



Comparative analysis of the biochemical parameters of the seminal plasma of *Oncorhynchus mykiss* and *Salmo trutta fario* in farm conditions in Kashmir Himalaya

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ABSTRACT

The aim of this research was to compare the biochemical parameters of seminal plasma of the rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta fario*) cultured in Laribal trout fish farm, Dachigam, Kashmir. During the study period, seminal plasma of rainbow trout was found to contain 73.99 ± 1.18 mg dl⁻¹ glucose, 2.22 ± 0.17 g dl⁻¹ total protein, 15.65 ± 0.84 mg dl⁻¹ triglycerides, 3.07 ± 0.27 mg dl⁻¹ cholesterol and 30.87 ± 0.65 mg dl⁻¹ urea, whereas in brown trout seminal plasma had 82.27 ± 0.52 mg dl⁻¹ glucose, 1.35 ± 0.11 g dl⁻¹ total protein, 10.66 ± 0.43 mg dl⁻¹ triglyceride, 1.65 ± 0.14 mg dl⁻¹ cholesterol and 33.30 ± 0.89 mg dl⁻¹ urea. The results revealed that total protein, triglycerides and cholesterol were higher in rainbow trout than the farmed brown trout. On the contrary, concentration of glucose and urea were found higher in farmed brown trout. These findings give us an idea on the effect of environment on milt quality of the two species of trouts. The higher concentration of urea and glucose in the milt of brown trout is an implication of its lower productivity under farmed conditions in Kashmir as compared to rainbow trout.

Keywords: Biochemical parameters, Brown trout, Milt quality, *Oncorhynchus mykiss*, Rainbow trout, *Salmo trutta fario*, Seminal plasma

Introduction

Fisheries sector occupies a very important place in the socio-economic development of Jammu and Kashmir (J&K). It has been recognised as a strong income and employment source of cheap and nutritious food, besides being an important source of livelihood for a large section of economically backward population of the state. The State Government is giving tremendous importance to the fisheries sector in the backdrop of employment opportunities offered. Efforts are afoot, both at central and at state level to strengthen the existing infrastructure and extension of successful aquacultural practices.

The total fish production of J&K was 18,467 t in 2000-2001, which has risen to 21350 t in 2019-20 (JKFD, 2020). The Kashmir Province is the leading producer of fish in the state and contributes about 80% to the total fish production (Qayoom and Bhat, 2015). Among all the fish species, the exotic trout species have thrived well in Kashmir as the climate, topography and environmental conditions are well suited for breeding, rearing, production and marketing of trout fishes. The production of trout has increased from 90 t during 2002-03 to 155 t during 2010-11 and has reached >650 t in 2019-20 (JKFD, 2020).

The water bodies of the state provide healthy environment for trout fishes as they are fed by the snow-fed and glacier-fed waters furnishing suitable water quality parameters for the growth of trouts. Two important and popular species of trouts farmed in Kashmir are brown trout (*Salmo trutta fario*) and rainbow trout (*Oncorhynchus mykiss*). The natural water resources of Kashmir in the form of lakes, rivers and canals, reservoirs, springs, tanks, ponds and floodplain areas provide abundant scope for increasing the production and productivity of trout in Kashmir. This can be achieved by increasing the number of hatcheries to provide enough seeds for stocking in various public and private farms and open water bodies. Besides this, interventions like detailed studies on the sperm quality to establish healthy and viable stocking material also assume importance for enhancing production from trout farming in the state.

Materials and methods

The present investigations on *S. trutta fario* and *O. mykiss* were carried out at Laribal Trout Farm, Dachigam and at the Fisheries Resource Management Laboratory, Faculty of Fisheries, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir (SKUAST-K), Rangil, Ganderbal, J&K.

Broodstock selection and collection of gametes

Adult male brooders of brown trout and rainbow trout (Fig. 1) in the age group of 3+ years were taken for experimentation from Laribal trout farm during the early reproductive season. In the pre-spawning period, the parental brood fish were kept separately in small ponds and were starved for 48 h prior to sperm collection. The brood fish used were first time spawners, raised in raceways, provided with close to natural environmental conditions without use of any hormonal injection. Milt was collected by manual stripping without anaesthetising the fish and was stored in labelled tubes at 4°C until analysis.

