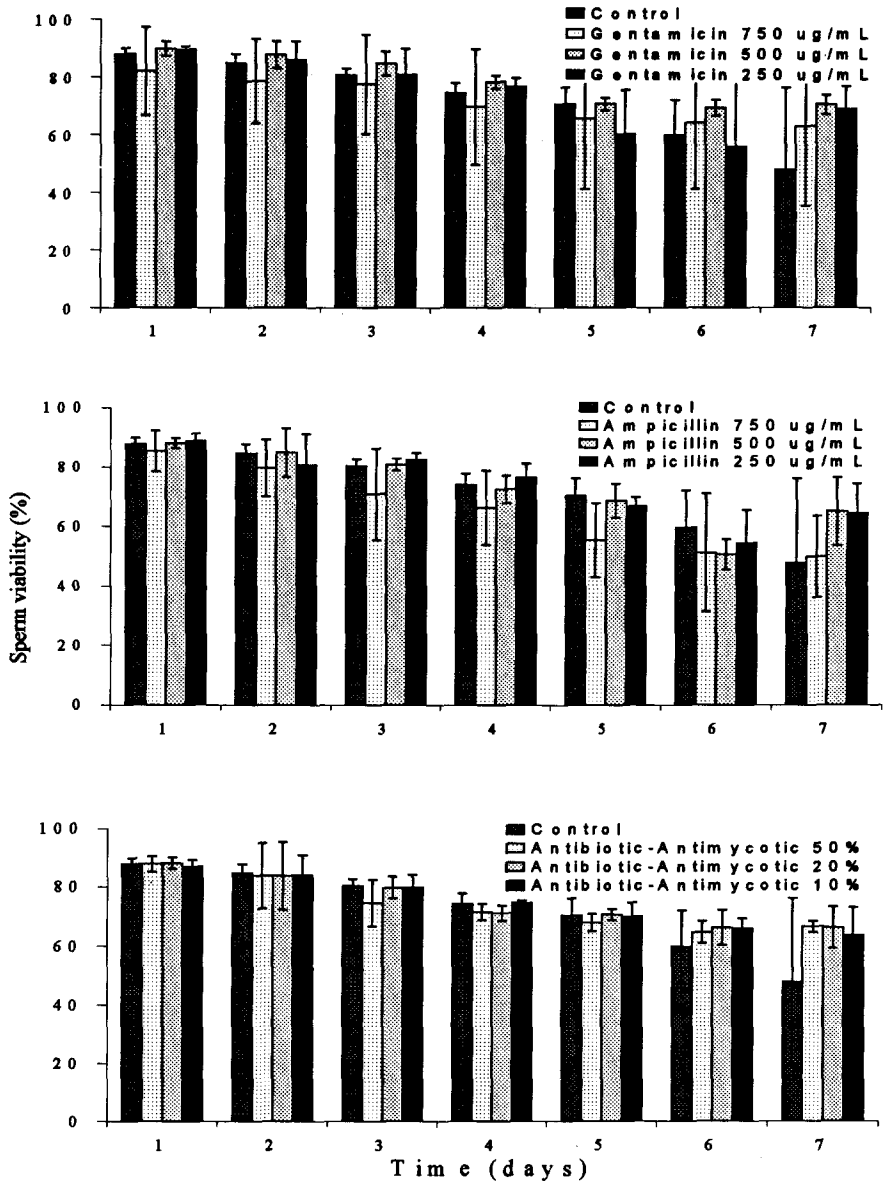


Figure 3. Viability (means ± SD) of Nile tilapia spermatozoa as determined by flow cytometry. Sperm cells were stored at 4 °C for 7 days without antibiotics (control) or with gentamicin (top panel), ampicillin (middle), or antibiotic/antimycotic (bottom).

