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Suitability of Citrated Egg Yolk Semen Extender for Short Term Preservation of the Milt of the African Catfish *Clarias gariepinus* Stored in Deep Freezer

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ABSTRACT

A study was conducted to test the viability of the milt of the African catfish *Clarias gariepinus* following extension in citrated egg yolk extender and stored in a deep freezer at -20°C. Two male brood stocks of Clarias *gariepinus* with average age of 14 months, average weight 1.5 kg and average measurement of 41.5 cm length were used. The experiment comprised of two groups via the test and control groups. The test group consisted of seven milt samples extended in citrated egg yolk extender and stored in deep freezer at -20°C while the control group consisted of seven milt samples diluted in 2.9% sodium citrate solution and stored in the fridge at 4°C. Both the test and control groups were stored for seven days and the milt samples were examined daily for percentage motility, percentage live and mass activity. Results showed that motility persisted in both groups up to day seven of the experiment with the test and control having motility of 10% and 20%, percentage live of 10% and 20% and mass activity of +(1) and +(1), respectively as at day seven. There was no statistically significant difference between the mean motility, mean percentage live and mean mass activity of the two groups. The mean percentage motility for the test and control groups were 41.43 ± 68 and 48.57 ± 65, respectively, mean percentage live were 41.42 ± 68 and 48.57 ± 65, respectively and mean mass activity were 1.86 ± 08 and 2.00 ± 10, respectively. It was concluded that citrated egg yolk extender at -20°C was suitable for short term storage of the milt. It was recommended that optimum cryoprotectant agents be established. It was also recommended that field trials be involved to test fertilizability and hatchability after such storage.

Keywords: Citrated egg yolk, Milt, Extender, Catfish

INTRODUCTION

An estimated 73.8 million tonnes of aquaculture production comes from fresh water aquaculture. Aquaculture has been reported as a fast growing sector in India with over 6.5 fold growth rate [1]. With regard to biological properties, the African walking catfish, *Clarias gariepinus* [2] is believed to be the most suitable species for aquaculture in Africa [3]. Its distribution is wide, performs excellently well in different environmental conditions (sub-tropical to tropical), it adjusts well to a wide range of water quality conditions primarily due to its breathing ability; it feeds on a wide array of natural prey under diverse conditions; its fecundity is great and it is easily reproduced under captivity [4]. The importance of *C. gariepinus* as an aquaculture species in Africa was first realized by Hey [5]. Before the mid 1970's, little research on its culture potential was conducted [6]; research interest in African catfish has grown since the past 10 decades and is now conducted in many parts of Europe and Africa with the most recent being in Nigeria (West Africa). These largely independent but parallel research initiatives have resulted in the rapid development of farming technology [4] and of techniques for reproduction and rearing [7]. Today, commercial and subsistence farming exist in many African countries. Nigeria, South Africa, Zambia and Ghana are the highest producers (in terms of annual tonnage). In Nigeria, catfish production is currently distributed among few big and a lot of small farms. The

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annual production is difficult to calculate due to poor records [8], but the nation is developing in technology (such as re-circulating systems) and hoping to be the largest African catfish producer in the world in the next 10-20 years, with 10-30,000 tons per year [9].

