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# Sperm motility determination of critically endangered mohashol fish, *Tor tor* (Hamilton) at various concentrations of the salt solution

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**Abstract**
Cryogenic gene banking is a prime necessity for conserving the genetic constitutes of vulnerable, endangered and critically endangered native and exotic fish species. Cryogenic freezing of fish gametes is the first step to develop such a gene bank. In the study, attempts have been taken to develop a cryogenic freezing protocol of spermatozoa of Mohashol, *Tor tor* as it is considered as a critically endangered fish species by IUCN. Juvenile and brood fish of *Tor tor* were collected from the Someshwari River of Netrokona district and domesticated in captive condition at Bangladesh Agricultural University campus. During breeding season, sperms were collected from hormone induced males by stripping. The milt was very sticky. The concentration of sperm was 4.47 x $10^{10}$ cells per ml and pH was 8.6±0.5. The sperm demonstrated Brownian and forward movement during motility. Activation of sperm motility has been evaluated in different concentrations (0.1% to 1.0%) of activation solution (NaCl) and found that sperm motility decreased with increasing concentration of NaCl. Motility of sperm at 0.4% NaCl was more or less stable and completely activated. Sperm motility was completely inhibited at 1.0% NaCl. A significant difference ($p = 0.001$) was observed between the complete activation and inhibition of sperm at 0.4% and 1.0% NaCl solution respectively. Though cryopreservation of sperm of *Tor tor* has been possible through this study, no breeding trial was conducted due to lack of ovulated eggs. So far it is the first cryopreservation work of *Tor tor* and more research work needs to be done to develop the complete protocol for *ex-situ* conservation of this examined fish species.

**Keywords:** Conservation, sperm motility, salt solution

## 1. Introduction
Bangladesh is enriched with numerous rivers, flood plains, low lands, haor, baor (ox-bow lakes), beels, lakes etc. The country is also enriched with endemic fish resources including 260 freshwater fishes and 24 prawn species, 16 exotic freshwater species; 475 marine fishes and 24 shrimp species (DoF 2016) [7]. Among 260 freshwater fish species of the country, 12 have been categorized as critically endangered, 28 as endangered and 14 as vulnerable (IUCN Bangladesh 2015) [11]. Normally some flora and fauna go into extinction in course of time. But the number of fish species designated as critically endangered, endangered and vulnerable compared to the total number of fish species is so high that it is hard to believe the situation as a normal one. The degradation brought about in the aquatic environment are due to unplanned road and dam construction giving less or no importance to fish pass and fish ways, erosion of soil and elevation of river beds, indiscriminate use of insecticides and pesticides for crop production, dumping of industrial wastes in aquatic environment, indiscriminate killing of fish paying minimum or no attention to abide by the fish laws and acts. In addition, freshwater resources are subjected to severe competition among multiple human stakeholders such as crop farming, aquaculture and industrial usage. Consequently, stocks of some fish species have been depleted to below replaceable levels.
Conservation programs require large populations to ensure biodiversity but for threatened and endangered species the numbers of fish are steadily decreasing. Cryopreservation can help in both of these situations. Cryopreservation is a process by which biological cells or tissues are preserved at subzero temperatures resulting in a radical decrease in the rate of metabolic

processes and the ability to store samples for extended periods (Armitage 1987) [3]. The availability of frozen sperm is a proven technique for developing, maintaining, and distributing genetic improvement in livestock, and provides great unexploited potential for fish breeding.


**Fig 1:** Mohashol, *Tor tor* (Hamilton 1822)

Mohashol (*Tor tor*) (Figure 1) has unique taste and deserves special mentioning. The abundance of *T. tor* has decreased to such an extent that very seldom they are reported to be caught in the region of Netrokona and Shunamganj districts. And as such not many people are lucky enough to taste the delicious flesh of *T. tor*. The mohashol (*T. tor*) including several other species of *Tor* have also been recognized as endangered fish in India, Pakistan, Nepal and Bhutan, in Southern Asia, Afghanistan, Thailand and Malaysia (Desai 2003, Rahman *et al*. 2005, Sharma 2003, Gurung *et al.* 2002, Bazaz and Keshavanath 1993) [6, 17, 18, 8, 4]. Several attempts have been made to conserve the fish in different countries (Patil and Lakra 2005, Nguyen *et al.* 2006, Ingram *et al*. 2005, 2007) [16, 15, 9, 10]. but in Bangladesh research work have yet been done.

