

# Spermatogenesis and related plasma androgen and progestin level in wild male golden mahseer, *Tor putitora* (Hamilton, 1822), during the spawning season

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**Abstract** Testicular development and plasma levels of sex steroid [11-ketotestosterone (11-KT), testosterone (T) and 17,20 $\beta$ -dihydroxy-4-pregnen-3-one (17,20 $\beta$ -P)] were studied for the first time in wild golden mahseer, *Tor putitora*. Testicular development was investigated by macroscopic observation and histology of the gonads, whereas steroids were measured by enzyme-linked immunosorbent assay. Based on macroscopic observation and germ cell types present in gonad histology, the testes of *T. putitora* were divided into five developmental stages: immature [stage I; spermatogonia (SPG)], early spermatogenesis [stage II; SPG and spermatocytes (SPC)], late spermatogenesis [stage III; SPG, SPC, spermatids (SPD) and spermatozoa (SPZ)], spermiation (stage IV; SPZ) and post-spawning (stage V; SPG, SPD and SPZ). During the stage I of the testes, the lowest levels of plasma sex steroid and gonadosomatic index ( $I_G$ ) were recorded. The highest plasma level of T was  $0.89 \pm 0.09$  ng/mL and 11-KT was  $4.23 \pm 0.54$  ng/mL, which was during the stage III and IV, respectively. The peak in 11-KT was coincident with the peak in  $I_G$  ( $1.65 \pm 0.12$  %). The lowest T and 11-KT levels were  $0.25 \pm 0.02$  ng/mL and  $0.47 \pm 0.09$  ng/

mL, respectively, which was at stage I. Plasma levels of 17,20 $\beta$ -P increased significantly at stage III ( $1.04 \pm 0.06$  ng/mL) and stage IV testes ( $1.28 \pm 0.03$  ng/mL) and then declined in post-spawned fish. This indicates that 17,20 $\beta$ -P could also be a possible maturation-inducing steroid in this fish. The condition factor ( $K$ ) significantly decreased during the testicular development and was lowest at spermiation stage ( $0.53 \pm 0.02$  %). The proportion of running male peaked concomitantly with the appearance of stage IV testes. Presence of germ cells of different developmental stages indicates that *T. putitora* male is a multiple spawner, and the information generated here is important for developing a captive breeding, culture and conservation programs for this endangered cold-water Himalayan fish species.

**Keywords** *Tor putitora* · Maturation-inducing steroid · Testicular development · Testosterone · 11-Ketotestosterone · 17,20 $\beta$ -Dihydroxy-4-pregnen-3-one

## Introduction

Golden mahseer, *Tor putitora* (Cypriniformes: Cyprinidae), is an endemic food and game fish of the Himalayan region of South and Southeastern Asia (Nautiyal 1984). Once, this fish was abundantly distributed in hill streams and lakes of India, Nepal, Pakistan, Afghanistan, Bangladesh, Bhutan, Sri

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Lanka, Myanmar, Thailand and Iran (Nautiyal 1984; Islam and Tanaka 2007; Jayaram 2010) and can grow up to 3 m in length in its natural habitat (Bhatt et al. 2000). However, due to overfishing and habitat destruction, its natural population is declining significantly over the past few decades (Oliver et al. 2007; Froese and Pauly 2011) and listed as endangered. Therefore, attempts to culture and revive *T. putitora* stocks have been initiated in trans-Himalayan regions (Ingram et al. 2005; Dinesh et al. 2010). The major bottleneck for its intensive culture is the inability of this *Tor* sp. to attain complete gonadal maturity in captivity (Ismail et al. 2011). Artificial propagation through inducing maturation and spawning by using sex hormones has been attempted (Pandey et al. 1998), with limited success. Therefore, seed production in captivity is still dependent on spawning brooders collected from wild, mainly taking place during months of May to August in accordance with the breeding season in nature.