Catfishes are economically important group of fresh and brackish water fish worldwide. Several species have been successfully introduced in aquaculture [10] and the African catfish, *Clarias gariepinus* (formerly *C. lazera*; [11]), is perhaps the most important, not only in Africa but also in South East Asia (e.g. Thailand) and in Europe (e.g. The Netherlands). The availability of gametes throughout the year is important to ensure a constant supply of fish all year round. Under captivity (25°C; 12 h light per day), *C. gariepinus* shows a continuous gametogenesis once sexual maturity is reached [2]. However, whereas females can be stripped of eggs after treatments with carp pituitary extracts cPE [12]; or gonadotropic hormones [13]; release of sperm and male reproductive behavior are unspontaneous [14], regardless of hormone treatment. Storing batches of germ cell or milt by cold storage would significantly improve the reproductive potential of the males. The use of adequate milt extenders that would guarantee long term storage becomes critical to the success of milt cryopreservation. Storage of gametes or germ cells remains a topic of discussion among scientists. Regardless of these efforts, the use of preservation and storage technologies to aquaculture practice is very limited [15]. Freezing, cryogenic storage and thawing of living material is addressed in cryopreservation [16]. Milt is a seminal fluid of fish containing single type of cells of the spermatozoa which are freely suspended in fluid medium (the seminal plasma) and the cells are not subject to growth or division. Production of milt marks onset of sexual maturity in males. Cryopreservation of milt or semen is being used worldwide. It is now applied in horse, pig, sheep and poultry breeding which has resulted in improved gene pool of native stocks [17]. Successful cryopreservation is well established for sperm cells from many fish species, but a standard technique for true cryopreservation of fish ova has proved elusive [18]. Considering the cost of keeping broodstock population, it would provide a method of retaining specific genetic line of fish. It would offer a secondary source of a genetic line in case of broodstock loss. It also enhances the preservation of endangered genetic lines in wild populations [19].

Diluents maintain inactivity of spermatozoa when semen is diluted before freezing due to their stabilizing potential on the physicochemical properties. Saline and sugar-based extenders have been created and used [20]. The extender is set on buffered physiological saline solutions. This is due to the resemblance of inorganic composition of seminal plasma of spermatozoa with such buffered solution [21]. In a short-term storage experiment, sperm was stored at different dilution ratios (1/10 and 1/50) and temperatures (4 and 20°C) and storage in constant agitation or unagitated. In addition, for the sperm cryopreservation of large volumes (cryovials of 2 and 5 ml), different cooling rates (1 and 3 cm above liquid nitrogen have been tested [22]. To minimize cryoinjuries during cooling and thawing process a large variety of cryoprotective agents of permeating and non-permeating categories are available for use. The widely used cryoprotective agents are DMSO and glycerol. Competency of extenders and cryoprotectants vary from one fish to another. Milt is usually packaged in cryovials, plastic straws of visotubes chilled over liquid nitrogen vapor or they are kept in programmable freezer and stored in liquid nitrogen [23]. Another way of cryopreserving the fish milt is cryopreserving it as pellets on dry ice blocks and then storing in caped cryovials in liquid nitrogen. For cryopreservation various cooling methods had been used successfully on fish sperm. Controlling the size, configuration and location of ice crystal demand a careful manipulation of temperature excursion. For any protocol development, choice and concentration of cryoprotectant and rate of cooling needs to be optimized for each species [24]. One universal protocol cannot be suggested from the current state of art of fish spermatozoa cryopreservation and species differences. This is because the response to cryoprotectant and freezing vary with the different biology. Although some common guides may be used for particular species of fish but effective utilization of the protocol is valuable for each peculiar species. Standardization for the development of any trustworthy protocols for fish milt cryopreservation should always be given priority [19].

Cryopreservation leads to the generation of reactive oxygen species (ROS), which impair post thaw motility, viability, intracellular enzymatic activity, fertility and other sperm parameters [25]. Antioxidants play an important role in sperm motility, integrity, metabolism and function, by protecting the cells against oxidative damage. Damage to sperm function has been successfully minimized in several mammalian species by the addition of antioxidants to the extender media prior to cryopreservation [3].

Egg yolk semen extender has performed excellently well in cryopreservation of bovine semen at -196°C. Establishment of a freezing protocol at -20°C using egg yolk extender would go a long way to encourage milt storage among subsistent fish farmers. Deep freezers are easily affordable and can be found anywhere among local farmers. Hence, the justification for suitable milt diluents, motility activators and cold shielding agents for deep freezing storage. This study therefore aims to assess the efficacy of a readily available semen extender (citrated egg yolk) on milt of African catfish, preserved at deep freezing temperature of -20°C by testing the quality of post - thawed milt extended in citrated egg yolk extender, with the objectives of having quality milt available to farmers all year round, in order to reduce the stress of having to search for and transport male brood stocks at each spawning period.

