Aquaculture Research, 2005, **36,** 422–430 doi: 10.1111/j.1365-2109.2004.01194.x

Some characteristics and short-term preservation of spermatozoa of Deccan mahseer, *Tor khudree* (Sykes)

Nagappa Basavaraja[1] & Shubaschandra Ninjoor Hegde[2]

[1]Department of Aquaculture, University of Agricultural Sciences, College of Fisheries, Mangalore, India

[2]VIDVATH, Mysore, India

Correspondence: Dr N Basavaraja, Department of Aquaculture, University of Agricultural Sciences, College of Fisheries, Mangalore-575 002, India. E-mail: basavarajan2002@hotmail.com

Abstract

Over the years, the natural stocks of *Tor khudree* (Cyprinidae) have depleted due to anthropogenic activities and hence it is considered a threatened species in India. Several *in situ* and *ex situ* conservation strategies have been suggested for the revival of *T. khudree* stocks. The total volume of milt obtained from hormone-injected (gonadotropin-releasing hormone analogue+domperidone) *T. khudree* were significantly higher (six to seven times) than that of uninjected fish. The total number of spermatozoa per fish was also higher in injected fish (6.5×10^8–7.6×10^8) than that obtained from uninjected counterparts (1.3×10^8–1.8×10^8). On the contrary, the spermatozoa density and spermatocrit were found to be lower in injected fish than that of the controls. Spermatozoa density and spermatocrit ranged between 4.1×10^8–4.4×10^8 spermatozoa mL^{-1} and 38.1–39.4%, respectively, in injected fish, whereas the figures fluctuated between 6.0×10^8–7.8×10^8 spermatozoa mL^{-1} and 61.5–63.1%, respectively, in uninjected fish. However, there was no significant difference in the spermatozoa motility rates between experimental and control fish. Different spermatozoa-activating media revealed no significant difference in spermatozoa motility between hormone-injected and uninjected mahseers. But motility duration was the longest with NaCl+urea (190–193 s) and the shortest with tap water (50–55 s) in the experimental and control groups. Short-term preservation of the spermatozoa of *T. khudree* indicated that spermatozoa stored at 4 °C had higher motility rates than those preserved at room temperature either in the presence or absence of oxygen.

Keywords: characteristics, preservation, spermatozoa, mahseer, *Tor khudree*

Introduction

Of the seven species of the genus *Tor* found in India, the Deccan or Malabar mahseer, *Tor khudree* Sykes is regarded as the most important mahseer in peninsular India. This riverine species was successfully induce-spawned through hypophysation (Kulkarni 1971). Successful commercial production of fry of *T. khudree* has, however, been hampered by several problems such as low fecundity, short spawning season, manual stripping of ova and spermatozoa, artificial fertilization, long egg incubation period and high mortality of eggs and fry (Nandeesha, Bhadraswamy, Patil, Varghese, Kamal Sarma & Keshavanath 1993; Keshavanath 2001).

The use of good-quality gametes is crucial for obtaining viable fry, but often gamete quality is difficult to assess. Different parameters are recommended for determining the quality of fish spermatozoa. Fish spermatozoa are usually immotile in the testes and the seminal fluid. In spermatozoa preservation studies, motility percentage (% motile spermatozoa) is the most commonly used parameter to evaluate spermatozoa quality (Billard, Cosson & Christen 1987; Miura, Yamauchi, Takahashi & Nagahama 1992). The duration of motility, including agitation of the flagellum without displacement (Billard & Cosson 1986) or duration of forward motion (Linhart, Peter, Rothbard, Zohar & Kvasnicka 1995), the distance traversed by the sperm or swimming intensity (Baynes, Scott & Dawson 1981) are also used as criteria for judging quality.

Spermatozoa count in milt is important, as it may influence fertilization rate. Spermatozoa density and spermatocrit are two estimates of concentration of spermatozoa. A significant positive relationship between spermatozoa density and spermatocrit was found in yellow perch, *Perca flavescens* Mitchell

(Ciereszko, Ramseyer & Dabrowski 1993), coho salmon, *Oncorhynchus kisutch* Walbaum (Bouck & Jacobson 1976), Atlantic salmon, *Salmo salar* M. (Piironen 1985), rabbit fish, *Siganus guttatus* Bloch (Garcia 1991) and in Atlantic cod, *Gadus morhua* L. (Rakitin, Ferguson & Trippel 1999). In rainbow trout (*Oncorhynchus mykiss* Walbaum), Munkittrick and Moccia (1987) observed a decline in spermatocrit and spermastozoa motility as the spawning season progressed. High spermatozoa motility, spermatozoa density and spermatocrit were observed during July, i.e. middle of the spawning season, in the fringe-lipped carp, *Labeo fimbriatus* Bloch (Akash 2001). He reported a high correlation between spermatozoa density and spermatocrit ($r = 0.84$).