Biochemical analysis of milt

Biochemical evaluation of milt was carried out at Fisheries Resource Management Laboratory at Faculty of Fisheries, Rangil, SKUAST-K. After storage at 4°C for 18 h, the seminal plasma from semen of each fish was separated by centrifuging samples at 3000 rpm for 10 min at 4°C and was stored in Eppendorf tubes. The biochemical analysis of seminal plasma was done employing biochemical kit (Coral Clinical System, Tulip Diagnostics (P) Ltd.) as per the instructions of the manufacturer.

Glucose by glucose peroxidase (GOD-POD) method

Working glucose reagent (1000 µl) was pipetted into three test tubes marked as blank (B), standard (S) and test (T) followed by addition of 10 µl each of distilled water, glucose standard and milt plasma to the test tubes, respectively. After mixing well, the samples were incubated at 37°C for 10 min in an incubator. The absorbance of the standard (Abs.S) and test (Abs.T) were measured against blank (Abs.B) in a spectrometer at 505 nm within 60 min.

Total glucose concentration (mg dl⁻¹) was calculated using the formula:

$$\text{Glucose (mg dl}^{-1}\text{)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 100$$



(a)

Cholesterol by cholesterol Oxidase/phenol + Aminophenazone (CHOD/PAP) method

Cholesterol reagent (1000 µl) followed by 10 µl of milt plasma, 10 µl of standard cholesterol or 10 µl of purified water were added to prepare test (T), standard (S) and blank (B) respectively. All the tubes were incubated at 37°C for 5 min in an incubator. The absorbances of test (Abs.T) and standard (Abs.S) were noted against blank (Abs.B) at 505 nm in a spectrophotometer.

Cholesterol in mg dl⁻¹ was measured using the following calculations:

$$\text{Cholesterol (mg dl}^{-1}\text{)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 200$$

Triglycerides by glycerophosphate oxidase - peroxidase (GPO-PAP) method

Enzymes reagent (1000 µl each) was added to three test tubes followed by 10 µl distilled water, standard reagent and milt plasma to prepare blank, standard and test respectively. After incubation at 37°C for 15 min, the absorbance of standard (Abs.S) and milt plasma (Abs.T) was measured against blank (Abs.B) at 505 nm in a spectrophotometer.

Triglycerides (mg dl⁻¹) was calculated as follows:

$$\text{Triglycerides (mg dl}^{-1}\text{)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 200$$

Urea by modified Berthelot method

The buffer reagent (1 ml) and the enzymes reagent (10 µl) were pipetted into three test tubes marked blank, standard and test. Distilled water, urea standard and milt plasma each in 10 µl quantity were added to each test tube respectively. After mixing and incubating at 37°C for 10 min, chromogen reagent (200 µl) was added to each test tube. The absorbance was measured in a spectrophotometer



(b)

Fig. 1. Male specimens of (a) *O. mykiss* and (b) *S. trutta fario*

for the standard (Abs.S) and test sample (Abs.T) at 570 nm using spectrophotometer.

Urea (mg dl⁻¹) was calculated as follows:

$$\text{Urea (mg dl}^{-1}\text{)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 40$$

Total protein by Biuret method

The biuret reagent was pipetted into three test tubes marked as blank (B), standard (S) and test (T) followed by addition of 20 µl distilled water, protein standard and milt plasma to blank, standard and test samples respectively. After mixing and incubating at 37°C for 10 min, absorbance was measured at 550 nm.

Total serum protein (mg dl⁻¹) was estimated using the following calculations:

$$\text{Total protein (mg dl}^{-1}\text{)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 8$$

Statistical analysis

The statistical analysis of data was done using Microsoft Excel, PAST 3 and SPSS windows (Version 16).

Results and discussions

The total length of rainbow trout ranged from 30-60 cm (mean±SD 45.1±8.96 cm) and for brown trout from 25-55 cm (mean±SD 37.56±8.05 cm). Body weight of rainbow trout ranged from 399-1886 g (mean±SD 1018.76±415.39 g) and in brown trout it was found to be between 205-1459 g (mean±SD 502.03±267.46 g) in case of brown trout. Rainbow trout in particular, showed significantly higher length and weight as compared to brown trout (Table 1).

