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An approach for determination of semen quality of golden mahseer (*Tor putitora*) From Central Himalayan

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Abstract
Semen has a unique composition containing spermatozoa and substances supporting the spermatozoa. The evaluation of seminal quality is essential in aquaculture for planning efficient artificial fertilization protocols in restoration programs involving supportive breeding together with other innovative conservation strategies as gamete cryopreservation. The evaluated semen quality parameters of Golden Mahseer, *Tor putitora* were: colour, volume, sperm density, spermatocrit value, sperm motility percentage and motility duration. Semen samples from ripe male brooders were collected during the period from April to July 2017 from the River Bhilangana and its impoundment (Tehri reservoir). The observations related to semen characteristics are based on 70 fish samples of *T. putitora* ranging from 220mm to 620mm in total fish length and weight ranging between 200grams to 900 grams. The colour of semen is milky white to creamy white. Semen volume varies from 0.4ml to 4.5ml in different individual brooders. The sperm density ranges from 1.28-2.23x10^9/ml per individual brooders. The sperm motility duration ranges from 38-59 seconds and the spermatocrit value range is 35.93% to 98.07%. The present study is aimed to evaluate the semen quality of *Tor putitora* for estimating the reproductive potential of male brooders and timing of optimal fertilization for seed production in the hatcheries.

Keywords: semen quality, golden mahseer, sperm motility

1. Introduction
The Golden Mahseer (*Tor Putitora*), one of the well-known large freshwater game fish, forms an important fishery resource in coldwater areas of Himalaya regions, India. The population of golden mahseer is reported to be declining in their natural habitats (Nautiyal and Lal, 1982; Sharma and Mishra, 2002) [19, 26]. The main threats to the fish are from overfishing, and loss and deterioration of habitat including the breeding grounds (Singh *et al.*, 1992; Bhatt *et al.*, 2000; Agarwal *et al.*, 2011) [27, 7, 3]. The threat of declining population of golden mahseer in natural waters can be reduced through the adequate knowledge of reproductive biology including gamete characteristics and semen biology for its successful reproduction and high seed production in the hatcheries. It is of interest not only for fish farming but also for the conservation and genetic improvement of resources. Semen is composed of spermatozoa (sperm) and seminal plasma. Seminal plasma in the semen has a vital role in sperm metabolism, function, survival and sperm motility. Sperm are freely suspended in seminal plasma and not subjected to cellular growth and division. Viable sperm is an essential component in any successful breeding operation. Without viable sperm, no egg fertilization will occur.
Therefore, the characterization of fish semen provides a strong basis for understanding the effective fertilization success. Semen quality defines those traits of the sperm that determine its capacity to fertilize eggs estimate male reproductive success (Billard *et al.*, 1995) [8]. Some physical parameters such as sperm density, semen volume, colour, pH, spermatocrit value and motility related factors are considered important spermatological issues for fish semen characterization. Therefore the aim of the present study is to determine some physical parameters of the semen of Golden mahseer (*Tor putitora*) that will determine the fertilization capability of spermatozoa and are often used to estimate the semen quality.

2. Material and method
2.1 Semen Collection: Seventy ripe male brooders of Golden Mahseer (*Tor putitora*) were procured from the River Bilangana and its impoundment (Tehri reservoir) during April-July 2017. The brooders having 220mm to 620mm in total fish length were stripped by gentle pressing the abdomen from anterior to posterior direction.

4.5ml graduated cryovials were used for the collection of semen samples. To avoid contamination with water and body mucous, the urinogenital pore was wiped with tissue paper to make it dry. First 1-2 drops of semen were always discarded. After the collection of the semen in the cryovials, these vials were tightly closed and kept in crushed ice (0-4^{o}c) for semen quality assessment within two hours of collection.

2.2 Semen Quality Evaluation: The semen quality including semen volume (ml), sperm motility duration (s), spermatozoa density (spz/ml), spermatocrit value (%) and pH were evaluated.

2.2.1 Semen volume: The semen was collected in cryovials graded in millimeters and sperm volume was registered immediately following collection of abdominal massage.

2.2.2 Spermatozoa motility: The motility of sperm in each sample was evaluated within two hours following sperm collection. About 10 microlitre semen was placed on a glass microscopic slide and 100 microlitre activation solutions (distilled water) were added. Under inverted microscope spermatozoa motility and percentage of motile spermatozoa were observed. Only forward movement by the spermatozoa was assessed as motility, where as simple vibrating motion of sperm cells were assessed as immotile.

2.2.3 Duration of spermatozoa movement and sperm pH: The duration of motility (sec) was also recorded on monitor connected with the inverted microscope from the initial contact between the activation solution and milt until almost all of the spermatozoa (up to 20%) were immotile. Sperm pH was measured with digital pH meter within five minutes of sampling.

