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Biological Characteristics of Milt and Environmental Factors Affecting Motility of Spermatozoa of Blue Gourami *Trichopodus trichopterus*

Sandra, B.S.* and Benno Pereira, F.G.

Department of Zoology, University of Kerala, Kariavattom Campus, Thiruvananthapuram -699581, Kerala, India
**Email: sandra.balachandran@gmail.com*

Abstract
The quality of sperm is one of the predominant factors determining the success of fertilization in fish breeding programs. In most of the teleost fishes, motility of spermatozoa is influenced by various environmental factors such as temperature, pH, salinity, osmolality and ionic concentration. The present study is undertaken to investigate various biological parameters of milt and major environmental factors affecting sperm motility in the freshwater fish, *Trichopodus trichopterus.* In *Trichopodus trichopterus*, the milt showed spermatozoa concentration (7.36x 10^9± 0.51), viability (91.6%), percentage of motile spermatozoa (90.2) and duration of motility (56.2±0.68 sec). Spermatozoa of *Trichopodus trichopterus* showed a higher percentage of motility and longer duration of motility at a lower temperature. The highest percentage of motility was observed at 5°C (95%) and the maximum duration of motility was observed at 15°C (72.14 sec). Spermatozoa of *Trichopodus trichopterus* were motile in a range of pH 5 to 8, with the highest percentage of motility at pH 5.5 (80%) and maximum duration of motility (137± 32sec) in pH 7.5. *Trichopodus trichopterus* sperm exhibited motility in a range of 0-20% salinity. The effect of various ions like K^+, Na^{2+} and Ca^{2+}on sperm motility was evaluated by activating with KCl, NaCl and $CaCl_2$ solutions. Maximum sperm motility was observed in concentration 120 mM K^+, 100mM Na^{2+} and 50mM Ca^{2+}, respectively. From the present study, it was observed that milt of *Trichopodus trichopterus* showed optimum sperm motility at a lower temperature, alkaline pH and dilute salt solutions. The ions such as K^+, Na^{2+} and Ca^{2+} significantly increase the motility parameters. Supplementation of motility activation solutions with K^+, Na^{2+} and Ca^{2+} improve motility which might be beneficial for experimental purpose and specific handling procedures in aquaculture.

Keywords: *Trichopodus trichopterus*, Sperm motility, Duration of motility, pH, Temperature, Salinity, Osmolarity

1. Introduction

The utilization of high-quality gametes is of great importance for enhancing the rate of fertilization and ensuring the production of viable larvae in aquaculture (Kjorsvik *et al.*, 1990; Bromage and Roberts, 1995). The evaluation of biological parameters of milt such as sperm motility and sperm density can determine the fertilization capability of spermatozoa and often are used to estimate the milt quality (Suquet *et al*., 1982; Billard *et al*., 1993; Linhart *et al*., 1994a; Krol *et al*., 2006)

Since teleost fishes have external fertilization, several endogenous factors such as paternal genetic heritage (Simmons, 2005), the spermiation period, and sperm storage conditions in the testes and exogenous factors such as environmental conditions can influence sperm motility (Billard, 1986). Knowledge of the factors influencing the motility of sperms has huge practical application in enhancing the fertilization and ensuring the production of viable larvae for aquaculture.

The Blue gourami (*Trichopodus trichopterus*) belongs to the order Anabantidae can be an ideal species for ecotoxicological study because of its wide environmental tolerances, ability to colonize anthropogenically disturbed habitats, opportunistic trophic nature and fast growth rates. It is highly tolerant of hypoxic conditions because of the presence of auxiliary respiratory organ (McKinnoon and Liley, 1987). With this aim, the present study has been conducted to investigate the biological characteristics of milt of *Trichopodus trichopterus* and analyse how the various environmental factors influence sperm motility in *Trichopodus trichopterus*.

