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Raghuvanshi SK
Department of Zoology, Bareilly College, Bareilly, Uttar Pradesh, India

Sapna Rani Charak
Fish Reproductive Biology Research Lab, Department of Zoology, School of Life Science, H.N.B. Garhwal Central University, Campus Badshahithaul, Tehri Garhwal, Uttarakhand, India

Agarwal NK
Fish Reproductive Biology Research Lab, Department of Zoology, School of Life Science, H.N.B. Garhwal Central University, Campus Badshahithaul, Tehri Garhwal, Uttarakhand, India

Corresponding Author:
Agarwal NK
Fish Reproductive Biology Research Lab, Department of Zoology, School of Life Science, H.N.B. Garhwal Central University, Campus dshahithaul, Tehri Garhwal, Uttarakhand, India

Semen characteristics and extenders competency during refrigerated storage of snow trout (*Schizothorax richardsonii*) semen

Raghuvanshi SK, Sapna Rani Charak and NK Agarwal

Abstract
The present study has investigated the semen characteristics and competency of different extenders for refrigerated storage of snow trout (*Schizothorax richardsonii*) semen. Semen samples were collected by stripping ripe male brooders during the spawning season. The pH (7.31 ±0.07), sperm concentration (3.77 ±0.78x10^8/ml), spermatocrit value (63.13 ±10.27%), and sperm motility duration (59.70 ±16.55 s) were determined by analysing 98 semen samples from different breeding phases. The short-term preservation of *S. richardsonii* semen was made under refrigerated conditions. Four extenders (Mounib's medium, KCl medium, extender modified from Buyukhatipoglu & Holtz, 189 M of Horton) each in two dilution ratios (1:4 & 1:10) were scrutinized for their competency. During refrigerated storage, sperm motility percentage and motility duration was evaluated after every 24 hours until the viability of semen samples. Mounib's medium was found significantly superior to the KCl medium ($P<0.01$), extender modified from Buyukhatipoglu & Holtz ($P<0.02$) and extender 189 M of Horton ($P<0.002$) in retaining sperm motility up to 9 days at 0-4 °C. It was also observed that the dilution ratio of 1:4 was not significantly different than the 1:10 ratio in all extender media (P ns, n=10).

Keywords: semen quality, extender, short-term storage, *S. richardsonii*, snow trout

1. Introduction
Early fish development is the most important thing that should be known before producing any Good quality of semen is an essential component for successful seed production in aquaculture systems [1] and it is typically monitored by determining the number of motile sperm, the success in fertilizing the eggs or by measuring some aspects of cell metabolism. The poor sperm quality may result from the effects of genetics, diet, environmental stress or disease [2]. The study of fish semen characteristics is a prerequisite for the preservation of semen for propagation of species to facilitate seed production in hatchery and ex-situ conservation of gene pool [3, 4, 5, 6]. The existing knowledge on the semen characteristics of freshwater fishes of Indian subcontinent is very limited [7, 8, 9, 10, 11, 12, 13, 14, 15, 16]. Adequate knowledge of semen characteristics provides information for storage protocols for the semen used in artificial fertilization [17]. Storage of fish gametes below their culture or ambient temperature can be used to prolong their viability [18]. The majority of the publication is related to the storage of semen under chilled conditions, usually in domestic refrigerators [19, 20, 21, 22, 23]. The availability of a suitable method to preserve *S. richardsonii* semen for several days may be of considerable value in terms of mass fertilization and thereby seed production. While reviewing the literature, it appears that almost no work has been conducted on the Semen characteristics and refrigerated storage of snow trout semen [10, 11, 13]. Therefore, the present study was conducted for evaluating semen characteristics and to develop a simple procedure for the refrigerated storage of *S. richardsonii* semen by using different extender media.

2. Materials and Methods
2.1 Sample collection
Live ripe male brooders were selected from wild collection from river Bhagirathi during breeding season. Semen samples were collected into graduated cryovials of 4.5 ml capacity. By stripping the brooders. Extra care was taken to avoid contamination of semen with urine blood or water by discarding initial 1-2 drop of ejaculate. Semen samples on ice packs were

Immediately brought to the laboratory at 0-4 ^{0}C for quality analysis and further processing for refrigerated storage.

2.2 Semen evaluation
Semen samples (n=98) were evaluated for pH, sperm density, motility percentage, motility duration and spermatocrit value in order to characterize the semen. Pocket-sized digital pH meter was used for pH measurement.

Sperm motility analysis: The percentage of sperm exhibiting rapid and forward movement was determined subjectively under phase-contrast microscope (x 400) by diluting semen sample with activation solution at a ratio of 1:100. The percentage of motile sperm was assessed by giving a motility score corresponding to an arbitrary scale of criteria from 0 to 5 [24, 25]. The sperm motility duration was evaluated using a sensitive chronometer started simultaneously with the addition of activating medium into semen sample under microscope.

