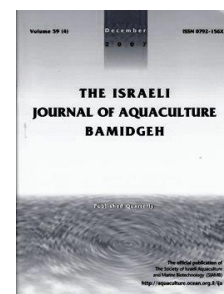




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Effect of Extender Compositions, Glycerol Levels, and Thawing Rates on Motility and Fertility of Cryopreserved Wild African Catfish (*Clarias gariepinus*) Sperm

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Keywords: African catfish; *Clarias gariepinus*; sperm; cryopreservation; glycerol; fertilization

Abstract

The aim of this study was to determine the effect of different extenders, glycerol levels, and thawing rates on post-thaw sperm motility and fertilization ability of cryopreserved African catfish (*Clarias gariepinus*) sperm. Having determined the main spermatological properties (volume, motility, motility duration, spermatozoa concentration and pH), the pooled ejaculates were diluted with 3 different extenders containing different glycerol levels (5, 10 and 15%) respectively. Dilution ratio was 1:10 and the diluted sperm was packaged in 0.25 ml straws and left for 10 min equilibration at 4°C. Following equilibration, the straws were exposed to liquid nitrogen vapor for 10 min and plunged into liquid nitrogen (-196°C), and then exposed to different thawing rates (30°C/20s and 40°C/20s) to determine sperm motility and post-thaw motility duration. The highest post-thaw sperm motility, motility duration, and fertilization rate was 85%, 81s, and 95% respectively when sperm was frozen with the extender (ACSE 3) containing 15% glycerol ($p < 0.05$). The protocol reported in this study can be successfully used for cryopreservation of African catfish sperm.

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Introduction

African catfish (*Clarias gariepinus*) is one of the most important African benthopelagic fish species cultured in South East Asia, Africa, and Europe (Fishbase, 2016). It has also been considered one of the most suitable species for aquaculture. The availability of gametes throughout the year is an important factor to ensure a continuous supply of fish. In captivity, African catfish gametogenesis is continuous once sexual maturity is reached (Omitogun et al., 2012). At present, it is necessary to kill male brood fish or surgically remove part of their testes to obtain spermatozoa. Thus, storing spermatozoa via cryopreservation can significantly improve the reproductive potential of this species.

Cryopreservation provides many benefits such as conservation of genetic diversity, selective breeding, hybridization, and maintaining continuous and stable supply of gametes for hatchery seed production or laboratory experimentation (Lubzens et al., 1997). In addition, cryopreserved sperm can also benefit commercial aquaculture by allowing fertilization of spawn when males are not available. Development of cryopreservation for sperm of *Clarias* species can aid the recovery of threatened and endangered species as well as genetic selection and maintenance of selected stock lines.

Research on sperm cryopreservation in African catfish is limited regarding the use of extenders in combination with various cryoprotectants and freezing methods (Rurangwa et al., 2004). Thus, standardization and simplification of cryopreservation procedures for African catfish sperm is needed. Before freezing sperm, evaluation of different extenders, cryoprotectants, cooling and thawing rates are needed to obtain optimum cryopreservation protocols (Yavas and Bozkurt, 2011; Rurangwa et al., 2004). In addition, evaluation of the fertilizing capacity of frozen and thawed sperm is necessary to assess cryopreservation success.

The main purpose of this research was to investigate the effect of different ionic extenders, glycerol concentrations and thawing rates on post-thaw sperm motility and fertilization ability of frozen-thawed African catfish sperm.

Materials and Methods

Broodstock management. Mature wild African catfish were captured from Golbasi lake in Hatay (Turkey) and maintained in brackish-water ponds (salinity ranging from 1‰ to 5‰). Sexually mature broodfish were kept separately in these shaded ponds supplied with continuously (2.5L/min) well-aerated water (24°C, 10 mg O₂/L). Broodfish were fed commercial dry feeds twice a day *ad libitum* and monitored daily.

Gamete collection. Before each injection and gamete collection, the broodstock were anesthetized in clove oil water bath 10 ml/L (Gabriel et al., 2011). Spermiation and ovulation were induced with an intraperitoneally injected GnRH analogue, Ovopel, which is obtainable as pellets weighing approximately 25 mg. Each pellet contains 18-20 µg [D-Ala⁶, Pro⁹] NEt-mGnRH and 8-10 mg dopamine D₂ receptor antagonist (metoclopramide) (Horvath et al., 1997). The ovopel pellets were pulverized using a mortar and dissolved in 0.7% NaCl solution.