Storage temperature is a major factor that affects the viability of fish gametes *in vitro* studies. Viability can be prolonged by maintaining gametes and embryos close to 0 °C to reduce metabolic rate. However, the ability to tolerate low temperature may vary between temperate and tropical species (Leung & Jamieson 1991). In salmonids, sperms stored *in vitro* survived for one to several days at 1–4 °C (Carpentier & Billard 1978). Buyukhatipoglu and Holtz (1978) reported that the spermatozoa of rainbow trout were fertile for 21 days at 4 °C under oxygen or air. When salmonid spermatozoa were stored under air, the fertilizing capacity (80–100%) was retained up to 8 days at 0–5 °C, 2–3 days at 5–10 °C and was reduced to less than a day at more than 12 °C (Scott & Baynes 1980). In common carp (*Cyprinus carpio* Linn.), spermatozoa stored *in vitro* at 2–5 °C, high motility was retained up to 2 days (Hulata & Rothbard 1979). However, the motility percentage and beat frequency of the flagellum continued to decline with an increase in storage period and the fertilizing capacity was completely lost after 6 days of storage. Harvey and Kelley (1984) found that post-activation motility of undiluted milt of *Sarotherodon mossambicus* (Peters) stored at 5 °C declined to zero in 60–120 h. The milt of *Mystus gulio* Hamilton could be stored up to 7 days at 4 °C without a significant reduction in motility (Sunitha & Jayaprakas 1997). In *L. fimbriatus*, the spermatozoa stored at 4 °C and room temperature retained motility up to 84 and 24 h respectively (Akash 2001).

Air, especially pure oxygen, is necessary for maintaining cellular activity. Scheuring (1925) stored trout milt under oxygen and air at 0 °C and found a motility duration up to 4 days and 24 h respectively. Truscott, Idler, Hoyle and Freeman (1968) reported that milt samples of *S. salar* stored at 2–3 °C in the presence of air retained full fertility for 5 days, but in the absence of air, fertility was reduced to 1 day. Rainbow trout sperm survival was improved after storage in an oxygen atmosphere in comparison with storage in air (Billard 1981). Prolonged viability of spermatozoa stored in plastic bags inflated with oxygen was also reported in *Silurus glanis* Linnaeus (Babiak, Glogowski, Kozlowski & Chyboski 1996), *Ictalurus punctatus* Rafinesque (Christensen & Tiersch 1996) and Indian major carps, catla (*Catla catla* Ham.), rohu (*Labeo rohita* Ham.) and mrigal (*Cirrhinus mrigal* Ham.) (Jayaprakas & Bimal Lal 1996). However, no significant effect could be seen in the motility of *L. fimbriatus* spermatozoa stored in polythene bags under oxygen (Akash 2001).

Seasonal effects on spermatozoa characteristics and the importance of spermatocrit, which is used to determine the stage of spermiation of individual males, have not been investigated in *T. khudree* (the Deccan mahseer). Also, no published reports are available on the short-term preservation of spermatozoa of *T. khudree*. The present study was undertaken with a view to test the effect of spawning agents on spermatozoa production, the utility of spermatocrit as an indicator of spermatozoa density and to evaluate seasonal changes in spermatozoa density and spermatocrit and the possibility of short-term preservation of *T. khudree* spermatozoa.

Materials and methods

The present study was conducted at the fish farm of the College of Fisheries, Mangalore. *Tor khudree* fingerlings procured from the Harangi fish farm were used for raising broodstock at the fish farm of the college. The broodstock was maintained in manured ponds and was fed on a pelleted diet (ingredients: ground nut oil cake 24%, rice bran 20%, rice flour 20%, fish meal 25%, tapioca flour 10% and minerals and vitamins 1%) at 1–2% of their body weight once daily.

Collection of spermatozoa

Spermatozoa were collected from healthy and mature *T. khudree* (200–500 g body weight) males after sedating them in 20 ppm quinaldine (Sigma, St Louis, MO, USA) for 1–2 min. The fish was released to fresh water soon after recovery. Before collection of spermatozoa, the region around the genital opening was cleaned with a filter paper/small cotton gauze to remove water, mucus, urine and feacal material. While applying gentle abdominal pressure, the sper-

matozoa were stripped either into chilled, sterile cryovials (1.8 mL) or first drawn into chilled disposable syringe (2.0 mL) and then transferred to chilled vials. The collected milt was held on ice and immediately brought to the laboratory for observation.

Effect of hormone injection on spermatozoa characteristics

In order to compare the volume of spermatozoa (milt) produced and motility parameters between hormone-injected and uninjected mahseer, uniform-sized (400 g) males oozing milt upon pressure on abdomen were selected and released in separate nylon hapas (2 × 1 × 1 m), tied in a cement cistern containing running water. After their acclimation in hapas for 2–3 h, two males were injected intramuscularly with a synthetic ready-to-use salmon gonadotropin-releasing hormone analogue (Gn-RH-a)+domperidone (DOM) (trade name: ovaprim) at 0.1 mL per fish, while two males that received no hormone injection served as the control. After 12 h, the milt was separately collected, following the procedure described above. The spermatozoa collected from both groups were analysed for the total volume produced, motility percentage and duration, density and spermatocrit. Each treatment was replicated three times.