2. Materials and Methods

The fish, *Trichopodus trichopterus* used in the study were collected from fish farms in Ernakulam. Fishes were acclimatizatised for two weeks under laboratory conditions, and mature male fishes were used for the experiments. Milt was collected from the fishes by dissecting and slicing the testis. Colour and nature of milt were recorded. The concentration of the spermatozoa was assessed using an improved Neubauer counting chamber following standard clinical methods (Buyukhatipogulu and Holtz, 1978). The milt volume was measured using a graduated capillary tube. Viability was determined using eosine- nigrosin dye exclusion method (Chao *et al.*, 1975). The percentage of motile spermatozoa and duration of motility was measured by following methods of (Goodall *et al.*,1989) with slight modification. Effect of temperature on percentage and duration of motility of spermatozoa was estimated in different temperatures from 5°C to 30°C by adjusting the temperature of A/C in an air-conditioned room. Effect of pH on percentage and duration of sperm motility was determined by preparing media of different pH, i.e. 5, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0 by adding disodium hydrogen phosphate and sodium dihydrogen phosphate to distilled water. Effect of salinity on percentage and duration of sperm motility was determined by preparing artificial seawater of different concentrations of salinity such as 10%, 20%, 30%, 50% and 100% based on the formula of Lyman and Flemming (1940), with modifications. Effect of ions such as K^+, Na^{2+} and Ca^{2+} on sperm motility were determined by diluting and activating semen with solutions containing 0, 10, 50, 100, 110, 120,

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130, 140, 150 and 200 mM KCl, 0, 10, 50, 100, 110, 120, 130, 140 and 150 mM NaCl and 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 mM $CaCl_2.2H_2O$. Six observations were recorded in each concentration, and the mean motility was recorded. Statistical analysis was done by one-way ANOVA.

3. Results

The spermatozoa of *Trichopodus trichopterus* was viscous in nature and white in colour. The mean spermatozoa concentration ($x10^9 ml^{-1}$) of *Trichopodus trichopterus* was 7.36x 10^9±0 .51. The mean milt volume (mL) recorded was 0.0135± 0.005. Viability is a reliable index of fertility which denotes the physiologically functional spermatozoa in a given sample. Viability of spermatozoa of *Trichopodus trichopterus* was 91.6± 0.56. Percentage of motile spermatozoa denotes the number of spermatozoa which can actively move in the medium and duration of motility is the time up to which spermatozoa remain motile in the external medium. Percentage of motile spermatozoa in *Trichopodus trichopterus* was 90.2± 0.63, and duration of motility (seconds) of sperm of *Trichopodus trichopterus* was 56.2± 0.68 (Table 1).

Table 1. Biological Characteristics of the milt of *Trichopodus trichopterus*

Parameter	Mean	SD
Milt volume (mL)	0.0135	0.005
Spermatozoa concentration ($x10^9 mL^{-1}$)	7.36x 10^9	0.51
Viability (%)	91.6	0.56
Percentage of Motile Spermatozoa	90.2	0.63
Duration of Motility (seconds)	56.2	0.68

3.1. Effect of temperature on sperm motility

In *Trichopodus trichopterus*, spermatozoa motility lasted longer at lower temperature and duration of motility decreased with increasing temperature. The mean duration of motility of spermatozoa was 72.14 sec at 15°C. After 15°C, duration of motility decreased reaching 55secs in 30°C. Percentage of motile spermatozoa also decreased with increasing temperature (Fig. 1, 2, Table 2).

3.2. Effect of pH on sperm motility

Spermatozoa of *Trichopodus trichopterus* were motile in pH 5 to 8, with a maximum duration of motility (137 sec) in pH 7.5. The least duration of motility (60 sec) was in pH 5. As pH increased from 5.0 to 7.5, duration of motility increased and then showed a decrease. Percentage of motile spermatozoa (80%) was maximum in 5.5, and the least percentage of motile spermatozoa (48%) was in 6.5. (Fig. 3,4, Table 3)

3.3. Effect of salinity on sperm motility

In *Trichopodus trichopterus*, the maximum duration of motility (64 secs) was found at 10% salinity and least duration of motility at 0% salinity. No motility was found at 30% - 100% salinity. (Fig. 5, 6, Table 4)

3.4. Effect of ions on sperm motility

3.4.1. Effect of K^+ on sperm motility

In *Trichopodus trichopterus*, duration of motility was highest at 120mM K^+ and lowest at 0mM K^+. Percentage of motility was highest at 120mM K^+ and lowest at 140 mM K^+. No motility was observed above 150 mM K^+ (Fig. 7,8, Table 5).

