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Cryopreservation of the Sperm of the African Catfish for the Thriving Aquaculture Industry in Nigeria

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1. Introduction

The production of fish in Nigeria is still very small and cannot sufficiently satisfy the increasing demand of its population of 140 million. To solve the populace's high demand for fish, Nigerians resort to aquaculture which is currently faced with major constraints including lack of fish seed and quality of feed. The scarcity of good broodstock has necessitated the need to conserve the fish genetic resources which are wasted during natural and artificial induced spawning process of fish breeding. One way of expanding aquaculture in Nigeria is by devising a means of preserving genetic resources of our broodstock for all year round supply of fish seed through cryopreservation (Omitogun *et al*., 2006).

The African catfish *Clarias gariepinus* Burchell, 1822 is one of the most suitable species for aquaculture in Africa. Since the 1970's, it has been considered to hold a great promise for fish farming in Africa. Some other merits of African catfish are: high growth rate reaching market size of 1 kg in 5–6 months under intensive management conditions: highly adaptable and resistant to handling and stress; can be artificially propagated by induced spawning techniques for reliable mass supply of fingerlings; commands a very high commercial value where it is highly cherished as food in Nigerian homes and hotels (Olaleye, 2005.).

The Clariid freshwater fishes belong to the family Clariidae with a wide geographical distribution in Africa consisting of 14 genera (Teugels, 1986a) and 32 species (Teugels, 1986b) in Nigeria. Syndenham (1980) reported that the family consists of 5 subgenera namely: Clarias, Clarioides, Anguilloclarias, Platycephaceloides, and Brevicephaloides. *C.*

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gariepinus is the species native to Africa where it is grown although mostly on a subsistence level for food. The fish is hardy and adaptable to diverse environments even with poor water quality with its air breathing ability (Hecht *et al.,* 1996*). C. gariepinus* is a typically non-aggressive stalking predatory omnivore that hunts at night using non-visual primary sense organs especially the senses of touch through the barbels and tactile organs on the mouth and skin (Bruton, 1996).

The availability of gametes throughout the year is important to ensure a constant supply of fish. In captivity (25^0C; 12h light per day*), C. gariepinus* gametogenesis is continuous once sexual maturity is reached. However, whereas females can be stripped of eggs after treatment with pituitary extracts, spermatogenesis and male reproductive behavior do not take place spontaneously, even after hormonal therapy. To obtain spermatozoa it is necessary to kill male brood fish or surgically remove the testes. Storing batches of spermatozoa by cryopreservation would significantly improve the reproductive potential of male catfish. The procurement of reliable broodstock (of good genetic quality), fingerlings and as juvenile fish for stocking ponds and fish farms has been a major set back in the development of catfish culture in Nigeria. This is because these cultivable species are not easily obtained from the wild. The development of cryopreservation procedures for sperm of Clarias species will aid in the recovery of threatened and endangered species as well as in the genetic selection and maintenance of lines of selected stocks. Cryopreserved sperm can also benefit commercial aquaculture industry by allowing females to be spawned when males are not available, decreasing the need to hold captive male as broodstock.

1.1 Success of cryopreservation in African catfish in Nigeria

Cryopreservation of fish spermatozoa has been the subject of many investigations. Successful cryopreservation depends not only on the right choice of cryoprotectant and extender, but also on the freezing protocol used. Cryoprotectant and freezing rate together determine the damage to spermatozoa due to intracellular ice crystallization.

The first two years of the NACGRAB- OAU Department of Animal Sciences cryopreservation project (2005-2007) were dedicated to optimization of cryopreservation protocols of the catfish sperm under short-term condition in deep freezer at -10 to -30°C (Oyeleye and Omitogun, 2007) and testing the viability of cryopreserved sperm by studying the ability of these cryopreserved sperm in fertilizing freshly spawned eggs (Omitogun *et al.*, 2006).

The second phase of the project (2008-2010) was dedicated to cryopreservation of the catfish sperm under long term conditions in liquid Nitrogen (-290^0C) and testing the motility and ability to fertilize eggs. Evaluation of optimization and economic feasibility of cryopreserved sperm was also carried out (Omitogun *et al.*, 2010). To this end, cryopreserved sperm in liquid nitrogen in Dewar container was further diluted and cryopreserved from 3-8 months, then was taken to an identified and willing commercial catfish farm with the objective of testing the ability of cryopreserved sperm of African giant catfish to fertilize a whole clutch of eggs from a mature female catfish, normally used by commercial farmers and consequently confirm the viability of using cryopreserved sperm in normal commercial hatchery operations. Our hypothesis was that if cryopreseved sperm is practically tested on

commercial scale and is proven economically feasible, being a true reflection of what was obtained in the laboratory (Oyeleye and Omitogun, 2007) then this will help to conserve male brood stock (Omitogun *et al*., 2010) which are normally slaughtered for fry production of catfish, and likewise ensure all-year round artificial propagation, helping the fish farmers in overcoming the problem of scarcity of male catfish breeders which are often encountered in the dry season.

1.2 Background information: Sperm: Egg ratio for optimum fertilization of catfish eggs

Cryopreservation of African catfish semen in liquid Nitrogen (LN_2) will invariably help us to conserve the genetic resources of our desirable male fish breeders for all year round artificial propagation and also help in overcoming the problems of scarcity of desirable male catfish breeders often encountered by the farmers most especially in the dry season and to meet high demand for catfish consumption (Oyeleye and Omitogun, 2007).

Sperm collection in African catfish as mentioned requires killing the male fish in order to excise the testes, it is important to maximize the use of a single male by optimization of sperm: egg insemination ratio. For fresh spermatozoa, the effective insemination ratio was estimated as 245 x 10^3 spermatozoa per egg in *C. gariepinus* (Steyn, 1987) and 50 x 10^3 spermatozoa per egg in *Heterobranchus longifilis* (Otenne *et al.,* 1996.). Because a percentage of spermatozoa die during freezing and thawing processes, the effective insemination ratio for frozen spermatozoa should be higher. In channel catfish, 50 x 10^6 frozen-thawed spermatozoa per 0.5 ml straw enabled fertilization of 250 eggs (200 x 10^3 spermatozoa per egg; Tiersch *et al.,* 1994). In blue catfish, *Ictalurus furcatus*, a minimum of 13,000 x 10^3 frozen-thawed spermatozoa per egg were needed to achieve 54% of control fertilization. In *C. gariepinus*, 49 x 10^3 live frozen-thawed spermatozoa per egg achieved a hatching rate (51.2%) equal to the control (51%).The insemination ratio was within the range 6 to 24 x 10^3 spermatozoa per egg. (Steyn, 1993). During ovulation the belly of the female will swell considerably due to water absorption of the ovary. The speed of the ripening process is dependent upon water temperature and likewise, the development process from fertilized egg to hatching is dependent upon water temperature (Coppens, 2009).

African catfish spermatozoa were first successfully cryopreserved by Steyn *et al.* 1985 who obtained 40% motility 24h after storage in LN_2. Glucose in combination with glycerol has been most widely used cryoprotective solution. Recently, glucose in combination with DMSO was also shown to be effective (Urbanyi *et al.*, 1999). Freezing rates can be rapid (*e.g.*, pellet freezing on dry ice or in LN_2 vapor) or slow (*e.g.*, at fixed rates in programmable freezer (Steyn, 1993). In most cases, sperm quality was only evaluated in terms of motility after thawing. When fertilization was included in the evaluation, sperm: egg ratio was not optimized and was often excessive (Padhi *et al.*,1995). Using excess spermatozoa for fertilization obviously masks the quality of cryopreserved spermatozoa, making comparison of protocols difficult (Viveiros *et al.*, 2000).