Estimation of motility of fresh spermatozoa

The quality of fresh spermatozoa, pooled from four to six males, was determined by placing a small drop of milt (5–10 μL), with a micropipette, on a clean glass slide, mixing with 50 μL unchlorinated tap water and observing under a pre-focused inverted microscope (× 400, Olympus CK2; Olympus, Tokyo, Japan) or on a TV monitor using a colour CCTV tube (Panasonic, Yokohama, Japan), attached to an inverted microscope (Olympus CK2). Spermatozoa showing no motility before activation, but with 90% or more motility after activation, were selected for preservation studies. The percentage motility (motile spermatozoa) was determined arbitrarily on a 0 to 10 point scale, 0 denoting 0% motility and 10 denoting 100% motility. The duration of motility was estimated by recording the time taken from activation to the complete cessation of activity of the last spermatozoan in that field.

Spermatozoa density

Spermatozoa were counted using a Neubauer haemocytometer following the method of Quinito, Gabasa, Sunaf, Reyes and Delapena (1984). Spermatozoa density was determined immediately after their collection. For the haemocytometer counts, the milt was diluted 40–80 times in an extender solution. The spermatozoa were then counted by taking aliquots and expressed as the total number of spermatozoa per mL milt, using the formula adopted by Quinito and colleagues (1984).

Spermatozoa density = total number of spermatozoa in four counting blocks/number of counting blocks × 10 000.

Spermatocrit

Plastic straws (75 mm length and 2 mm internal diameter) were filled (approximately 70%) with spermatozoa pooled from four to six males, sealed with a polyvinyl chloride (PVC) sealing gel and were centrifuged for 10 min at 5350 *g*. Spermatocrit was reported as the proportion of solid packed spermatozoa and expressed as a percentage of the total volume.

Influence of spermatozoa-activating solution on motility

To evaluate the effect of spermatozoa-activating solution on motility of fresh spermatozoa, tap water (unchlorinated, drawn from a well), NaCl (0.3%), glucose (1%) and NaCl+urea (0.3+0.4%) were selected as sperm-activating solutions. The spermatozoa were activated on a pre-focused slide by placing a small drop (5 μL) of freshly collected milt and mixing it quickly with an activating solution (200 μL). Motility rates were recorded in triplicate.

Monthly variation in spermatozoa motility, density and spermatocrit

To determine monthly variation in spermatozoa characteristics, spermatozoa were collected from three males every month from May to December and analysed for spermatozoa motility percentage and duration, their density and spermatocrit, as described above.

Short-term preservation of spermatozoa

To study the effect of temperature and oxygen on the characteristics of stored spermatozoa, triplicate samples of freshly collected spermatozoa pooled from four to six males were taken in polythene bags (8 in. ×

5 in., 800-mL capacity), inflated with pure oxygen (from an oxygen cylinder) and tightly secured with a rubber band. Triplicate samples of spermatozoa without exposure to oxygen served as a control. Experimental and control samples were stored either at room temperature (RT) or 4 °C in a refrigerator. In addition, smaller experimental and control spermatozoa samples were also taken in sterile polyethylene vials (1.8 mL) and stored at room temperature as well as at 4 °C. At 2 h intervals, motility percentage was recorded and this was continued until cessation of motility. At each trial, the bags and vials of experimental samples were freshly topped with pure oxygen. The number of males used in each treatment/control was low because of difficulty in procuring mature individuals.

Data analysis

Spermatozoa motility expressed as a percentage were first angular transformed and then analysed using ANOVA. Statistical analyses were carried out using a computerized statistical package (Minitab, State College, PA, USA).

Results

Effect of hormone injection on spermatozoa characteristics

Certain characteristics of spermatozoa derived from hormone-induced and control *T. khudree* are presented in Table 1. It is seen that the volume of milt obtained from the experimental fish (1.5–1.9 mL) was higher than that of controls (0.2–0.3 mL) in both trials. However, there was no significant difference ($P > 0.05$) in respect of spermatozoa motility or duration between the experimental and control groups. Spermatozoa density values between the injected (4.4×10^8) and uninjected (6.0×10^8) fish were not significantly different ($P > 0.05$) in Trial I. However, in trial II, the spermatozoa density was significantly lower ($P < 0.05$) in the injected fish. The total spermatozoa count (6.5×10^8–7.6×10^8) obtained from hormone-injected fish was much higher than that of uninjected fish (1.3×10^8–1.8×10^8). In contrast, the spermatocrit values (38.7–39.4%) were lower ($P < 0.05$) in treated fish than that of the untreated fish (61.5–63.1%), in both trials. There was no correlation between spermatozoa density and spermatocrit of injected ($r = 0.42$, $P > 0.05$) and uninjected ($r = 0.18$, $P > 0.05$) fish.

Effect of activating media on motility of fresh spermatozoa

Data on the effect of activating media on motility of fresh spermatozoa are given in Table 2. No significant difference ($P > 0.05$) was observed in spermatozoa motility between injected and uninjected mahseer. Whereas motility percentage values obtained with four activating media were not significantly different ($P > 0.05$), the motility duration was the longest (190.0–193.3 s) with a combination of NaCl (0.3%)+urea (0.4%) and the shortest (50–55 s) with tap water in either groups. Motility duration did not vary much between NaCl (0.3%) and glucose (1%).