Table 1. Body length and weight of rainbow trout and brown trout (male)

Species	Total length (cm)		Body weight (g)	
	Range	Mean±SD	Range	Mean±SD
<i>O. mykiss</i>	30-60	45.1±8.96	399-1886	1018.76±415.39
<i>S. trutta fario</i>	25-55	37.56±8.05	205-1459	502.03±267.46
p value	<0.01		<0.01	

Table 2. Comparative statistical analysis of the biochemical parameters of milt plasma of *O. mykiss* and *S. trutta fario*

Parameters (mg dl ⁻¹)	Mean±SD		t value	p value
	Rainbow trout	Brown trout		
Glucose	73.99±6.46	82.27±2.86	6.41	<0.01
Protein	2.22±0.93	1.35±0.61	4.26	<0.01
Triglyceride	15.65±4.62	10.66±2.40	5.24	<0.01
Cholesterol	3.07±1.50	1.65±0.78	4.61	<0.01
Urea	30.87±3.58	33.30±4.88	2.19	>0.01

Biochemical analysis of milt plasma

Comparative statistical analysis of biochemical parameters of milt between the rainbow trout and brown trout are given in Table 2 and Fig. 1. The levels of glucose in brown trout were significantly higher (p<0.01) than those in the rainbow trout. In contrast, the concentration of total protein, cholesterol and triglyceride in brown trout were significantly lower than that in rainbow trout (p<0.01). Though higher in brown trout, urea displayed insignificant variation between the two groups (p>0.01).

The Pearson correlation between biochemical parameters of milt plasma in rainbow trout and brown trout is given in Tables 3 and 4. The data reveals that most of the biochemical parameters of milt showed non-significant correlation except glucose which showed significant negative correlation with cholesterol (r=-0.338, p<0.05) and total protein (r=-0.591, p<0.01), whereas

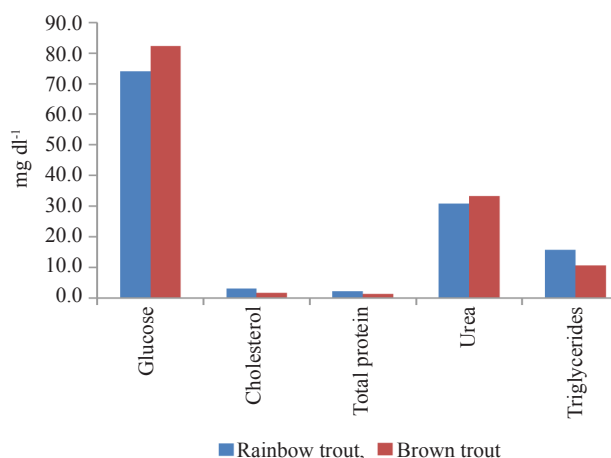


Fig. 1. Mean values of biochemical parameters of rainbow trout and brown trout

Table 3. Pearson correlation between biochemical parameters of milt in rainbow trout

	Glucose	Cholesterol	Total protein	Urea	Triglycerides
Glucose	1	-.338*	-.591**	-.138	.018
Cholesterol		1	.138	.318*	-.286
Total protein			1	.270	-.133
Urea				1	-.076
Triglycerides					1

*. Correlation is significant at the 0.05 level (1-tailed).

** . Correlation is significant at the 0.01 level (1-tailed).

Table 4. Pearson correlation between biochemical parameters of milt in brown trout.

	Glucose	Cholesterol	Total protein	Urea	Triglycerides
Glucose	1	-.200	.364*	.033	-.079
Cholesterol		1	-.164	.167	.207
Total protein			1	-.154	-.003
Urea				1	.167
Triglycerides					1

*. Correlation is significant at the 0.05 level (1-tailed).

cholesterol showed significant positive correlation with urea ($r=0.318$, $p<0.05$). On the other hand, in brown trout, glucose showed significant positive correlation with total protein ($r=0.364$, $p<0.01$).