Sperm density: Sperm density was estimated using hemo cytometer by two-step dilution method [8, 11]. Spermatocrit was determined using micro-hematocrit capillary tubes (75x1.1mm, 0.1 ml capacity). These tubes were filled with semen followed by sealing and centrifugation at 7500 rpm for 30 minutes. The spermatocrit value was calculated as the percentage of packed cell volume out of total volume [8].

2.3 Extenders preparation
Four extender media *viz.* Mounib's medium (1000 mg $KHCO_3$, 200 mg reduced glutathione, 4270 mg sucrose, 100 ml distilled water), KCl medium (1147 mg KCl, 100 ml distilled water), Extender modified from Buyukhatipoglu & Holtz (592 mg NaCl, 172 mg KCl, 68 mg $CaCl_2$, 15 mg $MgSO_4.7H_2O$, 2420 mg TRIS, 400 mg bovine serum albumen, 100 mg Promina-D, 100 ml distilled water) and Extender 189 M of Horton (730 mg NaCl, 500 mg $NaHCO_3$, 500 mg fructose, 750 mg vegetable lecithin, 500 mg mannitol, 100 ml distilled water) were prepared and examined for refrigerated storage of *S. richardsonii* semen.

2.4 Refrigerated storage
Semen samples from multiple brooders were pooled to avoid individual variability. Thereafter, nine aliquot of pooled semen samples were prepared. One was preserved as such and remaining eight were extended with four extenders under evaluation in two dilution (1:4 & 1:10) ratio (4x2=8) and kept under refrigeration (0-4 °C). The extended semen samples were evaluated for sperm motility percentage and motility duration for every 24 hours interval.

2.5 Statistical analysis
The mean values of multiple observations were calculated with their standard deviations. One-way analysis of variance (ANOVA) and student's t-test were used to reach on meaningful conclusions.

3. Results and Discussion
3.1 Semen characteristics: The observations on physical characteristics of semen of *S. richardsonii* are summarized in Table-1. The pH is an important seminal plasma characteristic influencing the potential for the motility of fish spermatozoa [26]. It was found slightly alkaline with its value ranging between 7.2 - 7.4 (mean 7.31 ±0.07) throughout the breeding season. The semen of most of the freshwater fish species also exhibit slightly alkaline pH [15, 16]. The mean sperm density for *S. richardsonii* was calculated as 3.77 ±0.78x10^8 sperm^{-ml}. It is comparatively lower than the other cold water fish species [7, 12]. The sperm density was also found varying in different phases of breeding (2.14 - 6.08 x10^8, F=13.944, P<0.05, n=98). It was usually high with the start of breeding (4.22 ±0.86x10^8 sperm^{-ml}), but fairly similar in middle (3.48 ±0.47x10^8 sperm^{-ml}) and late phase (3.43 ±0.86x10^8 sperm^{-ml}) of breeding (Table-1). A decrease in sperm density throughout the breeding season has also been reported in rainbow trout [27], brown trout [28] and Atlantic salmon [29]. The number of sperm^{-ml} of semen also varies with the individuals and may depend on the diet and up to some extent on their spawning state. Sperm density affects the fertilization rate and has vital importance in hatchery management and storage of semen.

Table 1: Characteristics of *S. richardsonii* semen during different phases of breeding

Breeding phase		pH	Density (x10^8 /ml)	Motility rating	Motility Duration (s)	Spermatocrit value (%)
Early (n=39)	Range	7.2-7.4	2.56-6.08	0-4	27-104	48.00-89.71
	Mean	7.35 ±0.06	4.22 ±0.86	3.03 ±1.38	57.05 ±18.44	71.08 ±8.76
Middle (n=47)	Range	7.2-7.4	2.14-4.18	0-4	24-99	42.00-70.15
	Mean	7.28 ±0.06	3.48 ±0.47	3.29 ±1.12	61.96 ±15.71	58.08 ±5.35
Late (n=12)	Range	7.2-7.4	2.14-4.87	1-4	42-85	34.00-79.37
	Mean	7.31 ±0.05	3.43 ±0.86	3.08 ±1.16	59.42 ±13.11	57.06 ±13.11
Grand Mean (n=98)		7.31 ±0.07	3.77 ±0.78	3.16 ±1.23	59.70 ±16.55	63.13 ±10.27

The considerable variation in spermatocrit value (34.00 to 89.71%, mean 63.13 ±10.27%) was also observed during the breeding season. It was maximum (71.08 ±8.76%) during the beginning of breeding. Its value decreases with the middle (58.08 ±5.35%) and late phase (57.06 ±13.11%) of breeding. The variation in spermatocrit is found statistically significant (F=31.856, P<0.05, n=98). The observation on the significant decrease in the spermatocrit with the progression of spawning season in *S. richardsonii* is in accordance with the earlier observations [27, 30]. Contrary to these, no significant difference in spermatocrit was reported in rainbow trout [1]. The spermatocrit and viscosity of semen was also found varying between conspecific males, between species and across the reproductive season [30, 31]. Spermatocrit appeared to be directly proportional to the viscosity of semen.