Variation in spermatozoa characteristics

Data on monthly variation in spermatozoa motility percentage and duration, spermatozoa density and spermatocrit are presented in Table 3. Higher ($P < 0.05$) spermatozoa motility (95%) observed in August was maintained through November, followed

Table 1 Spermatozoa production, motility, sperm density and spermatocrit in hormone injected* and uninjected mahseer, *Tor khudree* (values are mean ± SE)

Trial	Fish	Milt volume ($n = 2$)	Motility: Percentage ($n = 3$)	Motility: Duration (s) ($n = 3$)	Spermatozoa density (no. mL^{-1}) ($n = 3$)	Total no. of spermatozoa per fish ($n = 2$)	Spermatocrit (%) ($n = 3$)
I	Injected	1.5 ± 0.5	96.67^{a} ± 1.36	55.0^{a} ± 2.36	$4.40 \times 10^{8a} \pm 0.16 \times 10^{8}$	$6.48 \times 10^{8} \pm 2.32 \times 10^{8}$	39.37^{a} ± 2.29
	Uninjected	0.23 ± 0.03	95.0^{a} ± 0.0	61.67^{a} ± 4.41	$5.97 \times 10^{8a} \pm 0.46 \times 10^{8}$	$1.31 \times 10^{8} \pm 0.18 \times 10^{8}$	61.52^{b} ± 0.87
II	Injected	1.88 ± 0.63	96.67^{a} ± 1.36	56.67^{a} ± 3.33	$4.07 \times 10^{8a} \pm 0.09 \times 10^{8}$	$7.56 \times 10^{8} \pm 2.62 \times 10^{8}$	38.67^{a} ± 1.26
	Uninjected	0.28 ± 0.03	95.0^{a} ± 0.0	55.0^{a} ± 0.0	$7.74 \times 10^{8b} \pm 0.27 \times 10^{8}$	$1.83 \times 10^{8} \pm 0.02 \times 10^{8}$	63.07^{b} ± 2.27

*0.1 mL sGnRH-a+domperidone per fish (i.m.), activating solution: tap water, means with different letters within columns are significantly ($P < 0.05$) different. Sample size (n): 3.

Table 2 Effect of activating media on motility of fresh spermatozoa of *Tor khudree* (mean ± SE)

	Injected fish		Uninjected fish	
Activating media	**Motility (%)**	**Duration (s)**	**Motility (%)**	**Duration (s)**
Tap water	$96.67^{a1} \pm 1.67$	$50.0^{a2} \pm 5.77$	$96.67^{a1} \pm 1.67$	$55.0^{a2} \pm 2.87$
NaCl (0.3%)	$96.67^{a1} \pm 1.67$	$93.33^{b2} \pm 3.33$	$96.67^{a1} \pm 1.67$	$86.67^{b2} \pm 3.33$
Glucose (1%)	$95.0^{a1} \pm 0.0$	$93.33^{b2} \pm 3.33$	$95.0^{a1} \pm 0.0$	$90.0^{b2} \pm 0.0$
NaCl+urea (0.3+0.4%)	$96.67^{a1} \pm 1.67$	$190.0^{c2} \pm 5.77$	$96.67^{a1} \pm 1.67$	$193.33^{c2} \pm 3.33$

Means with different letters within columns and numbers within rows under motility percentage and motility duration are significantly ($P < 0.05$) different. Sample size (n): 3.

Table 3 Monthly variation in spermatozoa motility, their density and spermatocrit (mean ± SE)

	Motility			
Month	**Percentage**	**Duration (s)**	**Spermatozoa density (no. mL^{-1})**	**Spermatocrit (%)**
May	$90.0^{a} \pm 0.0$	$48.33^{a} \pm 1.67$	$8.10 \times 10^{7a} \pm 4.16 \times 10^{6}$	$53.13^{a} \pm 1.55$
June	$91.67^{ab} \pm 1.67$	$50.0^{a} \pm 2.89$	$8.21 \times 10^{7a} \pm 4.74 \times 10^{6}$	$54.72^{ab} \pm 3.64$
July	$93.33^{ab} \pm 1.67$	$53.33^{ab} \pm 1.67$	$2.60 \times 10^{8b} \pm 2.23 \times 10^{7}$	$60.04^{bc} \pm 1.45$
August	$95.0^{b} \pm 0.0$	$60.0^{b} \pm 0.0$	$3.94 \times 10^{8c} \pm 3.17 \times 10^{7}$	$63.96^{c} \pm 2.03$
September	$95.0^{b} \pm 0.0$	$61.67^{b} \pm 4.41$	$7.09 \times 10^{8d} \pm 1.85 \times 10^{7}$	$67.01^{cd} \pm 0.63$
October	$95.0^{b} \pm 0.0$	$63.33^{b} \pm 3.33$	$6.82 \times 10^{8d} \pm 2.92 \times 10^{7}$	$66.74^{c} \pm 1.22$
November	$95.0^{b} \pm 0.0$	$65.0^{b} \pm 2.89$	$6.37 \times 10^{8d} \pm 1.16 \times 10^{7}$	$71.09^{d} \pm 0.94$
December	$93.33^{ab} \pm 1.67$	$60.0^{b} \pm 0.0$	$6.25 \times 10^{8d} \pm 1.75 \times 10^{7}$	$68.59^{d} \pm 0.76$