3.4.2. Effect of Na^{2+} on sperm motility

In *Trichopodus trichopterus*, duration of motility was maximum at 100mM $Na^{2+}$and lowest duration of motility was found at 0 mM Na^{2+}. Percentage of motility was maximum at 100mM Na^{2+} and lowest at 130 mM Na^{2+}. (Fig. 9, 10, Table 6)

3.4.3. Effect of Ca^{2+} on sperm motility

In *Trichopodus trichopterus*, highest duration of motility was obtained at 50mM Ca^{2+}, and the lowest duration of motility was obtained at 0mM Ca^{2+}. Percentage of motility was maximum at 10mM Ca^{2+} and Lowest percentage of motility at 100mM Ca^{2+}(Fig. 11, 12, Table 7).

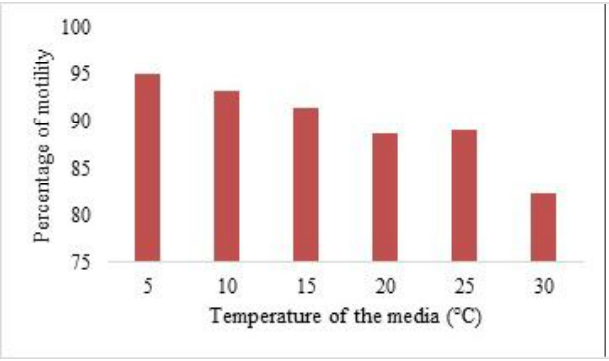

Fig. 1. Effect of temperature on the percentage of sperm motility in *T. trichopterus*

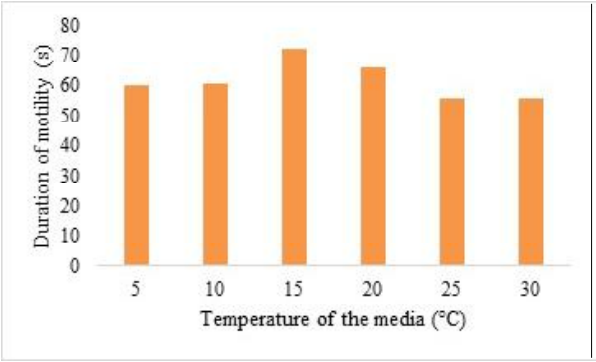

Fig. 2. Effect of temperature on the duration of sperm motility in *T. trichopterus*

Table 2. ANOVA table on the effect of Temperature on the percentage of motile spermatozoa and duration of motility in *T. trichopterus*

		Sum of Squares	df	Mean Square	F	Sig.
Time_Seconds	Between Groups	1362.857	5	272.571	1.553	0.198
	Within Groups	6317.143	36	175.476		
	Total	7680	41			
Per cent	Between Groups	693.357	5	138.671	3.492	0.011
	Within Groups	1429.429	36	39.706		
	Total	2122.786	41			

Table 3. ANOVA table on the effect of pH on the percentage of motile spermatozoa and duration of motility in *T. trichopterus*

		Sum of Squares	df	Mean Square	F	Sig.
Per cent	Between Groups	5131.905	6	855.317	8.071	0
	Within Groups	3709.167	35	105.976		
	Total	8841.071	41			
Time_Seconds	Between Groups	32018.143	6	5336.357	6.763	0
	Within Groups	27615.5	35	789.014		
	Total	59633.643	41			