In teleost, gonads have two major functions: firstly to produce the germ cells through the process of oogenesis (in females) and spermatogenesis (in males) and secondly to produce sex steroids for the initiation and regulation of reproduction. In fishes, the testes can be categorised into two major types based on the morphology of germinal compartment and distribution of germ cells within this compartment (Santana and Quagio-Grassiotto 2014). In higher teleost, the germinal compartment is organised into branching lobules, while those of lower fish are organised into anastomosing tubules (Weltzien et al. 2002). Both the testes types can be further categorised into restricted spermatogonial type, where spermatogonia (SPG) are confined to the periphery of the testis and unrestricted spermatogonial type, where SPG can be found all along the length of the tubule (Schulz and Miura 2002; Schulz 2003). In the testes of a fish, germ cells and Sertoli cells are organised in spermatocysts, and each spermatocyst contains germ cells clones, all at the same stage of development (Billard et al. 1982). During spermatogenesis, where spermatogonial stem cell (SSC) differentiates into mature spermatozoa (SPZ), three major events take place. Initially, the SSC undergoes mitotic division to produce new undifferentiated SPG (type A) and differentiated SPG (type B) (De Rooij 2006; Schulz et al. 2010). Then, the differentiated SPG undergo meiosis, which leads to the production of haploid spermatids (SPD). Finally,

the haploid SPD differentiate into matured SPZ by the process of spermiogenesis (Schulz 2003; Schulz et al. 2010).

Like other vertebrates, the gonad maturation of fish is regulated by several sex hormones (Lee and Yang 2002; Estay et al. 2003; Ismail et al. 2011). In males, androgen and progestin such as testosterone (T), 11-ketotestosterone (11-KT) and 17,20 $\beta$ -dihydroxy-4-pregnen-3-one (17,20 $\beta$ -P), respectively, are known to regulate testicular development by their direct influence on germ cell and also by exerting feedback effects in brain–pituitary–gonad (BPG) axis (Weltzien et al. 2002). Increase in 11-KT stimulates spermatogenesis (Aramli et al. 2014), whereas T stimulates the hypothalamus and pituitary, leading to the development and maturation of the testes (Chaves-Pozo et al. 2008). The progestins such as 17,20 $\beta$ -P are known to promote testicular hydration and spermiation (Miura et al. 1992; Nagahama 1994). Although 11-KT is considered as main androgen in teleost fish (Borg 1994; Koya et al. 2002; García-López et al. 2006; Ismail et al. 2011), sporadically T is found to be the main sex steroid in certain fish (Matsuyama et al. 1991). The temporal variation in plasma sex steroid levels in relation to testis development has been studied in Senegalese sole, *Solea senegalensis* Kaup (García-López et al. 2006), Atlantic halibut, *Hippoglossus hippoglossus* L. (Weltzien et al. 2002), Pacific herring, *Clupea pallasii* (Koya et al. 2002), spotted halibut, *Verasper variegates* (Koya et al. 2003), African catfish, *Clarias gariepinus* (Cavaco et al. 1997a, b), Japanese eel, *Anguilla japonica* (Miura et al. 1991), winter flounder, *Pleuronectes americanus* (Harmin et al. 1995), English sole, *Pleuronectes vetulus* (Sol et al. 1998), rainbow trout, *Salmo gairdnerii* Richardson (Scott et al. 1980) and mahseer *Tor tambroides* (Ismail et al. 2011). However, to our knowledge, there are no detailed studies, which focus on spermatogenic cycle of *T. putitora* in terms of testicular development in relation to plasma 11-KT, T and 17,20 $\beta$ -P.

Therefore, the aim of this study was to describe the testicular development of *T. putitora* in relation to plasma level of circulating androgens (T and 11-KT) and progestins (17,20 $\beta$ -P). So far little is known about the reproductive physiology of species from the genus *Tor*, and none of them correlate the testicular development with sex steroid dynamics.