Activating solution: tap water. Means with different letters within columns are significantly ($P < 0.05$) different. Sample size (n): 3.

by a decrease thereafter. In contrast, the motility duration steadily increased from May to November and decreased thereafter. The lowest motility (90%) and duration (48.3 s) were recorded in the month of May. Spermatozoa density was higher in September, October, November and December than that of other months. Maximum and minimum spermatozoa densities of 7.1×10^{8} and 8.1×10^{7} spermatozoa mL^{-1} were found in the months of September and May respectively. Like spermatozoa motility, spermatocrit values exhibited an increasing trend (barring October) from May to December. The highest and lowest spermatocrit values of 71.1% and 53.1% were found in the months of November and May respectively. There was a high correlation between spermatozoa density and spermatocrit ($r = 0.80$, $P < 0.05$).

Short-term preservation of spermatozoa

Results of the effects of oxygen, storage period and storage temperature on motility of spermatozoa are given in Table 4. At both RT and 4 °C, spermatozoa motility percentage decreased gradually. At RT, spermatozoa stored in polythene bags in the presence of oxygen did not show much change up to 12 h. After this period, motility decreased significantly ($P < 0.05$) with increasing storage period, such that cessation of motility ensued at 60 h of storage. In contrast, those spermatozoa stored in the absence of oxygen showed a more rapid decline ($P < 0.05$) in motility with increasing storage period. Complete cessation of motility took place at 36 h of storage. Secondly, spermatozoa stored under oxygen showed significantly better motility than those stored without oxygen, at 12 and 24 h of storage, notwithstanding that the initial motilities were similar. Motility of spermatozoa stored at 4 °C with oxygen was seen until 120 h, although they underwent a significant ($P < 0.05$) decrease up to 36 h and remained so until 60 h. The motility rate further decreased as storage period increased. Cold storage (at 4 °C) of spermatozoa without oxygen showed a trend in motility that was slightly inferior to that with oxygen.

Data on the effects of oxygen, storage period and storage temperature on motility of spermatozoa stored in polyethylene vials are presented in Table 5. At RT, spermatozoa stored in the presence of oxygen retained motility up to 24 h, while those preserved in the absence of oxygen showed motility only up to

Table 4 Effect of oxygen, storage period and storage temperature on motility (%) of spermatozoa stored in polythene bags (mean ± SE)

	At room temperature		At 4 °C	
Storage period (h)	**With O_2**	**Without O_2**	**With O_2**	**Without O_2**
0	$96.67^{a1} \pm 1.67$	$96.67^{a1} \pm 1.67$	$96.67^{a1} \pm 1.67$	$96.67^{a1} \pm 1.67$
12	$95.0^{a1} \pm 0.0$	$85.0^{b2} \pm 0.0$	$90.0^{b1} \pm 0.0$	$90.0^{b1} \pm 0.0$
24	$73.33^{b1} \pm 1.67$	$18.33^{c2} \pm 1.67$	$76.67^{c1} \pm 1.67$	$71.67^{c2} \pm 1.67$
36	$45.0^{c} \pm 2.89$	0.0	$60.0^{d1} \pm 0.0$	$48.33^{d2} \pm 1.67$
48	$6.67^{d} \pm 1.67$	0.0	$51.67^{de} \pm 1.67$	$40.0^{de2} \pm 0.0$
60	0.0	0.0	$43.33^{e1} \pm 1.67$	$31.67^{ef2} \pm 1.67$
72	0.0	0.0	$31.67^{f1} \pm 1.67$	$21.67^{fg2} \pm 1.67$
84	0.0	0.0	$30.0^{f1} \pm 0.0$	$16.67^{gh2} \pm .67$
96	0.0	0.0	$21.67^{fg1} \pm 1.67$	$8.33^{h2} \pm 1.67$
108	0.0	0.0	$11.67^{h} \pm 1.67$	0.0
120	0.0	0.0	$5.0^{h} \pm 0.0$	0.0
132	0.0	0.0	0.0	0.0

Activating solution: tap water. Means with different letters within columns and numbers within rows are significantly ($P < 0.05$) different. Sample size (n): 3.

Table 5 Effect of oxygen, storage period and storage temperature on motility (%) of spermatozoa stored in polyethylene vials (mean ± SE)

	At room temperature		At 4 °C	
Storage period (h)	**With O_2**	**Without O_2**	**With O_2**	**Without O_2**
0	$98.33^{a1} \pm 1.67$	$98.33^{a1} \pm 1.67$	$100.0^{a1} \pm 0.0$	$100.0^{a1} \pm 0.0$
12	$70.0^{b1} \pm 0.0$	$53.33^{b2} \pm 3.33$	$91.67^{b1} \pm 1.67$	$93.33^{b1} \pm 1.67$
24	$21.67^{c} \pm 1.67$	0.0	$70.0^{c1} \pm 0.0$	$48.33^{c2} \pm 1.67$
36	0.0	0.0	$51.67^{d} \pm 1.67$	0.0
48	0.0	0.0	$45.0^{de} \pm 2.89$	0.0
60	0.0	0.0	$40.0^{e} \pm 0.0$	0.0
72	0.0	0.0	$35.0^{e} \pm 0.0$	0.0
84	0.0	0.0	$21.67^{f} \pm 1.67$	0.0
96	0.0	0.0	$6.67^{g} \pm .67$	0.0
108	0.0	0.0	0.0	0.0

Activating solution: tap water. Means with different letters within columns and numbers within rows are significantly ($P < 0.05$) different. Sample size (n): 3.