Methods for cryopreserving spermatozoa and optimizing sperm: egg dilution ratio in African catfish *Clarias gariepinus* was first developed by Viveiros *et al*., 2000) where 5 to 25% DMSO and methanol were tested as cryoprotectant, by diluting semen in Ginzburg fish

ringer and freezing in 1-milliliter cryovials in a programmable freezer. To avoid an excess of spermatozoa per egg, post-thaw semen was diluted 1:20, 1:200 or 1:2000 before fertilization. Even frozen- thawed spermatozoa with low numbers of live cells yielded adequate hatching rates. They found out that the maximum sperm dilution ratio to achieve hatching rates similar to control was 1:200 without losing fertilization ability. However, at 1:2000 the hatching rates produced with frozen spermatozoa were lower than the control African catfish. Similarly *Heterobranchus longifilis* spermatozoa were diluted 1:3 before freezing and 1:10 after thawing and had the same fertilization ability (78.9%) as the control (81.1%). On the contrary for *Cyprinus carpio*, no spermatozoa survived when diluted higher than 1: 5 before and after freezing (Lubzens *et al.*, 1997).

Cryopreservation of catfish spermatozoa is useful as a routine method of gamete storage and management. However, the economic factor should also be considered. The technology of cryopreservation with the use of liquid nitrogen though desirable but is cost intensive. Therefore there is a need to study how the cryopreserved semen will be maximally utilized with good fertilizing results at the same time cost-effective and affordable for the farmers

To avoid wastage of cryopreserved spermatozoa per egg clutch after dilution with physiological salt solution, fertilization of various measures of egg clutches were tested in the present research with differently cryopreserved spermatozoa for optimization and for cost evaluation. In a second study the concentration of the semen was reduced, *i.e.*, diluted at a dilution ratio of 1:20 and 1:200 to verify the spermatozoa are not in excess and consequently be wasted.

Another study was carried out in order to assist the farmers to determine the approximate amount of egg clutch that will be adequate for a milliliter (ml) of cryopreserved semen without wasting spermatozoa in order to evaluate economic cost and profitability. The aim of this study was to verify the possibility of cryopreserving African catfish under long-term condition in liquid Nitrogen (LN_2) and evaluate the viability and fertility optimization of a specific amount (*i.e.*, 1 ml) of cryopreserved semen of African catfish cryopreserved in LN_2 using various cryoprotective agents with different measures of egg clutches. This paper aimed to establish a standard fertility ratio between a ml of semen and clutch of eggs in order to prevent wastage of semen; be able to maximize the resources and evaluate profitability of cryopreservation in liquid nitrogen by evaluating the effects on the cryopreserved semen as to motility and hatchability, the ability to hatch the eggs from a gravid female and survival of ensuing larvae.

2. Materials and methods

For the benefit of prospective users of cryopreservation of African catfish semen, the whole process is pictorially presented in Figures 1-12.

2.1 Husbandry of the broodstock

The broodstock of the African catfish, *C. gariepinus* were obtained from reliable farms in Ile-Ife and Ibadan, Nigeria and were transported in 25 litre tank opened at the top to the Wet Laboratory of the Department of Animal Sciences, Obafemi Awolowo University, Ile Ife.

The matured male and female broodstock were kept at constant temperature of 27^0C in a 1000 litre tank connected to a source of water by a pipe connected from the reservoir plastic tank placed in an elevated stand in the laboratory and its drainage was located at the bottom of the tank for easy flow by gravity.

The broodstock were fed with an imported floating palletized feed *i.e., Coppens*^{R} feed (42% protein, ISO -170 certified, Netherlands) containing a large percentage of high quality fish meal, which is especially important to facilitate repeated spawning at a maintenance level of 1.5% body weight on a daily basis by gradual hand broadcast. The water quality was regulated through proper monitoring and replaced weekly.

2.2 Selection of broodstock

The sexually matured female was selected according to their swollen, reddish genital papilla and a well distended, swollen soft abdomen. A slight pressure was applied on the abdomen towards the genital papilla after which ripe eggs oozed out which were green-brownish in color and ripe eggs are generally uniform in size. The female broodstock was stocked in the hatchery for about 2 days without feeding so that the alimentary tract was empty at the time of stripping. It is very important that the collected eggs did not get contaminated. Sexually matured male broodstock was selected based on a reddish or pinkish pointed and vascularized genital papilla. The temperature of the broodstock kept in the tank was maintained at 25–27^0C.

2.3 Preparation of extender-cryoprotectant

Two extenders were used in this study, phosphate buffered saline (PBS) and Ginzburg Fish Ringer (GFR) with pH of 7.4 and 7.6 respectively. The extenders were prepared as shown in Table 1 with Calcium-free Hanks Balanced Salt Solution (Ca-FHBSS) used in experiment 2. 14; after which they were sterilized for 20 min at 15 lbs/inch^2 using a pressure cooker to avoid contamination and deterioration of the spermatozoa.

2.4 Semen collection

A good quantity of the sperm of African catfish cannot be stripped and sperm can only be obtained after sacrificing it. Sexually matured male weighing 0.8$\pm$ 0.2kg were selected and kept in a different tank of about 50 l capacity for about 18 h prior to the time of sperm collection .The male broodstock was dried with clean towel and then made unconscious by breaking its backbone. The body cavity was carefully opened with a pair of sterilized scissors without damaging the testes after which the two testes were dissected out. It was then removed with a pair of forceps, the blood veins cleared out and rinsed in saline solution. The testes were lacerated with a new and sharp razor blade; the milt was gently squeezed out and collected in a sterilized Petri dish. The whole process was carried out in a disinfected environment to avoid bacterial contamination which can lead to degradation of samples, transfer of pathogens and inaccurate estimation of motility. Sterilized instrument and aseptic techniques for collection of sperm was incorporated to reduce the contamination by bacteria. The volume of the extracted sperm was measured with a 5.0 ml sterilized syringe.

2.5 Cryopreservation of sperm, cryoprotective agents used

Different combination of cryoprotective (CPA) agents are shown in Table 2 as CPA-DP (DMSO+PBS), CPA-DF (DMSO+GFR), CPA-GP (Glycerol+PBS), CPA-GF (Glycerol+GFR), CPA-DGP (DMSO+Glucose+PBS) and CPA-DGF (DMSO+Glucose+GFR). Before cryopreservation of semen, motility of the fresh semen was evaluated in two trials of dilution: 1:1 and 1: 20. In these trials two different extenders were used: Phosphate buffered saline (PBS) and Ginzburg Fish Ringer (GFR). The volume of the extracted semen was measured with a 5.0 ml syringe (DISCARDM(R) NIG) which was diluted with the extender PBS and GFR for first trial on a ratio 1:1 v/v and 1:10 v/v respectively, then mixed evenly with differently prepared cryoprotective agent combinations at a ratio 1:1 (PBS) for fertility and hatchability evaluation.

In the second trial, the two extenders were used but at different sperm dilution ratio for PBS and GFR at a ratio 1:20 for both extenders. The resulting semen-cryoprotective agent solution in each trial after thorough mixing was dispensed into labeled 1ml cryotubes with a 2-step freezing protocol of first freezing on the chilled water blocks at -10^0 C for 10 min before it was finally transferred into the liquid nitrogen for a long-term preservation.

Composition (g/1000 ml)	Phosphate buffer saline (PBS)	Ginzburg Fish Ringer	Calcium-Free HBSS 200mOsmol/kg
NaCl	8.0	7.0	5.26
KCl	0.02	0.28	0.26
$CaCl_2$	-	0.33	-
$NaHCO_3$	0.23	-	0.33
Na_2HPO_4	1.15	-	0.04
KH_2PO_4	0.20	-	0.04
$Mg\ SO_4\ 7H_2O$	-	-	0.13
$C_6H_{12}O_6$	-	-	0.66

Table 1. Composition of the extenders (g/l) tested for cryopreservation of catfish sperm in liquid Nitrogen.

Cryoprotective agent (CPA)	DMSO	Glycerol	PBS	GFR	Glucose/ Sucrose
DP	10	-	90	-	-
DF	10	-	-	90	-
GP	-	10	90	-	-
GF	-	10	-	90	-
DGP	10	-	85	-	5
DGF	10	-	-	85	5

DMSO =Dimethylsulphoxide, PBS =Phosphate buffered saline, GFR=Ginzburg Fish Ringer.: DP = DMSO and PBS; DF = DMSO and GFR; GP = Glycerol + PBS; GF = Glycerol + GFR; DGP = DMSO + PBS + Glucose/Sucrose; DGF = DMSO + GFR + Glucose/Sucrose

Table 2. Composition (%) of the cryoprotective agents used

Fig. 1. The African catfish broodstock.