MATERIALS AND METHODS

Study location

The study was carried out at physiology Laboratory of Faculty of Veterinary Medicine, University of Abuja which is situated within the Federal Capital Territory (FCT), Abuja (longitude 7009'54"E and 7010'38"E and latitude 8059'13"N and 8059'49"N [26].

Experimental design

The experimental design involved storage of semen in two experimental groups consisting the test group which was citrated egg yolk extended milt, shared into seven (7) properly labeled sterile containers, preserved at deep freezing temperature of -20°C and the control group which involved milt extended in sodium citrate shared into seven insulin syringes, properly labeled and stored at 4°C, a condition known to preserve semen of the African catfish. The two groups were observed for seven days and percentage motility, percentage live as well as mass activity of spermatozoa in these samples was recorded.

Experimental fish

Two male brood stocks of *Clarias gariepinus* with average age of 14 months, average weight 1.5 kg and average measurement of 41.5 cm length were purchased from a reputable farm (Olompet Holdings) in Abuja for the study. The male brood stocks were conveyed to the Theriogenology Laboratory, in well aerated containers. The choice of males was based on the possession of well vascularized genital papilla.

Preparation of semen extender

Preparation of buffer: About 250 ml of distilled water was heated to boiling point and allowed to cool. The preparation of 2.9% tri-sodium citrate solution was made by dissolving 2.9 g of the salt in 10 ml of distilled water in a flat bottom flask. This was shaken together until the salt dissolved completely. The solution was then made up to 100 ml by adding distilled water.

Preparation of egg yolk component (yolk separation): Two freshly laid eggs were washed and disinfected using 70% alcohol. They were cracked carefully into two, such that the albumen drained out from the crack until very little of it was left with the yolk. The yolk was then carefully dropped on Whartman® filter paper which absorbed what was left of the albumen. The yolks were collected into beakers.

Extender solution

The solution for the study was made using 80 ml tri-sodium citrate solution and 20 ml off egg yolk. This gave a total of 100 ml solution. To this 2 ml of penicillin and 2.5 ml streptomycin were added.

Milt collection

One of the male brood stocks was selected and sacrificed by spinal transection, after which the belly was dissected and the testes were removed. Blood clots and other tissues were rinsed away using tri-sodium citrate solution. The testes were cleaned and dried by placing on a Whartman® filter paper, which absorbed the excess fluid. Physical parameters which included weight, length and width were observed for the right testis. Semen was collected by gentle perforation of the testis with a sterile needle into a 5 ml sterile container. The content of one testis (the right) was extracted for mixture with the extenders.

Post collection examination

Pre-extension motility was determined by:

a) Mixing one drop of fresh milt with one drop of distilled water on a clean slide to check motility and mass activity using the light microscope at x40 (this was done for the two groups).

b) Using an insulin syringe to collect 0.1 ml of fresh milt, this was made up to 1 ml mark with trisodium citrate solution, giving a 1 in 10 dilution. A drop of this mixture was placed on a clean glass slide with a drop of neutral stain (eosin-nigrosin) for observation of percentage live. This was done with a light microscope using x100 objective lens. The color and volume of semen extracted were also observed.

Milt-extender mixture

Test group: 1 ml of freshly collected milt was carefully decanted into a sterile container with 4.5 ml of the diluents (citrated egg yolk extender solution). The second half 4.5 ml of the diluents containing 7% glycerol was added at room temperature to the first half constituting 3.5% glycerol in a 9 ml diluents. This mixture gives a dilution rate of 1 in 10 (1:10). The mixture was then gently rocked together to ensure proper mixing and then dispensed at 1 ml mark each into seven (7) 5 ml sterile containers. These containers were labeled accordingly and kept in an ice pack, before transfer to a deep freezer set at -20°C. Daily storage examination was done for motility; mass activity and percentage live for a period of 7 days.

