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[Cryogenic sperm banking of Indian major carps (*Catla catla, Labeo rohita and Cirrhinus cirrhosus*) and exotic carps (*Hypophthalmichthys molitrix*, *Hypophthalmichthys nobilis* and *Ctenopharyngodon idella*) for commercial seed production and brood banking]

Fish Innovation Lab

Final Technical Report [01/04/2020 – 31/08/2023]

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Final Technical Report

[01/04/2020 – 31/08/2023]

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Abbreviations and Acronyms

BAU	Bangladesh Agricultural University
BAURES	Bangladesh Agricultural University Research System
PI	Principal Investigator
Co-PI	Co-Principal Investigator
DMRT	Duncan Multiple Range Test
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DoF	Department of Fisheries
FAO	Food and Agriculture Organization of the United Nations
FIL	Feed the Future Innovation Lab for Fish
GO	Government Organization
IMCs	Indian major carps
LSU AgCenter	Louisiana State University Agricultural Center
AGGRC	Aquatic Germplasm and Genetic Resources Center
ME	Management Entity
MSU	Mississippi State University
MS	Master of Science
NGO	Non-Governmental Organization
PI	Principal Investigator
PhD	Doctor of Philosophy
USAID	U.S. Agency for International Development
USDA	United States Department of Agriculture

Glossary

Cryopreservation: Cryopreservation of fish deals with cryobiology that relates to the long-term preservation and storage of biological material at very low temperature, usually at -196^{o}C.

Cryogenic sperm bank: Establishment of germplasm repository through cryopreservation is called cryogenic sperm bank.

Extender: An extender is a solution consisting of inorganic and organic chemicals resembling that of blood or seminal plasma in which the viability of spermatozoa can be maintained during *in vivo* storage.

Cryoprotectant: Cryoprotectants are chemicals added to extenders to minimize the stress on cells during cooling and freezing.

Equilibrium period: Equilibrium period is the time allowed for cryoprotectant penetration into cells.

Lived gene bank: Maintenance of live fish of different sources in a confined place (ponds, baors, reservoirs etc.) is called live gene bank.

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Abstract

Catla (*Catla catla*), Rohu (*Labeo rohita*) and Mrigal (*Cirrhinus cirrhosus*) are the most popular indigenous aquaculture species of Bangladesh. Similarly, Silver carp (*Hypophthalmichthys molitrix)*, Bighead carp (*Hypophthalmichthys nobilis*) and Grass carp (*Ctenopharyngodon idella*) are also very popular exotic fish species for aquaculture. Presently almost 100% (99.68%) of seeds are artificially produced in hatcheries, but the seed quality is deteriorated due to some genetic reasons that results in poor performance. In this context, establishment of a cryogenic sperm bank of the species from quality broodstocks and use of cryopreserved sperm in seed production in commercial hatcheries could be a good option. With this view, a series of experiments including standardization of parameters of cryopreservation process, development of cryogenic sperm bank, production of seeds using cryopreserved sperm and their captive rearing, were conducted. Quality broodstocks of IMCs were developed by rearing of Halda and Padma river-origin fish and that of exotic carps was developed by rearing newly imported fingerlings from China by the Department of Fisheries, Bangladesh. For selecting suitable activation solution, motility and swimming duration of sperm were tested at various concentrations of NaCl solution (0.1 to 1.3% NaCl). Highest motility and swimming duration of sperm of Catla (95.0±2% and 37.61±0.6min), Rohu (96±1% and 37.46±0.8min), Mrigal (94±2% and 40.20±0.5min), Silver carp (97±1% and 28.12±1.5min), Bighead carp (96±1% and 20.6±0.6min) and Grass carp (96±1% and 21.89±0.7min) were observed at 0.4 % NaCl solution. For determination of suitable cryoprotectant solution, DMSO and methanol were tested with two extenders, Alsever's solution and egg-yolk citrate at 5, 10 and 15% of the extenders and the sperm was incubated with the diluents for 5-40 min. Cryoprotectants at 5% and 10% concentrations produced better motility during 5 and 10 min of incubation. For cryopreservation of sperm, diluents were prepared using Alsever's solution and egg-yolk citrate with DMSO and methanol, and Alsever's solution with 10% DMSO at 1:9 sperm-diluent dilution performed best producing highest equilibration (Catla- 93±2%, Rohu- 94±2%, Mrigal- 95±2%, Silver carp- 93±1%, Bighead carp- 95±1% and Grass carp- 93±2%) and post-thaw (Catla 86±2%, Rohu- 90±3%, Mrigal-

91±2%, Silver carp- 85±3%, Bighead carp- 89±2% and Grass carp- 85±3%) motility of sperm. The sperm samples were equilibrated at three time periods (5, 10 and 15 min) at 4 ºC and motility was evaluated at each time. Sperm equilibrated for 10 min found best for IMCs and for 15 min for the exotic carps. For freezing the sperm, 5, 10 and 15ºC/min cooling rates were tested and cooling at 10°C/min showed best for both IMCs and exotic carps. Sperm of all the six species were cryopreserved through standardized protocols and stored in liquid nitrogen Dewars as cryogenic sperm bank. The cryopreserved sperm demonstrated a satisfactory level of post-thaw motility (>80%) over the 12 months storage. Breeding trials using cryopreserved sperm and fresh sperm were conducted in the selected public and private hatcheries of four regions (Mymensingh, Jashore, Faridpur and Barishal), where the average fertilization and hatching rates from cryopreserved sperm were recorded as 38.09±3.17% and 31.03±3.10% in Catla, 38.63±2.35% and 31.09±2.15% in Rohu, 38.05±2.10% and 30.38±1.90% in Mrigal, 41.27±4.27% and 30.86±4.01% in Silver carp, 43.04±1.81% and 33.00±1.29% in Bighead carp and 37.96±4.38% and 29.72±3.49% in Grass carp respectively. Seeds of both cryopreserved and fresh sperm-origin were reared separately in the respected hatcheries for growth and DNA microsatellite analysis. A significantly ($P<0.05$) higher growth was observed in cryopreserved sperm-originated seeds than those produced with fresh sperm of hatchery-reared males due to introducing quality germplasm through cryopreserved sperm. DNA microsatellite analysis exhibited inheritance of parental alleles to all cryopreserved sperm-originated seeds following Mendelian rules. For hands-on training on cryopreservation techniques as well as for technology dissemination, a series of training workshops were arranged for stakeholders (hatcheries and nursery operators, fish farmers, Scientists and NGO personnel, MS and PhD students and Junior Faculty Members of Fisheries Faculty, BAU) in four regions. During selection of stakeholders, preference was given to involve at least 30% female and youth participants.

Introduction

Aquaculture production of Bangladesh has increased many fold over the past two decades and accounts for about 57.39% of total fish production of the country (DoF, 2023). Bangladesh ranked 5th in aquaculture production and 3rd in open-water capture fisheries in the world (FAO, 2022). Indian major carps (IMCs), Catla (*Catla catla)*, Rohu *(Labeo rohita)* and Mrigal *(Cirrhinus cirrhosus*) are the prime aquaculture species and contributed about 32% to aquaculture and 19% to total fish production. Exotic carps, Silver carp (*Hypophthalmichthys molitrix*), Bighead carp (*Hypophthalmichthys nobilis*) and Grass carp (*Ctenopharyngodon idella*) also contributed 18% to aquaculture and 10% to total fish production (DoF, 2023). Presently, the total fish production of the country is 4.76 million metric tonnes but more production is needed for an ever-increasing population. Although aquaculture production is coming from indigenous and exotic carps, catfishes, pangas, barbs, tilapia, prawn and shrimp, IMCs and exotic carps occupy the top positions. Bangladeshis have first preference for IMCs, but these three exotic carps are also very popular. These exotic carps are comparatively inexpensive and known as “poor people’s fish”.

Aquaculture production is increasing due to adoption of new technologies (presently around 5276 kg/ha), but not reached maximum production level compared to other major fish-producing countries like China and India (DoF, 2023). Sustainable production has not been ensured, and environmental and anthropogenic threats continue to cause reduced production. Limited availability of quality seeds of IMCs and exotic carps and inadequate supply to the farmers is a major problem leading to decreased production. Previously, seeds of indigenous carps were collected from natural sources but now essentially 100% of seeds of indigenous and exotic carps are produced in government and private hatcheries through induced spawning. Last year, the country produced 6,27,586 kg of seeds of IMCs and exotic carps from 110 government (15,799 kg) and 874 private hatcheries (6,11,787 kg) (DoF, 2023) but some genetic reasons such as inbreeding, interspecific hybridization, and negative selection diminished the quality of seeds (Simonsen et al., 2004, 2005; Hansen et al., 2006; Alam

and Islam, 2005). As a result, fry available for culture showing slow growth, high mortality and disease susceptibility. Therefore, many farmers became frustrated and are changing their professions. To overcome these problems, the government had begun brood banking program for IMCs in government hatcheries and distribution of broods to other public and private hatcheries. But in the real sense, it is difficult to supply broods to the large number of public and private hatcheries. The brood banks are developed by rearing naturally collected seeds of IMCs (from the Halda, Padma and Jamuna rivers) but availability and quality of wild seeds are a big concern. Wild seed production has been reduced over the years and contributes less than 1% of total production. Seed quality of exotic carps is also diminishing and replenishment of stocks is difficult and expensive. In addition, more than 99% of seed production occurs in hatcheries, and hybridization among silver carp, bighead carp and grass carp (and other carps), leading to inter-specific introgression among the IMCs is taking place. Establishment of a cryogenic sperm repository of IMCs and exotic carps and use of thawed sperm in hatcheries could resolve these existing genetic problems. It would also help brood banks in government and private sectors by providing a reliable source of quality germplasm.

Cryopreservation is an important *ex-situ* method of germplasm conservation, and FAO has endorsed it as a major strategy for conservation of fish resources (Khoshoo, 1997). Cryopreservation has been studied around the world and sperm cryopreservation protocols have been developed for more than 200 species of finfish and shellfish (Tiersch, 2000) including endangered fish species (Tiersch et al., 1998). Sperm banks have been developed in Europe, USA, Brazil, Australia and New Zealand (Martínez-Páramo et al., 2017). Cryopreserved sperm banks have been developed for the common carp in Hungary (Horváth, 2007) and Israel (Lubzens et al., 1997), Atlantic salmon in Norway (O'Reilly and Doyle, 2007), Tambaqui (*Colossoma macropomum*) and Cachara (*Pseudoplatystoma reticulatum*) in Brazil (Streit et al., 2013). The United States Department of Agriculture (USDA) operates the National Animal Germplasm Program (NAGP), which includes conservation of cryopreserved samples from several fish species. As a direct conservation program, cryopreservation

has been applied to restocking of the Adriatic lineage of the grayling (*Thymallus thymallus*) in Slovenia to prevent hybridization (Horváth et al., 2012).

Cryopreservation research was initiated in the Department of Fisheries Biology and Genetics, Bangladesh Agricultural University (BAU) in 2002, and protocols have been developed for IMCs (*Catla catla, Labeo rohita, Cirrhinus cirrhosus, Labeo calbasu*), and exotic carps (*Hypophthalmichthys molitrix*, *Hypophthalmichthys nobilis, Ctenopharyngodon idella, Cyprinus carpio)*, catfishes (*Clarias batrachus, Heteropneustes fossilis*), indigenous endangered fish species (*Ompok pabda, Mystus vittatus, Mystus tengra, Nandus nandus, Mastacembelus armatius, Tot tor)*, barb (*Puntius sarana, Barbonymus gonionotus*) and tilapia (*Oreochromis niloticus*) (Sarder, 2004; Sarder et al., 2010; Sarder et al., 2009, 2012, 2013; Hossain and Sarder, 2009; Khan et al., 2015, Kabir et al., 2022). Seed production using cryopreserved sperm of Rohu of Halda river origin in government and private hatcheries in Mymensingh showed 67% fertilization and 38% hatching (compared to fresh sperm 82% fertilization, 64% hatching) (Rashed, 2016). Similarly, cryopreserved sperm of Mrigal showed 64% fertilization and 53% hatching compared to 67% and 56% by fresh sperm (Hossain et al., 2018). In both cases, cryopreserved sperm of Rohu and Mrigal of Halda river-origin were used while the fresh sperm (control) were used from respective hatcheries where the experiments were conducted. Seeds produced by cryopreserved sperm of both species showed higher growth than those produced from fresh sperm. Also, F_1 males of Rohu produced from thawed sperm showed better breeding performance in terms of fertilization and hatching of eggs, and their progeny showed higher growth than those produced by hatchery-origin males (Ullah, 2018). This study suggested that cryopreservation does not impair the growth of fish and demonstrates that it can be used to provide improved genetics for better growth. Thus, cryopreservation is available for use in hatcheries for quality bloodstock development as well as seed production if the technology can be scaled up and linked to genetic improvement programs.

According to the plan of the research, improved donor broodstocks of Catla, Rohu and Mrigal were developed by rearing the Halda and Padma river-originated seeds at the BAU campus. It has been established through different studies that seeds of IMCs collected from Halda and Padma rivers showed better performance compared to other sources (Hansen et al., 2006). Similarly, improved broodstocks of three exotic carps were developed by rearing newly imported fingerlings from China by the Department of Fisheries. Sperm were collected from males of the IMCs and exotic carps broodstocks, cryopreserved and stored in liquid nitrogen dewars as cryogenic sperm bank. Breeding was conducted in 36 government and private hatcheries of four regions (Jashore, Barishal, Faridpur and Mymensingh) in which 28 hatcheries (breeding hatcheries) successfully produced seeds. Both cryopreserved sperm and fresh sperm of hatchery-origin males (control) were applied in breeding. Seeds of both cryopreserved and fresh sperm-origin were reared separately in nursery ponds in 22 hatcheries (breeding hatcheries) for at least six months for comparing their growth performance and eventually for brood production. DNA microsatellite markers as another quality assessment tool were also used to evaluate their genetic quality. This activity facilitated establishment of government and private brood banks and produced quality genetic seeds in hatcheries. Technology adoption was promoted through field trials of cryopreserved sperm-originated seeds on four demonstration hatcheries and fish farms in Barishal, Faridpur and Mymensingh regions, and the survival and growth performance of the seeds were evaluated. The technology were disseminated through training workshop of about 500 hatchery owners, nursery producers, and growers, scientists, NGO personnel and 50 graduate students and junior faculty members of Fisheries Faculty of Bangladesh Agricultural University.

Overall objective:

To increase production of Indian major carps and exotic carps by improving broodstock quality and seeds through establishing a national genetics program based on cryogenic sperm banking.

In order to achieve the target, the following objectives have been set up:

i) To develop donor broodstocks of Indian major carps and three exotic carps.

ii) To cryopreserve sperm of IMCs and exotic carps and develop a cryogenic sperm bank.

iii) To produce seeds of carps in hatcheries using cryopreserved sperm and characterize and assess their quality through growth study and DNA microsatellite analysis.

iv) To assess the performance of cryopreserved sperm for establishment of sperm banks in different regions.

v) To evaluate the adopting ability of technology by the stakeholders.

Research Methods

Experiment-1: Development of donor broodstocks of three Indian major carps (IMC) and three exotic carps

a) Development of donor broodstocks of IMCs and three exotic carps

Broodstocks of Indian major carps (IMCs) (Catla, Rohu and Mrigal) of Halda river-origin maintained in the Department of Fisheries Biology and Genetics, BAU was used for experiments. Besides the existing stock, a new donor broodstock of IMCs was developed at Field Laboratory Complex of Fisheries Faculty, BAU by collecting and rearing of Halda and Padma river-origin fish. Similarly, broodstocks of three exotic carps (Silver carp, Bighead carp and Grass carp) was developed by rearing newly imported (in 2019) fingerlings from China by the Department of Fisheries (DoF). The imported exotic carps were collected from a Government Fish Seed Multiplication Farm, Maskhanda, Mymensingh and stocked in a pond in the premises of Fisheries Faculty. The fish were stocked at 1200-1500 kg/ha and reared with supplementary feeds containing 25-30% protein provided twice a day at 4-5% body weight of the stocked fish. Fish health and water quality of ponds were monitored regularly during the experimental period.

b) Genetic characterization of donor broodstocks

Genetic characterization of broods of Indian major carps and exotic carps was conducted using DNA microsatellite markers (Tonny et al., 2014) following the procedures described below:

(i) Collection of fin samples and DNA extraction

Fin samples were collected from at least 20 individuals for each fish species and preserved in 95% ethanol. DNA was extracted from 30 mg of each fin sample following salt-isopropanol DNA extraction method.

(ii) Primer selection and amplification of DNA

Three to five (3-5) pairs of primers were selected for each species. Fifty ng DNA was used for 10 µl PCR reaction mixtures containing 0.25 mM each of the dNTPs, 2 µM of each primer, 1.5 mM $MgCl_2$, 1 µl 10-X reaction buffer and 1 unit of *Taq* DNA polymerase. Standard PCR thermal protocols were used and the amplified products were examined in 1% agarose gels.

(iii) Polyacrylamide gel electrophoresis and scoring of the bands

About 3 µl of PCR product of each sample was electrophoresed on a 6% denaturing polyacrylamide gel using vertical electrophoresis. The gel was stained with ethidium bromide and the electrophoretic bands corresponding to particular alleles at each locus were scored. Data were analyzed using GenAlEx (version 6.1) and POPGENE (version 1.31).

Experiment-2: Cryopreservation of sperm of IMCs and exotic carps and development of cryogenic sperm bank

a) Standardization of sperm cryopreservation protocols of IMCs and exotic carps

(i) Selection of mature male fish and induced with pituitary gland (PG) extract

Mature males of IMCs and exotic carps were collected from broodstock ponds. They were selected by observing their phenotypic characteristics such as rough pectoral fin, flat abdomen and pointed and extruded urogenital papillae. Moreover, a mature male discharged sperm upon applying a gentle pressure on its abdomen. Selected males were conditioned for 5-6h in circular tank with constant

water flow prior to hormone injection. Then the fishes were induced by injecting a single dose (2 mg/kg body weight) of pituitary gland extract (PGE)..

(ii) Collection of sperm and quality assessment

Sperm was collected after 6h of hormone injection through stripping and stored immediately on ice to prevent quality deterioration. Before collection of sperm, excess moisture, urine, gut exudates and mucus were wiped from the genital region with absorbent paper. For avoiding contamination of urine and water, a gentle pressure was applied on the abdomen to release a small amount of sperm before final collection. Concentrated whitish sperm was collected in 1.5-ml autoclaved eppendorf tubes and placed on ice for storage and further processing in the Fish Genetics and Biotechnology Laboratory, Department of Fisheries Biology and Genetics, BAU.