12 h of storage. At 4 °C, motility of spermatozoa stored with oxygen decreased gradually, compared with that of spermatozoa stored without oxygen, wherein it declined rapidly with storage period. Complete cessation of motility occurred at 108 and 36 h in the former and the latter respectively.

Discussion

The results indicate that the volume of milt obtained from *T. khudree* injected with sGn-RH-a+DOM is six to seven times higher than that from uninjected controls. In common carp, Clemens and Grant (1965) recorded an increase in milt volume from 0.1 to 2.9 mL on pituitary injection. Reenaselvi, Sukumaran and Rahman (1996) observed a similar increase in milt volume following injection of pituitary extract in common carp. A large quantity of milt (7–12 times the control) was obtained from sGn-RH-a+DOM-injected *L. fimbriatus* (Akash 2001). The increased volume of milt obtained from hormone-injected fish could be attributed to higher production of seminal fluid (spermatozoa hydration). While hormone injection did not influence spermatozoa motility rates, it resulted in a marginal decrease in spermatozoa density. In *C. carpio*, Reenaselvi and colleagues (1996) recorded lower spermatozoa density after pituitary injection, presumably due to spermatozoa hydration. Akash (2001) also observed that hormone adminis-

tration yielded reduced spermatozoa density in *L. fimbriatus*. However, mahseers injected with sGn-RH-a+DOM produced four to five times higher total spermatozoa count than the controls (Table 1). Linhart and colleagues (1995) found a larger number of spermatozoa when the common tench (*Tinca tinca* L.) was injected sGn-RH-a, compared with carp hypophysis.

Spermatocrit was significantly reduced in hormone-treated fish. Similarly, in fringe-lipped carp, Akash (2001) found lower spermatocrit on injection of an analogue of sGn-RH+DOM. Garcia (1991) reported high milt production, which was associated with low spermatocrit and sperm density in luteinizing hormone-releasing hormone analogue (LH-RH-a)-injected *S. guttatus*. Tvedt, Benfey, Martin-Robichaud and Power (2001) showed a significant positive relationship ($r^2 = 0.89$) between spermatocrit and sperm density in uninjected Atlantic halibut (*Hippoglossus hippoglossus* L.) They opined that implantation of males with Gn-RH-a decreased sperm density and spermatocrit, without affecting the relationship between sperm density and spermatocrit or that between sperm density and sperm motility.

In most fishes, spermatozoa are immotile in seminal plasma and are activated only by an external sperm-activating medium (Terner 1986; Goodhall, Blackshaw & Capra 1989). While spermatozoa motility (%) did not differ significantly among the four activating media, motility duration differred; NaCl (0.3%)+urea (0.4%) treatment resulted in the longest motility duration (Table 2). Akash (2001) made similar observations in *L. fimbriatus*. Saad, Billard,Theron and Hollebecq (1988) opined that the spermatozoa of freshwater fishes (e.g. common carp, 60 s) have a limited duration of motility. In *T. khudree*, spermatozoa motility in tap water lasts for about 60 s – a feature that compares well with other cyprinids such as *C. mrigal* (53 s) and *L. rohita* (45 s) (Jayaprakas & Bimal Lal 1996) and fringe-lipped carp (43 s) (Akash 2001). Ginsburg (1963) and Scott and Baynes (1980) observed that the duration of intense spermatozoa motility in a salt solution is generally twice that in freshwater, presumably because flagellar movement either exhausts the available energy early or the K^+ ion gradient falls to a low level. Goodhall and colleagues (1989) and Thorogood and Blackshaw (1992) also observed prolonged spermatozoa motility in a salt solution than freshwater in *Sillago ciliata* and *Acanthopagrus australis* respectively.

A progressive increase in spermatozoa motility and duration, spermatozoa density and spermatocrit was generally observed from May to November (Table 3). Higher values were mostly found during August–October/November, which coincides with the peak spawning period for *T. khudree*. High values of spermatozoa motility duration, spermatozoa density and spermatocrit were reported during July–August (peak spawning season) and lower values during both the pre-spawning and post-spawning period in the fringe-lipped carp (Akash 2001). He found a high correlation between spermatozoa density and spermatocrit ($r = 0.84$). The duration and intensity of sperm motility of rainbow trout were low at the beginning of the spawning season, increased towards mid-season and gradually dropped to low levels as the season drew to a close (Buyukhatipoglu & Holtz 1978; Munkittrick & Moccia 1987).