Fig. 2. Male catfish showing the genital openings.

Fig. 3. Removal of testes from male catfish

Fig. 4. Laceration of testes to extract milt

Fig. 5. Motility evaluation using a microscope and haemacytometer.

Fig. 6. Sperm cryopreserved in liquid Nitrogen for 4-8 months

Fig. 7. Removing the cryopreserved sperm and motility evaluated

Fig. 8. Rapid thawing process is employed.

Fig. 9. Stripping the eggs from gravid female catfish after injection with Ovaprim

Fig. 10. Eggs divided into various clutch weights in Petri dishes

Fig. 11. Fertilized eggs incubated in aerated plastic containers covered with nets

Fig. 12. The temperature was kept at 25-27° C and sometimes covered with black plastic sheets.

2.6 Induced spawning and stripping

The readiness of the female broodstock to be used for breeding was tested by holding it in a head-up vertical position and a slight pressure was applied by pressing its abdomen with a thumb from the pectoral fin towards the genital papilla after which eggs run out freely. The selected broodstock were kept separately in different tanks without feeding them, after they were injected with 0.35 ml Ovaprim ® (Syndel, Canada) per kg live weight (Oyeleye and Omitogun, 2007) and then left for 10-12 hours latency period as a post ovulatory maturation period and to ensure high hatching rates and low proportion of deformed larvae (Hogendoorn, 1979).

2.7 Method of female stripping

The female body surface was gently dried with clean towel. It was tightly held at both ends by two persons with wet towels and stripped by a gentle press on the abdomen with a thumb towards the rear. The first free running eggs obtained at a slight pressing of the induced female broodstock were collected for fertilization (Legendre and Oteme, 1995).

2.8 Egg clutch variation and fertilization

After inducing, the female fish was stripped and the clutch of eggs weighed (about 150–160g/kg of the body weight). The eggs were weighed in various measures of 1.0g, 2.0g, 3.0g, 4.0g and 5.0g based on the level of each experiment. That is for 1.0g, it was weighed seven times with replicates for the different cryoprotective agents and control. After which it was fertilized with the cryopreserved semen in liquid nitrogen after thawing in warm water at 35^0C for 5 min. Fresh semen was used to fertilize same amount of clutches of eggs to serve as the control for both trials.

2.9 Motility evaluation

The motility of the spermatozoa before and after the addition of the cryopreservative agents, CPA and after thawing was evaluated for each trial. The cryopreserved semen was also further evaluated for fertility, hatchability and survivability for each trial. The motility test was done by diluting a drop of post thawed or fresh spermatozoa either with PBS, GFR or 0.9 % saline solution at a ratio 1:100 from which one drop of the solution was put on the hemocytometer and viewed subsequently under the microscope 10X and 40X, low and high power objectives of the microscope. The result arrived at is converted to the total number of spermatozoa per ml by multiplying it by the dilution factor (100) and 10^4 (SIGMA, 1994) as follows:

$$\textit{Total no of spermatozoa per ml} = \textit{Average No. of counted spermatozoa} \times 10^4 \times \textit{of the cryopreserved semen} \quad (1)$$

2.10 Fertility and hatchability evaluation

The development process from fertilized eggs to hatching is dependent upon water temperature while hatching rate is, next to egg quality, dependent on the water quality;

temperature, oxygen level, pH and water hardness. After stripping of the induced female broodstock, the eggs were weighed in grams depending on the on-going experiment *i.e*, 1.0g (600±100 eggs), 2.0g, etc. The various measures of eggs (repeated 12 times together with replicates) were fertilized with cryopreserved semen thawed at 35^0C for 5 minutes, and a pair of egg clutches with fresh semen as control experiment. The mixture of eggs and semen was stirred gently for at least 1.0 min to allow contact and adequate fertilization. Within a few minutes after fertilization the eggs absorbed water and could become sticky so the eggs were distributed in a netted basket suspended in the hatching trough (50cm x 35cm x 30cm) containing contaminant-free (passed through a purification system with ultraviolet sterilization at 3000 µW/cm^2) well-aerated water in a single layer so that the eggs get sufficient oxygen during incubation. The hatching troughs were completely covered with mosquito net and black polythene materials placed under 200 Watt bulbs to prevent mosquitoes and other insects laying eggs and to increase level of heat generation. The system was supplied with an electric aerator to increase level of oxygen dissolved in the water.

The incubated eggs were monitored and temperature maintained between 26^0C -27^0C for incubation between 23– 25 h. Soon after hatching the larvae passed through the net and the dead eggs and shells remain on the net in the basket. The larvae were then simply separated from the unfertilized eggs and eggshells by lifting the basket and the nets out of the hatching trough.

The percentage, % fertility and hatchability were determined subjectively after 12–15 h of fertilization by identifying the healthy developing eggs which were transparent green brownish in colour (Coppens, 2007) while the dead eggs were also estimated:

$$\%\ Fertility = (No.\ of fertilized\ eggs / No.\ of inseminated\ egg)\ X\ 100\% \tag{2}$$

$$\%\ Hatchability = \frac{Total\ No.\ of\ fertilized\ eggs\ -\ No.\ of\ unhatcched\ eggs}{Total\ No.\ of\ Fertilized\ eggs} \times 100\% \tag{3}$$

2.11 Post-hatching survivability evaluation

This is done by allowing the newly hatched larvae of all the treatments and that of the control to live on the remains of their yolk sacs for the first 4 days (Heicht *et al.*,1996) after hatching out of the eggs and thereafter carefully removed from the hatching troughs and were fed with Artemia (Inve Aquaculture, USA) on a regular basis (*i.e.,* twice per day). Irregularities in the activities of the fry in terms of feeding, movement in water was observed at the same time taking note of the dead fry which were removed immediately to avoid contamination of water. Survivability evaluation which was observed for a period of about 3 – 4 weeks was done for each stage of the experiment together with fertility and hatchability for fresh (control experiment) and cryopreserved spermatozoa. The post-hatching survivability was evaluated as follows:

$$\%\ Survivability = \frac{Total\ No.\ of\ larvae\ -\ No.\ of\ dead\ larvae}{Total\ No.\ of\ larvae} \times 100\% \tag{4}$$

2.12 The control

The control for both trials was prepared by the use of fresh semen obtained from the lacerated testes from a normal gravid male broodstock with the use of sterilized dissecting knives but activated with saline solution in the ratio of 1:1 v/v and subsequently used to fertilized various measures of egg clutches ranging from 1.0g, 2.0g, 3.0g, 4.0g and 5.0g normal eggs from the same batch of eggs i.e. from the same fish. Control was set up for the evaluation of each parameter for trials *i.e.,* motility, fertility, hatchability and survivability.

The motility evaluation of the post-thawed cryopreserved sperm was evaluated after dilution with the extender on a ratio 1:100 using hemocytometer (SIGMA, 1994).

2.13 Further scaling up for commercial application of cryopreserved semen dilution and egg clutch fertilization

Motility of the fresh semen was evaluated in two different trials of dilution, before any cryopreservation of the semen. Phosphate-buffered-saline (PBS) and ordinary saline water were the two extenders used in the two different trials and were diluted 1:1 and 1:40. The extracted semen volume was measured with a 5.0ml syringe (DISCARDIM$^{(R)}$NIG) which was diluted with the extender PBS for trial on a ratio 1:20v/v and 1:200v/v respectively and thereafter mixed evenly with the cryoprotective agents at a ratio of 1:1 and cryopreserved for the next seven months in liquid Nitrogen stored in Dewar container

For the second trials, the same cryoprotectant (85% PBS+5% glucose+10% DMSO) was used but at different sperm dilution ratio of 1:40. In both trials, the resulting semen in each experiment after thorough mixing was then dispensed into labeled 1ml cryotubes while a 2-step freezing protocol, e.g. initial freezing onto frozen water (ice) blocks at -10^{0}C for 30min before the final transfer into liquid nitrogen for the next 4 to 7 months.