Control group (milt+sodium citrate): A one in ten (1: 10) dilution of milt was made with trisodium citrate. This was carefully dispensed into seven (7) insulin syringes at 1 ml mark each, labeled accordingly and kept in an ice pack before transfer and storage in a refrigerator at 4°C. This was also examined for a period of 7 days for the same parameters.

STATISTICAL ANALYSIS

Data were expressed as means and standard error of mean (SEM) and the differences between groups were analyzed using the Student t-test with SPSS/PC computer programme (version16.0). A confidence value of $p<0.05$ was considered

statistically significant. Moreover, Pearson correlation analysis was also done to establish strength of positive or negative relationship between the two groups.

RESULTS

Testicular parameters measured were weight, length and width and values obtained were 5.5 g, 7.8 cm and 1.5 cm respectively. The pre-dilution findings indicated a pre-dilution motility of 90%, percentage live of 90% and mass activity of +++ **(Table 1)**. Results for percentage live **(Table 1)** indicated that about 20% and 10% of spermatozoa were viable at day 7 post storage in the control and test groups, respectively **(Table 1)**. Mass activity results **(Table 1)** showed that in both groups, progressive mass activity of spermatozoa dropped with increasing days of storage. There was no significant difference (P>0.05) although Pearson correlation analysis, showed a strong positive relationship 0.924 for motility and percentage live and 0.904 for mass activity between the two groups **(Table 1 and Figures 1-3)** correlation was significant at the 0.01 level (2-tailed).

Table 1. Baseline data and post thawed sperm motility (%), percentage live (%) and sperm mass activity for test and control groups during the storage period (P>0.05).

Parameters	Baseline	Groups	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Mean
Color	Milky									
Volume (ml)	2.5									
Motility (%) correlation=0.924	90	Test	80	60	50	50	30	10	10	41.43 ± 68
		Control	80	70	70	50	30	20	20	48.57 ± 65
Mass activity correlation=0.904	+++(3)	Test	+++ (3)	+++ (3)	++ (2)	++ (2)	+ (1)	+ (1)	+ (1)	1.86 ± 08
		Control	+++ (3)	+++ (3)	+++ (3)	++ (2)	+ (1)	+ (1)	+ (1)	2.00 ± 10
Percentage live (%) correlation=0.924	90	Test	80	60	50	50	30	10	10	41.43 ± 68
		Control	80	70	70	50	30	20	20	48.57 ± 65

