STUDIES ON FRESH MILT PARAMETERS AND CRYOPRESERVATION OF SPERMATOZOA OF ENDANGERED, GOLDEN MAHSEER, *TOR PUTITORA* (HAMILTON)

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ABSTRACT

Physico-chemical parameters of fresh milt and mitochondrial activity of fresh spermatozoa of the endangered golden mahseer, Tor putitora (Hamilton) were investigated. Cryopreservation and fertility evaluation of cryopreserved-thawed spermatozoa of T. putitora were carried out. The concentration of spermatozoa and the spermatocrit values of the fresh milt were estimated to be 3.96±0.12 x 10^7 spermatozoa/ml and 70.00±1.53% respectively. The motility percentage of spermatozoa in the fresh milt was estimated to be 94.84±0.43. The elemental composition analysis revealed that the concentration of K^+ was higher i.e., 14.13±0.32 mg/l when compared to that of Na^+, Ca^+, Mg^+ and Zn^+. The concentration of total reducing sugars and total proteins were estimated to be 51.79±0.47 mg/100 ml and 40.57±0.75 mg/100ml respectively. The absorbance value of the fresh spermatozoa in Sperm Mitochnodrial Activity Index (SMAI) assessment by Nitro Blue Tetrazolium (NBT) assay was estimated to be 0.33±0.012. The optimum sperm:egg ratio was estimated to be 3.96 ±0.12 x 10^4 no./egg. Hatching percentage of 45.97±1.72 was recorded for the cryopreserved-thawed spermatozoa while the fresh milt control recorded a hatching percentage of 73.10±0.82.

Keywords *Tor putitora*, fresh milt, parameters, NBT, cryopreservation

INTRODUCTION

The ability of the spermatozoa to fertilize the ova is determined by the physico-chemical characteristics of fish milt (Rurangwa *et al*., 2004).Various organic and inorganic components of the fish seminal plasma support the sperm viability (Hajirezaee *et al*., 2010). Therefore, biochemical analysis of seminal plasma plays an important role in the assessment of milt quality (Billard *et al*., 1995). Studies on milt quality parameters has applications related to artificial fertilization and sperm preservation (Billard, 1978). Nitro Blue Tetrazolium (NBT) assay is widely used for assessing Sperm Mitochondrial Activity Index (SMAI), an indicator of functional

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integrity of mitochondria, in human spermatozoa (Gopalakrishnan *et al*., 1991). Any damage to the structural and functional integrity of enzymes of the mitochondrial-enzyme complex of the spermatozoa results either in decrease/cessation of generation of ATP required for motility of the spermatozoa in turn affecting their fertility. Golden mahseer, *Tor putitora* belonging to the family Cyprinidae, once formed a major fishery of the rivers and streams of northern parts of India and today it is enlisted as one of the endangered fish species (NBFGR, 2000). Hence, there is a need for its conservation using cryopreservation as one of the *ex situ* methods of conservation (Rao, 1989; Patil and Lakra, 2005). Hence, the present work was carried out with the objectives of studying the physico-chemical parameters of fresh milt of the endangered golden mahseer, *T. putitora*, to employ NBT test to determine the mitochondrial activity of fish spermatozoa, to cryopreserve the spermatozoa for a duration of 30 days and to evaluate the fertility of cryopreserved spermatozoa.

MATERIALS AND METHODS

The entire experiment was conducted in a mahseer ranching farm and hatchery complex of "Tata Power Company", at Lonavala, bordering Western Ghat mountain range, Maharashtra State, India. The region receives major rainfall during the South-West monsoon from mid-June to September and the ambient temperature of the water ranges from 18-24^{0}C. Even though, golden mahseer, *T. putitora* is not a native species of the western ghats, the stock of the species is maintained at the facility in the earthern ponds as *ex situ* method of conservation. Both the males and females of the species are found to be in oozing condition from October to December.