Glucose

Importance of glucose in fish semen has not been investigated in detail. High levels of glucose concentrations in the seminal plasma has been related to stress conditions (confinement/holding and handling) in captivity and subsequently, an increase in the glucose concentration of body fluids (such as seminal and blood plasma) follows because of the constant activity of glycolysis pathway in liver in response to stress conditions as reported by Portz *et al.* (2006) for Caspian brown trout. The glucose level recorded during the present study in the seminal plasma was 82.27 ± 0.52 mg dl⁻¹ in brown trout and 73.99 ± 1.18 mg dl⁻¹ in rainbow trout (Fig. 2). The glucose level recorded in the present study is higher than the level reported by Secer *et al.* (2004) for rainbow trout (1.33 ± 0.76 mg dl⁻¹ glucose). The higher level of glucose can be related to stressed environmental condition, frequency of stripping, hormonal stimulation of spermiation and sampling period and sampling methods (Billard *et al.*, 1995; Linhart *et al.*, 2003; Cierieszko, 2008). Brown trouts are more sensitive to their environments, therefore, higher concentration of glucose in brown trout than the rainbow trout under farmed conditions is believed to be caused by a wide range of environmental stressors such as hypoxic environment, starvation and captivity (Hardy and Audet, 1990; Torres *et al.*, 1991; Cech *et al.*, 1996; Santos and Pacheco, 1996 and Svoboda *et al.*, 2001). During the present study, the

increased levels of glucose may be attributed to stress caused by overcrowding.

Protein

Seminal protein (*i.e.* transferrin, anti protease) protect the spermatozoa against microbial attack, oxidative damage and premature activation (Dietrich *et al.*, 2010). White and Macleod (1963) indicated that proteins have a protective role. During the present study, the mean concentration of total protein was found as 2.22 ± 0.17 g dl⁻¹ in rainbow trout (*O. mykiss*) and 1.35 ± 0.11 g dl⁻¹ in brown trout (*S. trutta fario*) (Fig. 3) and these results are in conformity with the findings of Loir *et al.* (1990) who estimated the total protein of 1.737 ± 0.792 g ml⁻¹ for

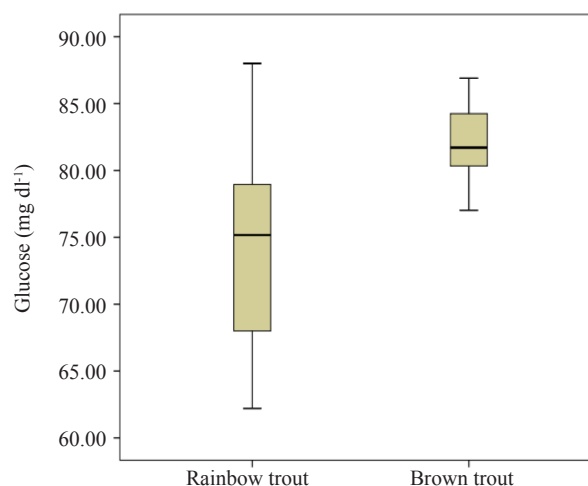


Fig. 2. Box plot depicting the glucose concentration of milt plasma in rainbow trout and brown trout

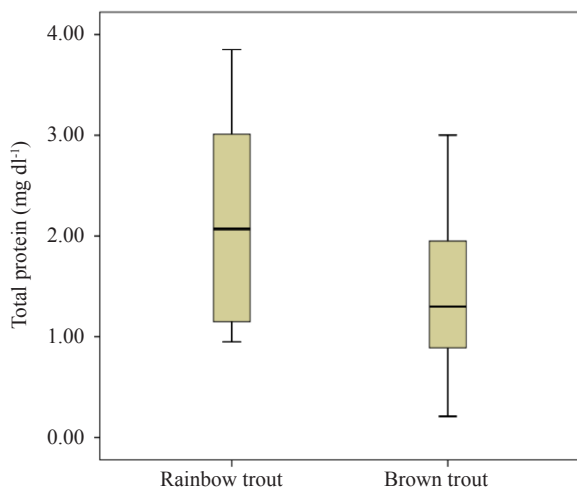


Fig. 3. Box plot depicting the total protein concentration of milt plasma in rainbow trout and brown trout

trout and Halimi *et al.* (2014) who estimated total protein as 1.29 ± 0.44 mg dl⁻¹ in Caspian roach (*Rutilus rutilus caspicus*). Secer (2004) recorded serum protein level of 0.15 ± 0.09 g dl⁻¹. A higher protein concentration was recorded in brown trout (3.0 ± 9.42 g dl⁻¹) by Bozkurt *et al.* (2006). Total protein concentration in the rainbow trout was found to be significantly higher than the brown trout under farmed conditions. Generally, high protein concentration has been reported as positive characteristic of fish semen. Therefore, the semen of rainbow trout can be suggested as better quality semen in comparison with the closely related species of brown trout raised under similar conditions. The results are in agreement with Butts *et al.* (2011) who worked on the seminal plasma proteins of cod species and reported the beneficial role towards spermatozoa viability.