Spermatozoa stored at 4 °C or RT with or without oxygen in polythene bags as well as polyethylene vials showed a slow to moderate decrease in motility (percentage) (Tables 4 and 5). The fall in motility rate, as expected, was much quicker at RT than at 4 °C. Air and pure oxygen in particular, are necessary for maintaining the viability of spermatozoa during *in vitro* storage and this was first demonstrated by Scheuring (1925), who successfully stored trout milt under air and oxygen at 0 °C and obtained motility of spermatozoa under oxygen up to 4 days, compared with those stored without oxygen, losing motility within 24 h. Okada, Ishikawa and Kimura (1956) estimated that sperms in 1 mL of *O. keta* semen at 10 °C consume 21 mm^3 oxygen h^{-1}. Buyukhatipoglu and Holtz (1978) stored *S. gairdneri* sperm under moisture-saturated oxygen and air and obtained motility up to 12 and 8 days of storage respectively. In *C. carpio*, Saad and colleagues (1988) observed that at 4 °C the percentage of motile spermatozoa was high for 2 days and then rapidly decreased to zero after 6–8 days. The viability of stored gametes can be prolonged by maintaining them at close to 0 °C to reduce their metabolic rate, and their ability to tolerate low temperature may vary between temperate and tropical species (Leung & Jamieson 1991). Prolonged viability of spermatozoa preserved in plastic bags inflated with oxygen was also observed by Babiak and colleagues (1996) in *S. glanis*, by Christensen and Tiersch (1996) in *I. punctatus* and by Jayaprakas and Bimal Lal (1996) in Indian major carps. However, pure oxygen did not have any favourable effect on the motility of spermatozoa of *L. fimbriatus* spermatozoa stored in polythene bags at 4 °C (Akash 2001). The author, in fact, observed a slight reduction in motility rate when spermatozoa were stored under oxygen in compari-

son with those preserved without oxygen and the effect was attributed to dehydration of stored milt and osmotic disturbance.

The results of this study could be summarized as follows: spermatocrit may be useful as a simple and rapid method for the estimation of spermatozoa density, period of the likelihood production of spermatozoa having optimum characteristics and spermatozoa could be preserved in a motile state for 4–5 days, in *T. khudree*.

Acknowledgments

This work was supported by the International Foundation for Science, Sweden (A/2288). The first author thanks Mr K. J. Palaksha for help in the preparation of the manuscript.

References

Akash N. (2001) *Preservation of gametes and fertilization methodology in fringe-lipped carp, Labeo fimbriatus* (*Bloch*). MFSc thesis, University of Agricultural Sciences, Bangalore, India, p. 54.

Babiak I., Glogowski J., Kozlowski J. & Chyboski L.D. (1996) Short-term preservation of European catfish (*Siluris glanis* L.) milt. *Archives Polish Fisheries* **4**, 85–90.

Baynes S.M., Scott A.P. & Dawson A.P. (1981) Rainbow trout, *Salmo gairdneri* Richardson, spermatozoa: effects of cations and pH on motility. *Journal of Fish Biology* **19**, 259–267.

Billard R. (1981) Short-term preservation of sperm under oxygen atmosphere in rainbow trout (*Salmo gairdneri*). *Aquaculture* **23**, 287–293.

Billard R. & Cosson M.P. (1986) Sperm motility in rainbow trout, *Parasalmo mykiss*: effect of pH and temperature on reproduction in fish. *Basic and Applied Aspects in Endocrinology and Genetics, INRA, Paris, les Colloques INRA* **44**, 161–167.

Billard R., Cosson M.P. & Christen R. (1987) Some recent data on the biology on trout spermatozoa. In: *Proceedings of the Third International Symposium on Reproductive Physiology of Fish* (ed. by D.R. Idler, L.W. Crim & J.M. Walsh), St John's, Newfoundland, Canada, pp. 187–190.

Bouck G.R. & Jacobson J. (1976) Estimation of salmonid sperm concentration by microhematocrit technique. *Transactions of the American Fisheries Society* **105**, 534–535.

Buyukhatipoglu S. & Holtz W. (1978) Preservation of trout sperm in liquid or frozen state. *Aquaculture* **14**, 49–56.

Carpentier P. & Billard R. (1978) Conservation a court time des gametes de salmonides a des temperatures voisines de 0 °C. *Annales Biologie Animale Biochimie Biophysique* **18**, 1083–1086.

Christensen J.M. & Tiersch T.R. (1996) Refrigerated storage of channel catfish sperm. *Journal of the World Aquaculture Society* **27**, 340–346.

Ciereszko A., Ramseyer L. & Dabrowski K. (1993) Cryopreservation of yellow perch semen. *The Progressive Fish-Culturist* **55**, 261–264.

Clemens H.P. & Grant F.B. (1965) The seminal thinning response of carp (*Cyprinus carpio*) and rainbow trout (*Salmo gairdneri*) after injection of pituitary extracts. *Copeia* **2**, 174–177.

Garcia L.M.B. (1991) Spermiation response of mature rabbitfish, *Siganus guttatus* (Bloch) to leutinising hormone-releasing hormone analogue (LHRHa) injection. *Aquaculture* **97**, 291–299.

Ginsburg A.S. (1963) Sperm-egg association and its relationship to the activation of the egg in salmonid fishes. *Journal of Embryology and Experimental Morphology* **11**, 13–33.

Goodhall J.A., Blackshaw A.W. & Capra M. (1989) Factors affecting the activation and duration of motility of the spermatozoa of the summer whiting (*Sillago sihama*). *Aquaculture* **37**, 391–395.