2.2.4 Sperm density: Sperm count was made by using a haemocytometer. Semen is diluted in two steps (step I: 1-5µl of semen in 2ml of NBF; step II: 50 µl of first diluted in 10ml of NBF) because undiluted semen has large population of sperm cells which are difficult to count. On haemocytometer, spermatozoa were counted in 8 squares at random after loading in counting chamber under an inverted microscope. The mean value for sperm No. is calculated. The no. of spermatozoa per ml of semen (spz/ml) was determined for each sample by using following formula

Sperm density (spz/ml) = X× 40×200×1000

Where, X= mean value of sperm number
40=first step dilution
200= second step dilution
1000= conversion factor from1mm^3 to ml or cm^3

Spermatozoa density was finally determined by taking the mean of sperm count in five aliquots of diluted semen samples.

2.2.5 Spermatocrit measurement: Microhaemocrit capillary tubes (75mm length, 1mm inner diameter and 0.1 ml capacity) were filled with semen and their both ends were sealed with haemoseal wax. The volume (length) of semen in capillaries was measured by meter scale in mm and centrifuged for 21min. at 7500 rpm (6315× g). Spermatocrit, defined as the percentage of white packed cells to the total volume of semen. Fifty semen samples were processed for spermatocrit measurement during the period of three months (May to July).
Centrifugation time for the stable reading of percentage of packed cell volume (sharp interfere between packed sperm cell and clear seminal fluid) was decided by conducting an initial experiment. Five aliquots of a semen samples were centrifuged at 7500 rpm for 7 min. interval upto 35 min. of centrifugation. Four replicates of a semen samples per male for each time interval were subjected for spermatocrit estimation and mean value were recorded.

Statistical Analysis: Results are presented by mean and standard deviation (mean ± S.D.) of each seminal parameter value.

3. Results

The *T. putitora* semen was observed creamy-white in colour during the period of sample collection. Sometimes, the colour of semen turned yellowish or pinkish due to the contamination of semen with urine, fecal matter or blood, which reduces the semen quality. The fish released 0.4 to 4.5ml semen (mean 0.92 ±0.88 ml) in one ejaculation from stripped brooder. The fluctuation in semen volume was highly variable and was observed depending on the condition of brooders. Most of the brooder collected from the river was found oozing out of semen very freely. In this state, fish was in partly spent condition and actual semen productivity of that individual could not be determined.
The observations made during the characterization of *S. progastus* semen revealed very slight variation (7.2 to 7.5) in pH value. It is slightly alkaline (7.37±0.08) as usually occurs in most of the fresh water fish species. The pH is an important seminal plasma characteristic influencing the potential for motility of fish spermatozoa (Duplinsky, 1982) [14]. It is an important parameter of consideration during preparation of extender medium for preservation of semen.
Spermatozoa motility under direct observation of undiluted semen by inverted microscopy showed that most spermatozoa in the seminal plasma of golden mahseer were immotile. Motility of golden mahseer spermatozoa was activated immediately after diluting the semen with distilled water. The range of motility duration was 38-59 seconds with mean value 48.1±8.09 seconds. The sperm density has traditionally been used for the assessment of semen quality. In *T. putitora*, mean sperm density was observed as 1.70±0.29 x10^9 sperm ml^{-1} of semen which varied from 1.28-2.23 x10^9 sperm ml^{-1} The spermatocrit value was found fluctuated between 35.93-98.07% with mean value as 63.11±19.26%.

Table 1: Spermatological parameters of *Tor putitora (n=70*)

	Length (cm)	Weight (gm)	Milt Vol. (ml)	pH	Density (x10^9/ml)	Motility Duration (sec)	Spermato-crit value (%)
Range(n=70)	22-62	200-900	0.4-4.5	7.2-7.5	1.28-2.23	38-59	35.93-98.07
Mean(n=70)	38.01±6.18	412.86 ±179.09	0.92 ±0.88	7.37 ±0.08	1.70±0.29	48.1±8.09	63.11±19.26

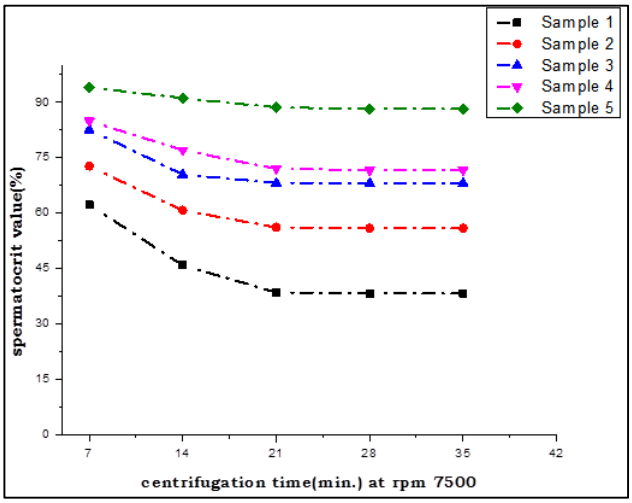

Fig 1: The influence of centrifugation time at 7500rpm on spermatocrit in Tor putitora. Each line represents data for milt collected from a different male, as mean values of four replicate samples per male at each time interval.