The quality of collected fresh sperm was examined under a photographic microscope by placing 1-2µl of sperm mixed with 18µl of water on a glass slide. The motility and swimming duration of activated sperm were observed immediately. Sperm with more than 90% motility was selected for cryopreservation. Moreover, for reproducibility of cryopreservation total concentration of sperm was also determined through standard haemacytometer counting. This concentration value was used to identify the sperm-egg ratio during fertilization of eggs.

(iii) Selection of suitable activation solution

For selecting suitable activation solution, motility and swimming duration of sperm were tested at various concentrations (0.1 to 1.2%) of NaCl solution (Sarder et al., 2013). Twelve graded dilutions of NaCl (0.1 to 1.2%) corresponding to an osmotic pressure ranging from 48 to 383 mOsmol/kg were prepared by dissolving NaCl in distilled water for each dilution. About 1-2 µl of sperm was placed onto a glass slide and 18 µl of each graded NaCl solution was added to activate the sperm. The percentage of motility and duration of swimming were determined under microscope at 5 min interval.

(iv) Determination of suitable cryoprotectant solution

The toxicity of cryoprotectants to sperm was evaluated following the method (Yang et al., 2007); Sarder et al., 2013). Two extenders, Alsever's solution and egg-yolk citrate and two cryoprotectants, DMSO and methanol were used. Each cryoprotectant was mixed with extenders to prepare final concentration of 5%, 10% and 15% in the diluents. Sperm was diluted with diluents at a ratio of 1:9 for Alsever's solution and 1:4 for egg-yolk-citrate solution. For each concentration, 1 to 2 µl of the milt-diluent solution was placed on a glass slide and sperm movement was tracked with a photography system (OLYMPUS, Model CX41 & DP22, Japan) at 10x magnification for 5 to 40 min at 5-min intervals.

(v) Determination of suitable diluent (extender + cryoprotectant) and sperm-diluent dilution ratio

Cryopreservation trials were conducted using Alsever's solution and Egg-yolk citrate as extender, and dimethyl sulfoxide (DMSO) and methanol as cryoprotectant to determine suitable diluent and sperm-diluent ratio. The diluent was prepared by mixing 10% cryoprotectant with 90% extender. The sperm sample was diluted with the diluents at the ratio of 1:9, 1:12 and 1:15 (sperm : diluent) for Alsever's solution and 1:4, 1:6 and 1:9 for egg-yolk citrate. The diluted sperm was equilibrated on ice for about 10 min after mixing. Sperm motility was evaluated during equilibration as described above. During equilibration period, 0.23 ml diluted sperm was drawn into each 0.25 ml plastic French straw using a micropipette and the unplugged ends of the straws were sealed manually by using a heated crucible tongs maintaining 1 cm air space. The sealed plastic straws containing diluted sperm were loaded into the cryochamber and placed in the cryobath containing liquid N_2. Then the sperm samples were cooled from the ambient temperature (4ºC) to -80ºC at a rate of 10ºC/min by using one-step freezing protocol through a computer-controlled freezer (CL-3300). After freezing, frozen straws were removed from the cryochamber and immediately loaded into a canister and placed into a liquid nitrogen dewar (-196°C) for long-term storage. For assessing post-thaw motility

of sperm, 1 or 2 frozen straws were retrieved from the liquid nitrogen container and thawed at room temperature for about 1 min or at 30-35ºC in a water bath for 15 sec. The straw was cut at both ends and 2 µl of sperm was placed onto a glass slide and 18 µl of water was added to activate for motility estimation. Then the post-thaw motility of the sperm was assessed under photographic microscope. On the basis of percent of motility evaluated during equilibration and post-thaw period, suitable diluent (extender + cryoprotectant) and sperm-diluent dilution ratio were selected for cryopreservation of sperm.

(vi) Determination of suitable equilibration time

Two extenders, Alsever's solution and egg-yolk citrate and two cryoprotectants, DMSO and methanol were evaluated for suitable equilibration time. Sperm-diluent ratio of 1:9 was maintained for Alsever's solution and 1:4 for egg-yolk citrate based on the result of the previous experiment. The sperm samples were equilibrated for a time periods (5, 10, 15 and 20 min) and motility was evaluated at each time period.

(vii) Determination of suitable cooling rate

For determining the effects of cooling rate on post-thaw motility of sperm, diluted samples were frozen from 0ºC to -80ºC with three different rates (5ºC/min, 10ºC/min and 15ºC/min) and the post-thaw motility of cryopreserved sperm was evaluated under microscope for each cooling rate. Samples were processed with two extenders (Alsever's solution and egg-yolk citrate) and two cryoprotectants (DMSO and methanol) and equilibrated for 10 min. The diluents were prepared by mixing 10% cryoprotectants with 90% extenders. Sperm dilution was maintained at 1:9 (sperm: diluent) for Alsever's solution and 1:4 for egg-yolk citrate

b) Cryopreservation of sperm and development of cryogenic sperm bank.

Based on the above investigations, sperm cryopreservation protocols of three IMCs and three exotic carps were standardized, and sperm were cryopreserved and stored in liquid nitrogen dewars using different canisters for long-term preservation as cryogenic sperm bank. To avoid confusion and

misidentification of cryopreserved samples, the canisters were marked by numbering and the straws were labeled by species and source.

Statistical analysis

Percentage data of sperm motility (fresh, equilibration and post-thaw motility) were subjected to arcsine transformation before statistical analysis. The effects of extenders and cryoprotectants on equilibration and post-thaw motility of spermatozoa, and their interaction were analyzed using one-way ANOVA of MSTAT followed by Least Significant Difference Test at 5% probability level.

Experiment-3: Production of seeds of IMCs and exotic carps in hatcheries using cryopreserved sperm and assessment of their quality through growth study and DNA microsatellite analysis

a) Production of seeds of carps in hatcheries using cryopreserved sperm

For production of seeds of IMCs and exotic carps using cryopreserved sperm of the cryogenic sperm bank, breeding trials were conducted in 36 government and private hatcheries in Jashore, Barishal, Faridpur and Mymensingh regions of Bangladesh. The cryopreserved sperm of Halda or Padma river-origin IMCs and China origin exotic carps were carried to the selected hatcheries in a 20-L liquid nitrogen dewar. Extra liquid nitrogen was also carried to the hatcheries in another 10-L dewar for supporting the cryopreserved straws if needed. Ovulated eggs were collected from hatchery-origin females after 6h of second hormone injection and fertilized with cryopreserved sperm. Generally, ten (10) straws containing cryopreserved sperm (230µl diluted sperm in each straw) were used for fertilizing a batch of approximately 5000 eggs (one tea spoon). Before fertilization, the post-thaw motility of cryopreserved sperm was checked under microscope and the sperm having more than 75% motility were used in breeding trials.

For fertilization, eggs of a hatchery-origin female were taken in to a small plastic bowl using a tea spoon and the required number of frozen straws were thawed at room temperature for 1 min and after cutting the straws at both ends, the thawed sperm were added to the egg mass and mixed thoroughly by a soft clean chicken feather for about 1 min. A similar number of eggs from the same donor

female were fertilized with fresh sperm of a hatchery-reared male. The eggs fertilized with cryopreserved sperm and fresh sperm were considered as treatment and control- group, respectively. After fertilization, eggs of both groups were incubated separately in two bottle incubators maintaining a constant water flow. The fertilization and hatching rates of eggs were calculated for both cryopreserved (treatment) and fresh sperm (control) groups.

b) Rearing of seeds and assessment of their quality by growth study

To examine the quality, seeds of both cryopreserved sperm and fresh sperm-origin were reared separately in the respective hatcheries. Firstly, the produced seeds were reared in two hapas for 2 to 3 weeks and then they were reared in two separate nursery ponds at the stocking density of 25000/ha with supplementary feeds. The standard management practices were followed to rear the seeds for at least 6 months. Monthly sampling was done by measuring the length and weight of fry/fingerlings to observe their growth performance and health condition. Water quality parameters of the rearing ponds were also measured at regular intervals. Growth performances of seeds produced by cryopreserved sperm (treatment) as well as fresh sperm (control) were compared.

c) Assessment of seed quality by DNA microsatellite analysis

The genetic quality of the seeds was determined by DNA microsatellite analysis. Fin samples were collected from donor males, females and their offspring (cryopreserved sperm-originated and fresh sperm-originated groups), and preserved with 95% ethanol in eppendorf tubes at -20°C. Genomic DNA was extracted from each fin sample and then amplified with specific DNA microsatellite markers through standard PCR thermal protocols. PCR amplifications were confirmed by running the PCR products in 1% agarose gel. Then the PCR product of each sample was electrophoresed on 6% denaturing polyacrylamide gel (PAGE) and stained with ethidium bromide. The electrophoretic bands corresponding to particular alleles at each locus were scored. Thus, microsatellite profiles of donor males, females and their offspring (F1 generation) were generated using specific DNA

microsatellite markers and inheritance of particular alleles of parents to F1 generation were determined.

Statistical analysis

The effects of cryopreserved sperm and fresh sperm on fertilization and hatching of eggs were analyzed by using Paired t-test followed by Duncan's Multiple Range Test (DMRT). Independent t-Test was used to determine the effects of cryopreserved sperm on the growth of fry compared to control. Genetic constitution of the seeds was examined through GenAIEx and POPGENE program (Peakall and Smouse, 2012).

Experiment-4: Assessment of the performance of cryopreserved sperm for establishment of cryogenic sperm bank

To study the feasibility for establishing cryogenic sperm bank, the effect of storage time on cryopreserved sperm was assessed. Sperm was cryopreserved and stored in liquid nitrogen Dewars. The post-thaw motility of cryopreserved sperm was determined for 12 months by thawing of 1 or 2 straws at each month under photographic microscope. As liquid nitrogen gets reduce through evaporation, the Dewars were refilled with liquid nitrogen at weekly basis to maintain the quality of cryopreserved sperm during storage. Moreover, the feasibility of transportation of cryopreserved sperm and liquid nitrogen to the far distant hatcheries were also assessed during breeding trials. These efforts would help to establish cryogenic sperm repository network of fish throughout the country.

Statistical analysis

The data generated from the evaluation of storage time effect on post-thaw motility of cryopreserved sperm were analyzed using one-way ANOVA of MSTAT followed by Least Significant Difference Test at 5% probability level.

Experiment-5: Evaluation of the adopting ability of technology by the stakeholders

Seeds produced using cryopreserved- and control sperm in the breeding hatcheries were reared in four technology adoption hatcheries and farms in Faridpur, Barishal and Mymensingh regions. A monthly sampling was done by measuring the length and weight of fry /fingerlings for observing their growth performance. Besides, an on-station trial was conducted by rearing a portion of cryopreserved and fresh sperm-originated seeds in ponds at Fisheries Faculty Field Laboratory Complex, BAU. Fry/fingerlings were sampled monthly to evaluate their growth performance. This on-station growth performance of seeds was compared with those reared in off-station farms.

For dissemination of the cryopreservation technology, a series of technology dissemination workshops were arranged for the stakeholders (hatchery owners, nursery operators, fish farmers, Scientists, NGO personnel) in the study areas. Details of fish sperm cryopreservation techniques, fertilization of eggs using cryopreserved sperm and rearing of seeds were presented in the workshops through lectures, power point presentations and video documentary demonstration.

Research Results

Experiment-2: Cryopreservation of sperm of IMCs and exotic carps and development of cryogenic sperm bank

For standardization of cryopreservation protocols of three Indian major carps and three exotic carps, different parameters such as selection of suitable extenders, cryoprotectants, combinations of extenders and cryoprotectants, suitable sperm dilution, suitable equilibration periods and cooling rates were tested and optimized. The findings of these parameters of all six species were more or less similar, so due to space limitation results of the parameters of Rohu (*L. rohita*) are presented here (Fig. 1-7) and results of the cryopreservation parameters of other five species are presented in the Appendix (Fig. 8-42).

Rohu (*Labeo rohita*)

i) Effects of osmotic pressure on sperm motility activation and swimming duration

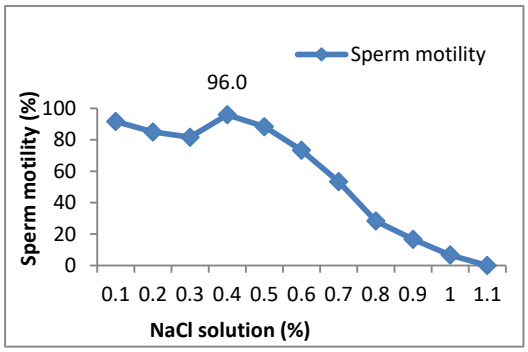

Fig.1 Motility of sperm of *Labeo rohita* along an osmotic gradient of NaCl concentrations.

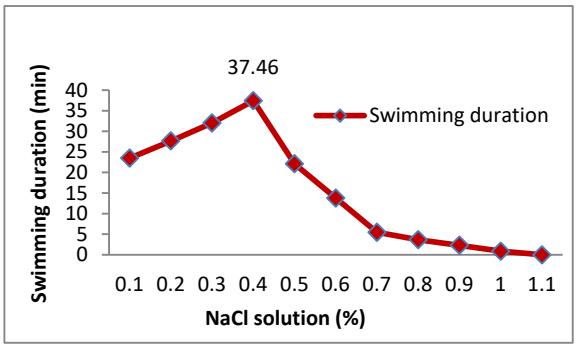

Fig.2 Swimming duration of *Labeo rohita* sperm at different concentrations of NaCl.

- Highest sperm motility (96±1%) and swimming duration (37.46±0.8 min) were observed at 0.4% NaCl solution.

ii) Evaluation of toxicity of cryoprotectants to sperm

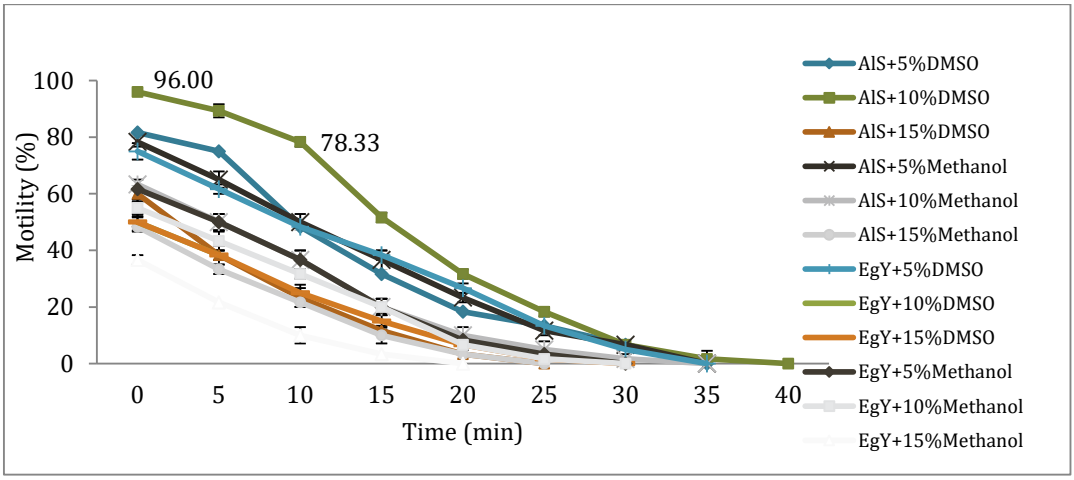

Fig. 3 Motility of *Labeo rohita* sperm at different concentrations of cryoprotectants and incubation times.

- DMSO and Methanol produced better motility at 5% and 10% conc. during 5 and 10 min incubation.
- Highest motility was observed with Alsever's solution plus 10% DMSO.

iii) Determination of suitable sperm-diluent ratio and diluent

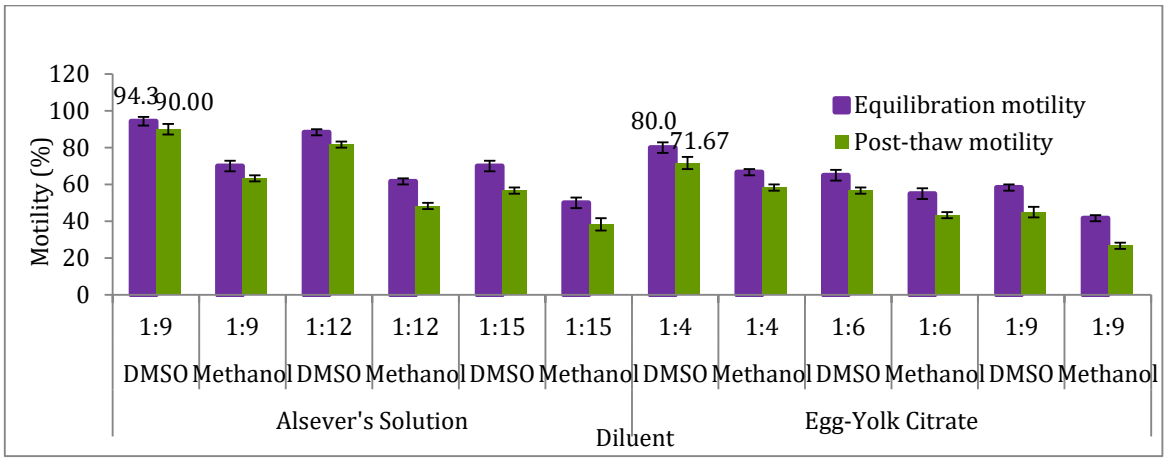

Fig. 4 Equilibration and post-thaw motility of sperm of *Labeo rohita* in different combinations of extenders and cryoprotectants and at different dilutions of milt.

- Alsever's solution with 10% DMSO at 1:9 sperm-diluent ratio produced best equilibration (94±2%) and post-thaw (90±2%) motility.