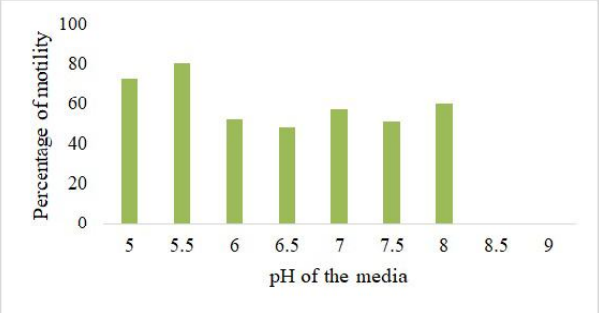

Fig. 3. Effect of pH on the percentage of motility in *T. trichopterus*

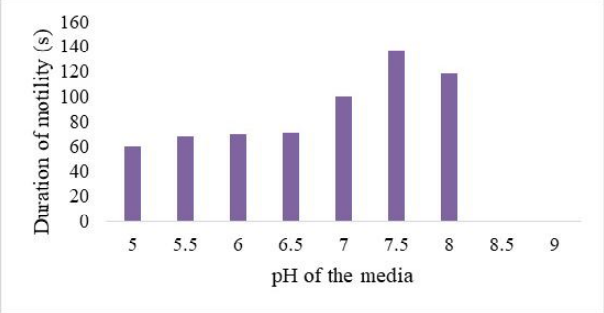

Fig. 4. Effect of pH on the duration of motility in *T. trichopterus*

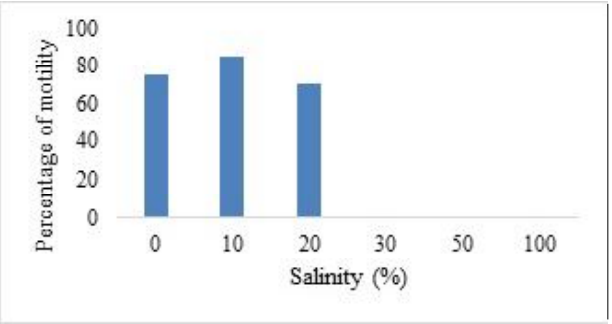

Fig. 5. Effect of salinity on the percentage of motility in *T. trichopterus*

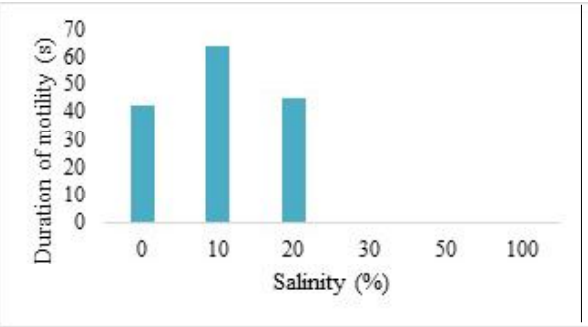

Fig. 6. Effect of salinity on the duration of motility in *T. trichopterus*

Table 4. ANOVA table on the effect of salinity on the percentage of motile spermatozoa and duration of motility in *T. trichopterus*

		Sum of Squares	df	Mean Square	F	Sig.
Time_Seconds	Between Groups	18808.467	4	4702.117	25.619	0
	Within Groups	4588.5	25	183.54		
	Total	23396.967	29			
Percent	Between Groups	40463.333	4	10115.833	40.01	0
	Within Groups	6320.833	25	252.833		
	Total	46784.167	29			