The quality of sperm is usually assessed by the intensity of motility, percentage of motile spermatozoa and the duration of forward movement [32, 33, 34]. Most semen samples of *S. richardsonii* have shown sperm motility rating above 3 (mean motility rating as 3.16 ±1.23, *i.e.*, >75% motility after activation). The highest motility rating (3.29 ±1.12) was observed in the semen samples of the middle phase of breeding, while it was lowest at the early phase (3.03 ±1.38). Though sperm motility percentage for early, middle and late phases of breeding were not significantly different (F=0.482, P ns, n=94). Very few semen samples have shown motility

rating less than 3. It may be due to contamination with body fluids.

The *S. richardsonii* sperm were completely inactive or immotile in seminal plasma at the time of collection. After activation, they showed progressively forward movement for the maximum duration of 61.96 ±15.71 s in the middle phase of breeding (Table-1). However, sperm rotate on its axis further for nearly 30-60 s without any displacement and thereafter cease to move. Now the chances of these sperm to approach the egg and fertilize them are rare. Thus, this period of sperm rotation on its axis is not considered for motility duration. The present study reveals that snow trout (*S. richardsonii*) sperm remain motile for 57-62 s (mean 59.70 ±16.55 s). In most of the freshwater fish species, spermatozoa usually remain motile for less than 2 minutes and in many cases are only highly active for less than 30 seconds [35, 36, 37, 38]. However, quite higher motility duration was observed in mirror carp [39], while it is 45 seconds in *Cyprinus carpio* [40]. Generally, longer sperm motility is observed in marine species [41]. It appears that the sperm motility duration in Coldwater species is very short in comparison to other freshwater and marine fish species [2]. Further, sperm motility duration in *S. richardsonii* semen was found varied with the breeding season. It was reported comparatively less in early and late phases of breeding (57.05 ±18.44 and 59.42 ±13.11 s, respectively), though statistically insignificant (F=0.890, P ns, n=94). In most of the fish species with external fertilization, the duration of motility was reported usually very short and the highest motility of sperm was observed at the peak of the breeding season [42]. In nature, the species with short motility duration, select such a mating strategy, which maximizes the chances of sperm-egg contact, e.g. gametes of both the sexes are released in close proximity. This also seems to be true for *S. richardsonii*.

Semen samples collected at the beginning or end of the permeation period may be of low quality [43]. However, our study revealed that the semen from the early phase of breeding is significantly superior over the other phases in terms of sperm density. But while analysing the semen characteristics for preservation, we always give priority to the sperm motility percentage and motility duration. Our observations support the views of Ballard and Cosson [14] by giving emphasis on the semen of the middle phase of breeding for its good quality.

3.2 Refrigerated Storage

The observations on motility percentage and duration for *S. richardsonii* sperm after different intervals of refrigerated storage in all extenders are summarized in Tables-2 and 3 respectively. The *S. richardsonii* semen quickly deteriorates within an hour after collection when kept undiluted at room temperature (20-25 ºC). The fresh undiluted semen of *S. richardsonii* stored at 4 ^{0}C showed motility up to 5 days but percentage of motile sperm and motility duration drastically drop down after 2 days. Various extenders screened in the present study were found able to retain motility for varying periods with a considerable decrease in motility percentage and duration (Tables-2 & 3). The present study reports that Mounib's medium is found significantly superior to the KCl medium (P<0.01), extender modified from Buyukhatipoglu and Holtz (P<0.02) and extender 189 M of Horton (P<0.002) in retaining the sperm motility in the semen at 0-4 ºC. Analysis of the result clearly indicated that Mounib's medium at a dilution ratio 1:4 is comparatively good for preserving the sperm motility in the *S. richardsonii* semen up to 9 days at 0-4 ºC.

Table 2: Extender competency in terms of preserving sperm motility (rating score*) during refrigerated storage of *S. richardsonii* semen