Collection of milt and ova : Mature individuals of *T. putitora* were caught by cast nets from the brood stock ponds maintained at "Tata Power Company", Lonavala. Both the males and females in oozing condition were selected. Sterile, modified Biggers Whitten Whittingham (BWW) extender (Ravinder *et al.,*1997) was used for rinsing the genital aperture, anal fin and belly region. Milt and egg samples were obtained by hand stripping and care was taken to collect the samples without contamination. Samples contaminated with faecal matter, blood or scales were discarded. Samples were collected separately in clean, dry, sterile vials/ beakers, stored on ice and analyzed immediately.

Estimation of sperm density, spermatocrit value and motility percentage : Density of spermatozoa in the fresh milt of each of the twelve males was estimated, by using improved Neubauer Haemocytometer (Germany) chamber. The milt samples were pre-diluted 1,000 times with 0.85% NaCl and the Haemocytometer chamber was charged using the RBC pipette and then the cells in the five group squares of the central square were counted according to the method of Ax *et al*.(2000) and the density of the spermatozoa was calculated by using the formula, Spermatozoa /ml = Cells in five group squares (in 16 x 5 = 80 squares) / (80 x 1/400 x 1/10 x 1/1000).

The spermatocrit value of the fresh milt of each of the twelve males was estimated as per the method of Rakitin *et al*.(1999). The micro-haematocrit capillary tubes (75 mm length with 1.1-1.2 mm inner diameter) were filled with milt (upto 70%). The end of the tubes were sealed with a sealing gel and the tubes were centrifuged at 7,500 rpm for 10 minutes. The spermatocrit value was calculated using the formula, Spermatocrit Value = (Volume of the white packed material / Total volume of milt) x 100.

A method involving two-step dilution of Billard *et al*.(1995) was used for the estimation of motility of spermatozoa in the fresh milt samples of each of the twelve males. Milt from each vial was diluted 100 times with sterile, modified BWW (Ravinder *et al*., 1997) extender(Appendix-1) and then about 1-2 µl of this pre-diluted milt was taken with the tip of a micropipette tip (equated

with a micropipette) and placed on a clean, non-greasy and dry glass slide which was pre-focussed on the stage of the microscope. About 8-10 µl of de-chlorinated tap water or filtered pond water was placed next to it and both were mixed on the stage of the microscope and observed immediately, under 40 X after placing a cover slip. The number non-motile spermatozoa were counted in any three randomly selected fields and were recorded. Their fields were marked and noted down. The total number of spermatozoa in these three marked fields under the same magnification after the motility has ceased, were recorded and the motility percentage was calculated by the formula, Sperm Motility %= [(Total no. of spermatozoa-Total no. of non-motile spermatozoa) / (Total number of spermatozoa)] x 100.

Analysis of elemental composition of seminal plasma: Aliquots from pooled fresh milt samples from males with ≥ 70% motile spermatozoa only, were centrifuged at 3600g for 20 minutes (Plouidy and Billard, 1982).The supernatant seminal plasma was analysed for the elements, Na^+, K^+, Ca^{2+}, Mg^{2+} and Zn^{2+} using Atomic Absorption Spectrophotometer, Electronics Corporation of India Ltd. – Make, Model No. AAS 4129 following the method of Gopalakrishnan *et al.* (1998). About 1.0ml of seminal plasma was mixed with 9.0 ml of 10% Tri-chloro Acetic Acid (TCA), boiled at 90^0C for 5 minutes, cooled on ice bath and centrifuged at 700g for 20 minutes to remove the protein precipitate. The supernatant was subjected to AAS analysis and the absorbance was measured at a wavelength of 589.0 nm for Na^+, 766.50 nm for K^+, 422.70 nm for Ca^{2+}, 285.20 nm for Mg^{2+} and 213.90 nm for Zn^{2+}. The absorbance of the standards of each of these elements at the corresponding wavelengths as mentioned above, were recorded. The samples were run in triplicates. 10% TCA was run as blank. The concentration of the element in the sample was found out from its standard curve depending on absorbance value in the sample at the specified wavelength.