) Deq

iv) Determination of suitable equilibration time

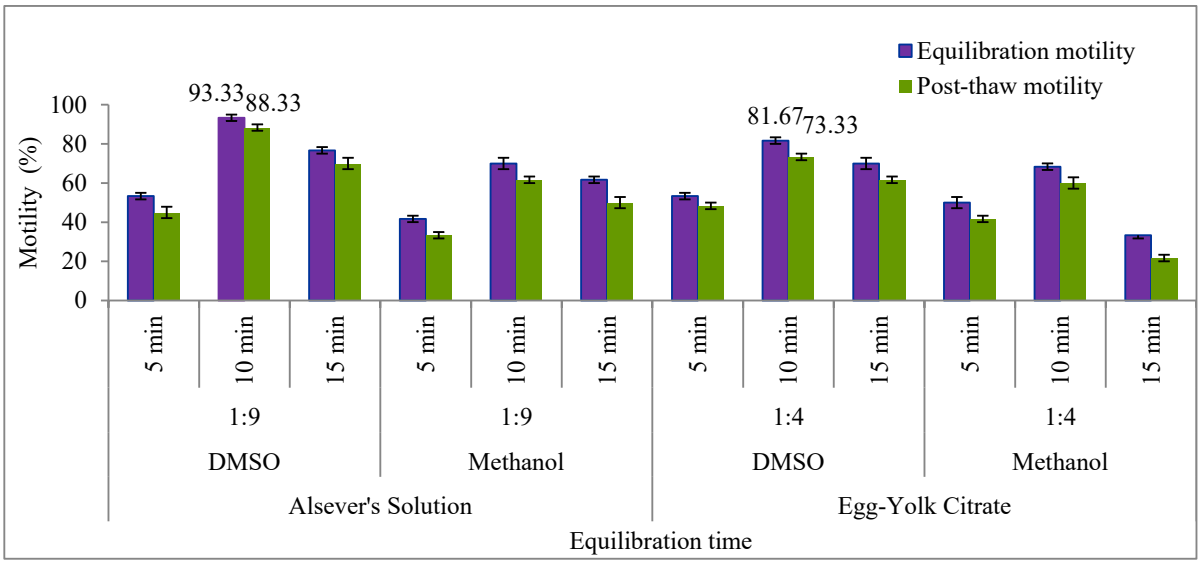

Fig. 5 Equilibration and post-thaw motility of sperm of *Labeo rohita* equilibrated at three different periods during cryopreservation.

- Highest equilibration (93.3±1.7%) and post-thaw (88.3±1.7%) motility during 10 min equilibration period with Alsever's solution plus 10% DMSO.

v) Determination of suitable cooling rate

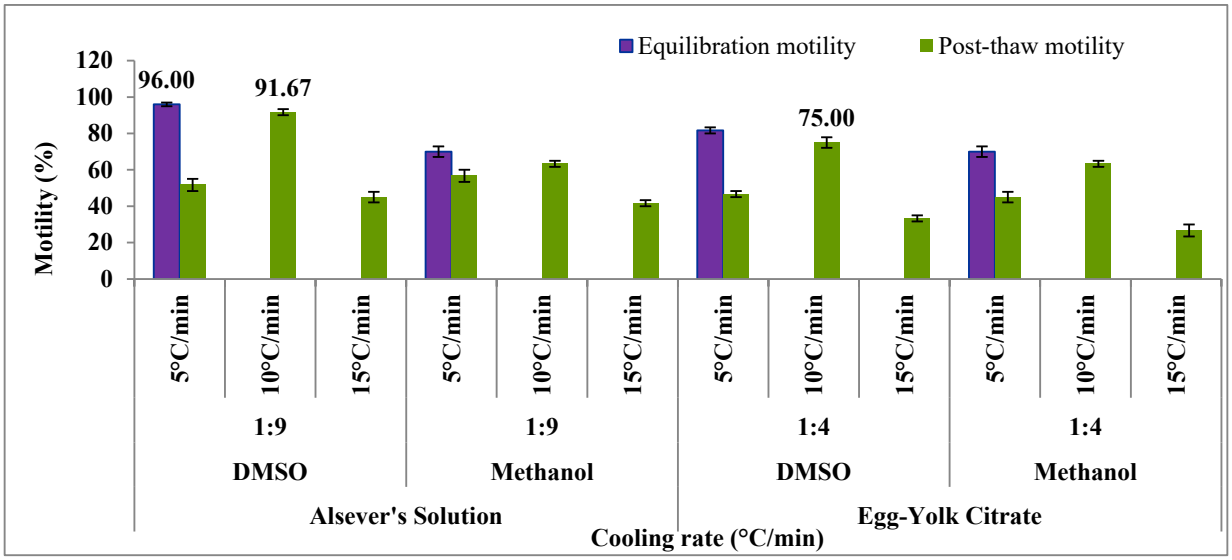

Fig. 6 Post-thaw motility of sperm of *Labeo rohita* at different cooling rates during cryopreservation**.**

- Highest post-thaw motility (91±2%) of sperm at 10°C/min with Alsever's solution plus 10% DMSO.

i) Storage time effect on post-thaw motility of cryopreserved sperm

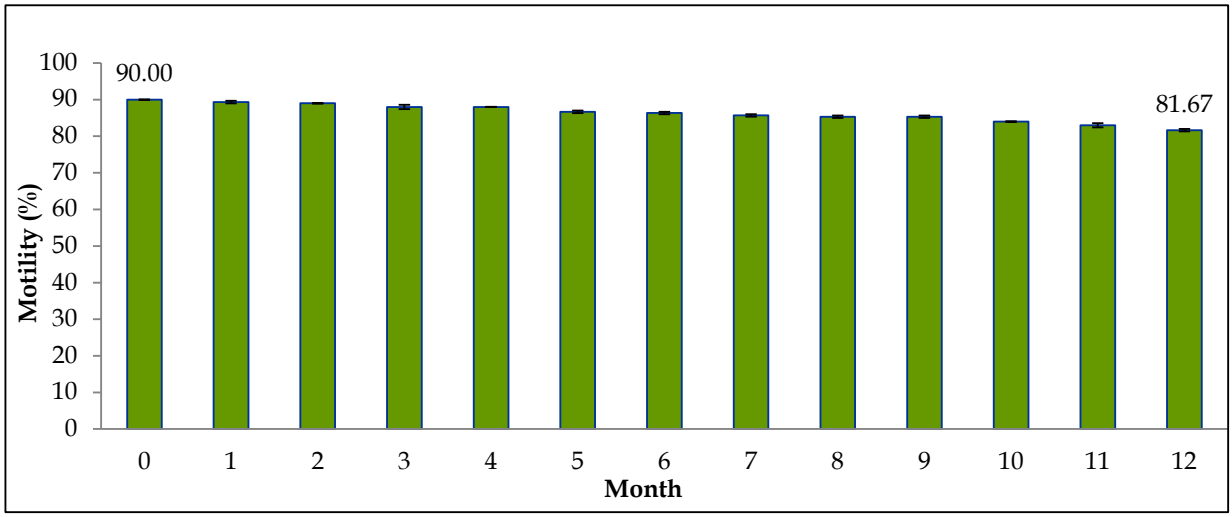

Fig. 7 Post-thaw motility of cryopreserved sperm of *Labeo rohita* for twelve months storage period.

- Post-thaw motility of cryo-stored sperm remained at satisfactory level (initial 90±0% and final 81±0.3%) for 12 months storage period.

Experiment-2: Production of seeds of Catla, Rohu, Mrigal, Silver carp, Bighead carp and Grass carp in the selected hatcheries using cryopreserved sperm

Fish breeding was done in both public and private hatcheries in Mymensingh, Jashore, Faridpur and Barishal regions in the three consecutive breeding seasons (2021, 2022, 2023). The following Table 1 and Table 2 showing the breeding trials conducted in four regions.

Table 1 Breeding trials, seed production and rearing of seeds in three breeding seasons

Year	Breeding trial (Hatchery no.)	Fry production (Hatchery no.)	Rearing (Hatchery/Nursery)
2021	9	7	6
2022	22	17	16
2023	5	4	4
Total	36	28	26

Table 2 Region-wise breeding trials conducted in public and private hatcheries

Activities	Mymensingh	Jashore	Faridpur	Barishal	Total (Govt. and Private hatcheries)
Breeding	16	8	3	9	36 (10+26)
Fry production	12	8	2	6	28 (8+20)
Rearing	11	8	2	5	26 (5+21)

Fertilization and hatching of eggs using cryopreserved and fresh sperm were calculated in each hatchery for each of the six species. Due to space limitation, the average fertilization and hatching results are presented here (Table 3) and species-wise breeding results are presented in the Appendix (Table 4-9).

Table 3 Species-wise average fertilization and hatching rates of eggs

Sl	Species	Fertilization (%)		Hatching (%)	
		Cryopreserved sperm	Fresh sperm	Cryopreserved sperm	Fresh sperm
1	Catla	38.09±3.17	77.25±4.15	31.03±3.10	65.20±4.30
2	Rohu	38.63±2.35	76.88±2.83	31.09±2.15	65.62±3.33
3	Mrigal	38.05±2.10	78.07±3.71	30.38±1.90	66.55±4.17
4	Silver carp	41.27±4.27	73.53±3.72	30.86±4.01	59.09±4.17
5	Bighead carp	43.04±1.81	77.79±4.14	33.00±1.29	63.46±5.00
6	Grass carp	37.96±4.38	72.21±2.98	29.72±3.49	60.82±5.04

Experiment-3: Production of seeds of carps in hatcheries using cryopreserved sperm and assessment of their quality through growth study and DNA microsatellite analysis.

i) Growth study

Fry/fingerlings of six species reared at different hatcheries and farms were monthly (for at least six month) sampled and the hatchery-wise growth data (weight) after 6th month of rearing of each species are presented in Fig. 43-48. A remarkable growth difference has been observed among the rearing facilities of each species due to variation in management practices, rearing facilities, water qualities, and feeding regimes. In most of the cases, cryopreserved sperm-originated fingerlings showed higher growth than those produced from fresh sperm (control).

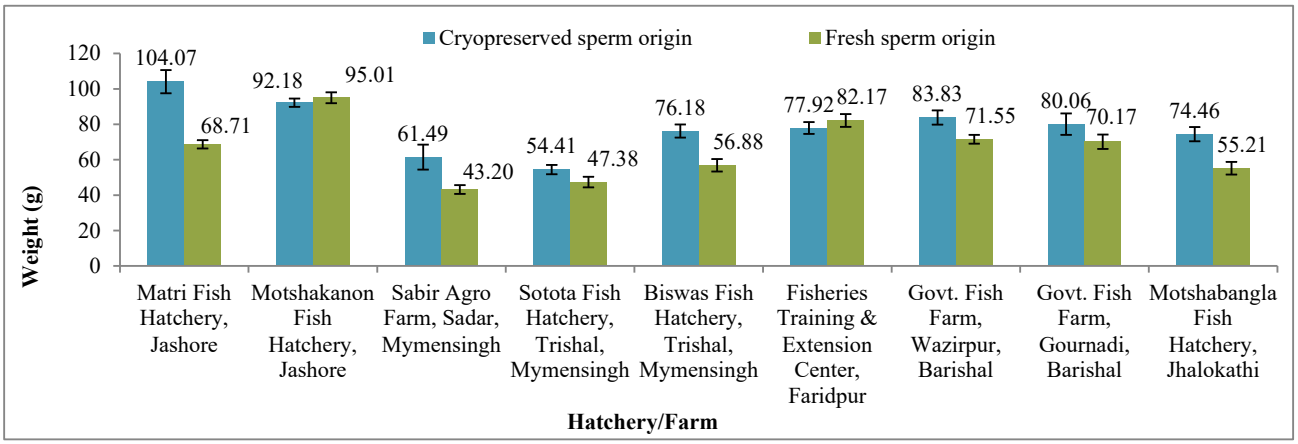

Fig. 43 Comparative growth performance of cryopreserved and hatchery- owned fresh sperm-originated seeds of Rohu (*L. rohita*). On an average, about 19.37% higher growth was observed in cryopreserved sperm-originated fingerlings.

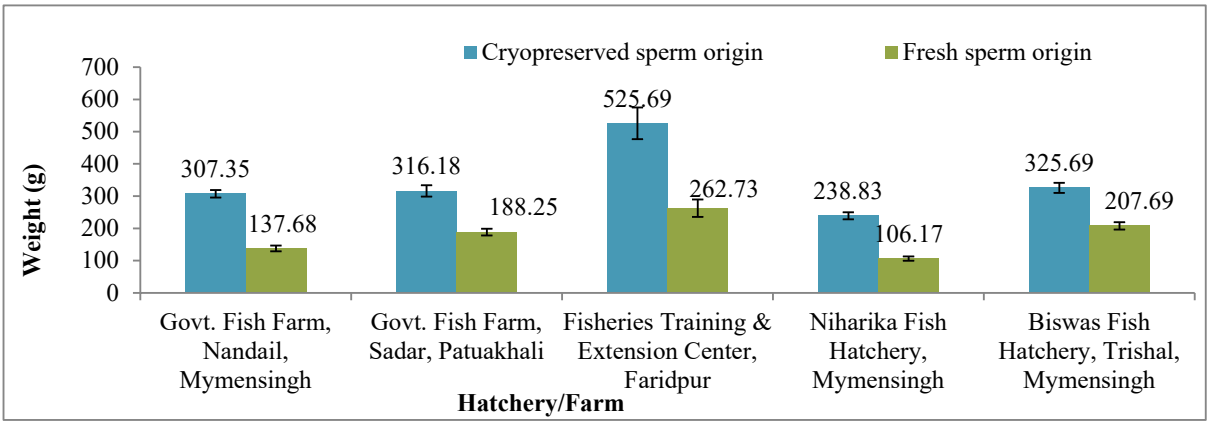

Fig. 44 Comparative growth performance of cryopreserved and hatchery- owned fresh sperm-originated seeds of Catla (*Catla catla*). On an average, about 89.88% higher growth was observed in cryopreserved sperm-originated seeds.

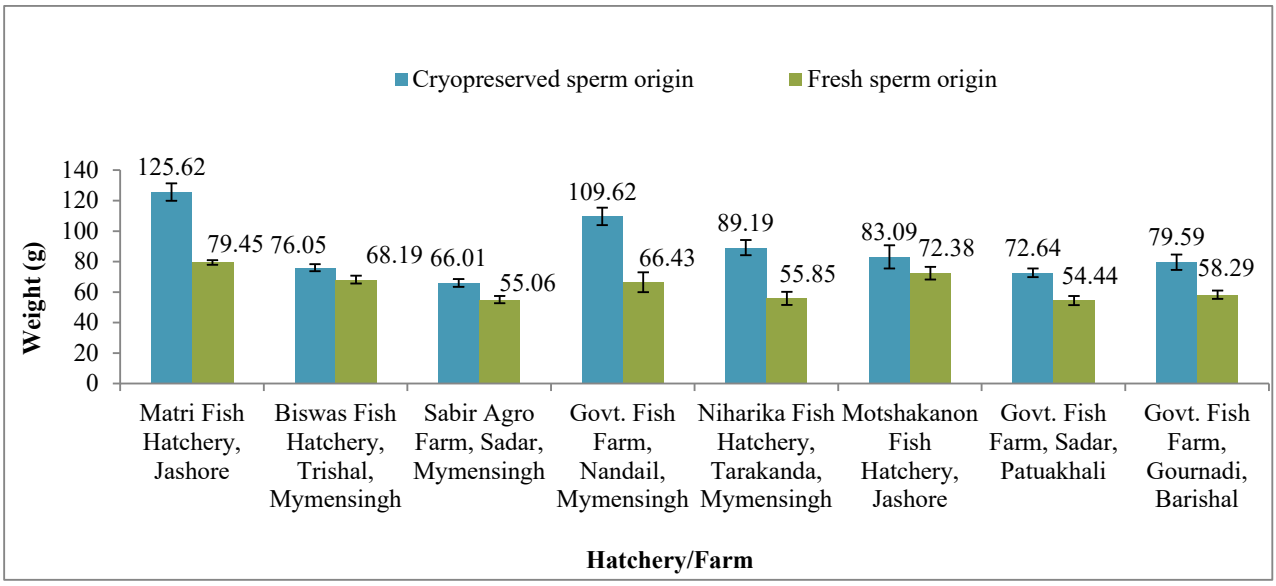

Fig. 45 Comparative growth performance of cryopreserved and hatchery- owned fresh sperm-originated seeds of Mrigal (*Cirrhinus cirrhosus*). On an average, about 37.59% higher growth was observed in cryopreserved sperm-originated seeds.

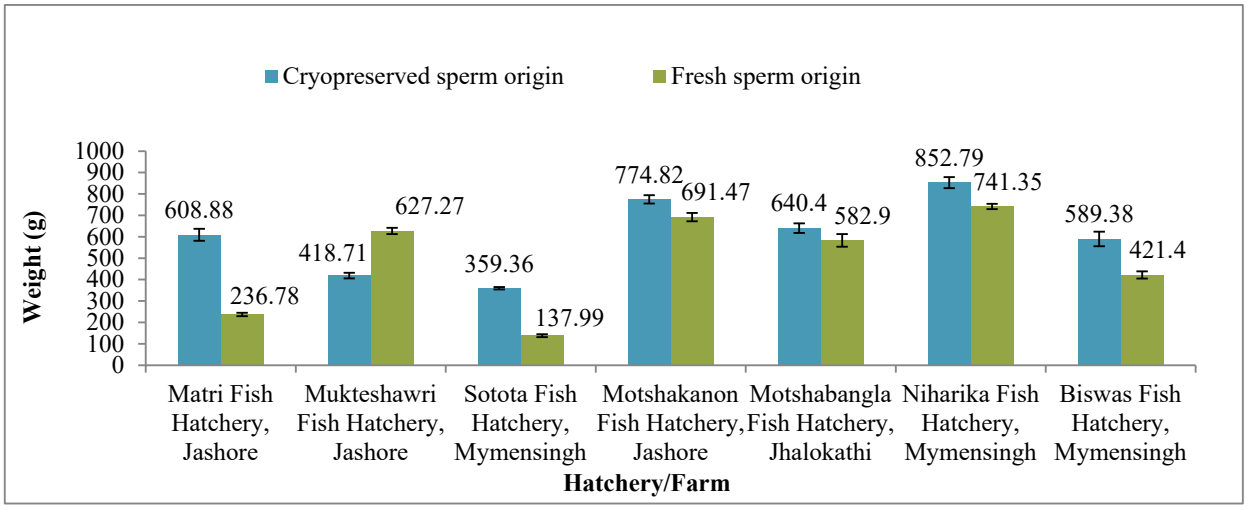

Fig. 46 Comparative growth performance of cryopreserved and hatchery owned fresh sperm-originated seeds of Silver carp (*Hypophthalmichthys molitrix*). On an average, about 19.37% higher growth was observed in cryopreserved sperm-originated seeds.