For sperm collection, 15 males (1424.6±28.2 g; 52.4±2.5 cm) were injected with one pellet (per kg body weight). The injected males were kept separately in 20L plastic tanks. After 24 h, testes were removed by dissection and perforated with a needle. Sperm was gently squeezed out and collected in dry tubes. The incisions were sutured by pulling the two sides together and stitching tightly with resorbable thread. The thread was knotted after each stitch. The male broodfish were disinfected with KMnO₄ (potassium tetraoxomanganate) at 1000 mg/L and returned to separate holder tanks as described by Adebayo et al., (2012). These males were starved for 1 week for proper healing of the incisions.

For egg collection, five females (1554.2±46.5 g; 48.6±4.6 cm) were injected with the contents of 1.5 pellet (per kg body weight) ovopel (Interfish, Hungary) at 24°C water temperature. For the first injection, 0.5 pellet (per kg body weight) was applied 12 h before and one pellet (per kg body weight) was given as second injection (Brzuska, 2001). The abdomens of the females and urogenital papillas were dried before stripping. The eggs from each female were stripped into a dry metal bowl. Care was taken to avoid

contamination with urine, mucus, feces, or water. Eggs were checked visually and those with homogenous shape, color, and size were used in the fertilization experiments.

Evaluation of sperm quality. Motility measurements were performed within 10s. following sperm activation. Sperm cells were activated by mixing 1 μ l of sperm with 20 μ l activation solution (0.3% NaCl) on a glass slide and spermatozoa motility was determined using a phase contrast microscope (Olympus, Japan) with 400x magnification. Spermatozoa motility was examined by counting and calculating percentages of forward moving sperm cells. (Vuthiphandchai and Zohar, 1999). Spermatozoa motility was assessed in three replicates. Sperm cells that vibrated but did not move were considered as non-motile. For cryopreservation experiments, sperm samples showing below 80% motility were discarded. Duration of sperm motility was determined using a sensitive chronometer (sensitivity: 1/100s) to record the length of time until all spermatozoa ceased moving following addition of the activation solution to the sperm samples.

Spermatozoa concentration was determined by the hemacytometric method. Semen was diluted at ratio of 1:1000 with Hayem solution (5 g Na_2SO_4 , 1 g NaCl, 0.5 g HgCl_2 , 200 mL bidistilled water) and 10 μ l of the dilution was counted on Thoma hemocytometer slides (depth 0.1 mm). These slides were kept in a moist atmosphere for at least 10 min before cell counting. Mean spermatozoa count was calculated from three replicate samples for each fish at magnification of 400x and expressed as $\times 10^9$ spermatozoa/ml. Sperm pH was measured with a portable pH meter (Hanna, USA) within 30 min of sampling.

Cryopreservation of sperm. Collected sperm from 15 males showing >80% motility was pooled into equal aliquots and chosen for cryopreservation experiments. Pooled semen was diluted at 1:10 (v/v) ratio with three different extenders containing glycerol at concentrations of 5, 10 and 15% separately. Following dilution, semen was equilibrated at 4°C for 10 min. The compositions of the extenders are shown in Table 1.

Table 1: The composition of extenders used in the experiment.

Chemicals	Extenders		
	ACSE 1	ACSE 2	ACSE 3
NaCl	4.68 g/L	7.5 g/L	8 g/L
KCl	2.98 g/L	0.2 g/L	0.5 g/L
CaCl_2	0.11 g/L	0.16 g/L	-
Tris-HCl	3.15 g/L	-	-
Glucose	-	-	15 g/L
pH	9.0	8.3	6.5
References	(Billard and Cosson, 1992)	(Kurokura et al., 1984)	(Chen et al., 1992)

ACSE: African Catfish Sperm Extender.

The diluted semen was drawn into 0.25 ml plastic straws (IMV, France) and sealed with polyvinyl alcohol (PVA). Straws were frozen for 10 min in liquid nitrogen vapor, 4cm above the liquid nitrogen surface, using an insulated box with an adjustable tray. Following freezing process, the straws were plunged into liquid nitrogen (-196°C) for storage. The semen was stored in a frozen state for at least two weeks. The frozen semen packaged into straws was thawed by plunging them directly into water bath at 30°C and 40°C for 20s. The sperm cells were activated by adding 100 μ l activation solution (0.3% NaCl) to determine spermatozoa motility (%) and motility duration (s).