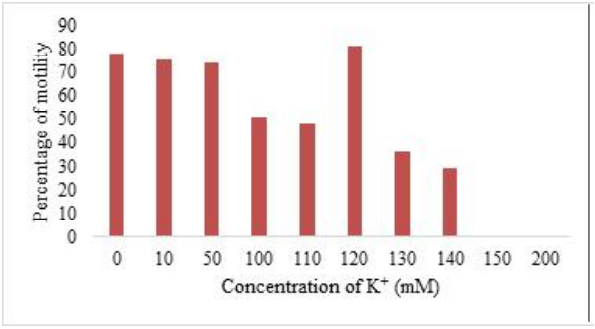

Fig. 7. Effect of K^+ on the percentage of motility in *T. trichopterus*

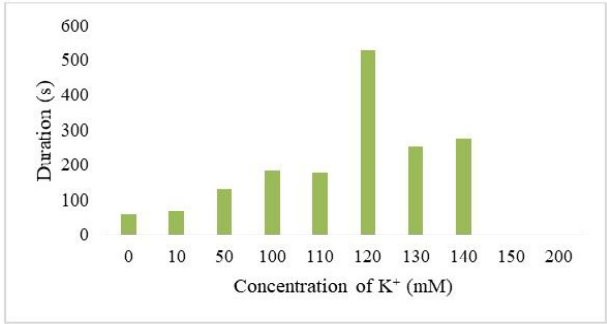

Fig. 8. Effect of K^+ on the duration of motility in *T. trichopterus*

Table 5. ANOVA table on the effect of K^+ on the percentage of motile spermatozoa and the duration of motility

		Sum of Squares	df	Mean Square	F	Sig.
Percent	Between Groups	23932.6	7	3418.943	20.711	0
	Within Groups	11885.6	72	165.078		
	Total	35818.2	79			
Time_Seconds	Between Groups	1587290.488	7	226755.784	64.163	0
	Within Groups	254452.9	72	3534.068		
	Total	1841743.388	79			

Table 6. ANOVA table on the effect of Na^{2+} on the percentage of motile spermatozoa and duration of motility in *T. trichopterus*

		Sum of Squares	df	Mean Square	F	Sig.
Percent	Between Groups	31283.143	6	5213.857	91.387	0
	Within Groups	3594.3	63	57.052		
	Total	34877.443	69			
Time_Seconds	Between Groups	2914633.486	6	485772.248	526.275	0
	Within Groups	58151.5	63	923.04		
	Total	2972784.986	69			

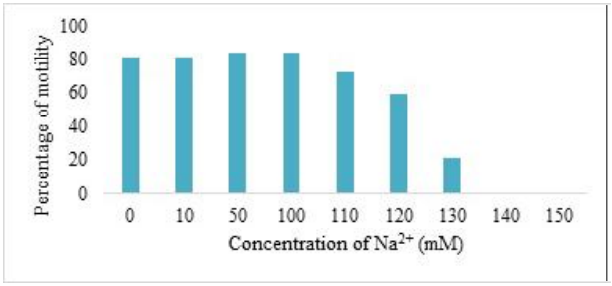

Fig. 9. Effect of Na^{2+} on percentage of motile spermatozoa in *T. trichopterus*

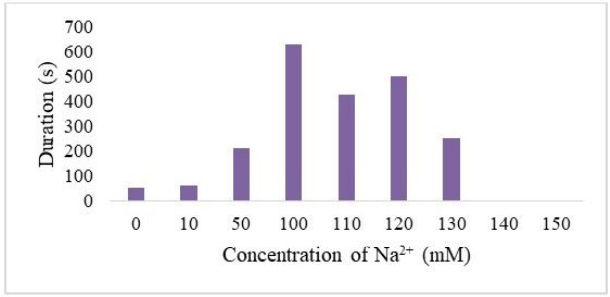

Fig. 10. Effect of Na^{2+} on duration of motile spermatozoa in *T. trichopterus*

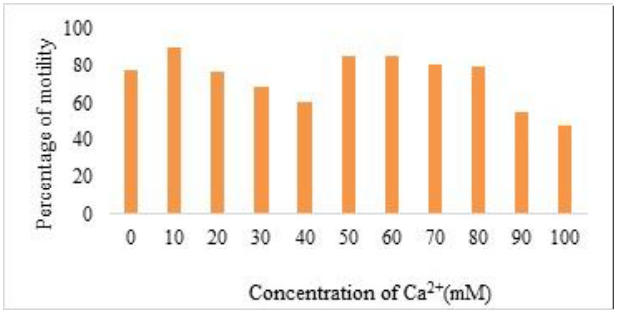

Fig. 11. Effect of Ca^{2+} on percentage of motile spermatozoa in *T. trichopterus*