Dilution ratio ☛ Storage period ☚	Control	Mounib's Extender		KCl Extender		Buyukhatipoglu & Holtz		189 M of Horton	
	Undiluted(i)	1:4(a)	1:10(b)	1:4(c)	1:10(d)	1:4(e)	1:10 (f)	1:4(g)	1:10(h)
Initial	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	3.8±0.5
24 h	3.8±0.5	4.0±0.0	3.8±0.5	4.0±0.0	4.0±0.0	4.0±0.0	3.8±0.5	3.3±1.0	2.3±2.1
48 h	2.8±1.3	3.8±0.5	3.8±0.5	3.5±0.6	3.5±0.6	3.8±0.5	3.3±0.5	2.8±1.0	1.5±1.7
72 h	2.3±1.5	3.0±0.0	2.8±0.5	3.0±0.0	2.8±0.5	2.8±0.5	2.5±0.6	1.5±1.0	1.3±1.5
96 h	1.5±1.3	3.0±0.0	2.8±0.5	2.5±0.6	2.3±0.5	2.5±0.6	2.3±1.0	0.8±1.0	0.0
120 h	0.8±0.5	2.5±0.6	2.3±1.0	2.3±0.5	1.8±0.5	1.5±0.6	1.3±1.0	0.0	-
144 h	0.0	2.0±0.0	1.8±1.0	1.5±0.6	1.0±0.8	0.8±0.5	0.5±0.6	-	-
168 h	-	1.5±0.6	1.0±1.2	0.8±0.5	0.8±0.5	0.0	0.0	-	-
192 h	-	0.8±1.0	0.5±0.6	0.3±0.5	0.0	-	-	-	-
216 h	-	0.3±0.5	0.3±0.5	0.0	-	-	-	-	-

***** Sperm Motility rating scores: 0 = 0-<1% motile, 1= 1-<25% motile, 2 = 25-<50% motile, 3 = 50-<75% motile And 4 = 75-100% motile.
Observed Probability Values: (a)x(i)P<0.002, (b)x(i)P<0.01, (c)x(i)P<0.01, (d)x(i)P<0.01, (e)x(i)P<0.02, (f)x(i)P<0.05, (a)x(c)P<0.01, (a)x(g)P<0.002, (a)x(e)P<0.02, (a)x(b)P<0.002, (c)x(d)P<0.05, (e)x(f)P<0.01, (g)x(h) P<0.05

Table 3: Extender competency in terms of sperm motility duration (in seconds) during refrigerated storage of *S. richardsonii* semen

Dilution ratio ☛ Storage period ☚	Control	Mounib's Extender		KCl Extender		Buyukhatipoglu & Holtz		189 M of Horton	
	Undiluted(i)	1:4(a)	1:10(b)	1:4(c)	1:10(d)	1:4(e)	1:10(f)	1:4(g)	1:10(h)
Initial	64.8±13.3	65.3±9.0	67.5±11.7	58.3±7.2	60.5±12.6	56.8±6.2	62.3±9.0	50.3±6.6	61.0±11.6
24 h	59.3±11.7	62.8±8.4	64.0±11.9	55.3±5.9	58.3±11.8	54.3±5.3	58.8±8.1	44.8±6.2	34.0±22.9
48 h	56.3±13.3	61.0±7.0	56.8±15.7	51.3±7.4	54.5±11.2	50.3±4.6	51.8±10.8	38.8±6.4	20.8±24.7
72 h	40.5±27.0	55.5±10.1	53.0±15.9	48.8±7.1	50.3±10.9	45.3±6.1	44.3±14.0	32.8±2.8	17.5±20.4
96 h	33.0±22.1	50.5±7.0	50.0±14.9	43.5±10.7	46.3±8.4	41.5±7.6	38.8±16.2	13.5±16.1	0.0
120 h	29.8±20.3	47.0±5.2	48.0±15.0	41.5±9.8	42.3±8.7	34.8±5.7	28.0±23.8	0.0	-
144 h	0.0	43.5±5.4	45.0±15.5	37.5±7.9	28.5±19.4	22.5±15.6	21.3±24.8	-	-
168 h	-	36.0±7.9	24.0±28.7	26.3±17.6	22.3±15.0	0.0	0.0	-	-
192 h	-	17.8±20.5	18.3±21.7	8.0±16.0	0.0	-	-	-	-
216 h	-	5.8±11.5	9.8±19.5	0.0	-	-	-	-	-

Observed Probability values: (a)x(i) P<0.01, (b)x(i) P<0.01, (a)x(c) P<0.001, (a)x(e) P<0.001, (a)x(g) P<0.001

However, nearly 50% of sperm remain motile up to 6 days of preservation. Mounib's medium is also capable to retain significantly superior sperm motility duration than the control or other extenders (P<0.001) under the screen, irrespective of two dilution ratios (1:4 & 1:10) for refrigerated storage.

Mounib's medium or its modified form has also been successfully used for the preservation of semen from different fishes and mammals [44, 45]. Several other extenders have also been successfully tested for extending the life span of semen of Indian freshwater fishes for short durations [12, 46, 47]. The present study reveals that sperm motility in *S. richardsonii* semen is reduced at higher dilution and semen: extender ratio 1:4 is optimal in Mounib's medium. These findings are in close proximity to observations of McAndrew *et al.* [48]. These results on extenders and refrigerated storage of *S. richardsonii* semen were very much promising for further study on cryopreservation.

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