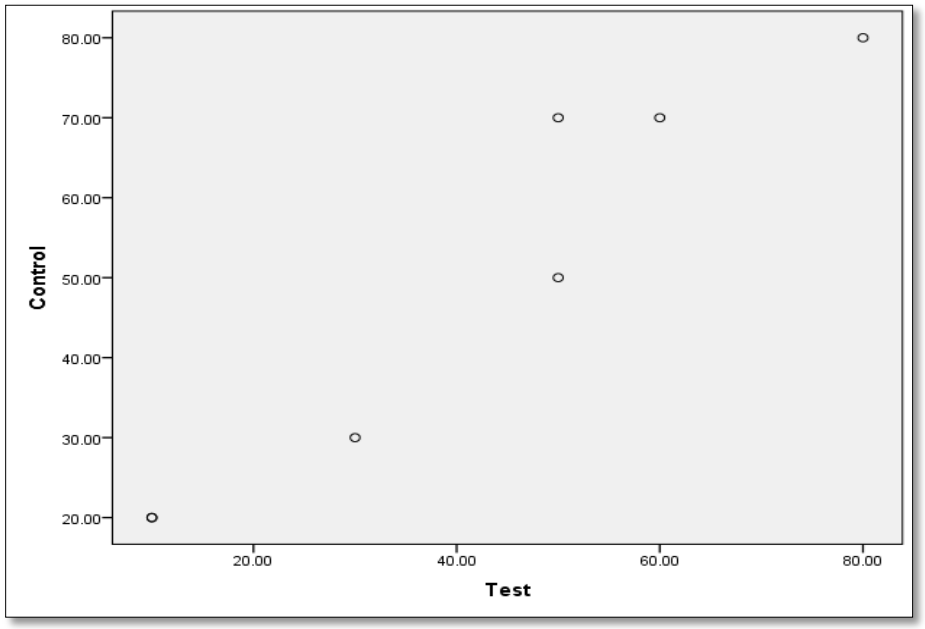

Figure 1. Scatter plot for correlation of motility between test and control.

*** Correlation is significant at the 0.01 level (2-tailed)*

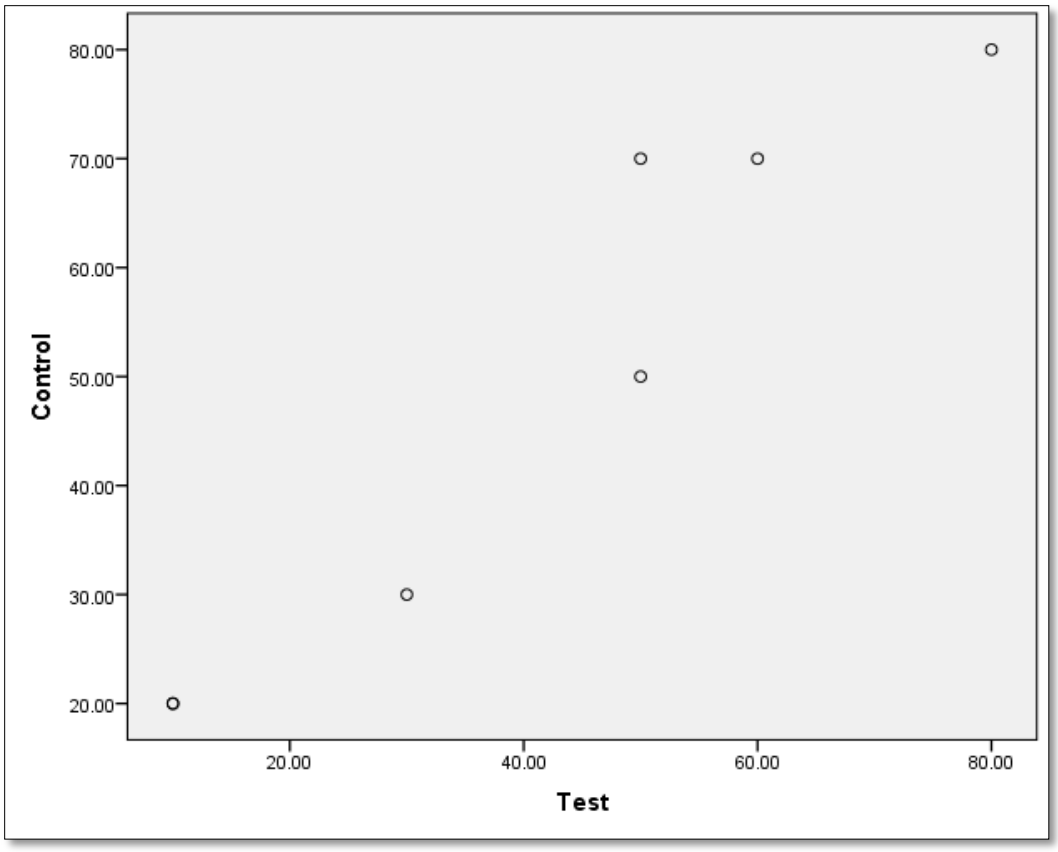

Figure 2. Scatter plot for correlation of percentage live between test and control.
*** Correlation is significant at the 0.01 level (2-tailed)*