After induction with Ovaprim, the female broodstock was stripped and the clutch of eggs weighed (which was about 150-200g/kg body weight) and divided into three portions of about 120 g each for the experiment on fertilization with cryopreserved sperm of diluted 1:1 and 1: 40 and for the control.

This procedure was repeated three times in a nearby commercial farm 5kms away from the University to serve as the replicates and to ensure the repeatability of the experiments.

2.14 Experiment on storing sperm in refrigerator

The various extenders used were

- 200mOsmol/kg Ca-F HBSS
- 300 mOsmol/kg Ca-F HBSS
- 400 mOsmol/kg Ca-F HBSS
- RPMI 1640 (SIGMA) Culture Medium in 0.9% NaCl Solution

The three different osmolalities of extender Ca-FHBSS (Calcium-free Hanks Balanced Salt Solution) were prepared according to Riley, 2002. The sperm with the 200, 300 and 400 mOsmol/kg Ca-F HBSS were kept in the refrigerator at 4^{0}C. The semen samples with RPMI and 0.9% NaCl solutions were kept at both room temperature and refrigerator. Two replicates were made for each treatment.

2.15 Statistical analysis

The data collected on the parameters, motility, fertility and hatchability was subjected to standard statistical analysis. The data collected were analyzed using analysis of variance (ANOVA) to find a level of significance at $p < 0.05$. The number of motile sperm counted per square of hemocytometer and percentage of motile sperm obtained after cryopreservation were subjected to Duncan's multiple range test to evaluate effects of types of cryopreservatives as well as egg clutch weights and sperm dilutions and period of cryopreservation on sperm motility. The data collected on motility, fertility and hatchability in the first trial were subjected to 2- way ANOVA at a significance level of $p < 0.05$. The bar charts and line chart showing the relationship between the period of refrigeration and the % motility for the various extenders were also employed for better understanding of the results.

2.16 Cost of production of cryopreserved semen

The costs of chemicals and other consumables used for the study were listed in Table 5. The rates per gram or per ml were calculated to determine the effective cost per ml of the cryopreserved sperm (Table 6).

3. Results and discussion

3.1 Effects of different cryoprotectants on fertility, hatchability, motility and survivability

In the first trial with dilution ratio of 1:1 (sperm: extender) the effect of nature of cryoprotectants on the parameters measured was significant ($p<0.05$). Though dimethylsulphoxide+5% glucose+ PBS (DGP) was higher, DGP and DP gave the best results and was not significantly different ($p>0.05$) from each other but significantly different ($p<0.05$) from other cryoprotectants. It was followed by GP but not significantly different ($p>0.05$) from other cryoprotectants. Fertility also followed the same trend, but DGP was significantly different from DP from their LSD values followed by GP. GF gave the least result but significantly different ($p<0.05$) from other cryoprotectants. Related trend was also observed for other cryoprotectants but that of control was higher ($p<0.05$) than other treatments for each parameter. A similar trend was also observed for motility and survivability.

In Table 3, control (fresh semen), DGP and DP were compared. It is obvious that control has highest mean value (significantly different), this may be expected as the control was not passing through any treatments and processes.

In the second trial when the semen was diluted at ratio 1:20 (sperm: extender) a very close trend was observed which shows that the spermatozoa seems to be too much and probably wasted in the first trial. Besides control which was significantly different ($p<0.05$) for all the parameters, DGP gave the best result before DP, this may be explained by extracellular protection offered by the glucose, but the mean values are not significantly different ($p>0.05$) from each other. Fertility also related to hatchability followed the same trend with hatchability. In fertility, DP, DGP and GP were not significantly different from each other. However, the mean values are significantly different from each other for other parameters in

decreasing order of C > DGP > DP > GP > GF. Generally, DGP gave the best followed by DP (without 5% glucose) while Glycerol in combination with Ginsburg fish ringer gave the lowest result.

A close means values were also discovered with trial 1 which indicate the results were better in trial 2, the further diluted semen which could supposedly be explained by addition of extenders.

The differences in fertility and hatchability with control and cryoprotectants tested may be due to the mild damage done to the spermatozoa during the process of lacerating the testes to extract the semen, and also due to the intracellular vitrification (Cryobiosystems, 2009)- a commonly occurring problem in the process of cryopreservation in liquid Nitrogen.

3.2 Effects of egg clutch weight on viability of African catfish gametes

There was a significant effect ($p<0.05$) of egg clutch weight on fertility, hatchability and survivability. In trial 1, although, the hatchability increases with increase in egg weight but the increment at egg weight 4.0g and 5.0g was not significantly different (LSD=88.749, $p>0.05$). There was fertility optimization at 4.0 g of egg clutch weight which though, close to the mean values of 5.0g which is higher but not a uniform increase. The same trend was observed for fertility of eggs but much higher than corresponding hatchability which may be due to loss of eggs to external factors like temperature, contamination and possible error during record taking. There is no significant difference for fertility at egg clutch weight 3.0g and 4.0g but there was significant difference ($p<0.05$) from 5.0g. Survivability was not significantly different from each other except for 5.0g ($p<0.05$).

In the second trial, a similar trend was observed; hatchability was highest ($p<0.05$) at egg clutch weight 4.0g. No significant difference ($p>0.05$) in survivability was observed except for egg weights 3.0g and 4.0g.

Generally, for both trials, egg clutch weight at 4.0 g gave the optimum viability value.

3.3 Effect of type of cryoprotectant and egg clutch weight interaction on hatchability and fertility

From the statistical analysis, it showed that there could also be effect of interaction of both cryoprotective agent (CPA) and egg clutch weight on fertility and hatchability.

The result, as observed shows a significant effect of ($p<0.05$) of interaction of CPA and egg clutch weight on fertility, hatchability for the first trial and only on fertility for the second trial.

Effect of interaction of cryoprotectant on hatchability was not different from the trend of results obtained in previous results. However, the cryoprotective agents were not significantly different ($p>0.05$) from each other but DGP, GP and DP still maintained the higher mean values while control took the highest. DP and DGP were not significantly different ($p>0.05$) from each other for egg clutch weight such as 1.0g to 5.0g. However, GP was significantly different ($P<0.05$) from DGP and DP for egg clutch weight 2.0-5.0g. For GF, DF and DGF, there was also no significant effect ($p>0.05$) with changes in egg weights. The effect of interaction of both CPA and egg weights on fertility was also significant ($p<0.05$).

The results for both trials were also very close, following the same trend, except for control changes in egg clutch weights from 1.0g to 5.0g was generally not significantly different for DP and DGP followed by GP and GF which were not significantly different ($p>0.05$) from means values of egg clutch weight 4.0g–5.0g for DGP and DP. However, GF gave the lowest value for the two trials.

3.4 Scaling up of the applications of the cryopreserved semen for commercial aquaculture

3.4.1 Effect of dilution ratio on viability of catfish gametes

Table 3 shows the comparison for the parameters measured among the control, dilution ratios 1:1 and 1:40. The fresh semen gave the highest fertility and hatchability rates ($P<0.05$). It is significantly different from ratio 1:1 and ratio 1:40. Comparing the fertility and hatchability rates of the two different dilution rates, ratio 1:1 gave the highest fertility and hatchability rates which was significant ($P<0.05$). Survival rate however, followed a different trend in which dilution ratio 1:1 gave the highest survival rate closely followed by ratio 1:40 while the control semen gave the least survival rate ($P< 0.05$).

Most importantly, the differences in fertility and hatchability may also be attributed to the condition of the farmer's hatchery environment in which many environmental and sanitation conditions were compromised for maximum profit (Amupitan *et al.,* 2010).