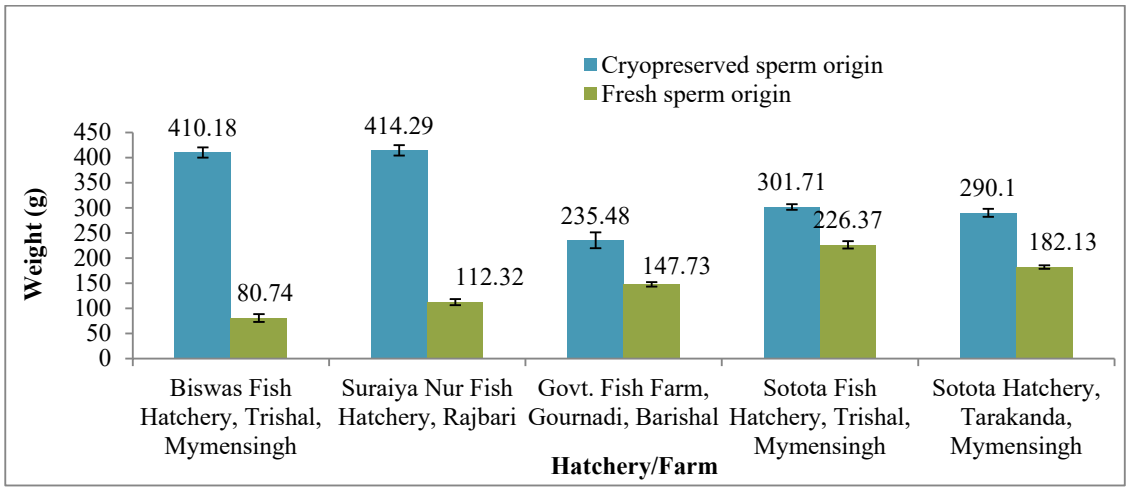

Fig 47 Comparative growth performance of cryopreserved and hatchery owned fresh sperm-originated seeds of Bighead carp (*Hypophthalmichthys nobilis*). On an average, about 120.44% higher growth was observed in cryopreserved sperm-originated seeds.

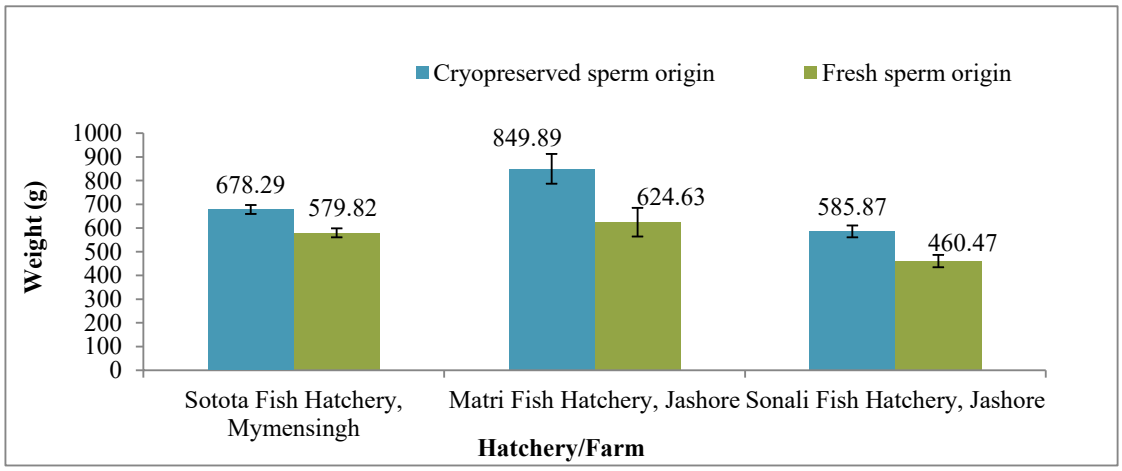

Fig. 48 Comparative growth performance of cryopreserved and hatchery owned fresh sperm-originated seeds of Grass carp (*Ctenopharyngodon idella*). On an average, about 26.98% higher growth was observed in cryopreserved sperm-originated seeds.

Outputs and Conclusions

Donor broodstocks (male and female) of Indian major carps (Catla, Rohu and Mrigal) of Padma and Halda river-origin were developed. Similarly, broodstocks of three exotic carps (Silver carp, Bighead carp and Grass carp) were developed by rearing newly imported fingerlings from China by the Department of Fisheries. However, the following outputs were achieved:

i) Basic parameters of sperm cryopreservation protocols (e.g. activation solution, sperm dilution, cryoprotectant concentration, combinations of extenders and cryoprotectants, equilibration period, cooling rate) of all six species were optimized.

ii) Sperm of Indian major carps and exotic carps were collected from the developed broods and cryopreserved following the optimized protocols. Thus, a cryogenic sperm bank of these species was developed.

iii) Seeds of three Indian major carps and three exotic carps were produced using cryopreserved sperm in public and private hatcheries in Mymensingh, Jashore, Faridpur and Barishal regions, and their growth performance was compared by rearing them in nursery facilities along with respective controls (fresh sperm-originated seeds). The quality of seeds was also assessed by DNA microsatellite markers. The seeds or fingerlings produced from cryopreserved sperm demonstrated higher growth compared to those produced from fresh sperm (control). The ultimate goal of rearing fingerlings was to convert them into quality broods.

iv) Performance of cryopreserved sperm was assessed and the feasibility for establishing the sperm bank was evaluated. Cryopreserved sperm was demonstrated to maintain viability for 12 months by assessing post-thaw motility. For conducting breeding trials in the selected hatcheries, cryopreserved sperm held in a 20-LN Dewar was carried to the hatcheries from Bangladesh Agricultural University, and some extra liquid nitrogen was also carried by a 10-litre LN Dewar for supporting the frozen sperm if necessary. Thus, transportation of cryopreserved sperm and liquid nitrogen to hatcheries demonstrated feasibility of a national germplasm repository system for IMCs and exotic carps in different regions.

v) To promote adoption, seeds produced with cryopreserved sperm in the breeding hatcheries were supplied to 4 technology adoption hatcheries and fish farms in Barishal, Faridpur and Mymensingh regions and reared with respective controls in separate nursery ponds. The fry/fingerlings of both groups were sampled monthly, and growth performance was compared.

The cryopreserved sperm-originated fry/fingerlings showed higher growth than control. Thus, the adoption ability of technology by the stakeholders was assessed.

Conclusions: The aim of the research project was to develop cryogenic sperm bank of three Indian major carps (Catla, Rohu and Mrigal) and three exotic carps (Silver carp, Bighead carp and Grass carp) and to produce seeds using cryopreserved sperm in public and private hatcheries for developing genetically quality broodstocks that will contribute to generating quality seeds for aquaculture facilities. Sperm of Indian major carps of Halda and Padma river-origin were cryopreserved, and cryogenic sperm bank was developed. Similarly, sperm of three exotic carps imported from China by the Department of Fisheries were cryopreserved and cryogenic sperm bank was also developed. Seeds of the six target species were produced by using cryopreserved sperm of the cryogenic sperm bank in public and private hatcheries in Mymensingh, Jashore, Faridpur and Barishal regions and reared in the respective hatcheries and a few other hatcheries and fish farms with the direct administration of hatchery and nursery operators. The six months rearing data revealed significantly higher growth of cryopreserved sperm-originated fish compared to control (seeds produce with fresh sperm of hatchery-reared males). The superiority of the former group confirmed their inheritance of good genetics from the cryopreserved sperm. In a few hatcheries, broodstocks (silver carp) developed from the cryopreserved sperm-originated seeds demonstrated their superior performance (high fecundity, healthy and strong spawn, and higher growth rate) in their first spawning. This result indicates that genetically quality brood fish could be produced in the F1 generation, and the hatchery operators are capable to produce quality broodstocks in their own hatchery and nursery facilities using the cryopreservation techniques.

Technologies/Innovations developed, and what phase was achieved

1. Cryopreservation technologies for sperm of three Indian major carps and three exotic carps have been developed.
2. Cryogenic sperm banking technology of all the six species was developed.

3. Technology for production of seeds using cryopreserved sperm at hatchery level was developed. Sperm of all six target species were cryopreserved and cryogenic sperm bank was developed in the laboratory. Seeds of all six species were successfully produced using cryopreserved sperm of the cryogenic sperm bank in public and private hatcheries of four different regions through artificial breeding in 28 hatcheries. It was the phase 1 (Under Research) study to assess the feasibility of producing seeds using cryopreserved sperm in the hatcheries.

Key Beneficiaries

1. Fish Hatchery Owners or Operators

The hatchery operators/owners will be able to produce seeds of Indian major carps and exotic carps using cryopreserved sperm in their hatcheries and sell them to nursery operators with good price.

2. Fish Nursery Operators

The nursery operators will purchase the cryopreserved sperm-originated seeds from the selected hatcheries and rear them in their nursery facilities. As the seeds will have higher growth and survivability they will be able to sell them to fish farmers with higher price.

3. Fish Farmers

The fish farmers will purchase the fry or fingerlings from the selected nurseries and rear them for table fish. As the fry/fingerlings will have higher growth, the farmers will get more production and be able to sell them with higher price.

4. MS and PhD students attached to the project

MS and PhD students attached to the project are the potential human resources and are direct beneficiaries. Three PhD students in which two are the employees of Department of Fisheries and another one engaged in teaching in an Agriculture Diploma College. Eleven MS students are also attached to the project. Four of them already completed their degrees, two are about to complete and rest of them are expecting to complete their degrees by the end of this year. Most of them are

potential candidates for recruiting by Fisheries related agencies and they will have opportunity to disseminate the technology to the stakeholders at different levels.

How the scientific results were disseminated.

The scientific results were disseminated through a series of technology dissemination workshops arranged for relevant stakeholders (Fish hatchery operators, nurserers and fish farmers, scientists, NGO personnel, Graduate students and Junior Faculties). Besides, the results were disseminated through several National and International Conferences and Seminars such as

1. Cryogenic sperm banking of mrigal (*Cirrhinus cirrhosus*) and production of seeds in commercial hatcheries. Presentation at 9th Biennial Fisheries Conference and Research Fair 2022, Dhaka;

2. Cryogenic sperm banking of Indian major carp, Rohu *(Labeo rohita)* and production of quality seeds in commercial hatcheries. Presented in World Aquaculture Singapore 2022, Singapore.

3. Development of cryogenic sperm bank of silver carp (*Hypophthalmichthys molitrix*) and seed production in commercial hatcheries for brood banking. Presented in World Aquaculture Singapore 2022, Singapore.

4. Cryogenic sperm banking of Catla (*Catla catla*), Bighead carp (*Hypophthalmichthys nobilis*) and Grass carp (*Ctenopharyngodon idella*) and production of seeds in commercial hatcheries. Presented in Aquaculture America 2023, New Orleans, Louisiana.

Scientific articles are prepared and to be submitted to the peer reviewed journals for publication.

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Experiment-2: Cryopreservation of sperm of IMCs and exotic carps and development of cryogenic sperm bank

a) Standardization of sperm cryopreservation protocols of IMCs and exotic carps

Catla (*Catla catla*)

i) Effects of osmotic pressure on sperm motility activation and swimming duration

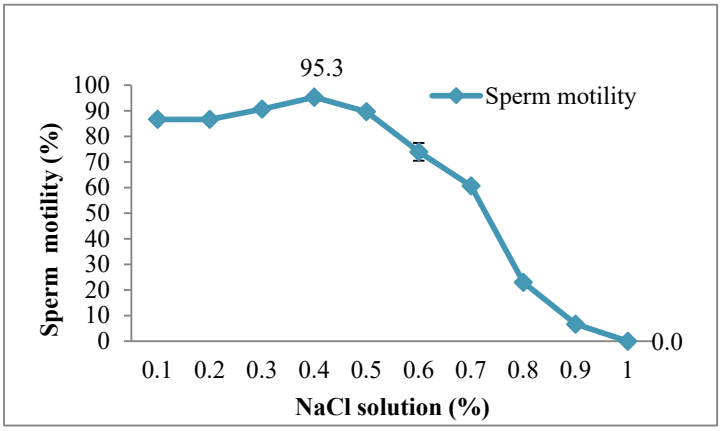

Fig.8 Motility of sperm of *Catla catla* along an osmotic gradient of NaCl concentrations.

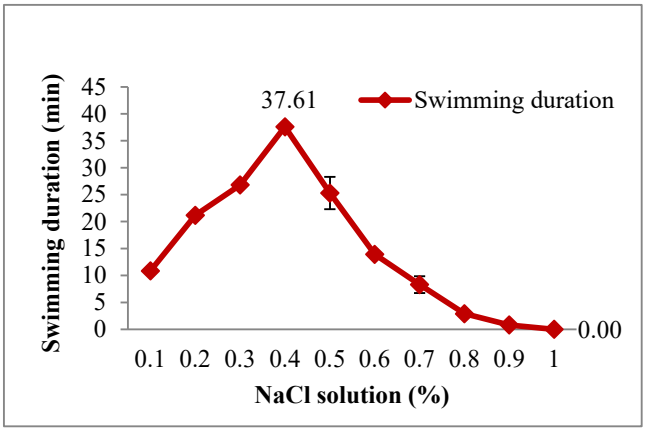

Fig.9 Swimming duration of *Catla catla* sperm at different concentrations of NaCl solution.

- Highest sperm motility (95.3±1.5%) and swimming duration (37.61±0.6 min) were observed at 0.4% NaCl solution.

ii) Evaluation of toxicity of cryoprotectants to sperm

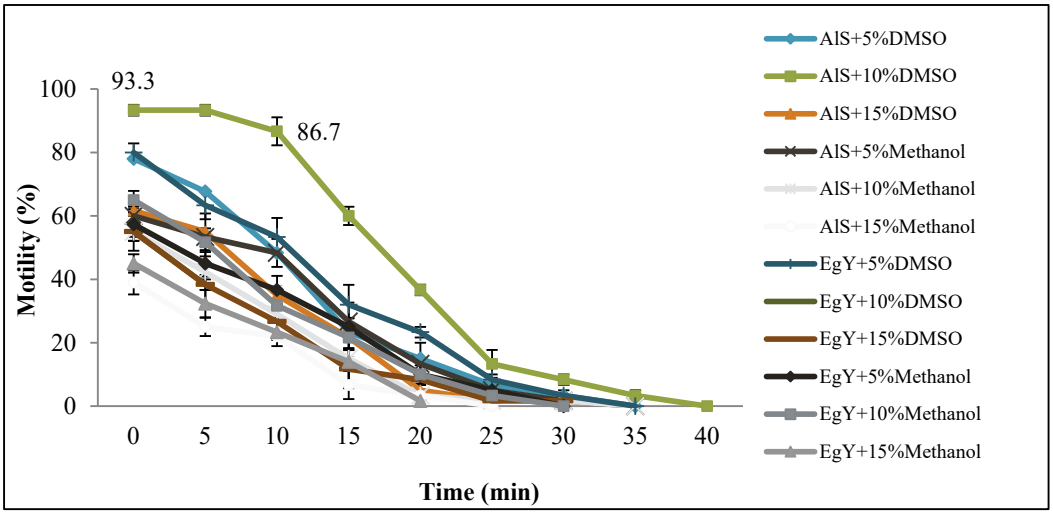

Fig. 10 Motility of *Catla catla* sperm at different concentrations of cryoprotectants and incubation times.

- DMSO and Methanol produced better motility at 5% and 10% conc. during 5 and 10 min incubation.
- Highest motility was observed with Alsever's solution plus 10% DMSO.

iii) Determination of suitable sperm-diluent ratio and diluent

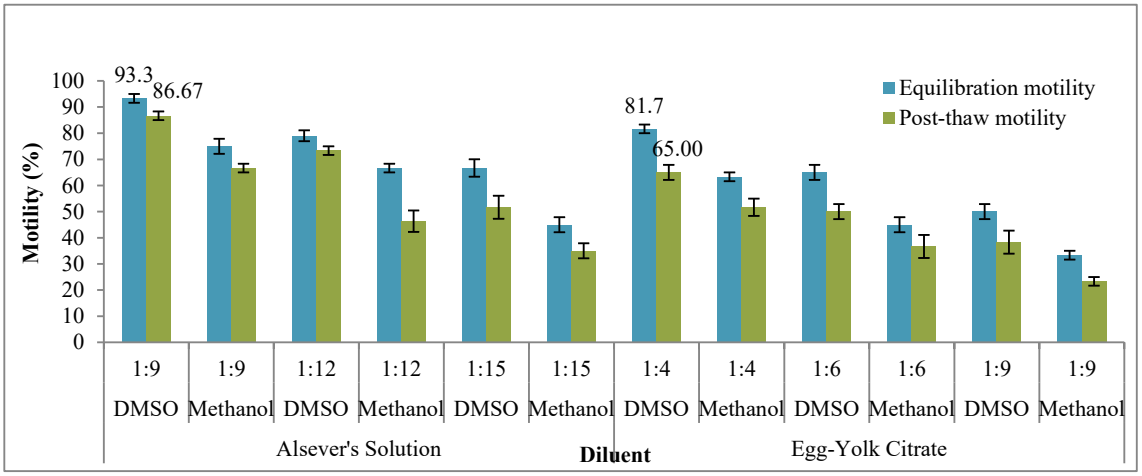

Fig.11 Equilibration and post-thaw motility of sperm of *Catla catla* in different combinations of extenders and cryoprotectants and at different dilutions of milt.