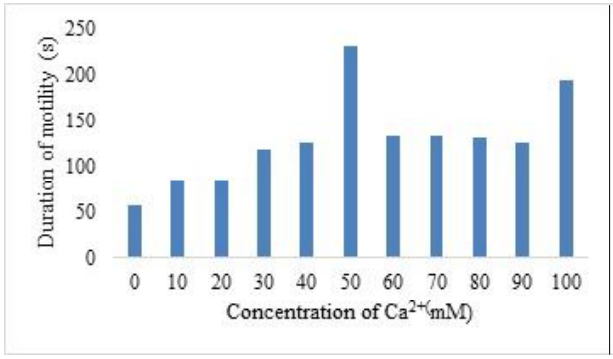

Fig. 12. Effect of Ca^{2+} on duration of motility in *T. trichopterus*

Table 7. ANOVA table on the effect of Ca2+ on the percentage of motile spermatozoa and duration of motility in *T. trichopterus*

		Sum of Squares	df	Mean Square	F	Sig.
Per cent	Between Groups	10954.091	10	1095.409	6.162	0
	Within Groups	9777.667	55	177.776		
	Total	20731.758	65			
Time_Seconds	Between Groups	142073.424	10	14207.342	12.423	0
	Within Groups	62898.833	55	1143.615		
	Total	204972.258	65			

4. Discussion

In the majority of teleosts fishes, fertilization is external, and spermatozoa produced by the gonads remain quiescent until they are released into the external environment, which is either freshwater or seawater. Therefore, sperm motility is considered as the most reliable indicator of semen quality in fishes (Terner 1986; Chao *et al*., 1987; Lahnsteiner *et al*., 1997).

In *Trichopodus trichopterus*, the fertilization is external, and the milt is released to the external medium, i.e., freshwater. In the majority of the fishes with external fertilization, spermatozoa concentration remains high due to short lifespan in the external medium. Studies by Aas *et al*. (1991) suggests that spermatozoa concentration has a direct effect on fertilization. However, in the present study spermatozoa concentration of *Trichopodus trichopterus* is low (7.36* 10^9±0 .51.) compared to other fishes. This is because, in an artificial environment, only a low number of spermatozoa is required (10^3 or 75,000 spermatozoa per egg) for successful fertilization (Billard, 1992; Erdahl and Graham, 1987). The volume of milt in fishes also varies with species and size of the fishes (Pereira, 2000). *T. trichopterus* reach sexual maturity at 7cm and 12 to 14 weeks of age (McKinnoon and Liley, 1987). In *Trichopodus trichopterus*, the mean milt volume is 0.014±005 mL**.**

In the present study, the mean viability of spermatozoa was 91.6%. Studies in freshwater fishes such as *Anabas testudineus* (93.12 %), *Ambassis commersoni* (94.85%), *Clarias batrachus (95.10%), Puntius filamentosus* (93.56%) and *Heteropneustes fossilis (94.26%)* (Paul, 1998) *Osphronemus goramy* (94.04%), *Puntius sarana* 95.36%, *Labeo fimbriatus* (96.92%) and *Etroplus suratensis* (94%) (Bindu., 1999) show comparable results. In the present study, the mean percentage of motile spermatozoa in *Trichopodus trichopterus* was very high (90.2±0.63). A high percentage of motile spermatozoa represents the success rate of fertilization. The duration of motility (seconds) of sperm of *Trichopodus trichopterus* was low (56.2±2.15). Similar observations were obtained by several authors (Terner, 1986; Erdahl, 1986; Saad *et al*., 1988; Billard and Cosson, 1992; Lahnsteiner *et al.*, 1997). In freshwater fishes having external fertilization, motility of spermatozoa is very short and less than 1 minute (Billard and Cosson, 1992; Lahnsteiner *et al.*, 1997).