Estimation of total reducing sugars: The protocol of Oser (1965), was followed for the estimation of the concentration of total reducing sugars in the pooled, fresh seminal plasma. 0.2ml of the seminal plasma was made up to 2ml with physiological saline, 0.5 ml of 5% $ZnSO_4$ and 0.5ml of 0.3N Barium hydroxide were added, boiled for 1 minute and centrifuged at 700g for 15 minutes. 0.5ml of the supernatant was added with 1ml of alkaline copper reagent and kept on boiling water bath for 20 minutes. The mixture was cooled in a cold water bath for 1 minute, 1ml of arseno-molybdate color reagent was added and the entire mixture was added with 7.5ml of distilled water. The absorbance was measured at 540 nm. The sample was run in triplicates. Physiological saline and D-Glucose @0.05mg/ml were run as blank and standards respectively. The concentration of the total reducing sugars in the seminal plasma was calculated from the standard curve.

Estimation of total proteins in the seminal plasma: The concentration of total proteins in the fresh, pooled seminal plasma was estimated following the procedure of Gopalakrishnan *et al.* (1998). 0.02% Bovine Serum Albumin (BSA) and physiological saline were run as standard and blank respectively. The absorbance was measured at 500 nm and the samples were analysed in triplicates. The concentration of the total proteins in the seminal plasma was calculated with the help of the standard curve.

Assessment of sperm mitochondrial activity by Nitro Blue Tetrazolium Assay (NBT): Physiological changes in the cryopreserved-thawed spermatozoa were evaluated using Sperm Mitochindrial Activity Index (SMAI) by Nitro Blue Tetrazolium (NBT) assay following the modified method of Gopalakrishnan *et al*. (1991) and Stasiack and Baumann (1996). The activity of the mitochondrial enzyme system was measured in terms of reduction of the dye, Nitro Blue Tetrazolium. The higher the intensity of

color developed, the higher will be the activity of the enzymes and better structural & physiological integrity of mitochondria of the spermatozoa. The absorbance was measured at 620 nm using ELISA reader (Lab Systems Multiskan MS) with the help of "Genesis" software version 3.03.

Optimization of sperm:egg ratio: Modified method of Suquet *et al*.(1995) was followed for the determination of optimal sperm:egg ratio and fresh milt and eggs were collected from mature males and females by hand stripping. Only milt samples with ≥70% motility were pooled and tenfold serial dilution was done with sterile, modified BWW extender upto 10^{-4}. Approximately, one hundred eggs by number (equivalent to 0.5 ml eggs not hardened by water) were distributed into clean, dry, sterile petri plates with the help of a syringe and fertilization was carried out with 0.5ml of neat as well as each of the milt dilutions. Triplicates were maintained for each dilution and for the neat. After about 30-60 seconds, excess milt was removed by adding excess water to all the petri plates. The eggs in each of the petri plates were counted and incubated in wooden trays with nylon mesh, floated on water in the cement cisterns with jets of fresh water spray on both the sides, for 72±6 hours. The hatchlings were counted and hatching percentage was calculated. The highest milt dilution which gives the highest hatching percentage is the optimum sperm: egg ratio.

Cryopreservation of spermatozoa: Freshly prepared, sterile, BWW extender, modified with 0.5% fructose, 0.5% mannitol and 0.3% tri-sodium citrate was used (Patil and Lakra, 2005). A combination of 9% DMSO and 11% glycerol was used as cryoprotectant solution (Patil and Lakra, 2005). All the materials used during cryopreservation including, milt samples, straws, sealing powder, glasswares, extender solution and the cryoprotectants solutions were chilled before use. Milt samples with ≥ 70% forward motility were pooled and the sperm density was estimated. The pooled milt sample was diluted with the cryoprotectant solution suitably diluted with BWW extender, by taking optimum sperm: egg ratio into consideration. The milt samples with the diluent were filled into medium (0.5 ml) straws (IMV India Pvt. Ltd., Haryana, India) with a syringe, sealed with Poly Vinyl Alcohol (PVA) powder (IMV India Pvt. Ltd., Haryana, India), equilibrated for 30 minutes in chilled water and wiped dry with a clean, dry towel. The straws were frozen over liquid Nitrogen (LN_2) surface at a height of 5 cm using straw rack for 10 minutes (Patil and Lakra, 2005). After freezing, the straws were removed with forceps and put into goblets in a canister with a tag and immersed into 26 liter LN_2 sample cryocans (IBP Ltd., Nashik, India) for storage.