DILUTION RATIO	MOTILITY	FERTILITY	HATCHABILITY	SURVIVAL
1:1	55%^{b}	30% ^{b}	35%^{b}	15%^{a}
1:40	49%^{b}	29%^{c}	34%^{c}	14%^{b}
C	72%^{a}	54%^{a}	62%^{a}	13%^{c}
LSD	13%	9.2%	25%	13%

Means in the same column with different letter are significantly different at $P<0.05$, C=Control, LSD = Least Significant Difference

Table 3. Effects of dilution ratio on viability of Catfish semen diluted at ratio 1:1 and ratio 1:40

3.4.2 Effect of dilution ratio on motility of catfish semen and survival of ensuing larvae

The fresh semen gave the highest motility at appreciably high percentage (71.00%) which was significantly different ($P<0.05$) from cryopreserved semen diluted at ratios 1:1 (50.52%) and 1:40 (49.05%) (Fig. 13). However, there was no significant difference between the two diluted cryopreserved semen ($P>0.05$). It was evident that the freezing process and cryopreservation decreased sperm motility after cryopreservation. It could be deduced that cryopreserved sperm still needs to be completely activated after thawing in order to fertilize the whole clutch of eggs since there is a direct relationship between motility and fertility.

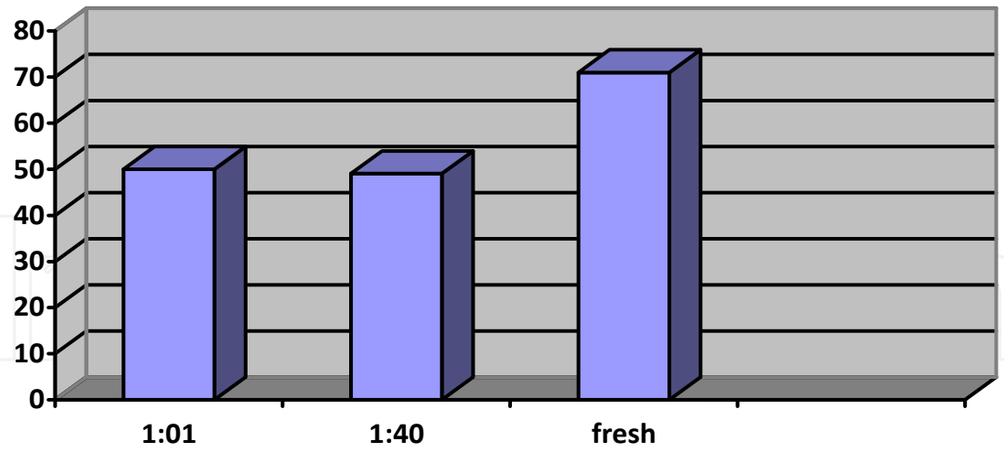

Fig. 13. Motilities of cryopreserved semen at ratios (1:1, and 1:40) compared with the control fresh semen

Cryopreservation in liquid Nitrogen did not have any effect on the survival of *C. gariepinus* larvae produced from cryopreserved semen as shown. However, larvae produced by the cryopreserved semen gave a higher survival, ratio 1:1 (65%), Ratio 1:40 (63%) and fresh semen (50%) ($P>0.05$).

The present study proves that sperm of African catfish cryopreserved aged up to 7 months in liquid Nitrogen and diluted more than 40 times with the extender is viable. The reason for low survivability rate in the control experiment using fresh semen may be attributed to high stocking density (because of the greater number of surviving fry) as practiced by many farmers, *i.e* the quantity of larvae per unit volume of water is less for cryopreserved sperm which in turn was favourable for survival.

3.5 Motility evaluation of refrigerated catfish sperm cells in different extenders

This experiment evaluated the effect of extenders and period of refrigerated storage on the sperm motility of *Clarias gariepinus* sperm cells with the intent to identify a suitable extender for the refrigerated storage of the sperm cells of *Clarias gariepinus*. Semen samples were collected from mature broodstock and were refrigerated with various different extenders at ratio 1:3 namely: Calcium-free Hanks' Balanced Salt Solution (Ca-F HBSS), RPMI 1640 culture medium and 0.9% NaCl. Ca-F HBSS extender was prepared at 3 different osmolalities: 200mOsmol/kg, 300mOsmol/kg and 400mOsmol/kg. Sperm in RPMI 1640 and 0.9% NaCl extenders were also kept at room temperature to assess the effect of refrigeration on motility of catfish sperm cells. Motility was monitored on a 24-hour basis and % motility was evaluated daily. Results showed that sperm cells of *Clarias gariepinus* using 200mOsmol/kg as extender ($p<0.05$) can be stored under refrigeration for 12 days. However, of all the extenders evaluated, RPMI 1640 proved to be the most effective extender ($p<0.05$) retaining higher motility of the refrigerated sperm cells of *Clarias gariepinus*.

3.5.1 Effect of refrigeration on % motility of sperm cells

The semen samples extended with 0.9% NaCl and the RPMI culture solution at room temperature did not have motile sperm cells after 48 hours. The motility of the semen

sample with 0.9% NaCl at room temperature dropped from the initial motility of 74.82% to 6.24% (Fig.3.6. 1) after the first 24 h while the semen sample with the RPMI culture solution at room temperature had 0.4% motility at the end of 24 h. The semen sample with 0.9% NaCl at room temperature had a fishy irritating smell after 24 h. This may be due to the production of waste since the sperm cells metabolised at the normal rate.

The semen samples with 0.9% NaCl and RPMI 1640 culture solution retained motility much longer when refrigerated (Fig. 14). The refrigerated semen sample with 0.9% NaCl retained motility for up to 7 days with motility after 24, 48, 72, and 168 hourly being 34.75%, 17.03%, 14.06% and 4.46% respectively. The refrigerated semen sample with RPMI however kept for 9 days with motility after 24, 48, 72, 168 and 216 hours being 53.47%, 37.62%, 25.64%, 8.32% and 5.25% respectively.