## 2 Materials and Methods

The cryopreservation of Mohashol (*Tor tor*) spermatozoa at liquid nitrogen temperature (-196$^0$C) by standardizing the suitable combination of extender and cryoprotectant, optimal milt-diluent ratio and cryoprotectant concentration. The duration of the experimental period was February to May 2016. The materials and methods are described under the following headings:

### 2.1 Experimental fish

To conduct this study, the collection of fish was the first step. The experiments were conducted in the Laboratory of the Department of Fisheries Biology and Genetics under the Faculty of Fisheries, Bangladesh Agricultural University (BAU), Mymensingh. One hundred and fifty fish of *Tor tor* were collected from wild sources (collection place) and maintained in circular tank with the provision of continuous water supply and aeration for conditioning for 6 h. Two rectangular earthen ponds each of 30.0 decimal area, having inlet and outlet facilities were used for stocking of fish. After conditioning *Tor tor* were stocked in a density of 2, 0000 kg/ha and reared in ponds for domestication. The ponds were dried and exposed to the sunlight for better mineralization, escape of toxic gases and to make free from aquatic weeds, harmful aquatic insects, predatory and weed fishes. Lime was used at the rate of 1kg per decimal. Then the pond was filled with water and after seven days, urea and triple super phosphate (TSP) were applied at the rate of 200g and 100g per decimal respectively. In pond, the fish were reared with the commercial supplementary feed having 30% protein at the rate of 2 to 6% of body weight up to maturation. The fish were fed two times a day at 09.00 and 17.00 h and the amount of feed were adjusted on the basis of fish weight. Broods of Mohashol were reared in ponds in the vicinity of Fisheries Faculty premises.

### 2.2 Collection of fish gametes

#### 2.2.1 Selection of mature male fish

Mature males were selected on the basis of their desired phenotypic characteristics mainly genital papilla. Before selecting male fish, a gentle pressure was applied on abdomen to remove some milt, which indicated the maturity of fish. Mature male broods were collected from the stock ponds for getting quality spermatozoa.

#### 2.2.2 Conditioning of brood fish

Selected male fishes were brought to the circular tank from the pond for conditioning before 6h of hormone treatment. During the period of conditioning, no feed was supplied to them. This was done for more effectiveness of hormone and to keep the fish in better condition after collection of milt.

#### 2.2.3 Inducing with hormone injection

The brood fishes were induced by pituitary gland (PG) extract. Male broods were induced so that sufficient amount of milt was collected easily at a time.

#### 2.2.4 Preparation of inducing agents

Dry carp pituitary glands (PG, available in the market) were used to induce the fish. The required amount of PG as per dose was weighed, homogenized in a tissue homogenizer with about 1.5 ml of distilled water. The suspension was centrifuged for 5 minutes at 3000 rpm. The supernatant was poured in a cubate and diluted with distilled water to obtain the desired quantity for injection. Then the solution was loaded into a 3 ml hypodermic syringe for injection.

#### 2.2.5 Collection of sperm and checking their motility

Firstly, excess moisture, urine, gut extrudes and mucus was wiped from the genital area of male *Tor tor* with absorbent paper. Gentle pressure was applied (Figure 2) on abdomen from anterior to posterior direction before collecting milt to remove some milt for avoiding contamination with urine and water. When the milt was concentrated and whitish in colour, 5 ml-sized glass tubes were held against the tip of the genital papillae and sperm were collected into the tubes and weighed. Watery or bloody milt and residues were discarded and tubes were weighed again to measure the actual quantity of milt. The collected milt was stored on ice to prevent quality deterioration during further processing. The quality of sperm in each tube was checked under the microscope taking about 1-2 µl of milt on a glass slide. Samples containing more than 80% motile cells by eye-observation under microscope were used for cryopreservation.

**Fig 2:** Collection of milt from *Tor tor* male

### 2.3 Determination of sperm motility in various osmolalities (%NaCl)

Sperm suspension was prepared individually with Alsever's solution, egg-yolk citrate and urea-egg-yolk solution. An aliquot (2 µl) of sperm suspension was taken into a glass slide and different concentrations of NaCl (01.%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9% and 1.0%) were mixed with the milt, and the motility was observed immediately by microscope. The per cent motility and duration of motility were recorded before the sperm stopped their movement.

### 2.*4* Statistical analyses

Data was presented as percentage of motile cells and all per cent motility values were converted to arcsine transformation before statistical analysis. Data was analyzed using Independent -samples T test of Statistical Package for the Social Science (SPSS v 16) and the means were separated by Least Significant Difference (LSD) at 5% probability level.

## 3. Results

The sperm was collected from *Tor tor* in a 1.5 ml eppendorf tube. During experiment mobility of sperm was examined all the time under microscope. Mobility of sperm can be categorized into two types: forward movement and Brownian movement. About 80-90% forward movement and 10-20% Brownian movement was recorded from the sample with different activation solution (0.1-1.0% NaCl). The number of spermatozoa per ml ranged from 4.47 x $10^{10}$ to 9.65 x $10^{10}$. The motility of sperm tested at various concentration of salt solution (% NaCl solution) demonstrated that sperm motility decreased with increase of NaCl concentration. Distilled water provided 92.0±2.7% motility of sperm. The motility of sperm was 80.0±3.5% at 0.1% NaCl solution which reached to 93.4±2.30 and 87.40±4.9 at 0.4% and 0.5% NaCl solution respectively. Then the motility decreased at a faster rate as the concentration of NaCl increased and it reached to 4.0±6.5 at 0.9% NaCl. The motility was completely inhibited at 1.0% NaCl solution (Figure 3). Motility of sperm at 0.4% NaCl was more or less stable and seemed completely activated. A significant difference (p = 0.001) was observed between the complete activation and inhibition of sperm at 0.4% and 1.0% NaCl solution respectively.