The thawed semen was then immediately used for fertilization experiments. Pooled eggs from five females were used to determine fertilization rates. Egg samples (1 g = about 750 eggs) were inseminated in dry Petri dishes with fresh (for control) or thawed sperm immediately at spermatozoa:egg ratio of $250 \times 10^3:1$. Eggs were inseminated according to the dry fertilization technique using 100 ml fertilization solution containing 3 g urea and 4 g NaCl in 1 L distilled water. The sperm and eggs were slightly stirred for 30 min and washed with hatchery water. Then eggs were incubated in plastic hatching vials supplied with flow-through hatchery water ($25 \pm 1^\circ\text{C}$, 10 mg O_2/L). Oxygen was provided by continuous aeration. The fertilization rates were calculated 12 h after incubation by

counting and screening the developing embryos under a stereomicroscope at 20X magnification. Fertilization experiments were conducted in triplicate.

Statistical analysis. Results are presented as means \pm SD. Differences between parameters were analyzed by repeated analysis of variance (ANOVA). Significant means were subjected to a multiple comparison test (Duncan) for post-hoc comparisons at a level of $\alpha=0.05$. Analyses were carried out with SPSS 10 for Windows Statistical Software Package.

Results

Fresh semen volumes ranged from 2-9 ml and mean volume was 6.2 ± 1.4 ml. Motility values ranged from 85% to 95% and mean values were determined as $89.6 \pm 2.5\%$. Mean spermatozoa movement duration (s), spermatozoa concentration ($\times 10^9/\text{ml}$), and pH values were 37.5 ± 1.2 s, $17.8 \pm 2.4 \times 10^9/\text{ml}$ and 7.2 ± 1.5 respectively.

High percentages of post-thaw motility were observed in sperm cryopreserved with 15% glycerol containing extenders. The highest mean post-thaw motility of African catfish sperm was 82.5 ± 0.7 when cryopreserved with the ACSE 3 extender. In general, sperm frozen with glucose based extender (ACSE 3) showed the highest post-thaw motility regardless of thawing temperature and glycerol levels (Table 2). Mean motility of fresh (control) African catfish sperm was $95.2 \pm 8.6\%$. Sperm cryopreserved with 5% glycerol in ACSE 1 extender thawed at 30°C and 40°C showed the lowest mean duration of motility (29.2 ± 0.4 and 30.6 ± 0.9 s, respectively).

The overall mean fertilization rate of frozen sperm was $85.0 \pm 3.3\%$ while the best fertilization rate was $92.7 \pm 1.5\%$ with sperm that was frozen with glucose based extender (ACSE 3) and thawed at 30°C for 20s. Mean fertilization rate of fresh (control) African catfish sperm was $98.6 \pm 2.4\%$. Motility features and fertilization rates of cryopreserved African catfish sperm was statistically different between the experimental groups ($p < 0.05$). (Table 2).

Table 2: Effect of extenders, glycerol levels and thawing rates on post-thaw motility, motility durations and fertilization rates in cryopreserved African catfish (*Clarias gariepinus*) sperm.

Extenders	Glycerol Levels (%)	Thawing Rates ($^\circ\text{C}$ / s) (30°C / 20 s)			Thawing Rates ($^\circ\text{C}$ / s) (40°C / 20 s)		
		Post-Thaw Motility (%)	Post-Thaw Motility Durations (s)	Fertilization Rates (%)	Post-Thaw Motility (%)	Post-Thaw Motility Durations (s)	Fertilization Rates (%)
ACSE 1	5	42.6 ± 1.5^a	29.2 ± 0.4^a	40.2 ± 4.6^a	46.2 ± 1.4^a	30.6 ± 0.9^a	42.3 ± 0.2^a
	10	48.9 ± 0.2^{ab}	32.5 ± 0.8^{ab}	60.8 ± 1.4^c	59.7 ± 0.6^{bc}	39.8 ± 1.6^b	70.2 ± 1.4^b
	15	57.2 ± 0.8^c	37.9 ± 1.2^{ab}	70.4 ± 0.2^d	54.3 ± 1.2^b	35.4 ± 0.2^{ab}	72.5 ± 1.2^{bc}
ACSE 2	5	55.4 ± 1.2^{bc}	39.2 ± 0.6^{bc}	50.4 ± 1.2^b	62.5 ± 1.7^{bcd}	42.3 ± 0.6^{bc}	70.4 ± 1.5^b
	10	54.2 ± 0.8^{bc}	38.6 ± 1.2^b	60.2 ± 1.5^c	67.3 ± 2.1^{cde}	48.2 ± 0.9^{cd}	80.2 ± 0.6^c
	15	69.5 ± 1.6^d	47.2 ± 1.9^{cd}	80.6 ± 0.2^e	72.6 ± 1.5^e	55.6 ± 0.1^{de}	80.6 ± 1.4^c
ACSE 3	5	72.6 ± 0.5^{de}	52.7 ± 1.8^d	88.4 ± 1.6^f	68.2 ± 1.7^{de}	54.2 ± 1.5^d	70.1 ± 0.2^b
	10	78.9 ± 1.6^{ef}	55.4 ± 1.4^d	90.2 ± 0.4^f	74.5 ± 0.6^e	63.5 ± 1.6^e	82.4 ± 0.6^d
	15	82.5 ± 0.7^f	64.2 ± 1.5^e	92.7 ± 1.5^f	75.2 ± 1.8^e	78.6 ± 1.4^f	86.2 ± 0.4^d

ACSE: African Catfish Sperm Extender. Values within column followed by different superscript letters indicate significant differences ($P < 0.05$).