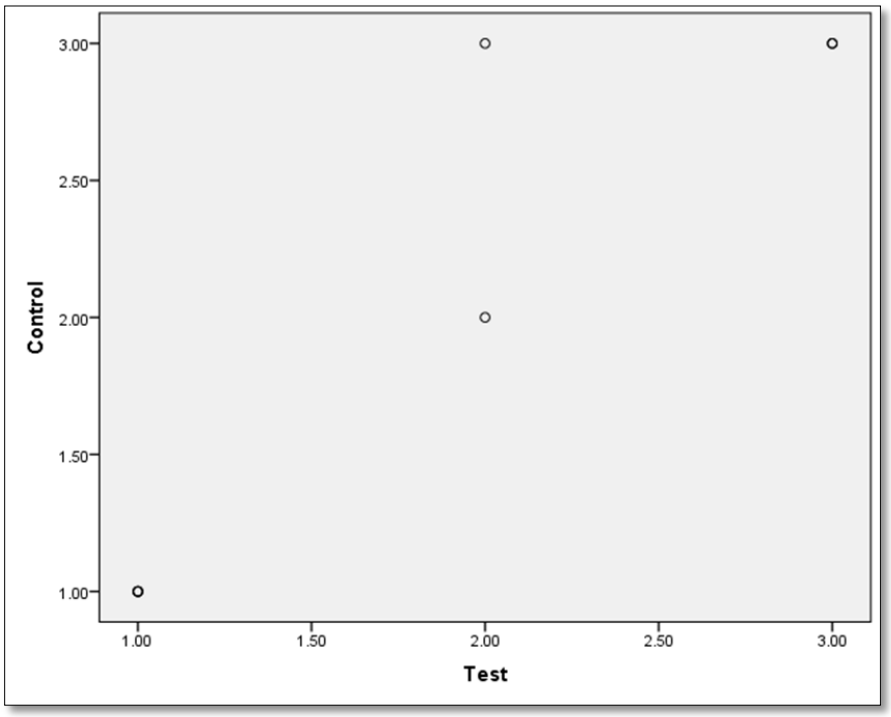

Figure 3. Scatter plot for correlation of mass activity between test and control.
*** Correlation is significant at the 0.01 level (2-tailed)*

DISCUSSION

This experiment suggests that citrated egg yolk extenders are suitable for short term preservation of the milt of the catfish when stored in deep freezer. Long term storage of the milt of the catfish in the deep freezer may be a possibility if adequate concentration of cryoprotectant agents like glycerol is added. Citrated egg yolk extender performed excellently well in enhancing survival within the first 24 h, motility recorded was 80% at 24 h post storage. Progressive motility dropped as the duration of storage (days) increased. The test group which involved storage of milt extended with citrated egg yolk at -20°C recorded motility as well as viability of 10% at day 7 post storage. This finding in the test group lends some support to the research published by Adeyemo et al. [27] who reported the observation of 10% post-thawed motility after one month with milt of *Clarias gariepinus* stored with 10% egg yolk at -40°C. It is a fact that cryoinjuries can result due to slower or faster than optimal cooling and thawing rates [9], therefore, the minor variation in motility, mass activity and percentage live observed in the experiment could be ascribed to the slow freezing rate in the deep freezer. Performance of sodium citrate in this work was expected. It has been standardized to preserve milt of *Clarias gariepinus* when refrigerated [27,28], thus it served as the control group. Results indicated that citrated water at 4°C gave 20% motility and with percentage live of 20% by day 7 of preservation. This observation agrees with the work of Mansour et al. [28] who observed that at optimal condition when buffered saline solution is used for storage of milt of *Clarias gariepinus* at 4°C, sperm viability persists longer than 7 days. Lardy and Philips [29] and Foote [30] also documented that physiological buffers have the ability to dilute semen and maintain its viability for some days when chilled.