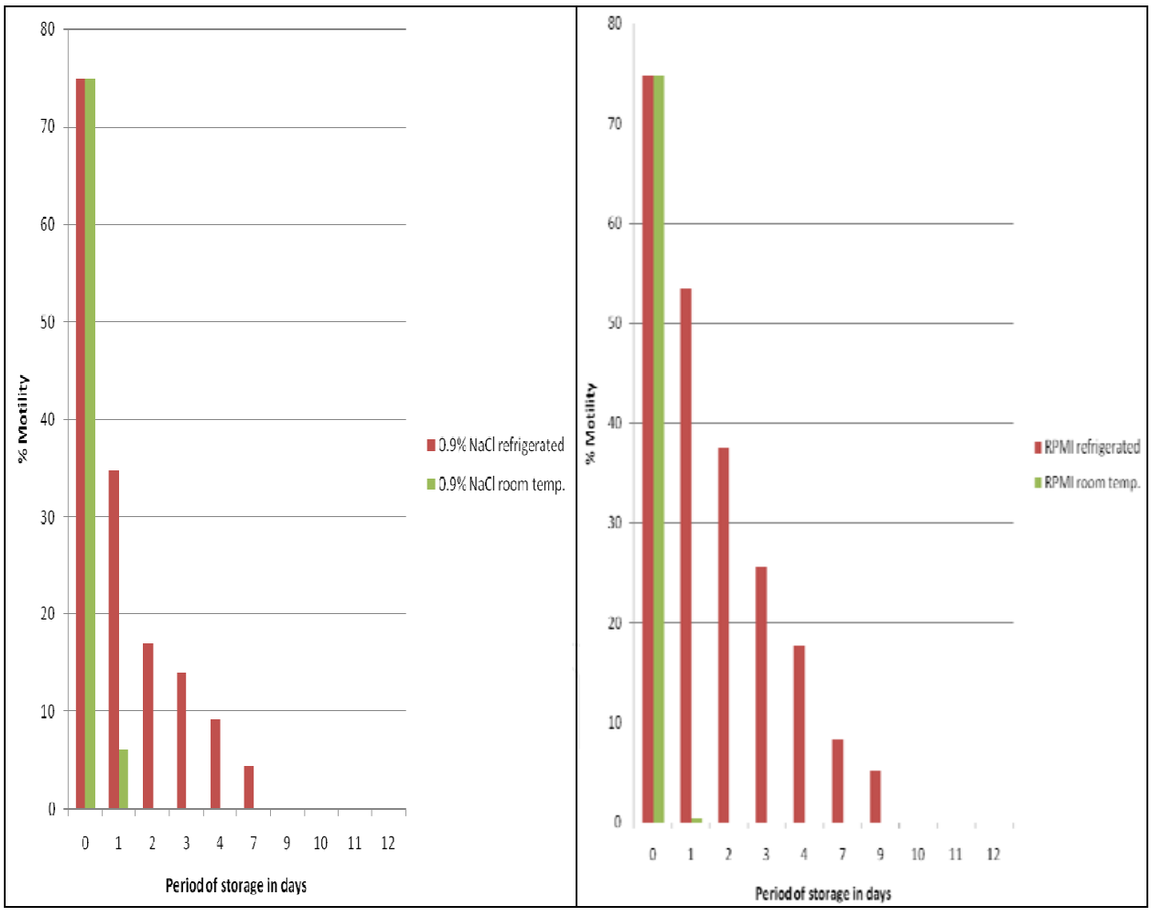

Fig. 14. Effect of refrigeration on motility of semen sample extended with NaCl (left) and RPMI (right). The refrigerated semen sample retained motility till the 7th (in NaCl) and 9th day (in RPMI) whereas semen samples at room temperature only lasted till the first day after storage.

The art of refrigeration provides a low temperature which lowers the metabolic rate of living organisms. The extended semen samples maintained at room temperature proceeded at the normal metabolic rate, hence the sperm cells could not survive up to 48 h. When the sperm cells were still within the fish in the testis, they were supplied with nutrients and the waste they produced are excreted out of the testis, they cannot be supplied with energy or nutrients except provided externally as in tissue culture. Their wastes also accumulate in the solution in which they are suspended in.

High metabolic rate means faster rate of using up available resources by living organism such as nutrients and energy. It also means that waste will be produced at a faster rate thereby causing fast accumulation of waste in the solution in which the sperm cells are suspended. This accumulation will immediately reach a toxic level causing fatality in the sperm cells. Whereas the low temperature provided by the refrigerator to the refrigerated semen sample reduced the metabolic rate of the sperm cells, thereby reducing the rate at which the available nutrients and energy in the semen-extender solution are used up. The nutrient and energy in the semen-extender solution lasted a much longer period when refrigerated, thus keeping the sperm cells alive for a longer period than in the semen samples extended at room temperature.

3.5.2 Effect of osmolality of Ca-F HBSS on % motility

After 24 hours, the motility of the refrigerated semen sample with the 200mOsmol/kg Ca-F HBSS dropped to 34.85% and motility was retained till the 12thday (288 hours) with 0.5% motility. The refrigerated semen sample with 300mOsmol/kg Ca-F HBSS retained motility for 10 days with % motility at 24 h being 31.88 and motility by the tenth day had dropped to 2.28%. The refrigerated semen sample with 400mOsmol/kg Ca-F HBSS retained motility also for 10 days but with a lower % motility at the 10th day (0.4% motility) but with motility at 24 h being 34.46% (Fig. 15).

Based on the length of days for which motility was retained, the 200mOsmol/kg Ca-F HBSS proved to be a good extender since it retained motility for 12 days but with a very low motility (0.5%) However the 300mOsmol/kg, although retained motility for only 10 days, is better since it had the highest motility at the fourth, seventh, ninth and tenth day (i.e. 16.93%, 10.73%, 2.77% and 2.28%).

A good extender should be isotonic to the seminal plasma of the fish. This is to keep the sperm cells immotile until ready for use. Sperm cells are immotile in the seminal plasma and when semen is released in aquatic environment, osmolality goes down and motility is initiated in freshwater species. (Maria *et al.*, 2006). Motility in freshwater species is initiated by exposure of the semen to a hypotonic solution (Morisawa and Suzuki, 1980). Use of extender solutions that are similar in chemical concentration and osmolality are essential to optimizing storage time (Baynes *et al.*, 1981). According to Mansour *et al.,* 2002, motility of *Clarias gariepinus* is completely but irreversibly suppressed in electrolytes and non-electrolytes with an osmolality of 200mOsmol/kg. This statement by Mansour *et al.,* 2002 proves that the osmolality of the seminal plasma of *Clarias gariepinus* is less than or equal to 200mOsmol/kg. This explains why the 200mOsmol/kg of Ca-F HBSS retained motility till the twelfth day as it is closer to being isotonic to the seminal plasma of *Clarias gariepinus*.

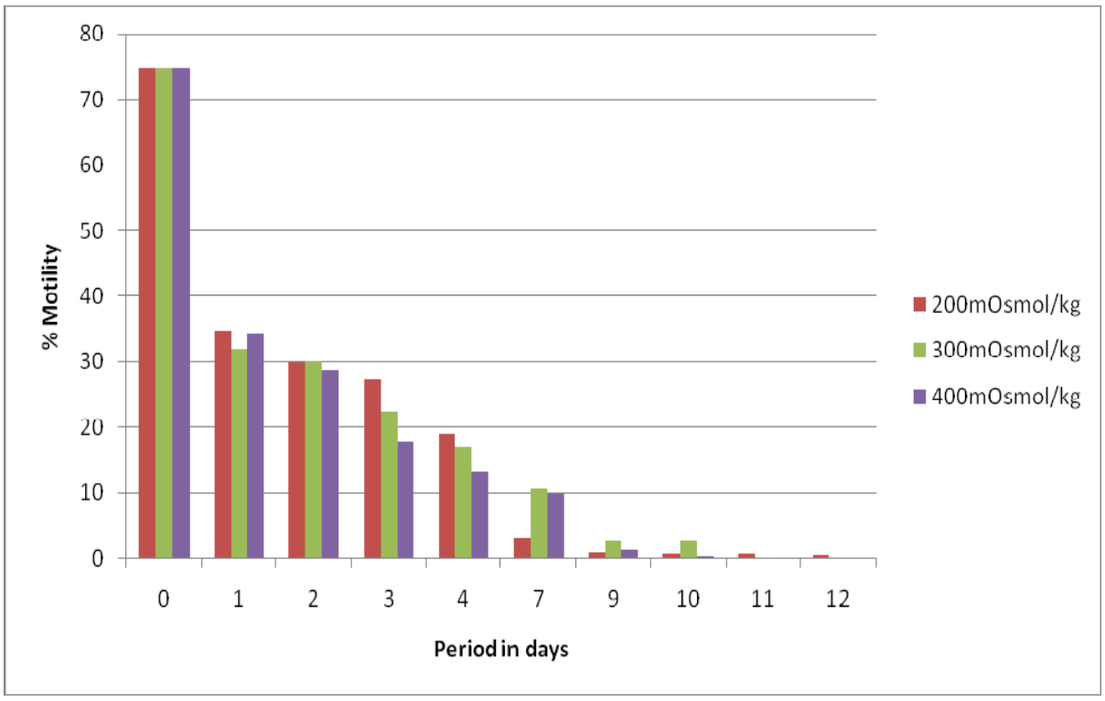

Fig. 15. Effect of osmolality on the sperm motility of refrigerated semen sample in Ca-F HBSS The 200mOsmol/kg Ca-F HBSS extended semen sample retained motility up to day 12 (0.5%)

3.5.3 Effect of extenders on % motility

The refrigerated semen sample with the RPMI culture solution had relatively very high motility at 24 hours after refrigeration; it had a motility of 53.47% as against the 34.85% motility of the semen sample with the 200mOsmol/kg Ca-F HBSS which comes next in rank with it after 24 h. The RPMI extended semen sample also had the highest motility after 48 h of refrigeration (37.62%) with the next in rank being semen sample extended with 300mOsmol/kg Ca-F HBSS with motility of 30.10%. However, the RPMI extended semen sample retained motility only till the fifth day with motility at the fifth day being 5.25% (Fig.16).