- Alsever's solution with 10% DMSO at 1:9 sperm-diluent ratio produced best equilibration (93.3±1.7%) and post-thaw (86.67±1.7%) motility of sperm.

iv) Determination of suitable equilibration time

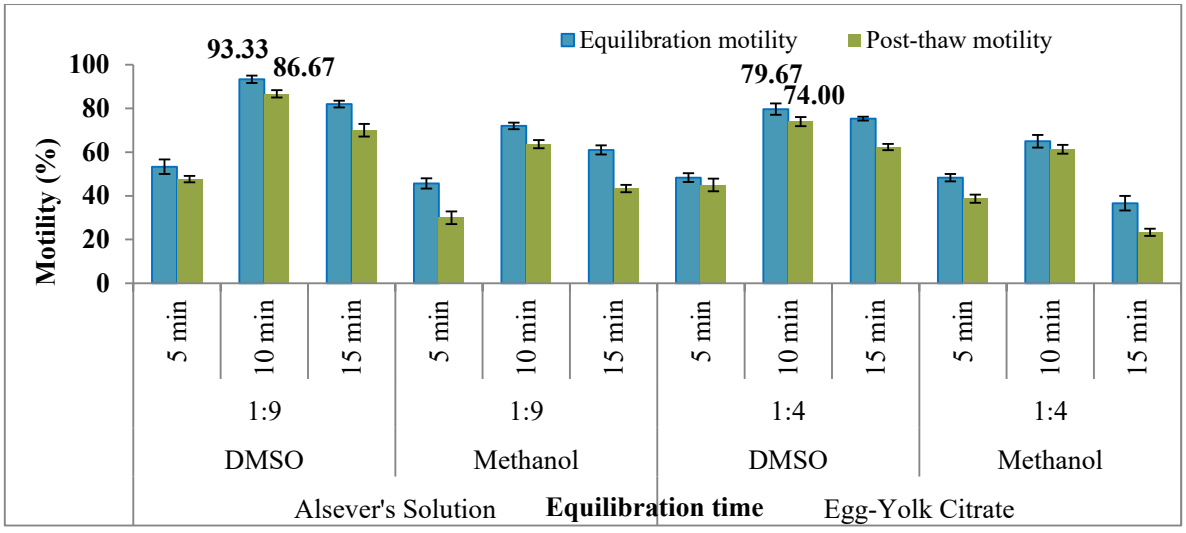

Fig.12 Equilibration and post-thaw motility of sperm of *Catla catla* equilibrated at three different periods during cryopreservation.

- Highest equilibration (93.3±1.7%) and post-thaw (86.67±1.7%) motility were recorded during 10 min equilibration period with Alsever's solution plus 10% DMSO.

v) Determination of suitable cooling rate

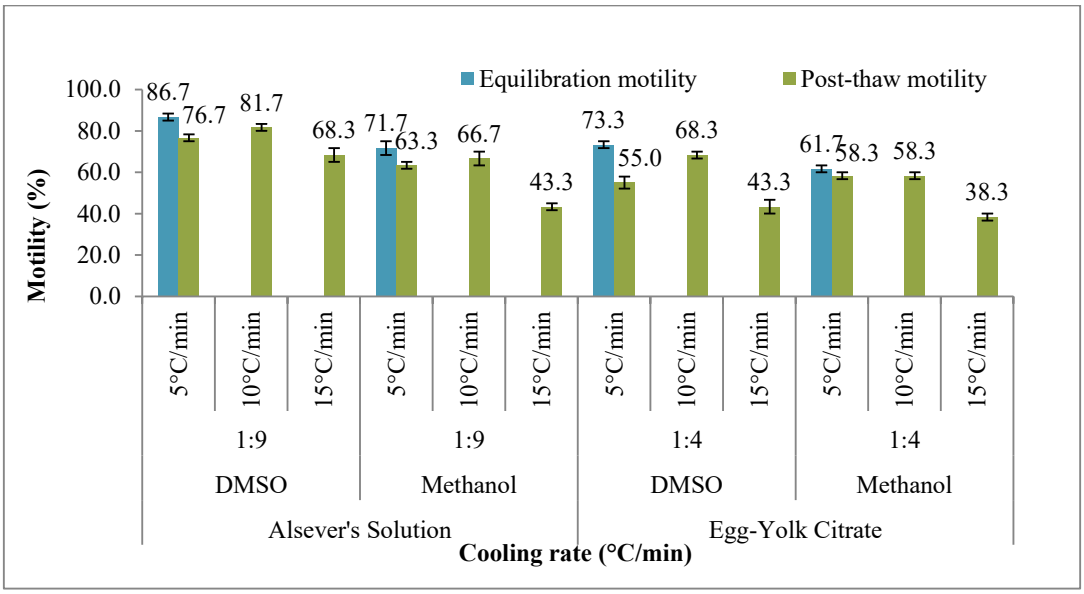

Fig.13 Post-thaw motility of sperm of *Catla catla* at different cooling rates during cryopreservation.

- Highest post-thaw motility (81.7±1.7%) of sperm at 10°C/min with Alsever's solution plus 10% DMSO.

vi) Storage time effect on post-thaw motility of cryopreserved sperm

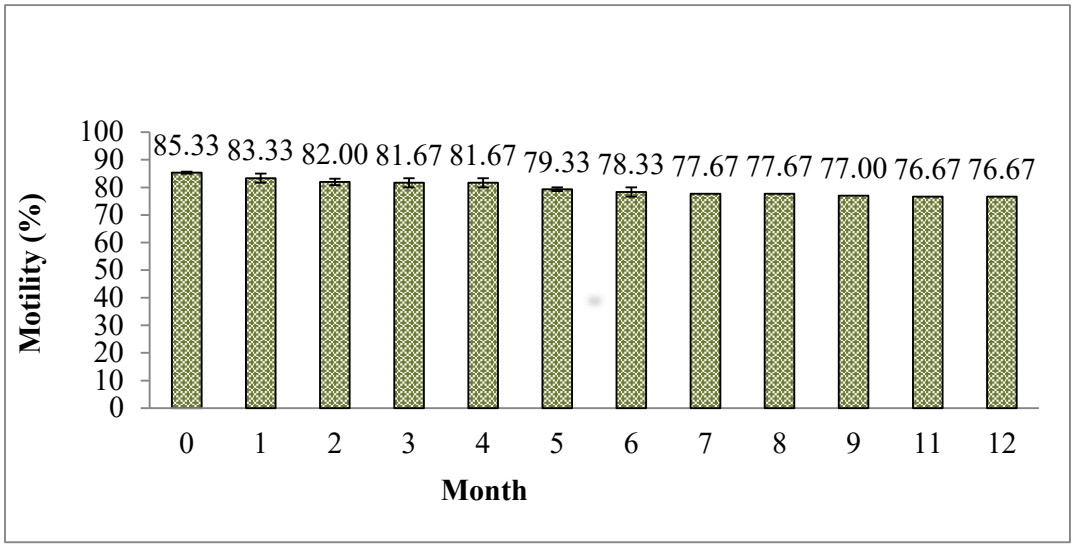

Fig. 14 Post-thaw motility of cryopreserved sperm of *Catla catla* for the twelve months storage period.

- Post-thaw motility of cryo-stored sperm remained at satisfactory level (initial 85.33±33 and final 83.67±1.67%) for 12 months storage period.

Mrigal (*Cirrhinus cirrhosus*)

i) Effects of osmotic pressure on sperm motility activation and swimming duration

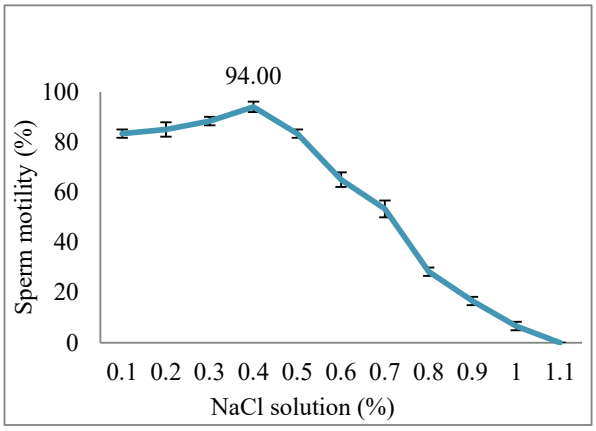

Fig. 15 Motility of sperm of *Cirrhinus cirrhosus* along an osmotic gradient of NaCl concentrations.

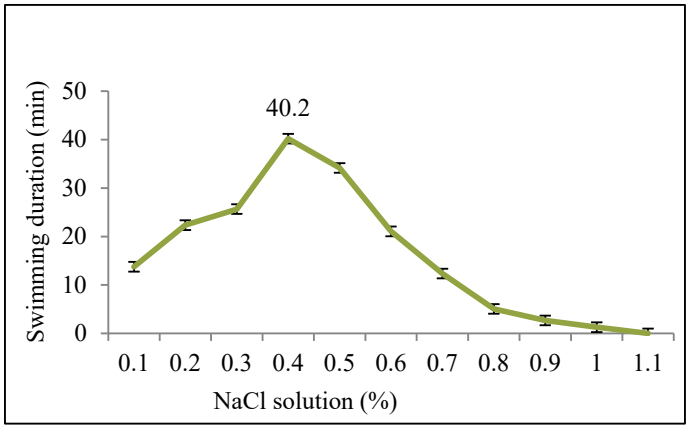

Fig.16 Swimming duration of *Cirrhinus cirrhosus* sperm at different concentrations of NaCl solution.

- Highest sperm motility (94.0±2.08%) and swimming duration (40.2±0.5 min) at 0.4% NaCl solution.

ii) Evaluation of toxicity of cryoprotectants to sperm

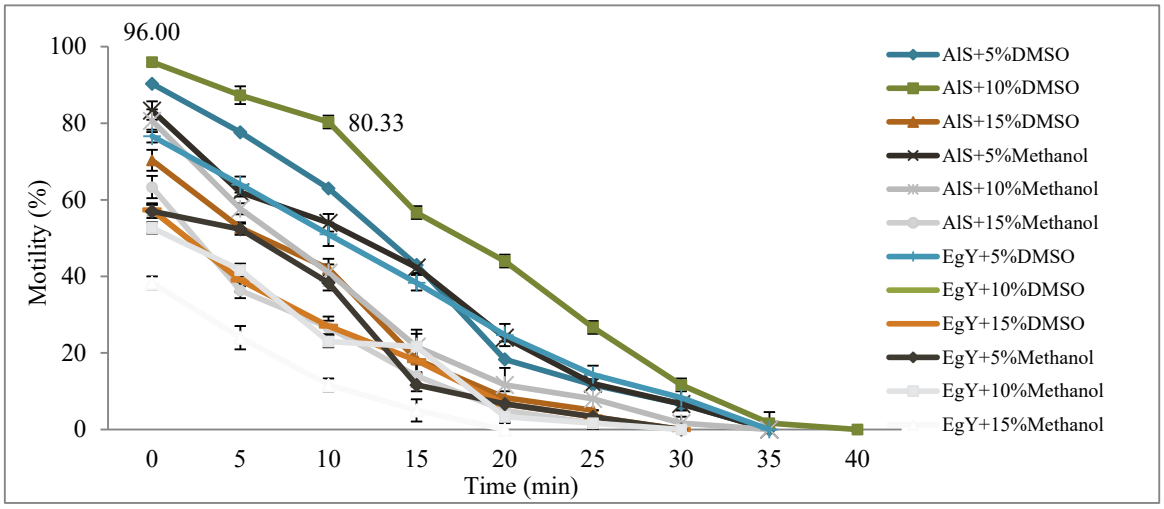

Fig. 17 Motility of *Cirrhinus cirrhosus* sperm at different concentrations of cryoprotectants and incubation times.

- DMSO and Methanol produced better motility at 5% and 10% conc. during 5 and 10 min incubation.
- Highest motility with Alsever's solution plus 10% DMSO.

iii) Determination of suitable sperm-diluent ratio and diluent

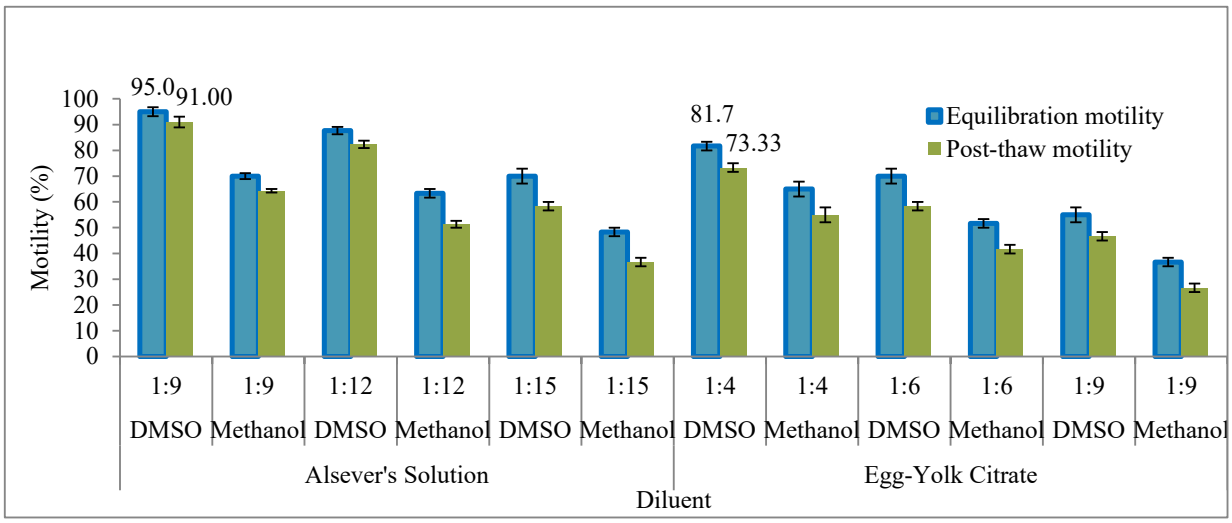

Fig. 18 Equilibration and post-thaw motility of sperm of *Cirrhinus cirrhosus* in different combinations of extenders and cryoprotectants and at different dilutions of milt.

- Alsever's solution with 10% DMSO at 1:9 sperm-diluent ratio produced best equilibration (95.00±1.7%) and post-thaw (91.00±2.1%) motility of sperm.

iv) Determination of suitable equilibration time

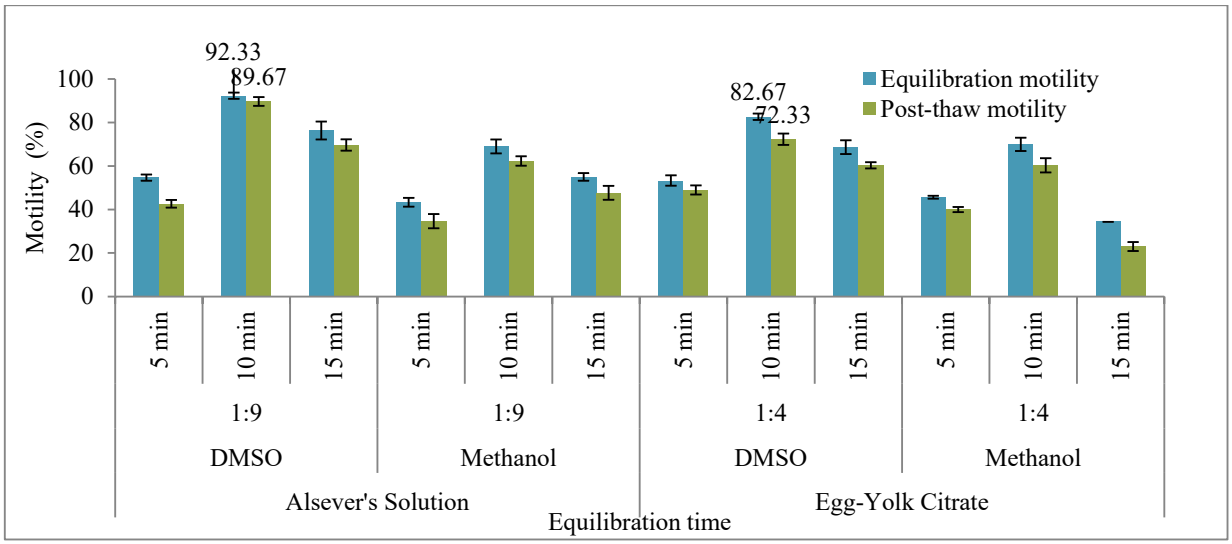

Fig.19 Equilibration and post-thaw motility of sperm of *Cirrhinus cirrhosus* equilibrated at three different periods during cryopreservation.

- Highest equilibration (92.33±1.5%) and post-thaw (89.67±2.0%) motility during 10 min equilibration with Alsever's solution plus 10% DMSO.

v) Determination of suitable cooling rate

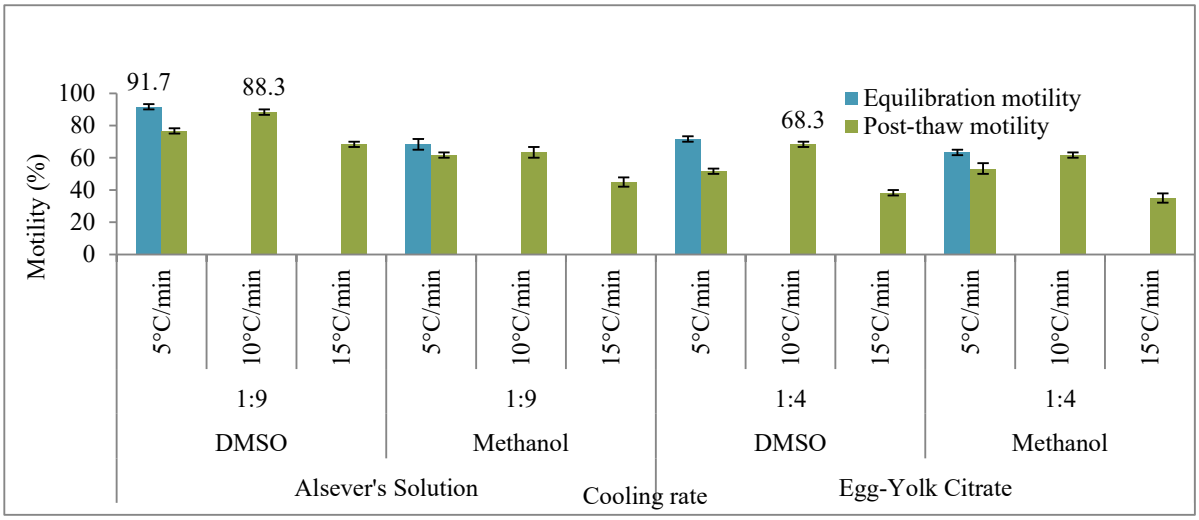

Fig. 20 Post-thaw motility of sperm of *Cirrhinus cirrhosus* at different cooling rates during cryopreservation**.**

- Highest post-thaw motility (88.30±1.7%) of sperm at 10°C/min with Alsever's solution plus 10% DMSO.

vii) Storage time effect on post-thaw motility of cryopreserved sperm

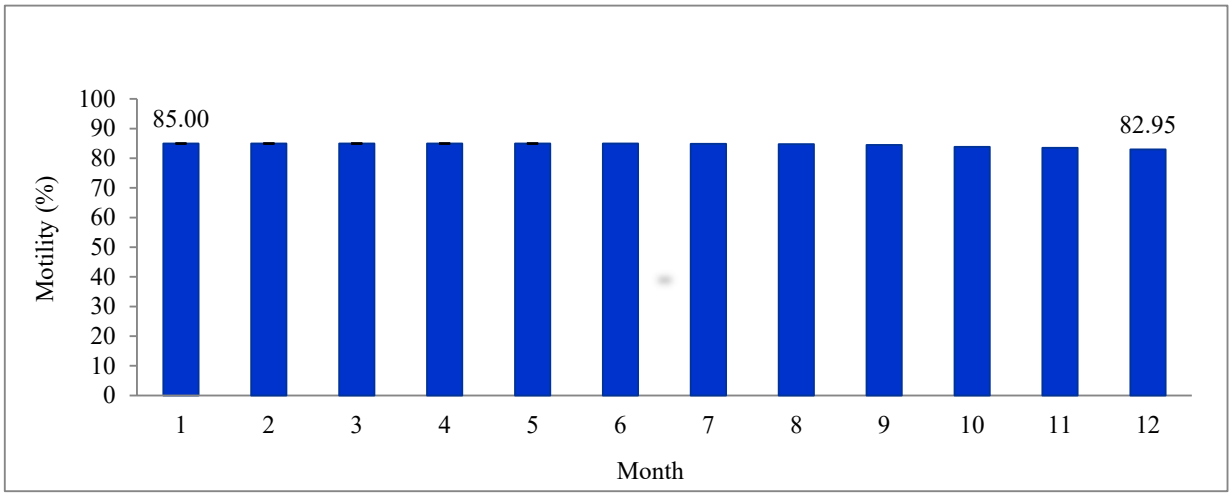

Fig. 21 Post-thaw motility of cryopreserved sperm of *Cirrhinus cirrhosus* for the twelve months storage period.