## Materials and methods

### Fish

On the first week of every month from February 2014 to September 2014, live *T. putitora* were procured from local fisherman and brought back to wet laboratory of the Directorate of Coldwater Fisheries Research, Bhimtal, India. The fish were captured by hook and line from Bhimtal (latitude 24°48'N and longitude 73°54'E) and Sattal Lakes (latitude 29°20'N and longitude 79°31'E) of mid-Himalayan region of India. The average weight and length of the sampled fish were  $429.62 \pm 84.91$  g and  $37.48 \pm 8.73$  cm (mean  $\pm$  SEM), respectively. The age of collected fish was determined by scale counting method (Nikolsky 1963), using the scale reader (Sipcon profile projector SP-300, Ambala cantonment, India). The fish were acclimatised in 1000-L capacity square fibreglass tanks with 0.45 m of maximum water depth for 7 days. During the acclimatisation, the tanks were continuously supplied with groundwater, and the water flow rate was 2.5 L/min. Body length (total length, standard length and fork length) was measured using a 60-cm measuring scale, and body weight and gonad weight were measured using a digital scale balance with 0.01 sensitivity. The temperature, dissolved oxygen and pH of the tank water during the acclimatisation period varied between 20.51 to 24.18 °C, 6.57 to 6.85 mg/L and 6.60 to 7.40, respectively. Feeding to satiation was done twice daily with a supplementary pellet diet (prepared by DCFR, Bhimtal), at the rate of 4 % of the body weight (35 % crude protein, 3 % moisture, 5 % fibre and 3 % crude fat).

### Blood sampling

From acclimatised fish, blood sampling and tissue sampling were carried out. Fish were anaesthetised using tricaine methane sulphonate (MS 222; 60 mg/L of water). Approximately, 1.5–2.0 mL of blood was drawn from the caudal vein of each fish using cold heparinised syringe (3 mL capacity) fitted with 22-gauge needle. The blood was collected in 2-mL Eppendorf tubes and before plasma separation allowed to stand at room temperature for 1 h. The plasma was separated from the blood by centrifugation at  $5000 \times g$  for 10 min at 4 °C. The blood plasma was

aliquot to 150  $\mu$ L in 0.5 mL Eppendorf tubes and stored at  $-80$  °C till further analysis.

### Gonad development and histology

After collecting the blood, the fish were euthanised by an overdose of MS 222 (150 mg/L of water). The ventral surface of the fish was cut open and liver and testes were excised, weighed to the accuracy of 0.01 g. For macroscopic determination of gonad maturity, features such as degree of opacity of the gonads, colour, size, volume, length and sperm visibility were recorded (Rosenblum et al. 1987). Transverse fragments of testis tissue (approximately  $0.4 \text{ cm}^3$ ) from midlobe were fixed in 4 % phosphate-buffered (0.1 M, pH 7.2) formalin for 48–96 h at room temperature. For histological examination, the tissues were rinsed in running tap water, dehydrated in an ascending series of ethanol and processed by standard histological methods (Allen 1993). Testis sections were cut at 4–5  $\mu$ m thickness on a semi-automatic microtome (Microm HM340E, Thermo Scientific) and stained with haematoxylin and eosin (H&E). The stained sections were examined and photographed on a Leica DM500 microscope (Leica, Germany). The testicular development stages were determined according to germ cell types present and their relative abundance (Schulz et al. 2010).

### Sex steroid immunoassay

Sex steroid quantification (11-KT, T and 17,20 $\beta$ -P) in collected plasma was conducted using commercially available enzyme-linked immunosorbent assay (ELISA) kits from Cayman Chemical Company, Michigan, USA. The sex steroids were quantified following the assay kit procedures and methods described by Cuisset et al. (1994) and Nash et al. (2000). Microtiter plates (MaxiSorp<sup>TM</sup>) for the assay were purchased from Nunc (Roskilde, Denmark). All samples and standards were assayed in triplicates, and the absorbance was read at 415 nm by using microplate spectrophotometer (EON, Bio Tek). The absorbance values were analysed using SoftMax Pro 5.4 software (Molecular Devices, LLC).