Evaluation of fertility of cryopreserved-thawed spermatozoa: Fertility evaluation of the cryopreserved milt was carried out after a storage period of 30 days in LN_2, as described in optimization of egg:sperm ratio. The cryopreserved milt samples were thawed with warm water maintained at 37±1^{0}C for 10 seconds (Patil and Lakra, 2005). Post-thaw motility percentage of the cryopreserved spermatozoa was estimated. Approximately, one hundred eggs by number (equivalent to 0.5 ml eggs not hardened by water) per petri-plate were fertilized with the cryopreserved-thawed milt sample. Triplicates were maintained including the control. A control was maintained with fresh spermatozoa diluted with sterile modified BWW extender. The eggs were incubated for 72±6 hours. The percentage of fertilization and hatching were calculated.

Statistical Analysis: The calculated means were expressed as mean ± standard error. Normality of the data was tested by Box-Plot method. Wherever needed, arcsine and logarithmic (to the base 10) transformations were carried out. Wherever necessary, Analysis of Variance test was performed to find out the significant differences

between the mean observations (P<0.05) (Sokal & Rohlf 1998). All the statistical analyses were performed using SAS Analyst Package (Version 8.2).

RESULTS AND DISCUSSION

Estimation of spermatozoa density, spermatocrit value and motility percentage: The mean density of spermatozoa in the fresh milt was estimated to be 3.96± 0.12 x10^7 sperm cells/ ml (Table 1). Similar findings were reported by Gupta and Rath (1996), in which the spermatozoa densities ranged between 2.0 - 2.5 x 10^7, 3.0-3.25 x 10^7 and 2.0-2.5 x 10^7 cells / ml for fresh water cyprinids, catla, rohu and mrigal respectively. Higher sperm concentrations of 1.70 ± 0.29 x 10^9 sperm cells/ ml for *T. putitora*, contradictory to the findings of present study were reported by Agarwal (2011). In the present study, mean spermatocrit value was estimated to be 70.00± 1.53 (Table 1). Similar spermatocrit values ranging from 65-75%, 75-85% and 65-75% for the carps, catla, rohu and mrigal respectively, were reported by Gupta and Rath (1996). On the contrary, Gasco *et al.* (1999) reported a very low spermatocrit value of 11.5% in rainbow trout. The mean motility percentile value for fresh spermatozoa was estimated to be 95.01±0.85 (Table 1) in the present study. The findings of the present study are in agreement with those of Basavaraja *et al.* (2002) who reported 95-100% of the motile sperm cells in case of fresh milt of the deccan mahseer, *T. khudree*. The variations in findings reported by different researchers in terms of sperm density, spermatocrit values and motility percentiles of fresh milt of fish occur due to differences in species, feeding conditions, husbandry practices, age, environmental factors, spawning time or dilution ratio (Tahoun *et al.,* 2008; Aliniya *et. al.,* 2013).

Analysis of elemental composition, total reducing sugars and total proteins of seminal plasma: In the present study, it was found that the concentration of K^+ was higher (14.13±0.32 mg / liter) when compared to that of Na^+, Ca^{2+}, Mg^{2+} and Zn^{2+} (Table 2) and this explains the fact that the spermatozoa remain non-motile in the seminal plasma as high levels of Potassium are found to inhibit sperm motility (Baynes *et al.,* 1981). Similar findings that the concentration of K^+ was higher than the other elements in the seminal plasma of common carp have been reported (Plouidy and Billard, 1982).

Higher concentrations of total reducing sugars at levels of 51.79±0.47 mg/100 ml in the seminal plasma were recorded in the present study (Table 2). There are some ambiguities with respect to the role of reducing sugars in the seminal plasma of fish. In fish species where the oxidative capacity of the mitochondria is low, ATP required for motility seems to be pre-accumulated in the sperm before its release from the testis (Billard *et al.,*1995). However, in some fish species, the spermatozoa remain motile longer, when extracellular source of glucose was provided and this indicates the ability of the spermatozoa to utilize external source of energy-substrates and this might explain the presence of total reducing sugars in the fish seminal plasma (Gardiner, 1978).