Triglycerides

Seminal plasma lipids are associated with metabolism in spermatozoa (Piironen, 1994). Triglycerides serve as energy sources for sperm motility in fish (Stoss, 1983; Lahnsteiner *et al.*, 1993). During the present work, the triglyceride concentration was recorded as 10.66 ± 0.43 mg dl⁻¹ in brown trout and 15.65 ± 0.84 mg dl⁻¹ in rainbow trout (Fig. 4). The mean level of triglyceride in *O. mykiss* has been reported as 8 mg dl⁻¹ by Secer *et al.* (2004) and in *Ctenophryngodon idella* as 14.58 mg dl⁻¹ by Bozkurt *et al.* (2008). Bozkurt *et al.* (2009) reported the triglyceride level in scale carp from 5-12 mg dl⁻¹. Bozkurt *et al.* (2006) reported the triglycerides level as 5.4 ± 3.17 mg dl⁻¹ for brown trout. Similarly, Lahnsteiner *et al.* (1998) determined semen quality of the rainbow trout, *O. mykiss* and estimated triglycerides concentration to be 188.37 ± 129.04 μmol l⁻¹. The concentration of

triglycerides during the present study was found higher as compared to study conducted by Bozkurt *et al.* (2009) in scale carp and Bozkurt *et al.* (2006) in brown trout but lower than *C. idella* as reported by Bozkurt *et al.* (2008). The variation in the level of lipid has been reported due to stressed environmental conditions, frequency of stripping, hormonal stimulation of spermiation, sampling period and sampling methods (Billard *et al.*, 1995; Linhart *et al.*, 2003; Ciereszko, 2008) and also due to contamination of sperm by urine (Perchec *et al.*, 1995). Generally, high triglyceride concentration is a positive characteristic of fish semen. The triglyceride level was recorded higher in rainbow trout than the brown trout but the difference in triglyceride level between the two was found to be statistically non-significant.

Cholesterol

The cholesterol level was found to be 1.65 ± 0.14 mg dl⁻¹ in brown trout and 3.07 ± 0.27 mg dl⁻¹ in rainbow trout during the present study (Fig. 5). There is insufficient information about the role of cholesterol in seminal plasma in spite of its identification in the seminal plasma of freshwater fishes (Billard *et al.*, 1995). Cholesterol has been reported to have protective effect against environmental changes especially in temperature that occurs when the fish semen is released (Bozkurt *et al.*, 2008). The cholesterol level in the fish during the present study was lower than those reported for *C. idella* (12.02 mg dl⁻¹) by Bozkurt *et al.* (2008), for *S. trutta fario* (19.2 ± 18.57 mg l⁻¹) by Bozkurt *et al.* (2006), for *Jundia' Rhamdia quelen* (13.9 ± 0.9 mg dl⁻¹) by Borges *et al.* (2007) and for *O. mykiss* (2.55 mg dl⁻¹) by Secer *et al.* (2004). The difference in the values of the present

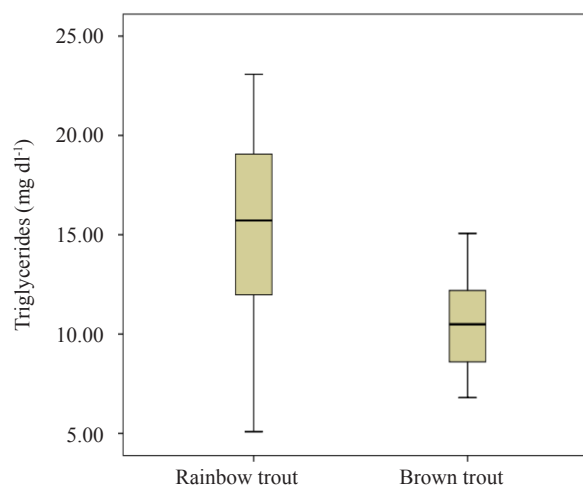


Fig. 4. Box plot depicting the triglyceride concentration of milt plasma in rainbow trout and brown trout