CONCLUSION

Citrated egg yolk extender has shown potential in short term storage of milt of the African catfish at a temperature of -20°C, but may not be suitable for long term storage at this temperature. Na citrate still remains a suitable buffer for short term storage of milt of African catfish but as used in this research has to be at 4°C. It is recommended that more work should be done on the effect of deep freezing at -20°C on citrated egg yolk extended milt of the African catfish with adequate cryoprotectant agents to explore its long term storage potentials. Finally it is recommended to take this research to field trials to test fertilizability and hatchability using the stored milt.

REFERENCES

1. FAO (2016) The State of World Fisheries and Aquaculture 2016. Food and Agriculture Organization of the United Nations, Rome, pp: 5-21.
2. Huisman EA, Richter CJJ (1987) Reproduction, growth, health control and aqua cultural potential of the African catfish, *Clarias gariepinus* (Burchell, 1822). Aquaculture 63: 1-14.
3. Martin P, Michaela F, Zuzana D (2016) Effect of extender supplemented with different antioxidants on motility, DNA damage and fertilizing ability of wild African catfish (*Clarias gariepinus*) sperm. 6th International Workshop on the Biology of Fish Gametes University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, Zátiší 728/II, 389 25 Vodňany, Czech Republic.
4. Hecht T, Oellermann L, Verheust L (1996) Perspectives on Clariid catfish culture in Africa. Aquatic Living Resources, pp: 197-206.
5. Hey D (1941) Practical fresh water fish culture. Department of Nature Conservation: Cape Town, p: 118.
6. Richter CJJ (1976) The African catfish, Clarias lazera. A new possibility for fish culture in tropical regions. In: Huisman EA (Eds.), Aspects of fish culture and fish breeding. Miscellaneous papers Landbouwhoge School; Veenman WH and Wageningen ZBV (edtr.), pp: 51-74.
7. Viveen WJAR, Richter CJJ, van Oordt PGWJ, Janssen JAL, Huisman EA (1985) Practical manual for the culture of the African catfish (*Clarias gariepinus*). Joint publication of Ministry of Foreign Affairs, The Hague; Dept Fish Culture and Fisheries of Wageningen Agricultural University, Wageningen and Research Group of Comparative Endocrinology, Department of Zoology, Utrecht University, Utrecht, The Netherlands, p: 94.
8. FAO (2001) Yearbook 1999 - Aquaculture Production. Food and Agriculture Organization of the United Nations, Rome, p: 178.
9. Viveiros ATM (2002) Semen collection and preservation in African catfish, *Clarias gariepinus*. PhD thesis, Fish culture and fisheries group, Wageningen Institute of Animal Science, Wageningen University, The Netherlands, pp: 3-15.
10. Teugels GG (1996). Taxonomy, phytogeny and biogeography of catfishes (Ostariophysi, Siluroidei): An overview. Aquatic Living Resources 9: 934.
11. Fishelson L, Van Vuren JHJ, Tyran A (1994) Ontogenesis and ultrastructure of seminal vesicles of the catfish, *Clarias garieninus*. J Morphology 219: 59-67.
12. Hogendoorn H (1979) Controlled propagation of the African catfish *Clarias lazera* (C&V). In: Reproductive biology and field experiments. Aquaculture 17: 323-333.
13. Eding EH, Janssen JAL, Staarman GHJ, Richter CJJ (1982) Effects of human chorionic gonadotropin on

maturation and ovulation of oocytes in the catfish *Clarias lazera* (C & V). In: Richter CJJ. Goos HJT (Ed), Proc Int Symp Reprod Physiol Fish, Wageningen: The Netherlands, p: 195.