### Calculations and data analyses

The gonadosomatic index ( $I_G$ ; %), condition factor ( $K$ ) and hepatosomatic index ( $I_H$ ; %) were calculated

as  $100 \times W_G \times (W_B)^{-1}$ ,  $10^5 \times W_B \times (L^3)^{-1}$  and  $100 \times W_L \times (W_B)^{-1}$ , respectively.  $W_G$  is gonad weight (g),  $W_B$  is total body weight (g), and  $W_L$  is total liver weight (g). Data were analysed for statistical differences within spermatogenetic stages and spermiation condition by one-way analysis of variance (ANOVA) using SPSS (version 19.0), followed by Student–Newman–Keuls (SNK) multiple comparison tests with a significance level of  $p < 0.05$ . The data compliance with homogeneity of variance and normality was tested by the Levene and Kolmogorov–Smirnov methods, respectively, and log transformation was carried out, when necessary. Data are presented as mean  $\pm$  standard error of mean (SEM).

## Results

Altogether, 52 male *T. punitora* of age 3.0–4.5 years were sampled during the study period (Table 1). The standard length and fork length of the collected fish were  $32.52 \pm 6.78$  and  $35.69 \pm 3.48$  cm, respectively.

### Testicular development

Five testicular development stages of spermatogenesis were defined in *T. punitora*, based on the macroscopic appearance, histological changes and relative abundance of various germ cell types present (Table 2 and Fig. 1).

**Table 1** Total number of male golden mahseer, *Tor punitora*, sampled for each testicular development stage during the sampling period from Bhimtal and Sattal Lakes

	Development stages of testes					
	I	II	III	IV	V	
February 2014	5					5
March 2014	3	3				6
April 2014		2	4			6
May 2014		2	5	3		10
June 2014				5		5
July 2014				5		5
August 2014				4	5	9
September 2014					6	6
	8	7	9	17	11	52

### Spermiation

In February and March, running males (RM) were not observed. RM was found from May to August (Fig. 2). The fraction of RM was low (25 %) in April and then gradually increased from May (around 64 %) to August (83 %). The percentage of RM was highest in August, which abruptly declined to 50 % in September. The proportion of RM was low in fish with stage II and III testes (10 and 25 %, respectively) and peaked in fish with testes at stage IV (90 %) (Fig. 3). At stage V testes, RM proportion was tremendously low (around 29 %). Non-RM (100 %) was observed at stage I of testicular development.

### Morphometric changes

For fish with stage I testes, the mean  $I_G$  was the lowest at  $0.43 \pm 0.01$  % (Fig. 4a). The  $I_G$  increased to  $0.65 \pm 0.04$ ,  $1.18 \pm 0.06$  and  $1.68 \pm 0.12$  % for fish with stage II, III and IV testes, respectively. Subsequently, the index decreased to  $1.20 \pm 0.41$  % for stage V. Mean  $I_G$  was higher in running male ( $1.68 \pm 0.12$  %) than in non-running male ( $0.73 \pm 0.09$  %). The  $I_H$  (mean  $1.03 \pm 0.32$  %) did not vary significantly in between testicular development stages (Fig. 4b). The  $K$  (mean  $0.76 \pm 0.06$  %) declined as the development of testes progressed from stage I testes (mean  $1.12 \pm 0.09$  %) to stage II (mean  $0.72 \pm 0.06$  %), stage III (mean  $0.67 \pm 0.03$  %) and stage IV testes (mean  $0.59 \pm 0.02$  %) (Fig. 4c). At stage V testes, the  $K$  increased significantly (mean  $0.93 \pm 0.10$  %).