- Post-thaw motility of cryo-stored sperm remained at satisfactory level (initial 85.00±0% and final 82.95±0.1%) for 12 months storage period.

Silver carp (*Hypophthalmichthys molitrix*)

i) Effects of osmotic pressure on sperm motility activation and swimming duration

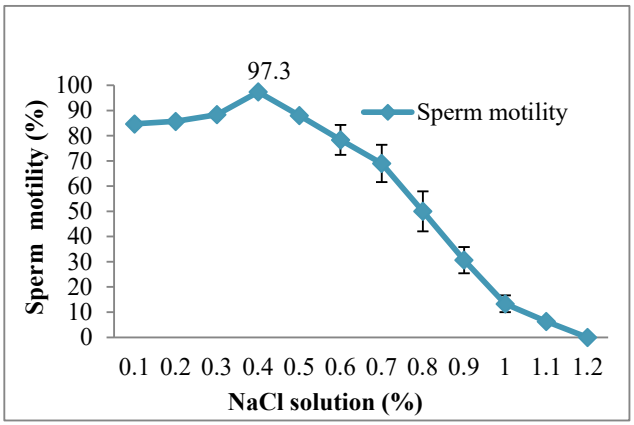

Fig.22 Motility of sperm of *Hypophthalmichthys molitrix* along an osmotic gradient of NaCl concentrations.

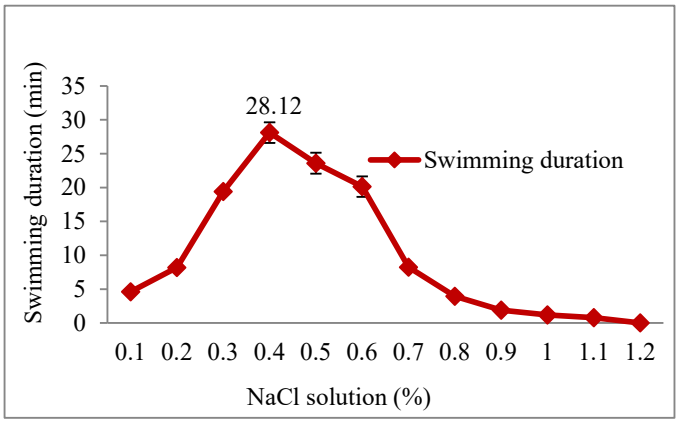

Fig. 23 Swimming duration of *Hypophthalmichthys molitrix* sperm at different concentrations of NaCl solution.

- Highest sperm motility (97.3±0.9%) and swimming duration (28.12±1.5 min) at 0.4% NaCl solution.

ii) Evaluation of toxicity of cryoprotectants to sperm

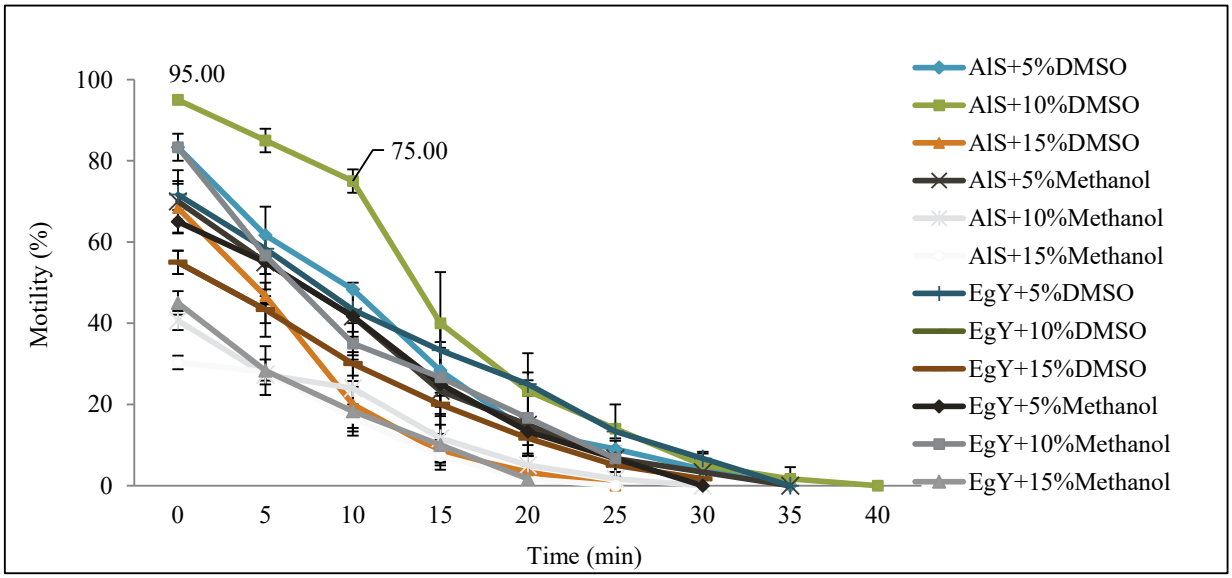

Fig. 24 Motility of *Hypophthalmichthys molitrix* sperm at different concentrations of cryoprotectants and incubation times.

- DMSO and Methanol produced better motility at 5% and 10% conc. during 5 and 10 min incubation.
- Highest motility with Alsever's solution plus 10% DMSO.

iii) Determination of suitable sperm-diluent ratio and diluent

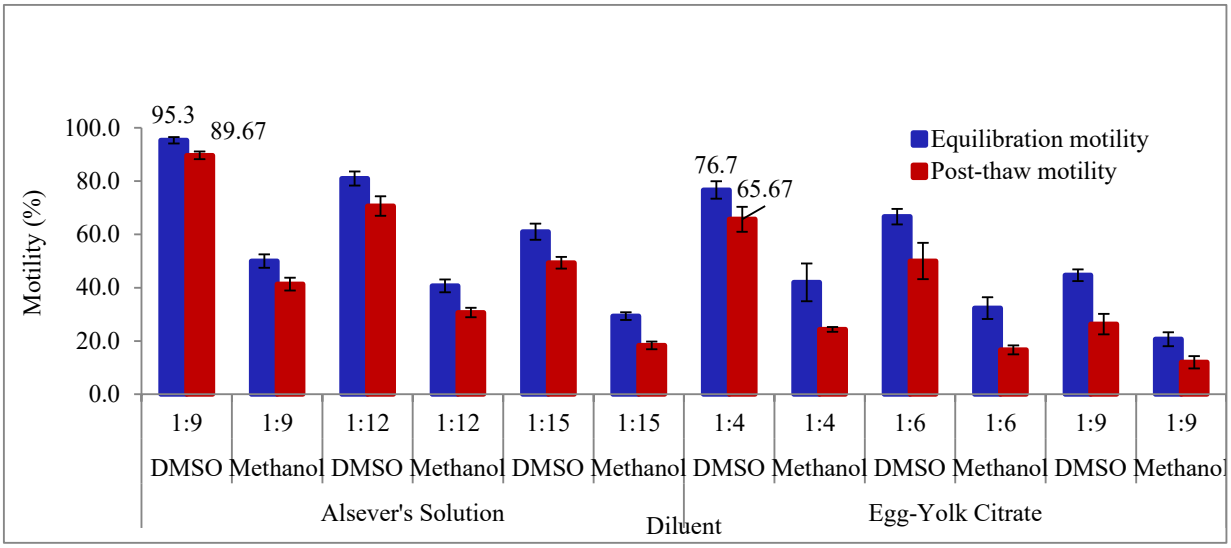

Fig. 25 Equilibration and post-thaw motility of sperm of *Hypophthalmichthys molitrix* in different combinations of extenders and cryoprotectants and at different dilutions of milt.

- Alsever's solution with 10% DMSO at 1:9 sperm-diluent ratio produced best equilibration (93.3±0.9%) and post-thaw (85.67±2.7%) motility of sperm.

iv) Determination of suitable equilibration time

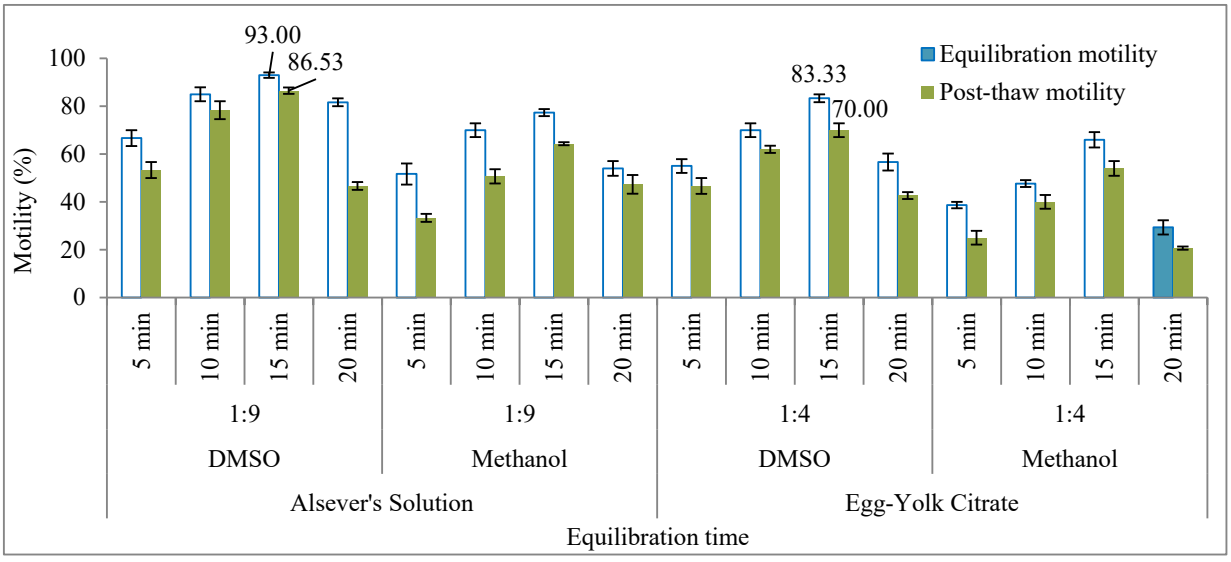

Fig. 26 Equilibration and post-thaw motility of sperm of *Hypophthalmichthys molitrix* equilibrated at three different periods during cryopreservation.

- Highest equilibration (93.00±1.2%) and post-thaw (86.63±1.3%) motility during 15 min equilibration with Alsever's solution plus 10% DMSO.

v.) Determination of suitable cooling rate

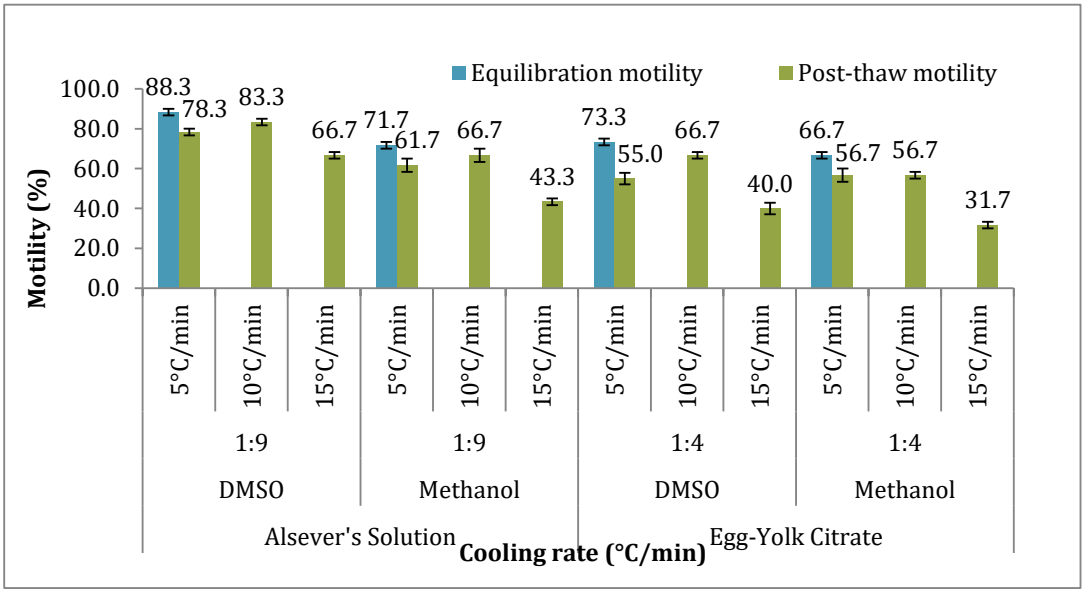

Fig. 27 Post-thaw motility of sperm of *Hypophthalmichthys molitrix* at different cooling rates during cryopreservation

- Highest post-thaw motility (83.3±1.7%) of sperm at 10°C/min with Alsever's solution plus 10% DMSO.

v) Storage time effect on post-thaw motility of cryopreserved sperm

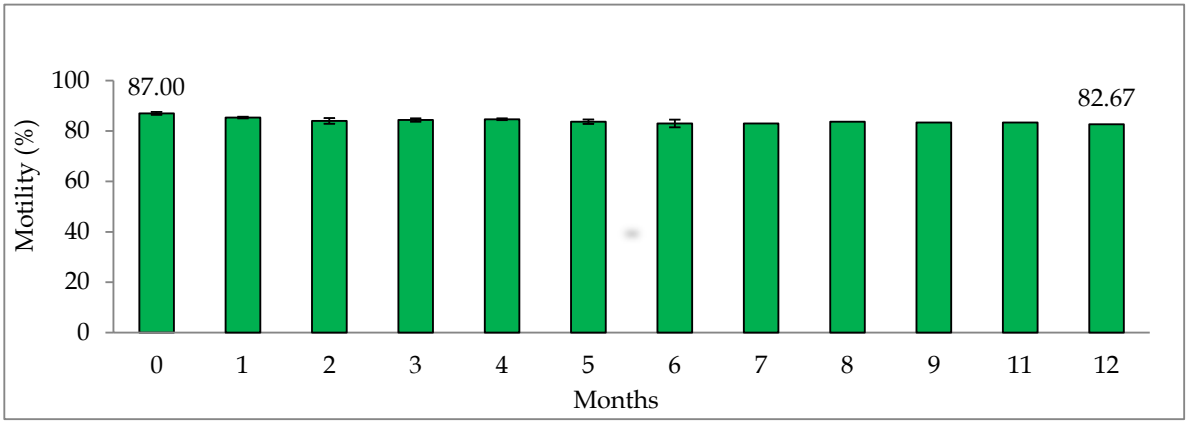

Fig. 28 Post-thaw motility of cryopreserved sperm of *Hypophthalmichthys molitrix* for the twelve months storage period.

- Post-thaw motility of cryo-stored sperm remained at satisfactory level (initial 87.00% and final 82.67%) for 12 months storage period.

Bighead carp (*Hypophthalmichthys nobilis*)

i) Effects of osmotic pressure on sperm motility activation and swimming duration

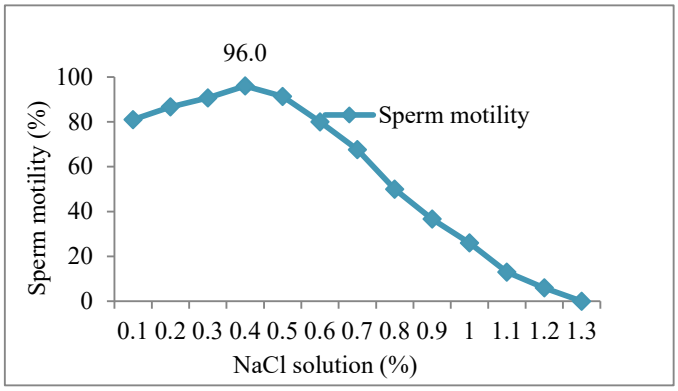

Fig. 29 Motility of sperm of *Hypophthalmichthys nobilis* along an osmotic gradient of NaCl concentrations.

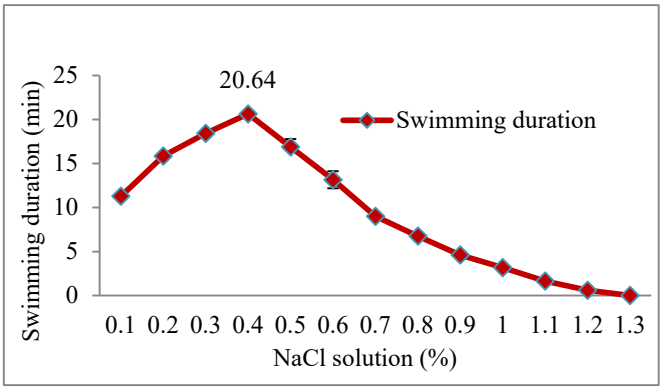

Fig. 30 Swimming duration of *Hypophthalmichthys nobilis* sperm at different concentrations of NaCl solution.