4. Discussion

Sperm quality assessment is important for estimating a stock's reproductive potential (Trippel 1999, 2003) [29, 30] and timing of optimal fertilization for hatchery production (Rana 1995) [23]. Similarly, the semen characteristic also varies from species to species. In the present study, we assessed the semen characteristics of *Tor putitora*. Density of spermatozoa is observed to be highly varying depending on the species as evident from the reports on carp where it has been reported to be from 1 to 5×10^9 cells/mL, in trout it was from 5 to 15×10^9 cells/mL and in sturgeon it was from 0.1 to 4×10^9 cells/mL (Cosson *et al.*, 1991; Redondo *et al.*, 1991; Tsvetkova *et al.*, 1996) [13, 24, 32]. Present study revealed that mean sperm density of *Tor putitora* is 1.70 ±0.29x10^9 sperm ml $^{-1}$ which is less than *Tor khudree* (4.90-7.45 x10^9 sperm ml $^{-1}$; Basavaraja *et al.*, 1998) [6], rainbow trout, *Oncorhynchus mykiss* (11.8 ± 6.19 × 10^9 ; Ciereszko and Dabrowski, 1993) [11], 8.9 ± 3.8 × 10^9 (Geffen and Evans, 2000), for Atlantic salmon, *Salmo salar*, 3.5 – 17.9 × 10^9 (Aas *et al.*, 1991) [1], 12 – 30 × 10^9 (Truscott and Idler, 1969) were reported. On the other hand, sperm density of *Tor putitora* is found higher than other cold water fishes *S. richardsonii* (3.77 ±0.78x10^8 sperm ml^{-1}) reported (Agarwal, 2011) [3].

The assessment of motility of fish sperm has basically relied on subjective estimates of the total duration of movement (Duplinsky, 1982) [14] The motility duration of fish spermatozoa ranges from 30 to 300 s (Stoss, 1983) [28]. Since many fish species have shorter motility period ranging from 30 to 120 seconds (Alavi and Cosson, 2005, 2006) [5, 6], it is necessary to avoid the preactivation of motility by urine contamination during stripping (Perchec *et al.*, 1995a; Dreanno *et al.*, 1998) [21, 16]. The present study reveals that *T. putitora* sperm remain motile for 38-59 seconds with mean value as- 48.1±8.09 seconds (table-1)). In most freshwater species, spermatozoa usually remains motile for less than 2 minutes and in many cases is only highly active for less than 30 seconds (Billard *et al.*, 1995; Kime *et al.*, 2001) [8, 19]. The duration of motility is very short in trout (20-25 seconds) and slightly longer than 1 minute in carp (Bromage and Roberts, 1995) [11]. However, quite higher motility duration of 9 minute 31 seconds ±0.90 was observed in mirror carp (Akcay *et al.*, 2004) [4], while it is 45 seconds in *Cyprinus carpio* (Billard, 1992) [10].

Spermatocrit has been used to estimate sperm concentration for several species of fish (Rakitin *et al.* 1999; Rideout *et al.* 2004) [23, 26]. such as yellow perch, *Perca flavescens* (Ciereszko and Dabrowski 1993) [11], haddock, *Melanogrammus aeglefinus* (Rideout *et al.*, 2004) [26], Atlantic halibut, *Hippoglossus hippoglossus* (Tvedt *et al.* 2001) [34], snow trout, *Schizothorax richardsonii* (Agarwal and Raghuvanshi 2009) [2], brown trout, *Salmo trutta* (Poole and Dillane 1998) [22], Atlantic salmon, *Salmo salar* (Aas *et al.* 1991) [1], rainbow trout, *Oncorhynchus mykiss* (Ciereszko and Dabrowski, 1993) [11] and lake whitefish, *Coregonus clupeaformis* (Ciereszko and Dabrowski, 1993) [11]. The milt pH in golden mahseer was found to be slightly alkaline (7.2-7.5) in the present study. It has been reported that pH is one of the major sperm activating factors in fish species (Stoss, 1983) [28]. With an increase in pH, the duration of sperm motility in *Petromyzon marinus* decreased, but over the pH range 6.0-9.0, the percentage of motile cells did not change (Ciereszko *et al.* 2002) [13]. According to Ingermann *et al* (2002) [18], the sensitivity of pH on sperm motility in *Acipenser transmontanus* demonstrated that sperm maintained at high pH (more that 8.2) had significant motility when added to water but the motility was inhibited when sperm was maintained at low pH (less than 7.5).

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