Discussion

Sperm cryopreservation using liquid nitrogen vapor is a commonly used biotechnological technique in aquaculture (Linhart et al., 2000). During this process, spermatozoa is subjected to drastic physical and chemical changes such as ice crystal formation, mechanical and osmotic stress, and destabilization of the plasmatic membrane (Lahnsteiner et al., 1992).

Motility is an important characteristic to estimate quality of fresh as well as cryopreserved sperm. The present research demonstrates that utilization of the glucose-based extender (ACSE 3) containing 15% glycerol produced significantly higher post-thaw motility and fertilization results. In the present study, a high positive interaction was observed between post-thaw spermatozoa motility and fertilization. This finding is consistent with results obtained with common carp (Linhart et al., 2000). To the best of our knowledge, this is the first report on utilization of glycerol as a successful cryoprotectant for African catfish sperm.

The simplified extender composition, low levels of dilution, and short holding time can increase success of the cryopreservation procedure. The success of carbohydrates can be explained by their cryoprotective effect against freezing damage as membrane stabilizers. In particular, saccharides serve as extenders in cryopreservation of fish sperm (Dabrowski and Ciereszko, 1996). In our study, better fertility results were obtained using sperm frozen in glucose-based extenders containing glycerol rather than in the ionic extenders. Glycerol is one of the most important internal permeable cryoprotectants. Success, indicated by higher fertilization rates can also be linked to the adequate penetration of glycerol through the cell membranes. This indicates that glycerol has a certain protective effect against freezing damage and also acts as a membrane stabilizer. Similar results for the motility parameters of frozen-thawed spermatozoa were reported in some experiments (Tekin et al., 2007; Bozkurt et al., 2011).

Thawing rate is critical in preserving viability of the spermatozoa. There is a lack of available data regarding thawing conditions. Generally, thawing rates should be high to avoid recrystallization (Lahnsteiner, 2000; Bozkurt et al., 2014). According to the results of the present study, higher temperatures are necessary to recover membrane stability or spermatozoa. Reducing ice crystal formation and reactivation of enzymatic activities were found to be best at higher temperatures (Lahnsteiner, 2000). In the present study, thawing of frozen sperm at 30°C and 40°C for 20 s. positively affected spermatozoa motility. However there were significant differences in fertilization rates obtained with frozen semen that were thawed at different periods and temperatures (Adebayo et al., 2012; Omitogun et al., 2012; Muchlisin et al., 2015).

Between 8,000-80,000 fresh spermatozoa per egg are required for artificial insemination in European catfish (*Siluris glanis*) (Linhart et al., 2004). On the other hand, in African catfish (*Clarias gariepinus*) the effective fertilization dosage was indicated to be 245×10^3 spermatozoa per egg for fresh sperm (Steyn, 1987). In the present study, higher fertilization rates were determined regardless of whether semen was frozen or fresh with an egg:spermatozoa ratio of 1:250.000.

Sperm quality is generally evaluated in fertilization experiments in terms of spermatozoa motility rate and percentage of live sperm (Viveiros and Godinho, 2009; Yildiz et al., 2015). It is important to note that egg quality is a crucial factor for hatchery success and fry production. One of the main reasons is difficulty in controlling of egg quality during fertilization experiments due to separation of viable from non-viable eggs. As a result of well adaptations and response of broodfish to captive conditions and induction protocol, the obtained egg quality from five females was good in the present study.

In conclusion, this study showed that the highest mean fertilization rate for frozen semen obtained with glucose-based extender (ACSE 3) containing 15% glycerol was $92.7 \pm 1.5\%$. According to the results, cryopreservation protocol developed in the present study was effective for the successful cryopreservation of African catfish sperm. On the other hand, further studies are needed to evaluate viability, survival, and development of larvae produced from cryopreserved African catfish sperm. The applied cryopreservation protocol in this study can also be used for the recovery of endangered species as well as maintenance of selected genetic lines of broodstock in hatcheries.

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