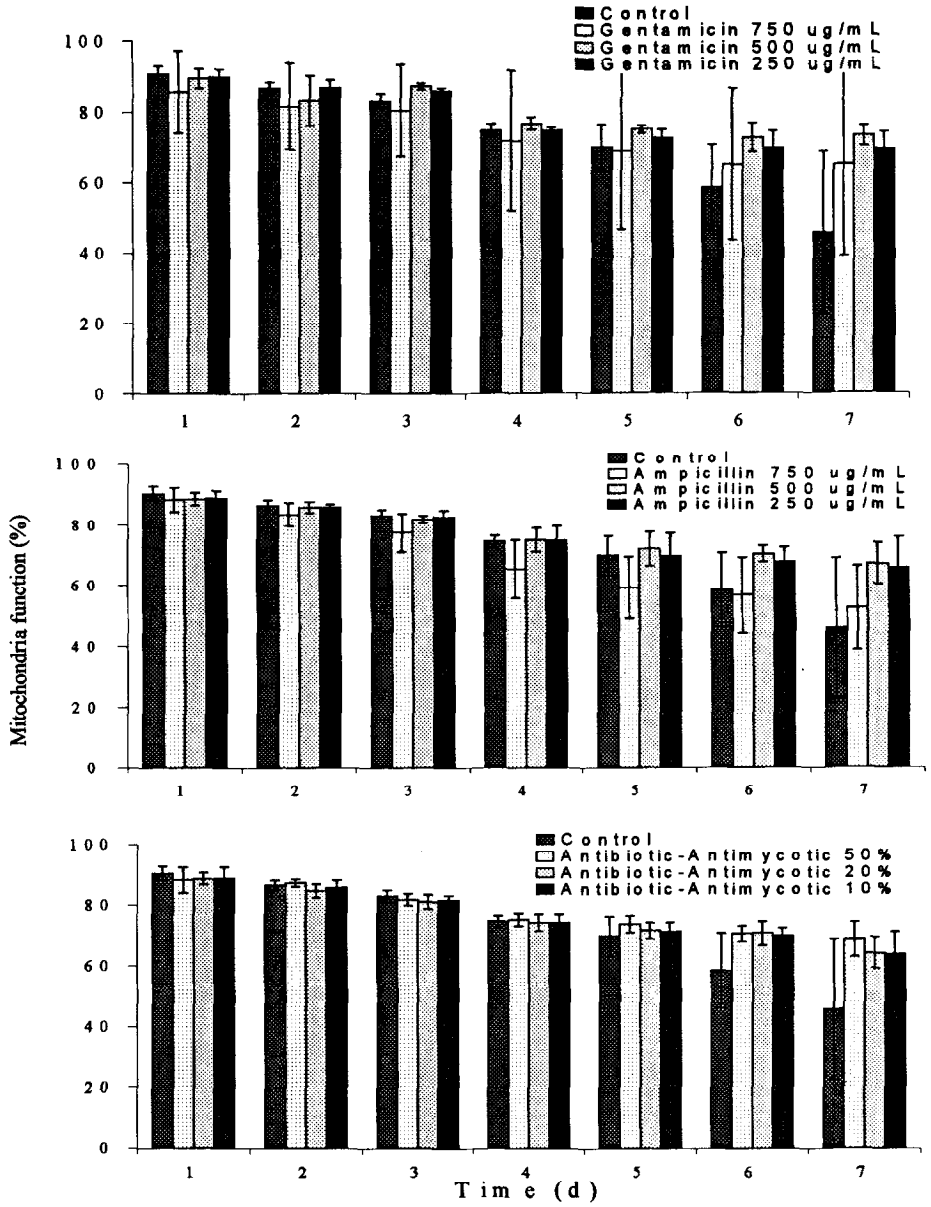


Figure 4. Mitochondrial function (mean ± SD) of Nile tilapia spermatozoa as determined by flow cytometry. Sperm cells were stored at 4 °C for 7 days without antibiotics (control), or with gentamicin (top panel), ampicillin (middle), or antibiotic/antimycotic (bottom).

Mitochondrial functionality was not different among the three antibiotic treatments (P = 0.3417; Figure 4). However mitochondrial function declined significantly during storage (P = 0.0001). The mitochondrial function in the highest concentration of each antibiotic treatment and the control (no antibiotic) were significantly different from the values of the medium and low concentrations (P = 0.0001). A weak correlation was found between mitochondrial function and motility (r = 0.3483).

## DISCUSSION

To control infectious agents that are transmissible with sperm, the addition of antibiotics is common with livestock species, and protocols have been delineated for the control of microorganisms that are not detrimental to seminal quality or fertility (12). However, such standard methods need to be applied with fish spermatozoa (12). Inhibition of bacterial growth by addition of antibiotics to refrigerated sperm cells has improved viability and storage time of spermatozoa from common carp (Cyprinus carpio), Atlantic salmon (Salmo salar) and brown trout (Salmo trutta) (20, 22, 23).

The standard curves generated to evaluate sperm viability and mitochondrial function demonstrated that each dye combination was effective in identifying viable and nonviable sperm cells. The large standard deviations in the motility results were likely due to individual variability among males in quality of spermatozoa (20). Factors such as fish condition, genetic background, level of stress and handling of the samples could have influenced sperm quality.

Sperm viability and mitochondrial function are two of several properties that can be determined using dual-staining techniques (4, 5, 7, 8, 9, 10). Combinations of Sybr 14 and PI and R 123 and PI, among other fluorescent dye combinations, have been used to estimate cell quality and viability with sperm of bulls (9) and rainbow trout spermatozoa (18). Sybr 14 is able to penetrate the cell membrane of the sperm head and stain the nucleic acids of viable cells. Propidium iodide is not able to pass through the membrane of living cells but is able to penetrate and stain the nuclear DNA of degenerated or dead sperm (8). Rhodamine 123 is able to stain functional mitochondria of viable cells (10, 14, 15). In either case, the dual-staining protocols provided rapid identification between viable and nonviable spermatozoa (9).

The viability of Nile tilapia spermatozoa during storage was affected by all 3 concentrations of ampicillin and the highest concentrations of gentamicin and the antibiotic/antimycotic. In this study, a weak correlation was found between sperm viability and motility. Mitochondrial function during the storage trial was reduced by the highest concentrations of ampicillin, gentamicin and antibiotic/antimycotic, which may damage eukaryotic cells at inappropriate concentrations (24). Other studies have demonstrated a relationship between functional mitochondria and sperm motility (1, 6), and this study found a weak correlation. In common carp, motility depended mainly on endogenous ATP stores, and stopped when 50 to 80% of the ATP was exhausted by hydrolysis (2). In this study at the end of 7 d of storage time, the level of motility decreased by 70% and percentage of functional mitochondria decreased by 30%. To improve and obtain more accurate estimates, a more objective and quantitative analysis could be performed with the aid of computer assisted sperm analysis (CASA)(16).

The use of flow cytometry to quantify the effect of antibiotics on sperm quality is a novel approach for aquatic species and can provide additional information to assess sperm function for applications such as breeding, and transport and cryopreservation of spermatozoa. The use of fluorescent dyes to evaluate viability and mitochondrial function should be compared with fertilization rates to further evaluate the utility of these flow cytometry procedures is assessing sperm quality of fish.

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