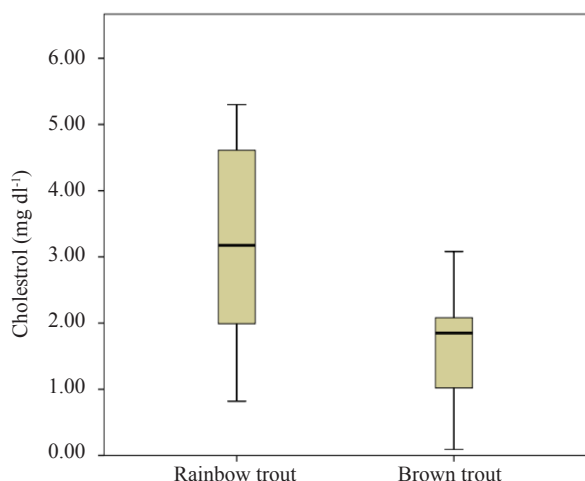


Fig. 5. Box plot depicting the cholesterol concentration of milt plasma in rainbow trout and brown trout

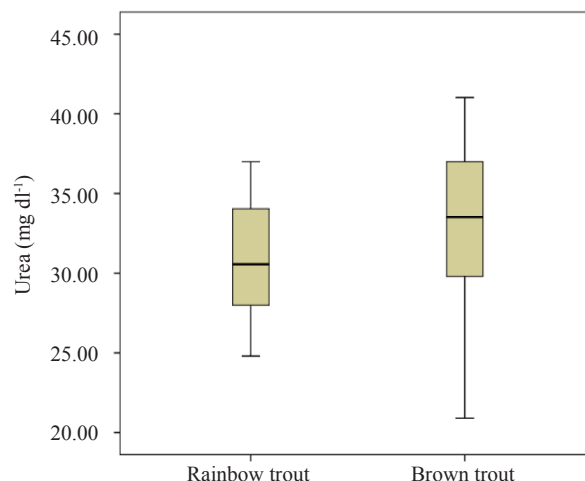


Fig. 6. Box plot depicting the urea concentration of milt plasma in rainbow trout and brown trout

study with other findings may be due to the difference in age, season, environment, and physiological conditions of fish as stated by Billard *et al.* (1995), Linhart *et al.* (2003) and Ciereszko (2008).

Urea

Urea contamination of semen may cause reduced sperm motility and fertilising ability (Dreanno *et al.*, 1998) influencing the variability of other semen parameters (Glogowski *et al.*, 2000). The mean concentrations of urea during the present study in brown trout was found as $33.30 \pm 0.89 \text{ mg dl}^{-1}$ and $30.87 \pm 0.65 \text{ mg dl}^{-1}$ in rainbow trout (Fig. 6). Secer *et al.* (2004) reported that the urea content in *O. mykiss* as $31.65 \pm 40.78 \text{ mg dl}^{-1}$ which is similar to the results obtained during the present study. Bozkurt *et al.* (2006) estimated the urea content in the milt of *S. trutta fario* to be $3.0 \pm 9.42 \text{ mg dl}^{-1}$, which is much less than the results obtained during the present study. Our results show that the mean levels of serum urea in

cultured individuals of *S. trutta fario* were a little elevated when compared to *O. mykiss*. Although similar methods were used for stripping in both the species taking every precaution to reduce contamination by urine, brown trout showed increased contaminations of urea indicating the species' sensitivity to captivity.