- Highest sperm motility (96±1%) and swimming duration (20.64±0.6 min) at 0.4% NaCl solution.

ii) Evaluation of toxicity of cryoprotectants to sperm

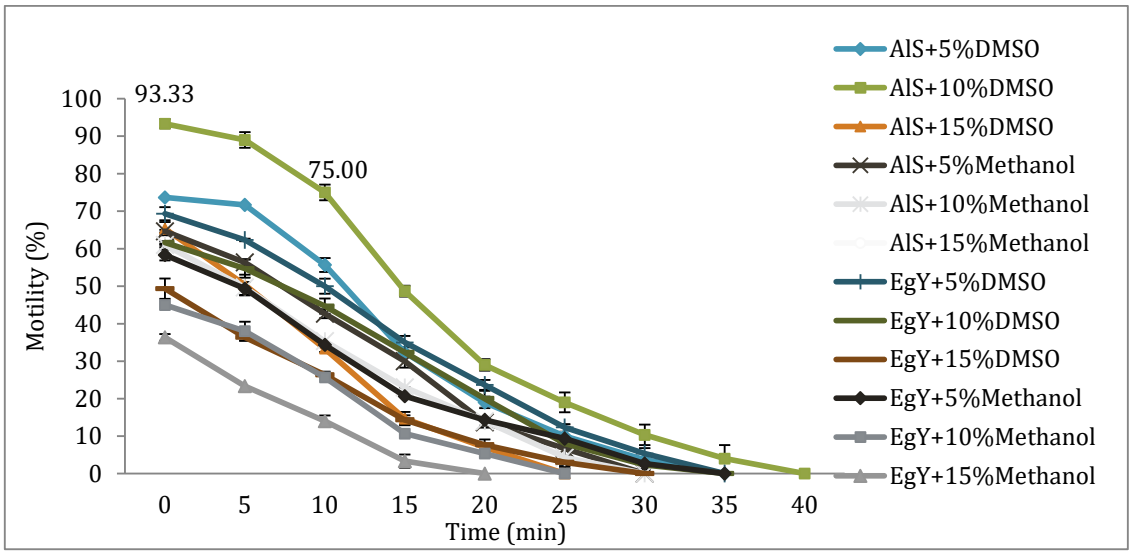

Fig. 31 Motility of *Hypophthalmichthys nobilis* sperm at different concentrations of cryoprotectants and incubation times.

- DMSO and Methanol produced better motility at 5% and 10% conc. during 5 and 10 min incubation.
- Highest motility with Alsever's solution plus 10% DMSO.

iii) Determination of suitable sperm-diluent ratio and diluent

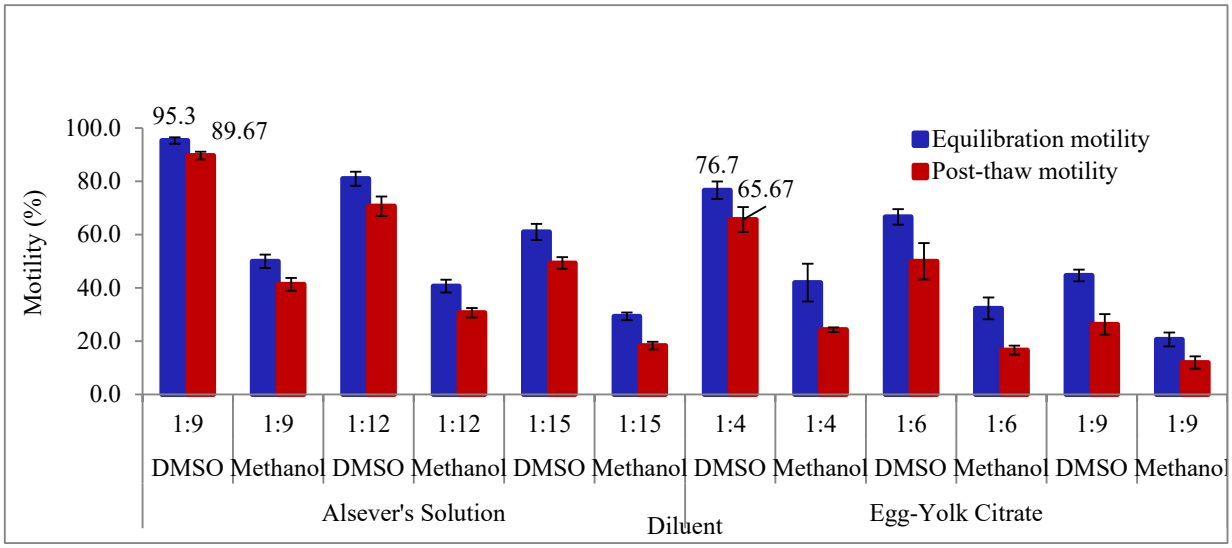

Fig. 32 Equilibration and post-thaw motility of sperm of *H. nobilis* at different sperm-diluent ratios and diluents.

- Alsever's solution with 10% DMSO at 1:9 sperm-diluent ratio produced best equilibration (95±1%) and post-thaw (89±2%) motility of sperm

iv) Determination of suitable equilibration time

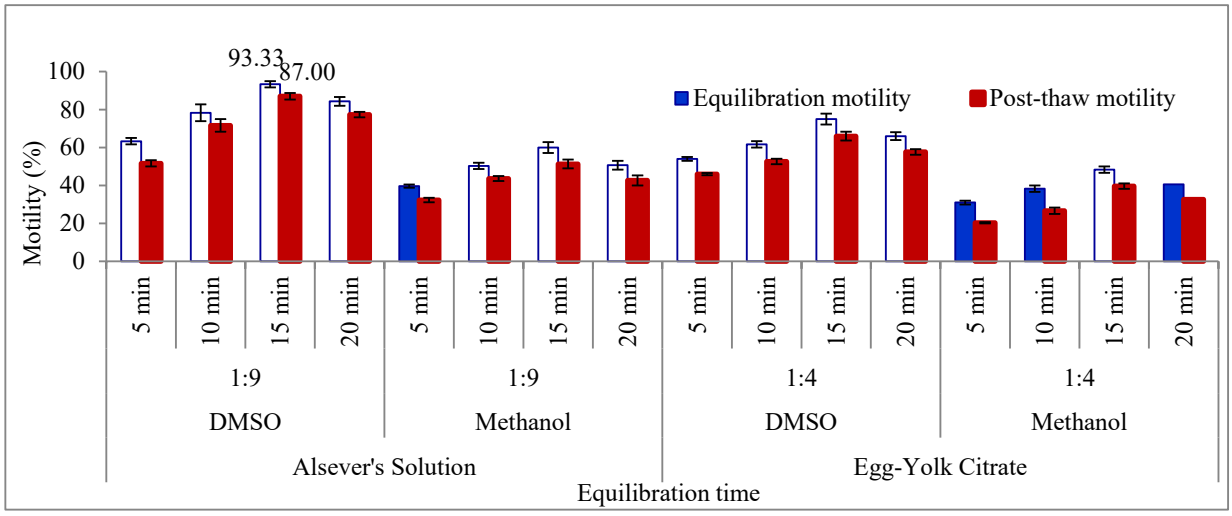

Fig. 33 Equilibration and post-thaw motility of sperm of *Hypophthalmichthys nobilis* equilibrated at three different periods during cryopreservation.

- Highest equilibration (93.3±1.7%) and post-thaw (87.0±1.7%) motility observed during 15 min equilibration with Alsever's solution plus 10% DMSO.

v) Determination of suitable cooling rate

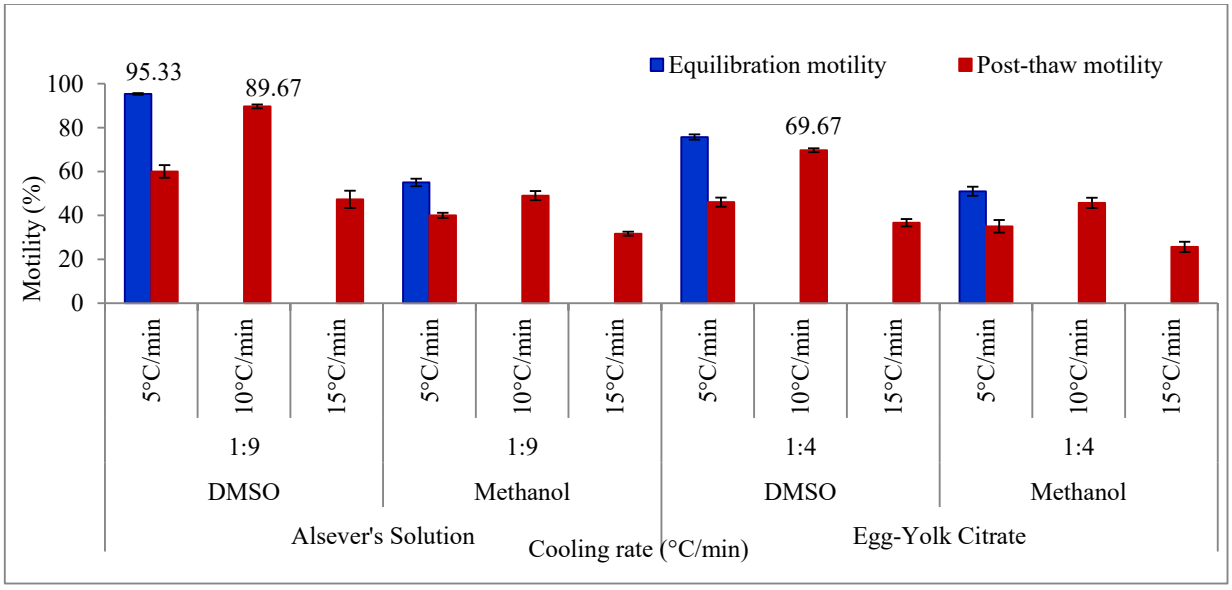

Fig. 34 Post-thaw motility of sperm of *Hypophthalmichthys nobilis* at different cooling rates during cryopreservation**.**

- Highest post-thaw motility (89.67±0.9%) of sperm at 10°C/min with Alsever's solution plus 10% DMSO.

vi) Storage time effect on post-thaw motility of cryopreserved sperm

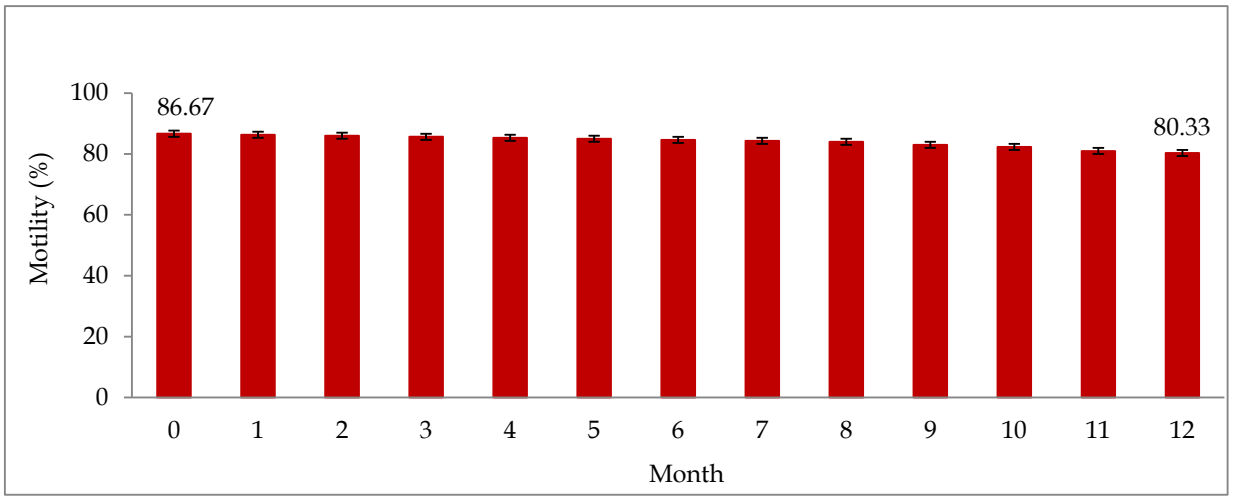

Fig. 35 Post-thaw motility of cryopreserved sperm of *Hypophthalmichthys nobilis* for the twelve months storage period.

- Post-thaw motility of cryo-stored sperm remained at satisfactory level (initial 86.67±0.88% and final 80.33±0.33%) for 12 months storage period.

Grass carp (*Ctenopharyngodon idella*)

i) Effects of osmotic pressure on sperm motility activation and swimming duration

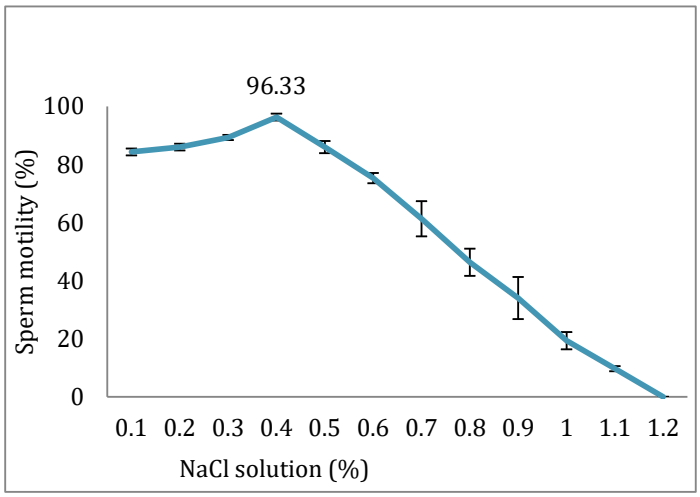

Fig. 36 Motility of sperm of *Ctenopharyngodon idella* along an osmotic gradient of NaCl concentrations.

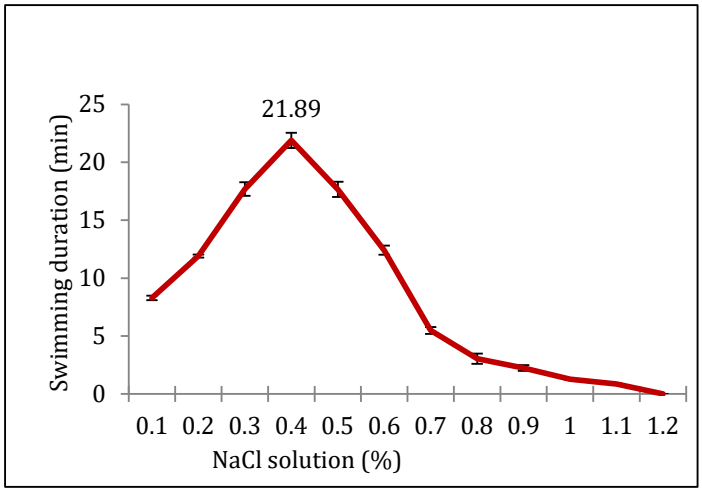

Fig. 37 Swimming duration of *Ctenopharyngodon idella* sperm at different concentrations of NaCl solution.

- Highest sperm motility (96.33±1.2%) and swimming duration (21.89±0.7 min) at 0.4% NaCl solution.

ii) Evaluation of toxicity of cryoprotectants to sperm

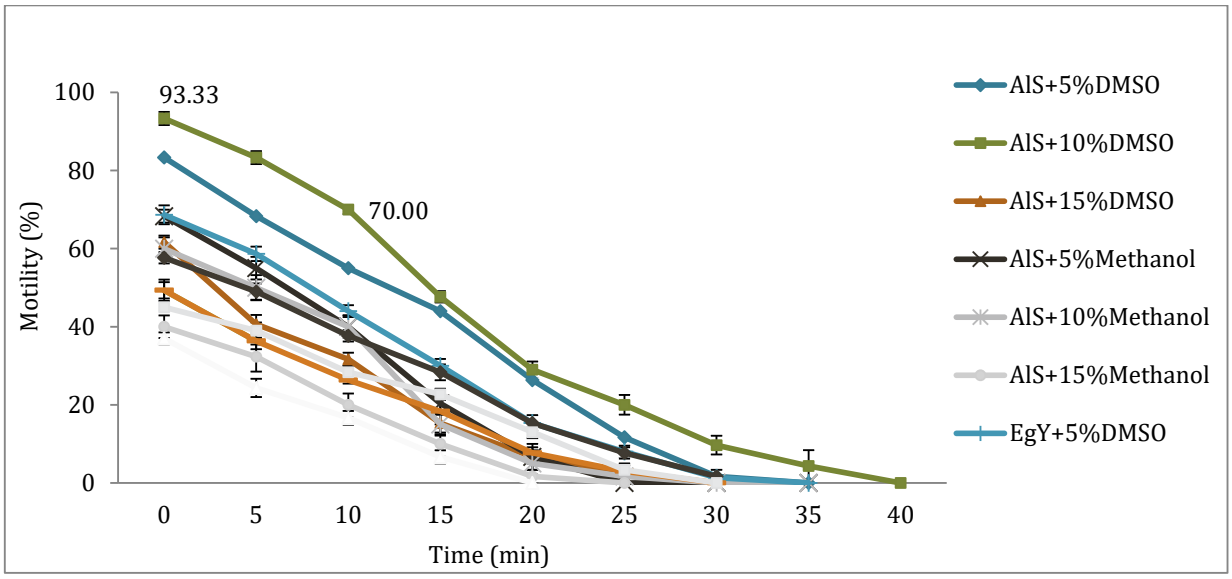

Fig. 38 Motility of *Ctenopharyngodon idella* at different concentrations of cryoprotectants and incubation times

- DMSO and Methanol produced better motility at 5% and 10% conc. during 5 and 10 min incubation.
- Highest motility with Alsever's solution plus 10% DMSO.

iii) Determination of suitable sperm-diluent ratio and diluent

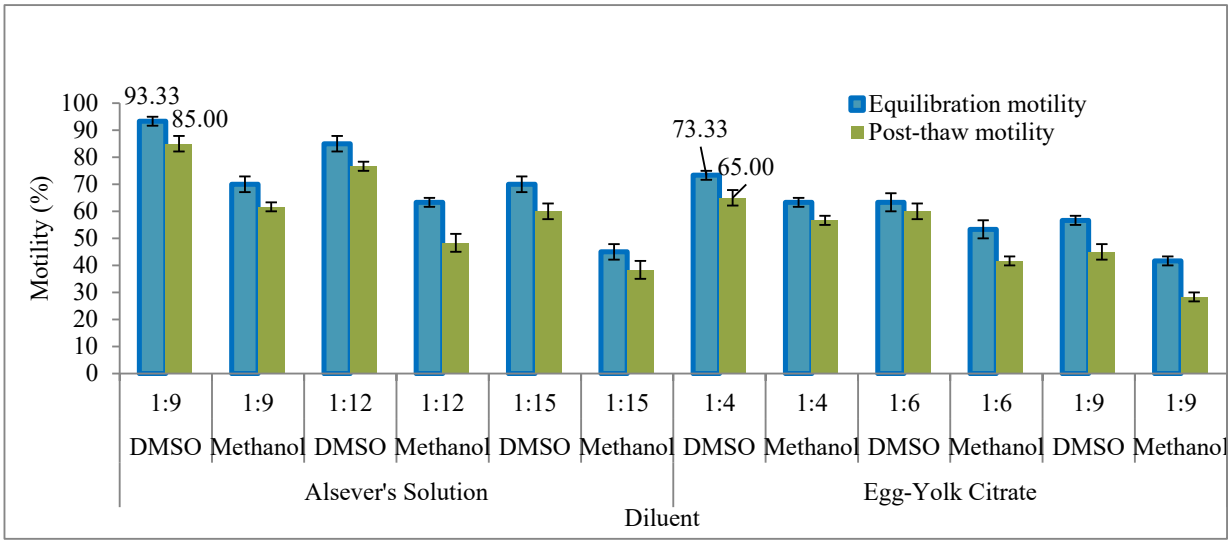

Fig. 39 Equilibration and post-thaw motility of sperm of *Ctenopharyngodon idella* at different sperm-diluent ratios and diluents.