In the present investigation, it was found that the concentration of total proteins in seminal plasma was 40.57±0.75 mg/100ml (Table 2). Similar values regarding concentration of total proteins have been recorded in the seminal plasma of muskellunge (35.2 mg/100ml) (Lin *et al.,*1996). Contradicting the findings of the present study, very low concentration of the total protein in the seminal plasma of common carp (0.04 to 0.38 mg/100ml) and tilapia (0.00 to 0.06mg/100ml) have been reported (Kruger *et al.,*1984). Proteins in the fish milt play a significant role by acting as buffers and help in maintaining the osmotic pressure, thereby protecting the spermatozoa (Kruger *et al.,* 1984).

Assessment of sperm mitochondrial activity by Nitro Blue Tetrazolium Assay (NBT):

NBT assay, widely used for assessing Sperm Mitochondrial Activity Index (SMAI) which is the indicator of functional integrity of mitochondria of human spermatozoa (Gopalakrishnan *et al*., 1991) was successfully employed to fish spermatozoa in the present study. The mean absorbance value for fresh spermatozoa was found out to be 0.33±0.01 (Table 3) in the present study. NBT assay plays a major role in quantitative quality assessment of fresh spermatozoa in terms of functional integrity of enzymes of the mitochondrial-enzyme complex, responsible for the generation of ATP required for the motility of the spermatozoa (Bergstrom *et al*., 2013).

Optimization of sperm:egg ratio: In the present study, optimum sperm to egg ratio was determined to be 3.96 ± 10^3 / egg. Similar observations of 10^3 sperm cells/egg, were reported in a related species, *T. khudree* (Basavaraja *et al.,* 2002). On the contrary, higher optimal sperm to egg ratio of 1.8-2.4 x 10^5 spermatozoa /egg in case of common carp have been reported (Linhart *et al.,* 2000). In another study, a very high sperm:egg ratio of 3.38 x 10^7 spermatozoa/egg was reported in brown trout. The optimum sperm: egg ratio values differ due to differences in species, environmental factors, diet and health condition of fish (Gopalakrishnan *et al.,*1999).

Evaluation of the fertility of cryopreserved-thawed spermatozoa: Post-thaw motility percentile values were very low when compared to fertilization percentile values and the hatching percentile values were lower when compared to the fertilization percentile values in the present study (Table 5). This trend of low post-thaw motility percentiles (98.33 ± 1.67), higher fertilization percentiles (99.56±0.15) followed by lower hatching percentiles (40.81±0.82) was also reported in case of *T. khudree* (Basavaraja *et al.,* 2002). However, a lower hatching percentage of 13.6% in case of *T. putitora* was reported when compared to the findings of the present study (Ponniah *et al.,*1999). In the present study, there was a drastic decline from higher fertilization percentages (70.60±1.40) to the lower hatching percentages (45.97±1.72). Lower post-thaw motility percentiles when compared to higher fertilization percentiles may be due to the cryo-damages resulting in loss of tail, thereby loss of motility of the spermatozoa while the nuclear material of some of the non-motile spermatozoa may remain structurally and functionally intact. The non-motile spermatozoa are still capable of fertilizing the eggs, assisted by the presence of micropyle over the egg surface (Jamieson and Leung, 1991). This is reflected in the higher fertilization percentiles in the present study. However, even though, the non-motile spermatozoa initiate embryonic development, it gets arrested after some stage, as the DNA of some of the non-motile spermatozoa may be damaged due to cryopreservation-thawing injury, (Gwo *et al.,* 2003) which is reflected in the lower hatching percentiles when compared to higher fertilization percentiles in the present study.