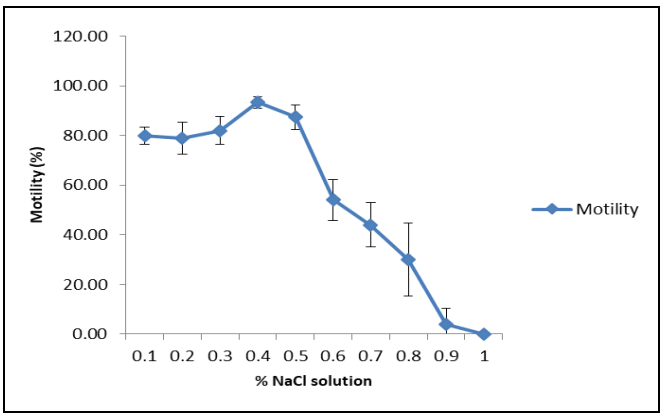


**Fig 3:** The motility of sperm of *Tor tor* in different concentration of NaCl solution (0.1 to 1.0%)

The swimming duration of activated sperm differed in different NaCl concentrations and the duration was severely reduced with the increase of NaCl. The duration of motility of sperm was estimated as 8.4±0.89 minutes at 0.1% NaCl and 68.0±16.08 min at 0.4% NaCl. Then swimming time was reduced sharply with the increase of NaCl concentration and it became zero at 1.0% NaCl (Figure 4). Statistical analysis showed that the swimming duration of activated sperm in 0.4% solution was significantly higher ($p<0.001$) than 1% NaCl solution.

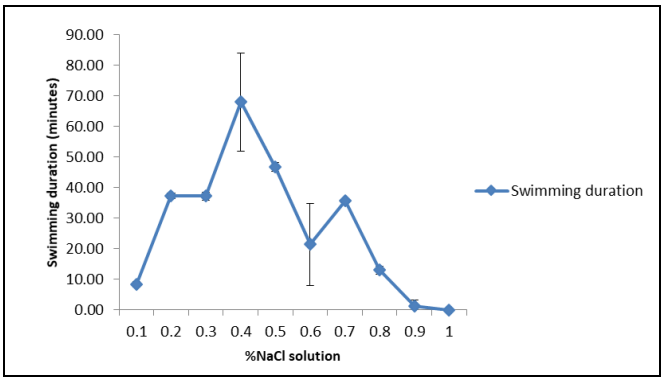


**Fig 4:** The swimming duration of sperm of *Tor tor* in different concentration of NaCl solution (0.1 to 1.0%)

## 4. Discussions

Identification of suitable activation solution is very important for successful cryopreservation. In the present study, experiment was conducted to determine the activation of sperm at different osmolalities of NaCl solution. It was found that the motility of sperm of *T. tor* decreased with the increase of NaCl concentration and it became zero (completely inhibited) at 1.0% NaCl. Motility of sperm at 0.4% NaCl was more or less stable and completely activated. A significant difference (p = 0.001) was observed between the complete activation and inhibition of sperm at 0.4% and 1.0% NaCl solution. Najmus *et al.* (2015) [14] reported that motility of sperm of Rohu decreased with increasing concentration of NaCl solution which is similar to the present findings. However, a reverse situation has been reported in Zebrafish where sperm were found to swim longer period in higher osmolalities (Yang *et al.* 2007) [20]. Morisawa *et al.* (1999) [13] and Alavi and Cosson (2006) [1] reported that several factors such as pH, temperature, ions and osmolality may affect sperm motility, where pH plays the major role in sperm activation (Stoss 1983) [19]. Although fish sperm are characteristically immotile in the testis (Morisawa and Suzuki 1980) [12], they become activated on release into aquatic environment (Billard and Cosson 1992; Alavi and Cosson 2005 and 2006; Yang *et al.* 2006) [5, 1, 2, 20]. These mechanisms of activation vary between species. Sperm of freshwater species are activated in hypotonic solutions but sperm of marine species become activated in hypertonic solutions (Morisawa and Suzuki 1980; Yang *et al.* 2006) [13, 21]. It can be uttered that the swimming activity of sperm of *T. tor* existed for a long period at lower osmolalities (about 50-55 min), which gradually reduced with the increase of concentration of the NaCl solution and the motility severely reduced at 1.0% NaCl.

## 5. Conclusions

In this experiment the activation of motility of sperm at various concentrations of the salt solution (% NaCl) was tested. It was found that the activation of sperm motility decreased with increasing concentration of NaCl. The swimming activity of sperm existed for a long period at lower concentrations which gradually reduced with the increase of concentration of the extending media.

**6. Acknowledgements**
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