14. Van Oordt PGWJ, Peute J, Van den Hurk R, Viveen WJAR (1987) Annual correlative changes in gonads and pituitary gonadotrophes of feral African catfish, *Clarias gariepinus*. Aquaculture 63: 27-41.
15. Horváth A (2016) Gamete and germ cell preservation and storage: Perspective and the reality, 6th International Workshop on the Biology of Fish Gametes, Session V. Gamete storage and preservation Plenary presentation, p: 74.
16. Basavaraju Y (2013) Amur: A new breed of common carp. Fishing Chimes 33.
17. Yildiz C, Bozkurt Y, Yavas I (2013) An evaluation of soybean lecithin as an alternative to avian egg yolk in the cryopreservation of fish sperm. Cryobiology 67: 91-94.
18. Ciereszko A, Dietrich GJ, Nynca J, Dobosz S, Zalewski T (2014) Cryopreservation of rainbow trout semen using a glucose-methanol extender. Aquaculture 420-421: 275-281.
19. Sharma A, Ram RN, Prasad S (2014) Cryopreservation: A promising tool for conservation of fish genetic resources. Biochem Cell Arch 14: 229-258.
20. Sharma A (2016) Comparative assessment of performance of cryopreserved sperm of common Carp to Amur Carp. Ph.D. Thesis submitted to G. B. Pant University of Agriculture and Technology Pantnagar-263145, (U. S. Nagar), Uttarakhand, India.
21. Kamaruding AN, Embong W, Abdullan B (2012) Frozen-thawed sperm motility characteristics of African catfish (*Clarias gariepinus*) by using glycerol or DMSO based extender. Int J Environ Sci Dev 3.
22. Germán HJ, Victor G, David SP, Christoffer R, Luz P, et al. (2016) For European eel sperm short-term storage and cryopreservation of large sperm volumes. Oral presentation, Session V. Gamete storage and preservation optimization and standardization of protocols.
23. Conget P, Fernandez M, Herrera G, Minguell JJ (1996) Cryopreservation of rainbow trout (*Oncorhynchus mykiss*) spermatozoa using programmable freezing. Aquaculture 143: 319-329.
24. Yavas L, Bozkurt Y, Yildiz C (2013) Cryopreservation of scaly carp (*Cyprinus carpio*) sperm: Effect of different cryoprotectant concentrations on post thaw motility, fertilization and hatching success of embryos. Aquacult Int 10499-013-9698-6.
25. Ping L, Zhi-Hua L, Boris D, Martin H, Marek R, et al. (2016) Oxidative stress induced by cryopreservation on common carp sperm. 3rd International Workshop on the Biology of Fish Gametes September Budapest, Hungary. 36: O-5/03 Book of Abstracts.
26. Dikedi PN (2012) Geo-electric probe for ground water in Giri, Nigeria. In: Global Journal of Science Frontier Research in Physics and Space, pp: 3-4.
27. Adeyemo OK, Adeyemo OA, Oyeyemi MO, Agbede SA (2007) Effect of semen extenders on the motility and viability of stored African catfish (*Clarias gariepinus*) spermatozoa. J Appl Sci Environ Manag 11: 13-16.
28. Mansour N, Lahnsteiner F, Patzner RA (2005) The spermatozoon of the African catfish: Fine structure, motility, viability and its behavior in seminal vesicle secretion. J Fish Biol, pp: 1095-8649.
29. Lardy HA, Phillips AH (1940) A yolk-buffer pabulum for the preservation of bull semen. J Dairy Sci XXXIII: 399-404.
30. Foote RH (1980) Reproduction in farm animals (E.S.E. Hafez, Ed.). 4th Edn, pp: 521-545.
31. Aondoaokaa E (2012) Effects of Climate Change on Agricultural Productivity. 5: 4.
32. Cuvier G, Valenciennes A (1840) Histoire naturelle des poisons. Tome quinzieme. Suite du livre dix-septieme. Siluroides 15: 1-540.
33. Nigerian Muse (2008) UniAbuja: Plight of 1500 students. In: categories of higher education in Nigeria, pp: 3-5.