4.1 Temperature

According to Ginsburg (1968), Stoss (1983) and Billard *et al*., 1995, the temperature of activation medium is the major factor controlling the duration of sperm motility, fertilizing capacity and sperm velocity. Several studies described that in the majority of freshwater fishes, percentage and duration of sperm motility decreased with increase in temperature of the swimming medium and maximum duration of motility is obtained in low temperatures ranging from 4 - 18°C and decrease gradually with the rise in temperature (Bindu., 1999; Mansour *et al*.,2002; Sheeja., 1994; Bombardelli *et al*.,2013; Ani Joseph and Jayaprakas., 2013). However, the optimum temperature of the medium for maximum spermatozoan motility vary between species.

In the present study, a longer duration of motility and a higher percentage of motility was found in lower temperature. The variation in the duration of motility coincides with observations of Ginzburg (1968). The duration of motility increased progressively with increase in temperature, till 15°C. Further increase in temperature resulted in a constant reduction of sperm motility duration. This is because the temperature of the medium changes the chemical composition of the seminal plasma and affects the enzymatic system of the spermatozoa, which in turn affects the physiological and morphological function of spermatozoa (Bindu., 1999). This can be attributed to oxidative damages induced by the reactive oxygen species (ROS) produced in the sperms in high temperatures (Nichi *et al*., 2006; Nandre *et al*., 2013). Oxidative stress in the sperms can also be formed by the lipid peroxidation of the membrane lipid constituents of the sperm, thereby affecting the motility of the sperms (Krasznai *et al*., 2003). These lipids may also serve as energy sources for the motile sperms. Hence, lipid peroxidation may cut off the energy supply to the sperms, thereby reducing the duration of their motility (Baeza *et al*., 2015).

4.2 pH

The pH of the activating medium has a profound effect on the duration of spermatozoan motility. Several studies revealed that in freshwater fishes, hypo-osmotic shock leads to Na^+/H^+ exchange through ion channels and intracellular alkalinization (Krasznai *et al.*, 1995). Extracellular and intracellular pH has an influential role in the initiation and duration of sperm motility (Marian *et al.*, 1997). The intracellular proton concentration is influenced by external pH, which in turn modifies the membrane potential, and motility behaviour (Boitano *et al.*, 1991, Boitano *et al.*, 1992).

In the present study, the percentage of motile spermatozoa was higher at acidic pH. This contradicts the observation by Billard *et al*., 1974 and Cosson and Linhart 1996, which states that the pH value above 8 increases the percentage of motile spermatozoa. In *Trichopodus trichopterus*, duration of motility was higher at alkaline pH. However, this is in accordance with the study conducted by Cosson *et al.* 1991 and Chao *et al.* (1992). In *Cyprinus carpio*, the optimum sperm motility has been reported at pH 7.0 and 8.0 (Cosson *et al.* 1991). Chao *et al.* (1992) found that spermatozoa are active when pH values were at 7.5 - 8.

4.3 Salinity

Salinity is one of the major factors ensuring male reproductive success in fishes (Atse *et al.*, 2002). Several investigators observed that in freshwater fishes, the motility of spermatozoa is longer in dilute salt solutions compared to freshwater (Buyukhatipoglu and Holtz, 1978; Goodall *et al*., 1989; Thorogood and Blackshaw, 1992). In the present study, the highest percentage of motile spermatozoa and maximum duration of motility was found in 10% seawater. Similar observations are reported in indigenous ornamental fishes, *Rasbora daniconius* and *Puntius filamentosus*, where the longest duration of sperm motility was observed at 1% salinity in *R. daniconius* and 10% salinity in *P. filamentosus (*Ani joseph and Jayaprakas, 2013).