Relationship between body weight, body length and spermatological properties of rainbow trout and brown trout

Correlation between the body weight, body length and spermatological properties of rainbow trout are presented in Table 5. It reveals that length has significant positive correlation with weight and milt volume ($r=0.848$, $p<0.01$, $r=0.715$, $p<0.01$). Significant positive correlation was also found between fish weight and milt volume ($r=0.799$, $p<0.01$). On the other hand, sperm pH has significant negative correlation with fish weight ($r=-0.319$, $p<0.05$), cholesterol ($r=-0.570$, $p<0.01$) and total protein ($r=-0.425$,

Table 5. Pearson correlation between body weight, length and sperm physical and biochemical parameters in rainbow trout

	Length	Weight	Milt volume	pH	Glucose	Cholesterol	Total protein	Urea	Triglycerides
Length	1	.848**	.715**	-.127	-.117	-.008	-.183	.159	-.140
Weight		1	.799**	-.319*	-.153	.146	-.122	.169	-.200
Milt volume			1	-.234	.076	.052	-.246	.194	-.069
pH				1	-.117	-.570**	.425**	-.029	.167
Glucose					1	-.338*	-.591**	-.138	.018
Cholesterol						1	.138	.318*	-.286
Total protein							1	.270	-.133
Urea								1	-.076
Triglycerides									1

**Correlation is significant at 0.01 level (1-tailed).

*Correlation is significant at 0.05 level (1-tailed).

$p < 0.01$). Also, glucose concentration has a significant negative correlation with cholesterol ($r = -0.338$, $p < 0.05$) and total protein ($r = -0.591$, $p < 0.01$). Significant positive correlation was found between cholesterol concentration and urea concentration in milt ($r = 0.318$, $p < 0.05$).

Correlation between the body weight, body length and spermatological properties of brown trout semen are presented in Table 6. The table reveals a significant positive correlation of fish length with fish weight ($r = 0.745$, $p < 0.01$) and milt volume ($r = 0.670$, $p < 0.01$). Negative significant correlation was observed between fish length and triglyceride concentration of milt ($r = -0.324$, $p < 0.05$). Fish weight shows significant positive correlation with milt volume ($r = 0.662$, $p < 0.01$) and milt pH ($r = 0.406$, $p < 0.05$). Negative significant correlation was observed between milt pH and total protein

procedures which would surely help in increasing the efficiency of broodstock and hatchery management practices.

Present data from the study can be used to select high quality mature males for fertilising eggs in a commercial aquaculture operation by providing the size and weight groups which produce good quality milt. With use of better quality milt, the productivity can be increased significantly as better grade milt elevates the chances of fertilisation success. The information on sperm biochemistry obtained from the present study can lead to more efficient gamete management and increased fry yields. Further studies are needed to increase the efficiency of sperm to fertilise the egg batches. In addition to this, better quality mature males can be selected after scientific broodstock management through maturation diet, health management, good water quality and appropriate stocking density.

Table 6. Pearson correlation between body weight, length and sperm physical and biochemical parameters in brown trout

	Length	Weight	Milt volume	pH	Glucose	Cholesterol	Total protein	Urea	Triglycerides
Length	1	.745**	.670**	.241	.230	-.298	-.277	.067	-.324*
Weight		1	.662**	.406*	.123	-.220	-.279	-.013	-.150
Milt volume			1	.468**	.243	-.116	-.083	.030	-.001
pH				1	-.089	-.010	-.319*	.301	-.006
Glucose					1	-.200	.364*	.033	-.079
Cholesterol						1	-.164	.167	.207
Total protein							1	-.154	-.003
Urea								1	.167
Triglycerides									1

** . Correlation is significant at 0.01 level (1-tailed).

* . Correlation is significant at 0.05 level (1-tailed).

($r = -0.319$, $p < 0.05$). Significant positive correlation was found between milt volume and pH ($r = 0.468$, $p < 0.01$), glucose and total protein ($r = 0.364$, $p < 0.01$).

Cultured fishes are densely stocked as compared to those in the wild and they depend upon artificial feeding. In addition, they frequently suffer physical stresses during management (Coz-Rakovac *et al.*, 2005). Such ecological conditions induce significant fluctuations in the level of various biochemical composition of semen. The composition of seminal plasma has a great influence on the biological quality of the milt and these factors are directly related to the fertilisation success (Rurangwa *et al.*, 2004). The knowledge of quantitative characteristics and chemical composition of sperm is a prerequisite for the successful evaluation of the reproductive ability of different fish species. This may also lead to better understanding of fertilisation mechanisms.

The present study provides information regarding the biochemical parameters of milt and the data generated provides the basis to improve the current fertilisation

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