- Alsever's solution with 10% DMSO at 1:9 sperm-diluent ratio produced best equilibration (93.33±1.7%) and post-thaw (85.00±2.9%) motility of sperm.

iv) Determination of suitable equilibration time

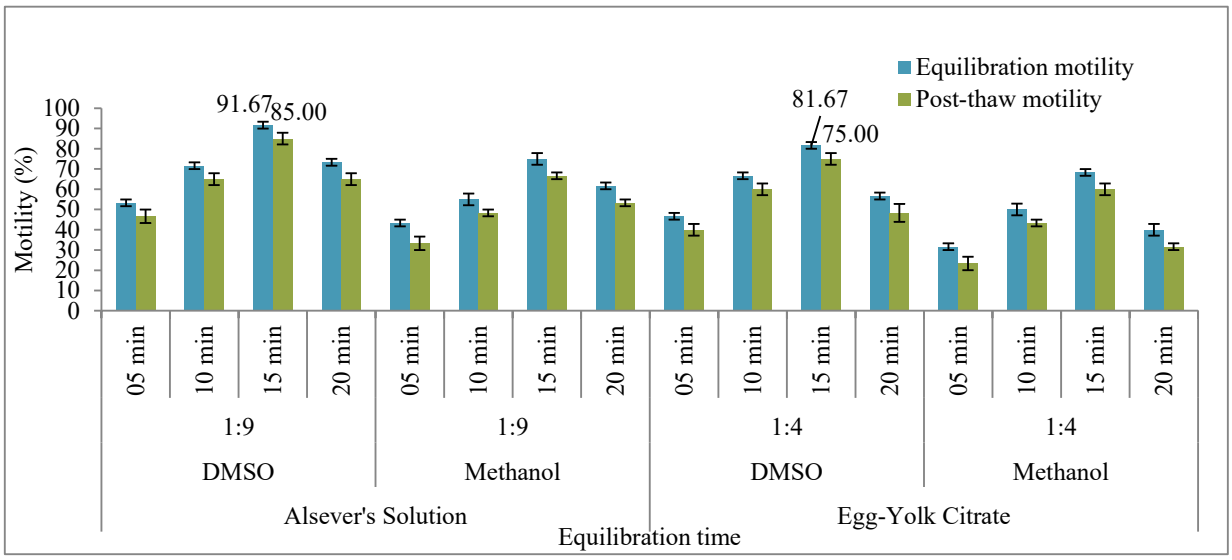

Fig. 40 Equilibration and post-thaw motility of sperm of *Ctenopharyngodon idella* equilibrated at three different periods during cryopreservation.

- Highest equilibration (91.67±1.7%) and post-thaw (85.00±2.9%) motility during 15 min equilibration with Alsever's solution plus 10% DMSO.

v) Determination of suitable cooling rate

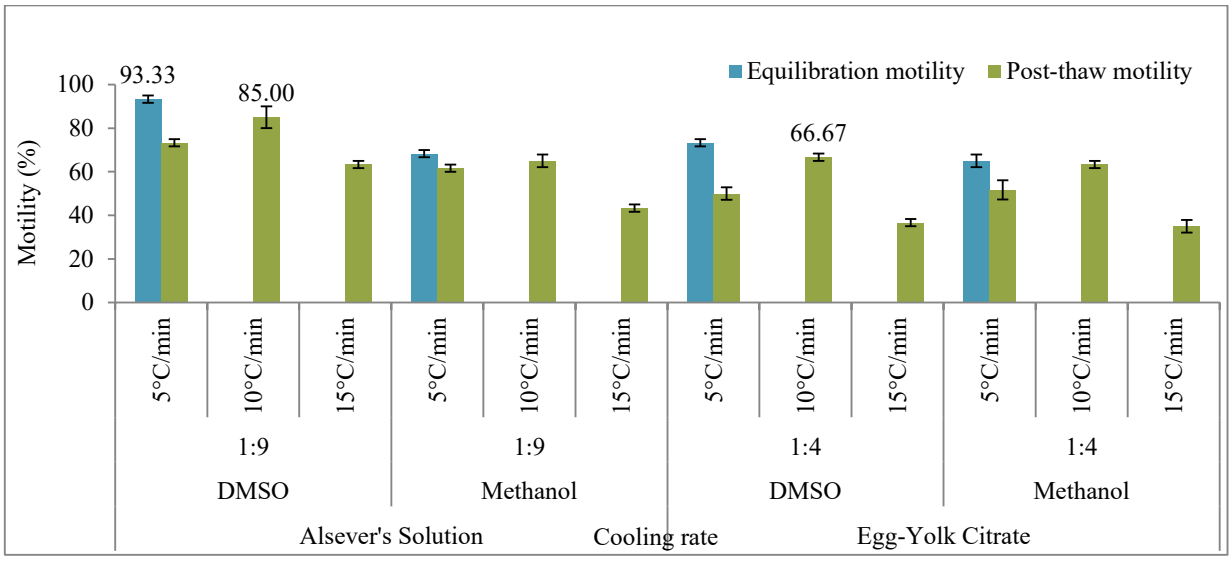

Fig. 41 Post-thaw motility of sperm of *Ctenopharyngodon idella* at different cooling rates during cryopreservation**.**

- Highest post-thaw motility (85.00±5.0%) of sperm at 10°C/min with Alsever's solution plus 10% DMSO.

vi) Storage time effect on post-thaw motility of cryopreserved sperm

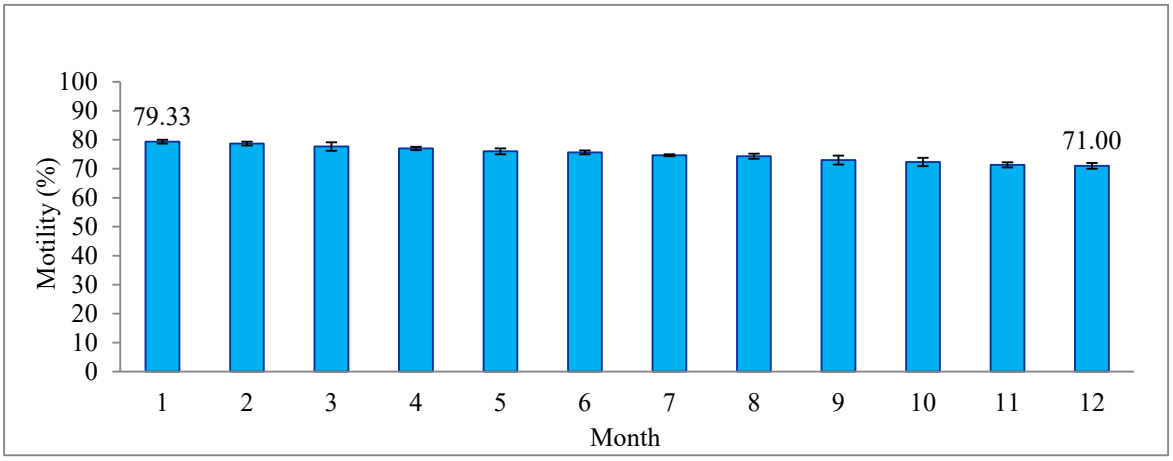

Fig. 42 Post-thaw motility of cryopreserved sperm of *Ctenopharyngodon idella* for the twelve months storage period.

- Post-thaw motility of cryo-stored sperm remained at satisfactory level (initial 79.33±0.7% and final 71.00±1.0%) for 12 months storage period.

Experiment-3: Production of seeds of carps in hatcheries using cryopreserved sperm and assessment of their quality through growth study and DNA microsatellite analysis.

Table 4 Hatchery-wise fertilization and hatching rates of eggs of *C. catla*

Sl	Hatchery Name	Fertilization (%)		Hatching (%)		Storage period (day)
		Cryo sperm	Fresh sperm	Cryo sperm	Fresh sperm	
1	Govt. Fish Seed Multiplication Farm, Nandail, Mymensingh	32.66	90.16	25.18	75.24	4
2	Shornolota Fish Hatchery, Fulbaria, Mymensingh	34.68	78.34	26.23	72.58	4
3	Mukteshawri Fish Hatchery, Sadar, Jashore	40.00	78.13	33.58	66.50	11
4	Govt. Fish Seed Multiplication Farm, Maskanda, Mymensingh	33.73	64.54	28.03	55.12	6
5	Govt. Fish Seed Multiplication Farm, Sadar, Rajbari	26.67	78.18	20.85	71.20	41
6	Sonali Fish Hatchery, Sadar, Jashore	57.14	73.82	50.00	64.72	5
7	Govt. Fish Seed Multiplication Farm, Sadar, Patuakhali	40.10	59.80	32.85	40.25	15
8	Bishwas Fish Hatchery, Trishal, Mymensingh	39.75	95.00	31.54	76.00	42
Average		**38.09**	**77.25**	**31.03**	**65.20**	

Table 5 Hatchery-wise fertilization and hatching rates of eggs of *L. rohita*

Sl	Hatchery Name	Fertilization (%)		Hatching (%)	Storage period (day)	
		Cryo sperm	Fresh sperm	Cryo sperm	Fresh sperm	
1	Field Lab. Complex, BAU, Mymensingh	23.00	64.00	20.00	50.00	2
2	Govt. Fish Seed Multiplication Farm, Maskanda, Mymensingh	33.00	94.00	27.00	90.00	3
3	Matri Fish Hatchery, Jashore	44.00	68.00	29.00	40.00	47
4	Govt. Fish Seed Multiplication Farm, Nandail, Mymensingh	39.44	81.66	32.85	72.72	4
5	Shapla Fish Hatchery, Trishal, Mymensingh	31.66	84.58	24.16	75.40	6
6	Sotota Fish Hatchery, Trishal, Mymensingh	34.18	85.37	25.66	67.47	9
7	Sharnolota Fish Hatchery, Fulbaria, Mymensingh	40.32	89.10	33.44	78.17	291
8	Motsha Kanon Fish Hatchery, Jashore	48.40	72.23	41.90	66.44	6
9	Govt. Fish Seed Multiplication Farm, wazirpur, Barishal	52.87	61.15	40.20	51.00	3
10	Motsha Bangla Fish Hatchery, Jhalokathi	62.43	53.56	56.00	45.86	14
11	Govt. Fish Seed Multiplication Farm, Maskanda,Mymensingh	31.11	74.00	24.55	62.40	6
12	Bishwas Fish Hatchery, Trishal, Mymensingh	35.79	84.38	27.22	71.43	7
13	Govt. Fish Seed Multiplication Farm, Sadar, Rajbari	25.86	86.20	20.10	80.00	3
14	Mukteshawri Fish Hatchery, Jashore	39.20	90.62	32.87	81.25	5
15	Shohel Fish Hatchery, Agailjhara, Barishal	42.23	65.13	34.26	58.70	16
16	Govt. Fish Seed Multiplication Farm, Shambuganj, Mymensingh	33.85	72.14	26.88	57.37	70
17	Sotota Fish Hatchery, Tarakanda, Mymensingh	39.44	80.85	32.50	67.40	48
	Average	**38.63**	**76.88**	**31.09**	**65.62**	

Table 6 Hatchery-wise fertilization and hatching rates of eggs of *C. cirrhosus*

Sl	Hatchery Name	Fertilization (%)		Hatching (%)		Storage period (day)
		Cryo sperm	Fresh sperm	Cryo sperm	Fresh sperm	
1	Govt. Fish Seed Multiplication Farm, Maskanda, Mymensingh	40.67	80.33	35.33	76.33	5
2	Bishwas Fish Hatchery, Trishal, Mymensingh	35.33	86.67	31.33	75.67	19
3	Matri Fish Hatchery, Sadar, Jashore	36.67	60.67	21.67	43.33	61
4	Govt. Fish Seed Multiplication Farm, Nandail, Mymensingh	36.85	97.72	31.88	83.33	4
5	Shapla Fish Hatchery, Trishal, Mymensingh	27.24	94.32	21.33	87.3	6
6	Sonali Fish Hatchery, Sadar, Jashore	50.77	77.78	40.15	66.3	11
7	Motsha Kanon Fish Hatchery, Jashore	39.3	71.5	32.63	63.7	12
8	Govt. Fish Seed Multiplication Farm, Maskanda, Mymensingh	45	75.52	32.31	68.28	4
9	Govt. Fish Seed Multiplication Farm, Sadar, Rajbari	23.35	81.23	17.85	72.32	34
10	Govt. Fish Seed Multiplication Farm, Sadar, Patuakhali	40.1	59.8	32.85	40.25	15
11	Shohel Fish Hatchery, Agailjhara, Barishal	39.33	61.81	32.45	55.16	16
12	Sotota Fish Hatchery, Tarakanda, Mymensingh	41.94	89.47	34.78	66.67	??
Average		**38.05**	**78.07**	**30.38**	**66.55**	

Table 7 Hatchery-wise fertilization and hatching rates of eggs of *H. molitrix*

Sl	Hatchery Name	Fertilization (%)		Hatching (%)		Storage period (day)
		Cryo sperm	Fresh sperm	Cryo sperm	Fresh sperm	
1	Matri Fish Hatchery, Jashore	27.33	63.00	18.67	35.67	02
2	Mukteshawri Fish Hatchery, Jashore	42.33	95.67	23.67	60.53	42
3	Sotota Fish Hatchery, Tarakanda,Mymensingh	37.33	81.33	28.33	68.67	276
4	Sotota Fish Hatchery, Trishal, Mymensingh	44.67	82.33	40.67	73.67	241

5	Shapla Fish Hatchery, Trishal, Mymensingh	31.33	77.33	22.00	70.00	01
6	Sharnolota Fish Hatchery, Fulbaria, Mymensingh	30.67	72.33	19.00	60.00	42
7	Sonali Fish Hatchery, Jashore	47.33	73.00	39.00	64.00	05
8	Motsha Kanon Fish Hatchery, Jashore	64.33	72.00	44.18	64.67	06
9	Motsha Bangla Fish Hatchery, Jhalokathi	61.33	65.33	54.08	57.67	08
10	Biswas Fish Hatchery, Trisal, Mymensingh	26.00	53.00	19.00	36.0	56
Average		**41.27**	**73.53**	**30.86**	**59.09**	

Table 8 Hatchery-wise fertilization and hatching rates of eggs of *H. molitrix*

Sl	Hatchery Name	Fertilization (%)		Hatching (%)		Storage period (day)
		Cryo sperm	Fresh sperm	Cryo sperm	Fresh sperm	
1	Matri Fish Hatchery, Jashore	27.33	63.00	18.67	35.67	02
2	Mukteshawri Fish Hatchery, Jashore	42.33	95.67	23.67	60.53	42
3	Sotota Fish Hatchery, Tarakanda, Mymensingh	37.33	81.33	28.33	68.67	276
4	Sotota Fish Hatchery, Trishal, Mymensingh	44.67	82.33	40.67	73.67	241
5	Shapla Fish Hatchery, Trishal, Mymensingh	31.33	77.33	22.00	70.00	01
6	Sharnolota Fish Hatchery, Fulbaria, Mymensingh	30.67	72.33	19.00	60.00	42
7	Sonali Fish Hatchery, Jashore	47.33	73.00	39.00	64.00	05
8	Motsha Kanon Fish Hatchery, Jashore	64.33	72.00	44.18	64.67	06
9	Motsha Bangla Fish Hatchery, Jhalokathi	61.33	65.33	54.08	57.67	08
10	Biswas Fish Hatchery, Trisal, Mymensingh	26.00	53.00	19.00	36.0	56
Average		**41.27**	**73.53**	**30.86**	**59.09**	

Table 9 Hatchery-wise fertilization and hatching rates of eggs of *H. nobilis*

Sl	Hatchery Name	Fertilization (%)		Hatching (%)		Storage period (day)
		Cryo sperm	Fresh sperm	Cryo sperm	Fresh sperm	
1	Bishwas Fish Hatchery, Trishal, Mymensingh	36.33	56.00	32.00	50.33	3
2	Suraiya Noor Fish Hatchery, Baliakandi, Rajbari	50.00	77.33	27.00	36.00	16
3	Sotota Fish Hatchery, Tarakanda, Mymensingh	40.00	94.67	32.33	75.67	256
4	Sotota Fish Hatchery, Trishal, Mymensingh	38.67	80.67	31.00	71.00	1
5	Mukteshawri Fish Hatchery, Sadar, Jashore	39.67	82.33	31.33	71.33	14
6	Shohel Fish Hatchery, Agailjhara, Barishal	44.33	67.33	37.67	61.33	23
7	Matri Fish Hatchery, Sadar, Jashore	49.33	85.67	38.00	77.67	6
8	Motsha Bangla Fish Hatchery, Jhalokathi	46.00	78.33	34.67	64.33	9
Average		**43.04**	**77.79**	**33.00**	**63.46**	