DMSO is observed to be a better cryoprotectant mainly because of the fact that it penetrates as well as leaves the cells much faster than glycerol. However, DMSO is found to be toxic when used at concentrations above 20% (v/v) or when the equilibrium time is extended (Stoss and Refestie, 1983). At equimolar concentrations, cryoprotection by glycerol is almost identical to DMSO but because of its slower permeation of cells, glycerol takes longer time to reach the equilibrium than DMSO (Ashwood-Smith, 1986).

A combination of cryoprotectants performing better in terms of better hatching percentiles is demonstrated in the present study. It may be due to the synergistic effect of the two cryoprotectants, DMSO and Glycerol, than either of these cryoprotectants used individually. The

present study also demonstrates that NBT assay can be successfully employed for the quantitative quality assessment of activity of mitochondrial enzyme complex of fish spermatozoa.

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REFERENCES

Agarwal N K. (2011). Cryopreservation of fish semen. In: Thapliyal M, Thapliyal A and Bhatt J P (Eds.). *Himalayan Aquatic Biodiversity Conservation & New Tools in Biotechnology.* Transmedia Publication, Srinagar (Garhwal), Uttarakhand, India. pp. 104-127.

Aliniya M, Khara H, Noveiri S B and Dadras H. (2013). Influence of age of common carp (*Cyprinus carpio*) broodstock on reproductive traits and fertilization. *Turkish Journal of Fisheries and Aquatic Sciences,* **13:** 19-25.

Ashwood-Smith M J. (1986). The cryopreservation of human embryos. *Human Reproduction*, **1:** 319-332.

Ax R L, Dally M, Didion B A, Lenz R W, Love C C, Varner D D, Hafez B and Bellin M E. (2000). Semen evaluation. In: Hafez E S E and Hafez B. (Eds.). *Reproduction in farm animals,* Lippincott Williams and Wilkins, New York. pp. 365-375.

Basavaraja N, Hegde S N, Akash N and Udupa K S. (2002). The fertility of cryopreserved deccan mahseer, *Tor khudree* (Sykes) spermatozoa. *Asian Fisheries Science*. **15:** 193-202.

Baynes S M, Scott A P and Dawson A P. (1981). Rainbow trout, *Salmo gairdneri* (Richardson), spermatozoa: effects of cations and pH on motility. *Journal of Fish Biology*, **19:** 259-267.

Bergstrom, B. and Jarkman, S. 2013. Correlation between sperm oxidative stress and sperm DNA damage in subfertile men . Masters Thesis Submitted to Lund University, Sweden, 34p.

Billard R. (1978). Changes in structure and fertilizing ability of marine and freshwater fish spermatozoa diluted in media of various salinities. *Aquaculture*, **14:** 187-198.

Billard R, Cosson J, Crim L W and Suquet M. (1995). Sperm physiology and quality. In: Bromage N R and Roberts R J. (Eds.). *Broodstock management and egg and larval quality.*Wiley, Oxford. pp. 25-52.

Gardiner D M. (1978). Utilization of extracellular glucose by spermatozoa of two viviparous fishes. *Comparative Biochemistry and Physiology*, **59A:**165-168.

Gasco L, Zoccarato I, Lussiana C and Amaral H Jr. (1999). Effect of dietary lipid source on semen fatty acids profile and sperm motility after cryopreservation in rainbow trout (*Onchrhynchus mykiss*). *Rivista Italiana Acquacoultura,* **34:** 61-69.

Gopalakrishnan A, Thakur K L, Ponniah A G, Kumar K and Dayal R. (1999). Cryopreservation of Brown Trout (*Salmo trutta fario*) sperm: the influence of extender composition and fertilization procedure. *Fishery Technology,* **36:** 104-109.

Gopalakrishnan, K., Hinduja, I. N., Mehta, R. H. and Kumar, T. C. A. 1991. Assessment of mitochondrial activity of human spermatozoa: Motility/Viability in fertile/ infertile men. *Mol. Androl.*, 3: 243-250.

Gopalakrishnan K, Hinduja I N, Mehta R H and Kumar T C A. (1998). Human semen

analysis. In: Gopalakrishnan K, Hinduja I N, Mehta R H and Kumar T C A. (Eds.). *Laboratory manual for human semen analysis*. Institute for Research in Reproduction and WHO Collaborating Center, Mumbai. pp. 1- 34.