Harvey B. & Kelley R.N. (1984) Short-term storage of *Sarotherodon mossambicus* ova. *Aquaculture* **37**, 391–395.

Hulata G. & Rothbard S. (1979) Cold storage of carp semen for short periods. *Aquaculture* **16**, 267–269.

Jayaprakas V. & Bimal Lal T.S. (1996) Factors affecting the motility and short-term storage of spermatozoa of the Indian major carps, *Labeo rohita* and *Cirrhinus mrigal*. *Journal of Aquaculture in the Tropics* **11**, 67–78.

Keshavanath P. (2001) *Development of hatchery technology for the conservation of mighty mahseer, Tor khudree in Karnataka.* Final Report of the Project funded by Department of Fisheries, Government of Karnataka, India, p. 23.

Kulkarni C.V. (1971) Spawning habits, eggs and early development of Deccan mahseer, *Tor khudree* (Sykes). *Journal of Bombay Natural History Society* **67**, 510–521.

Leung L.K.P. & Jamieson B.G.M. (1991) Live preservation of fish gametes. In: *Fish Evolution and Systematic: Evidence from Spermatozoa* (ed. by B.G.M. Jamieson), pp. 245–269. Cambridge University Press, Cambridge, UK.

Linhart O., Peter R.E., Rothbard S., Zohar Y. & Kvasnicka P. (1995) Spermiation of common tench (*Tinca tinca* L.) stimulated with injection or implantation of GnRH analogues and injection of carp pituitary extract. *Aquaculture* **129**, 119–121.

Miura T., Yamauchi K., Takahashi H. & Nagahama Y. (1992) The role of hormones in the acquisition of sperm motility in salmonid fish. *Journal Experimental Zoology* **261**, 359–363.

Munkittrick K.R. & Moccia R.D. (1987) Seasonal changes in the quality of rainbow trout (*Salmo gairdneri*) semen: effect of delay in stripping on spermatocrit, motility, volume and seminal plasma constituents. *Aquaculture* **64**, 147–156.

Nandeesha M.C., Bhadraswamy G., Patil J.G., Varghese T.J., Kamal Sarma & Keshavanath P. (1993) Preliminary re-

sults on induced breeding of pond-raised mahseer, *Tor khudree*. *Journal of Aquaculture in the Tropics* **8**, 55–60.

Okada S., Ishikawa Y. & Kimura G. (1956) On the variability of the sperm and the egg left in the dead body of Dog-salmon, *Oncorhynchus keta* (Walbaum). *Science Report of Hokkaido Fish Hatchery* **11**, 7–18.

Piironen J. (1985) Variation in the properties of milt from the Finnish landlocked salmon (*Salmo salar M. Sebago Girrad*) during a spawning season. *Aquaculture* **48**, 337–350.

Quinitio E.T., Gabasa P.G., Sunaf F.P., Reyes E.P. & Delapena D.T. (1984) *Prawn Hatchery Design and Operation*. Extension Manual No. 9, SEAFDEC Aquaculture Department, Tigbauan, Iloilo, Philippines.

Rakitin A., Ferguson M.M. & Trippel E.A. (1999) Spermatocrit and spermatozoa density in Atlantic cod (*Gadus morhua*): correlation and variation during the spawning season. *Aquaculture* **170**, 349–358.

Reenaselvi P., Sukumaran N. & Rahman M.D. (1996) Properties of common carp (*Cyprinus carpio*) milt after homoplastic pituitary administration. *Journal of Aquaculture in the Tropics* **11**, 285–289.

Saad A., Billard R., Theron M.C. & Hollebecq M.G. (1988) Short-term preservation of carp (*Cyprinus carpio*) semen. *Aquaculture* **71**, 133–150.

Scheuring L. (1925) Biologische und physiologsche Untersuchungen an Forellensperma. *Archives Hydrobiologie, Supplement* **4**, 181–318.

Scott A.P. & Baynes S.M. (1980) A review of the biology, handling and storage of salmonid spermatozoa. *Journal of Fish Biology* **17**, 707–739.

Sunitha M.S. & Jayaprakas V. (1997) Influence of pH, temperature, salinity and media on activation of motility and short-term preservation of spermatozoa of an estuarine fish, *Mystus gulio* (Hamilton) (Siluridae-Pisces). *Indian Journal of Marine Sciences* **26**, 361–365.

Terner C. (1986) Evaluation of salmonid sperm motility for cryopreservation. *The Progressive Fish-Culturist* **18**, 230–232.

Thorogood J. & Blackshaw A. (1992) Factors affecting the activation, motility and cryopreservation of the spermatozoa of yellowfin bream, *Acanthopagrus australis* (Gunther). *Aquaculture and Fisheries Management* **23**, 337–344.

Truscott B., Idler D.R., Hoyle R.J. & Freeman H.C. (1968) Subzero preservation of Atlantic salmon spermatozoa. *Journal of Fisheries Research Board of Canada* **25**, 363–372.

Tvedt H.B., Benfey T.J., Martin-Robichaud D.J. & Power J. (2001) The relationship between sperm density, spermatocrit, sperm motility and fertilization success in Atlantic halibut, *Hippoglossus hippoglossus*. *Aquaculture* **194**, 191–200.