4.4 Potassium ions (K^+)

Several studies observed that K^+ ions play an important role in keeping the sperm in an inactivated or quiescent state within the milt (Baynes *et al*., 1981). In salmonids and sturgeons, seminal plasma K^+ concentration is the major inhibitor of sperm motility (Morisawa *et al*., 1983; Billard *et al*., 1987: Cosson and Linhart, 1996). However, in freshwater fishes, the K^+ ions do not have an inhibitory effect. K^+ is a major component of seminal plasma, and K^+ ions increase sperm viability, sperm velocity and motility (Morisawa *et al*.,1983; Billard and Cosson, 1992). The K^+ concentrations in diluents used for cryopreservation strongly affect the motility of carp sperm (Linhart and Cosson, 1997).

In the present study, sperm motility increased with increasing concentration of K^+ and lower values of duration of motility were observed at low levels of K^+ ion concentration. The duration of sperm motility showed a sudden and drastic increase when the K^+ concentration increased to 120 mMol L^{-} It was also observed that spermatozoan motility was completely inhibited at K^+ concentration of 150 mMol L^{-1}. The isotonicity of the activation medium with seminal plasma might be the reason for the inhibition of motility. This is because the isotonic solution prevents the hyperpolarization of the sperm cell membrane and prevents activation of sperms (Christen *et al*., 1987).

4.5 Sodium ions (Na^{2+})

In freshwater fishes, sperm motility is initiated by the alkalization of the intracellular medium by Na^+/H^+ exchanger (Krasznai *et al*., 1995; Perchec *et al*., 1995a, b; Alavi and Cosson, 2005a; Tron et al., 1990; Marian et al., 1993). However, studies conducted by Krasznai *et al*., 1995 in carp spermatozoa show that sodium channel inhibitors do not affect the motility of carp spermatozoa (Krasznai *et al*., 1995) and higher NaCl concentrations are less favorable and did not increase the percentage of motile spermatozoa.

In the present study, the percentage of motile spermatozoa did not show a significant difference and is decreased in higher Na^{2+} concentrations. The present study confirmed that the duration of motility in *Trichopodus trichopterus* increased (values below the seminal plasma) with the increase in the concentration of Na^{2+} ions up to 100mM Na^{2+} and decreased after that. Motility is inhibited at 140 mM Na^{2+}.

4.6 Calcium ions (Ca^{2+})

In freshwater fishes, extracellular Ca^{2+} plays a prominent role in sperm motility initiation. The influx of extracellular Ca^{2+} through specific channels causes the release of Ca^{2+} from intracellular stores and the initiation of sperm motility. (Krasznai *et al*., 2000). In the present study percentage of motile spermatozoa did not change significantly with the increase in Ca^{2+} ions. Still, the duration of motility increased with increasing concentration of Ca^{2+} up to 50 mM Ca^{2+} compared to 100 mM Ca^{2+}. Several studies showed the role of millimolar concentrations of calcium cause an increase in sperm motility parameters, including the total period of activity, percentage of motile sperm and sperm velocity (Alavi and Cosson., 2006; Cosson., 2004; Baynes *et al*., 1981). However, studies conducted by Alavi *et al*. (2007) showed that Ca^{2+} concentration of 2.5 mM increased both the percentage of motile sperm and the sperm velocity in perch, but lower percentage of motile sperm and sperm velocity was observed at Ca^{2+} concentration of 5.0 mM compared to 2.5 mM.

5. Conclusion

The biological parameters of milt of *Trichopodus trichopterus* showed high values of viability, percentage of motile spermatozoa and duration of motility. This proves that *Trichopodus trichopterus* can be developed as a model for ecotoxicological studies. In *Trichopodus trichopterus*, optimum sperm motility is observed in lower temperature. Motility is optimum at alkaline pH and in dilute salt solutions compared to freshwater. The ions such as K^+, Na^{2+} and Ca^{2+} significantly increase the motility parameters and supplementation of motility activation solutions with K^+, Na^{2+} and Ca^{2+} can improve motility and enhance fertilization which might be beneficial for aquaculture.

Acknowledgements

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