The control experiment being semen samples extended with 0.9% NaCl solution retained motility for 7 days with motility at the seventh day being 4.46% and its motility at 24 hours was 34.75% which is exceeded by the semen sample extended with the 200mOsmol/kg Ca-F HBSS which had 34.85% motility at 24 h (Fig. 17).

The relatively high motility retained by the refrigerated semen sample with RPMI culture medium may be due to the additional nutrient supply provided by the RPMI culture medium solution. The RPMI medium culture contains many amino-acids, vitamins and growth factors.

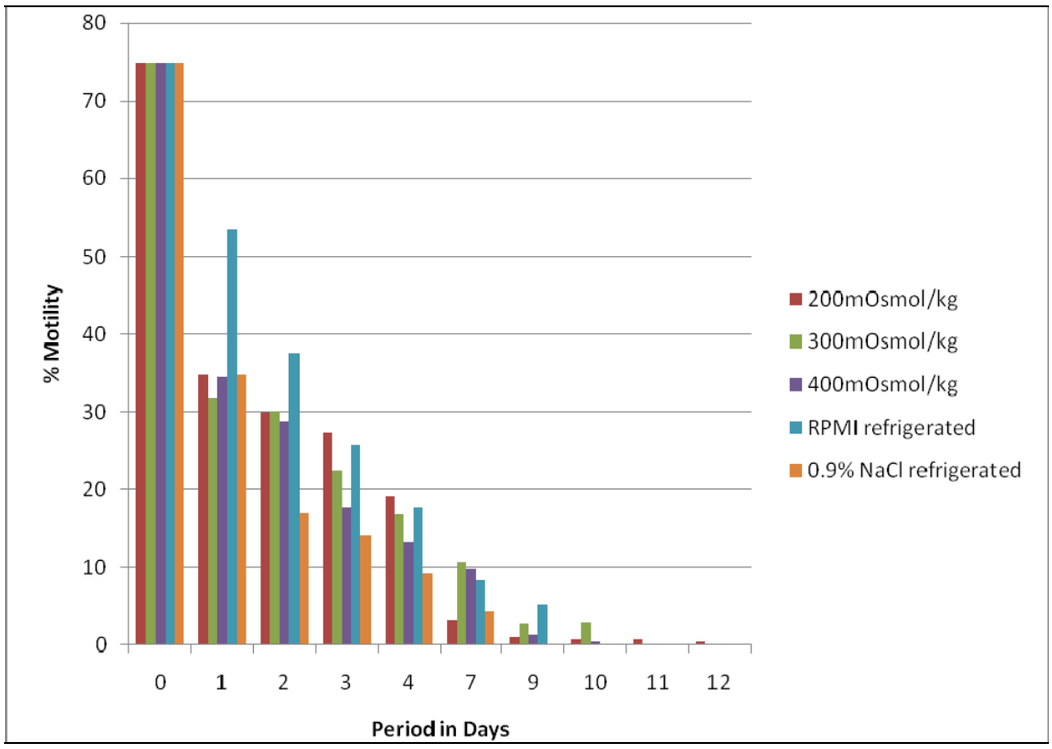

Fig. 16. Effect of the different extenders on the motility of the sperm cells of *Clarias gariepinus.*

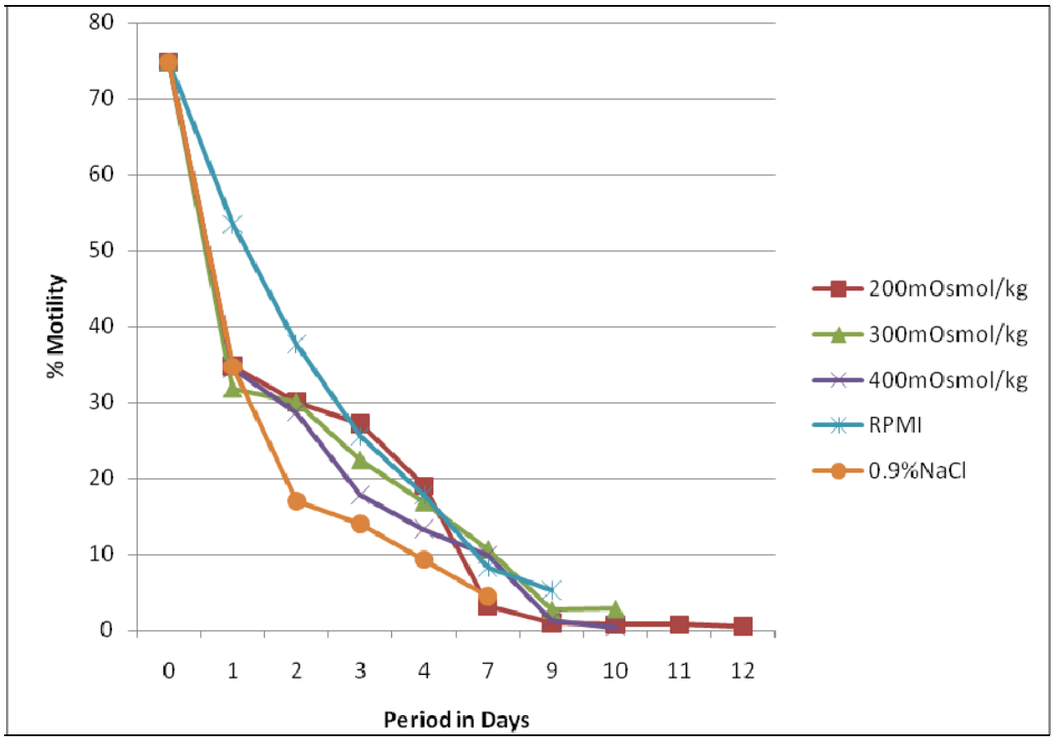

Fig. 17. The decline in motility as the period of storage of refrigerated sperm increased in different extenders.

3.6 Cost of production of cryopreserved catfish sperm for the aquaculture industry

The cost analyses for each reagent and materials used in cryopreservation per ml, g or piece is shown in Table 5. Table 6 shows the cost estimates for the 4-best cryoprotecting agents (DP,

ITEMS	COST (in Naira)	RATE
Liquid Nitrogen LN_2	₦16000/20 l Dewar	₦ 800/l
DMSO	₦ 3700/100/ml	₦ 37/ml
NaCl	₦ 1800/500g	₦ 3.6/g
KCl	₦ 2200/500g	₦ 4.4/g
Na_2HPO_4	₦ 3400/500g	₦ 6.8/g
KH_2PO_4	₦ 2600/500g	₦ 5.2/g
Distilled water	₦100/l	₦ 0.1/ml
$NaHCO_3$	₦ 6028/500g	₦ 12.056/g
$CaCl_2$	₦ 2200/500g	₦ 4.4/g
Glycerol	₦ 2500/2.5l	₦ 1.00/ml
Glucose	₦ 4,100/500g	₦ 8.2/g
Cover Slip	₦175/box (100pcs)	₦ 1.75/slip
Cryovials	₦ 8500/1000	₦8.50/tube
Broodstock (male)	₦ 1200	₦ 1200

Table 5. The cost of reagents and other consumables used to cryopreserve African catfish semen in liquid Nitrogen (LN_2) and listed and the rates per l or ml, g, or per piece are estimated

Ingredients	DP	DGP	GP	GF
Broodstock	1200	1200	1200	1200
Liquid Nitrogen	5000	5000	5000	5000
Glycerol	-	-	1.00	1.00
NaCl	5.76	5.76	5.76	5.76
KCl	0.0176	0.0176	0.0176	0.0176
Na_2HPO_4	1 .564	1 .564	1 .564	-
KH_2PO_4	0.208	0.208	0.208	-
$CaCl_2$	-	-	-	0.2904
$NaHCO_3$	-	-	-	0.5546
Distilled Water	50.00	50.00	50.00	50.00
Glucose	-	4.10	-	-
Cover slip	1.75	1.75	1.75	1.75
Cryovials (80 paces)	680.00	680.00	680.00	680.00
Saline Solution	3.24	3.24	3.24	3.24
Miscellaneous	300.00	300.00	300.00	300.00
TOTAL	7279.5396	7293.6396	7243.5396	7192.1214
Cost/unit of 80 cryovials	7279.5396	7293.6396	7243.5396	7192.1214
Cost per 1ml cryovial	₦ 90.9942	₦ 91, 0455	₦ 90.5442	₦ 89.9015
Proposed Selling price	₦ 100.00	₦ 100.00	₦ 100.00	₦ 100.00
Profit /cryovial	₦ 9.00	₦ 8.95	₦ 9.46	₦ 11.01

Estimation based on 200 ml extender for each cryoprotective agent: DP:DMSO-PBSS, DGP: DMSO-Glucose-PBS; GP: Glycerol-PBS; GF: Glycerol-Fish Ringer .