Gupta S D and Rath S C. (1996). Cryogenic preservation of carp milt and its utilization in seed production. In: Joseph M M and Mohan C V (Eds.). *The Third Indian Fisheries Forum Proceedings*. Asian Fisheries Society Indian Branch, Mangalore. pp. 77-79.

Gwo J C, Wu C Y, Chang W S P and Cheng H. Y. (2003). Evaluation of damage in Pacific oyster (*Crassostrea gigas*) spermatozoa before and after cryopreservation using comet assay. *CryoLetters*, **24:** 171-180.

Hajirezaee S, Amiri B M and Mirvaghefi, A. (2010). Fish milt quality and major factors influencing the milt quality parameters: A review. African Journal of Biotechnology, **9**: 9148-9154.

Jamieson B G M and Leung L K P. (1991). Introduction to fish spermatozoa and the micropyle. In: Jamieson B G M. (Ed.). *Fish evolution and systematics:Evidence from spermatozoa.* Cambridge University Press, Cambridge. pp. 56-72.

Kruger J C De W, Smit G L, Vuren J H J V and Ferreira J T.(1984). Some chemical and physical characteristics of the semen of *Cyprinus carpio* L. and *Oreochromis mossambicus* (Peters). *Journal of Fish Biology,* **24 :** 263-272.

Lin F, Ciereszko A and Dabrowski K. (1996). Sperm production and cryopreservation in Muskellunge after carp pituitary extract and human chorionic gonadotropin injection. *The Progressive Fish Culturist,* **58:** 32-37.

Linhart O, Rodina M and Cosson J. (2000). Cryopreservation of sperm in common carp, *Cyprinus carpio*: sperm motility and hatching success of embryos. *Cryobiology,* **41:** 241-250.

NBFGR. (2000). Threatened fishes of India. In: NBFGR (Ed.). *Annual Reports.* National Bureau of Fish Genetic Resources, Lucknow. pp 16-17.

Oser B L. (1965). Estimation of total reducing sugars. In: Oser B L. (Ed.). *Hawk's Physiological Chemistry.* 14th Ed. McGraw Hill Publishers, New York. pp. 1054-1055.

Patil R and Lakra W S. (2005). Effect of different cryoprotectants, equilibration periods and freezing rates during cryopreservation of spermatozoa of mahseer, *Tor khudree* (Sykes) and *T. putitora* (Hamilton). *Aquaculture Research,* **36:**1465 – 1472.

Plouidy M G and Billard R.(1982). The chemical composition of the companion fluids of the gametes in the common carp. In: Richter C J J and Goos H J Th. (eds.). *Reproductive physiology of fish.* PUDOC, Wageningen. pp.127-134.

Ponniah A G, Lakra W S and Ogale S N. (1999). Effects of cryoprotectant and fertilization protocol on viability of mahseer *Tor khudree* cryopreserved spermatozoa. *Journal of Aquaculture in the Tropics,* **14:** 153-158.

Rakitin A, Ferguson M M and Trippel E A. (1999). Spermatocrit and spermatozoa density in Atlantic cod (*Gadus morhua*): correlation and variation during the spawning season. *Aquaculture*, **170:** 349-358.

Rao K G. (1989). Cryopreservation of carp sperm. In: Das P and Jhingran A G. (Eds.). *Fish genetics in India.* Today's and Tomorrow's Printers and Publishers, New Delhi. pp. 193-198.

Ravinder K, Nasaruddin K, Majumdar K C and Shivaji S. (1997). Computerized analysis of motility, motility patterns and motility parameters of spermatozoa of carp

following short-term storage of semen. *Journal of Fish Biology,* **50:** 1309-1328.

Rurangwa E, Kime D E, Ollevier F and Nash J P. (2004). The measurement of sperm motility and factors affecting sperm quality in cultured fish. *Aquaculture*, **234**: 1-28.

Sokal R R and Rohlf F J. (1998). Biometry. In: Sokal R R and Rohlf F J. (Ed.). Introduction to the Analysis of Variance, 3rd edn W. H. Freeman, NewYork, USA. pp.179-203.

Stasiak S A and Bauman P C. (1996). Neutrophil activity as a potential bioindicator for contaminant analysis. *Fish and Shellfish Immunology*, **6:** 537-539.

Stoss J and Refestie T. (1983). Short-term storage and cryopreservation of milt from Atlantic salmon and sea trout. *Aquaculture,* **30:** 229-236.

Suquet M, Billard R, Cosson J, Normant Y and Fauvel C. (1995). Artificial insemination in turbot (*Scophthalmus maximus*): determination of the optimal sperm to egg ratio and time of gamete contact. *Aquaculture,* **133:** 83-90.

Tahoun A M, Ibrahim M A R, Hammoudi Y F, Eid M S, El-Din M M A Z and Magouz F I. (2008). Effect of age and stocking density on spawning performance of nile tilapia, *Oreochromis niloticus* broodstock reared in hapas. In: Proceedings of 8th International Symposium on Tilapia in Aquaculture. The Central Laboratory for Aquaculture Research, Cairo. pp.329–343.

Table 1 Spermatozoa density, spermatocrit value and motility percentage of the fresh milt of Golden Mahseer, *Tor putitora*

Sampled Individual Males	Sperm Cell Density (x 10^7)		Spermatocrit Value (%)		Spermatozoa Motility (% Motility)	
	Individual Observation	Mean ± SE	Individual Observation	Mean ± SE	Individual Observation	Mean ± SE
1	4.05	3.96 ± 0.12	66	70.00± 1.53	94.69	95.18±0.29 (only from ten males with ≥ 70% motility)
2	4.68		78		54.49	
3	3.95		75		96.32	
4	4.16		72		95.96	
5	3.6		67		49.37	
6	4.44		74		94.50	
7	3.35		64		96.41	
8	4.19		65		94.93	
9	3.21		62		95.16	
10	3.87		69		93.94	
11	4.22		71		56.54	
12	3.82		77		94.72	

Table 2 Biochemical composition of the seminal plasma of fresh milt of Golden Mahseer, *T. putitora*

Chemical Constituent		Concentration (Mean ± SE)
Elements	Sodium (mg/l)	8.79±0.03
	Potassium (mg/l)	14.13±0.32
	Calcium (mg/l)	1.24±0.03
	Magnesium (mg/l)	0.36±0.01
	Zinc (mg/l)	0.02±0.01
Total Reducing Sugars (mg/100ml)		51.79±0.47
Total Proteins (mg/100ml)		40.57±0.75

Table 3 Absorbance values cryopreserved-thawed spermatozoa of Golden Mahseer, *T. putitora* in SMAI assessment by NBT assay

Milt Sample	Fresh Spermatozoa
R1	0.322
R2	0.357
R3	0.321
Mean± SE	0.33±0.01

Table 4 Hatching percentages observed at different dilutions of fresh milt of Golden Mahseer, *T. putitora*

Dilutions	**Replications**			**Mean Percent Hatching (Mean ± SE)**
	R_1**	**R_2**	**R_3**	
Neat Milt	76.42	80.22	75.25	77.29±1.50[a*]
10^{-1}	75.00	75.65	71.57	74.07±1.27[a]
10^{-2}	67.62	64.65	72.04	68.10±2.15[a]
10^{-3}	39.53	45.37	38.14	41.02±2.21[b]
10^{-4}	39.66	39.81	38.95	39.47±0.26[b]

***** Values with the same superscripts are not significantly different

****** R_1, R_2, R_3 are the replications

Table 5 Mean percentile values of post-thaw motility, fertilization and hatching of cryopreserved-thawed spermatozoa of *T. putitora*

Milt Sample	Post-thaw Percent Motility (Mean ± SE)	Percent Fertilization (Mean±SE)	Percent Hatching (Mean±SE)
Cryopreserved-thawed Milt	23.58±0.44	70.60±1.40	45.97±1.72
Fresh Milt (Control)	90.40±1.49	84.40±0.73	72.79±0.69