Table 6. Cost estimates/ml in Naira (₦) for preparation of cryopreserved African catfish semen using different cryoprotectants

DGP, GP and GF. Though, they were not all procured from the same source, all gave almost similar total cost which ranges between ₦7,192. 00 – ₦7,280. 00 and cost/vial ranges between ₦ 89.00 and ₦ 91.00 (1 $ = ₦ 150.00; ₦, naira is the Nigerian currency). Consequently, the proposed economical selling price (in case a cryobank in the University or the collaborating research agency is to be established) considering the present inflation rate is the same for the type of cryoprotectant to be used, but net profit per vial is slightly different.

The selling price for GF is expected to be reduced irrespective of its total cost because of lower viability rate of semen cryopreseved with it. Profit per vial was also calculated which is highest for GP at ₦100/vial and lowest for GF at ₦95/vial. The selling price ranges from ₦95.00 – ₦100.00 which would encourage the buyers to buy it affordably with reasonable profit for the institute (cryobank).

However, the total cost of GP and GF are lower because of the lower cost price of glycerol, but GF gave the least total cost because of a slight difference in the type and amount of chemical used. Total cost of DGP is higher than that of DP, because of cost of glucose inclusion and higher cost of DMSO.

4. Conclusion

From this study it can be concluded that at further dilution of semen together with different cryoprotecting agents, viability of the semen is still maintained but it varies depending on the type of cryoprotecting agents used. DMSO-dimethylsulphoxide proved to be more efficient than other cryopreservatives in preserving sperm viability. Its potential in cryopreservation can be increased when used in combination with a 5% glucose solution, i.e. DGP and DP proved to be the best even for both trials.

Also, viability of African catfish *C. gariepinus* semen cryopreserved in liquid nitrogen can be maintained for a relatively long time provided ideal protocols are strictly followed. From economic feasibility perspective of cryopreservation of catfish semen, cryopreserved semen is economically feasible and profitable for the cryobank institute or company. The farmers are also assured of the viability of the sperm cells they are buying.

The ability of the African giant catfish (*Clarias gariepinus* Burchell, 1822) semen cryopreserved from 4-8 months with different combinations of extender and cryoprotecting agents, dimethylsulphoxide (DMSO) and glycerol with two extenders: GFR (Ginzburg fish ringer) and PBS (Phosphate buffer saline) to fertilize various egg clutch weights were investigated to evaluate the optimum clutch of egg a milliliter of cryopreserved semen can fertilize. DMSO + glucose with PBS and DMSO+ PBS only proved to be the best cryoprotectant-extender combination in maintaining viability of catfish semen. The optimum viability of the semen was also observed at 4.0-5.0 g of clutch of eggs/ml of semen with little deviation. The first trial was on dilution ratio of 1:1 but in the second trial, the semen was diluted further at a ratio of 1:20 and tested on various egg clutch weights (1, 2, 3, 4, 5 g) to evaluate the viability of cryopreservation even at further dilution. There was a significant effect of different cryoprotecting agents ($p<0.05$) on egg clutch weights. There was a significant effect ($p<0.05$) for hatchability and fertility in the first trial but only fertility in the second trial.

In another experiment we tried higher dilution ratio 1:40 and with bigger clutch of eggs (120g) of a standard female breeder (1.0 kg + 0.2 kg) simulating the practices of many commercial farmers, this time with the cryopreserved sperm. Compared with the control

fresh semen that gave the highest motility at appreciably high percentage (71%) which was significantly different ($P<0.05$) from cryopreserved semen diluted at ratios 1:1 (50.52%) and 1:40 (49.05%). However, there was no significant difference between the two diluted cryopreserved semen ($P>0.05$). It was evident that the freezing process and cryopreservation decreased sperm motility after cryopreservation. It could be deduced that cryopreserved sperm still needs to be completely activated after thawing in order to fertilize the whole clutch of eggs since there is a direct relationship between motility and fertility.

In order to assist subsistence fish farmers who may not be able to obtain cryopreserved semen, the motility of sperm cells stored under refrigerated conditions in different extenders was studied. This research evaluated the effect of extenders and period of refrigerated storage on the sperm motility of *Clarias gariepinus* sperm cells with the intent to identify a suitable extender for the refrigerated storage of the sperm cells of *Clarias gariepinus.* Semen samples were collected from mature broodstock and were refrigerated with various different extenders at ratio 1:3 namely: Calcium-free Hanks' Balanced Salt Solution (Ca-F HBSS), RPMI 1640 culture medium and 0.9% NaCl. Ca-F HBSS extender was prepared at 3 different osmolalities: 200mOsmol/kg, 300mOsmol/kg and 400mOsmol/kg. Sperm cells in RPMI 1640 and 0.9% NaCl extenders were also kept at room temperature to assess the effect of refrigeration on motility of catfish sperm cells. Motility was monitored on a 24-hour basis and % motility was evaluated daily. Results showed that sperm cells of *Clarias gariepinus* using 200mOsmol/kg as extender ($p<0.05$) can be stored under refrigeration for 12 days. However, of all the extenders evaluated, RPMI 1640 proved to be the most effective extender ($p<0.05$) retaining higher motility of the refrigerated sperm cells of *Clarias gariepinus.*

The viability of sperm preserved under ordinary refrigerated conditions is possible for a short period of time of 2-7 days depending on the amount of extender used. A culture medium like RPMI 1640 used in this study may give longer life span for sperm cells under refrigerated conditions. Extenders like RPMI 1640 or the cheaper Ca-F HBSS is an alternative that can be recommended for farmers who may have excess of sperm cells from slaughtered male fish for more female gravid eggs that can be sourced within a time period of one week.

The cost of production of a cryotube of sperm (cost of materials, reagents, liquid nitrogen, etc.) was carried out to determine the cost of a milliliter of cryopreserved sperm with a view to selling cryopreserved semen by the research laboratory to farmers who may not be able to afford to buy a male broodstock yielding an affordable cost of ₦100/ml compared to current cost of a male breeder which is ₦ 1000 -1500 each (1 $ = 165 ₦).This also ensures the farmer that the cryopreserved sperm cells they might alternatively buy are viable and will be able to induce the spawning of the female broodstock.

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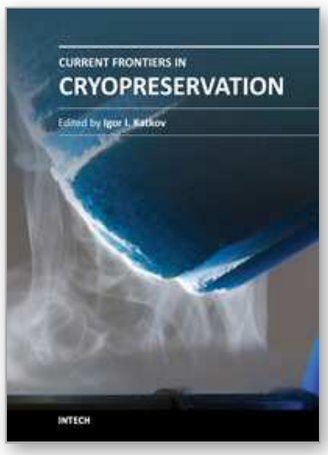

Current Frontiers in Cryopreservation

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Almost a decade has passed since the last textbook on the science of cryobiology, Life in the Frozen State, was published. Recently, there have been some serious tectonic shifts in cryobiology which were perhaps not seen on the surface but will have a profound effect on both the future of cryobiology and the development of new cryopreservation methods. We feel that it is time to revise the previous paradigms and dogmas, discuss the conceptually new cryobiological ideas, and introduce the recently emerged practical protocols for cryopreservation. The present books, "Current Frontiers in Cryobiology" and "Current Frontiers in Cryopreservation" will serve the purpose. This is a global effort by scientists from 27 countries from all continents and we hope it will be interesting to a wide audience.

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