### Plasma sex steroid

Plasma 11-KT, T and 17,20 $\beta$ -P in male *T. punitora* ranged from 0.47 to 4.23 ng/mL serum, 0.26 to 0.89 ng/mL serum, and 0.71 to 1.289 ng/mL serum, respectively, during the study period. 11-KT level significantly increased from the stage II testes (mean  $0.56 \pm 0.21$  ng/mL) to stage III (mean  $2.89 \pm 2.81$  ng/mL) and IV (mean  $4.23 \pm 0.51$  ng/mL) (Fig. 5a). T level significantly increased from fish with the stage I testes (mean  $0.25 \pm 0.009$  ng/mL) to stage III (mean  $0.89 \pm 0.02$  ng/mL) (Fig. 5b). 17,20 $\beta$ -P in serum of fish increased from the stage II testes ( $0.71 \pm 0.09$  ng/mL) to stage III ( $1.04 \pm 0.06$  ng/mL) and IV ( $1.28 \pm 0.03$  ng/mL) (Fig. 5c). At stage V, the level of all the three sex steroids in fish

**Table 2** Stages of gonad maturity in male golden mahseer, *Tor putitora*

Maturity stages	Macroscopic appearance of testes	Histological observations
Immature (I)	Testes asymmetrical thin, elongated, paired with smooth edges, and grey in colour. It extends less than half the length of the body cavity and tightly adhered to body wall and swim bladder with connective tissue. Testis mass (mean $\pm$ SEM) is $0.64 \pm 0.02$ g	Testis cortex was completely occupied with mitotic SPG surrounded by Sertoli cells. Few SPC were also present, but SPD and SPZ were completely absent (Fig. 1a, b). Numerous circular empty lumen could be seen at this stage (Fig. 1a). Fish with stage I testes were found in February and March
Early spermatogenic (II)	Testes asymmetrical elongated, whitish, translucent, extending a little more than half the length of body cavity. Testis mass (mean $\pm$ SEM) is $1.15 \pm 0.18$ g	Meiosis was initiated, which results in decline in the number of SPG and the increase in population of SPC I and SPC II (Fig. 1c). SPD and SPZ were completely absent. Empty space was not seen. Fish with testes at stage II were found in March to May
Late spermatogenic (III)	Testes asymmetrical, opaque, enlarged and occupy 2/3 of the length of body cavity. Blood vessels visible on the surface. Small amount of milt can be expressed. Testis mass (mean $\pm$ SEM) is $2.51 \pm 0.14$ g	Germ cells of all developmental stages were present (SPG, SPC, SPD and SPZ). The lumen of the cortical seminiferous lobules was mainly occupied by SPZ (Fig. 1d, e, f). Few SPG were seen, as meiosis initiated and progressed. At this stage, the SPD were the most abundant cell inside the testis. Testes at this stage prevailed from April to May
Spermiation (IV)	Testis enlarged, asymmetrical, soft, milky white, soft and anterior part is filled with white colour milt. Viscous fluid present in the anterior part. Blood vessels visible on the surface. Testes occupy almost entire length of the body cavity. Milt could be stripped from more than 80 % of the males in this stage. Testis mass (mean $\pm$ SEM) is $6.05 \pm 0.27$ g	Sperm duct and lobular lumen are completely occupied with SPZ. The diameter of the medullar efferent ducts showed a considerable increase in size and change in shape. SPD, SPC and SPG were completely absent. The ripe SPZ occupy more than 95 % of the total area of medullar efferent ducts (Fig. 1g, h). Fish with testes at this stage are found from May to August
Post-spawning (V)	Testes in regression, flabby, blood red and deflated sacs. Milt could not be stripped from any of the male. Testis mass (mean $\pm$ SEM) is $3.35 \pm 0.72$ g	The number of SPZ decreased significantly at this stage in the medullar efferent ducts compared with spermiation stage (Fig. 1i). SPD were the abundant germ cell present. Actively proliferating SPG and Sertoli cells were also present (Fig. 1j). Empty space and vacuoles were more visible at this stage. Stage V testes were observed after August

was significantly lower ( $1.23 \pm 1.01$  ng/mL for 11-KT,  $0.34 \pm 0.001$  ng/mL for T and  $0.89 \pm 0.04$  ng/mL for 17,20 $\beta$ -P) than those of fish with stage IV testes. Average intra-assay and inter-assay coefficients of variations of 11-KT, T and 17,20 $\beta$ -P are shown in Table 3.

## Discussion

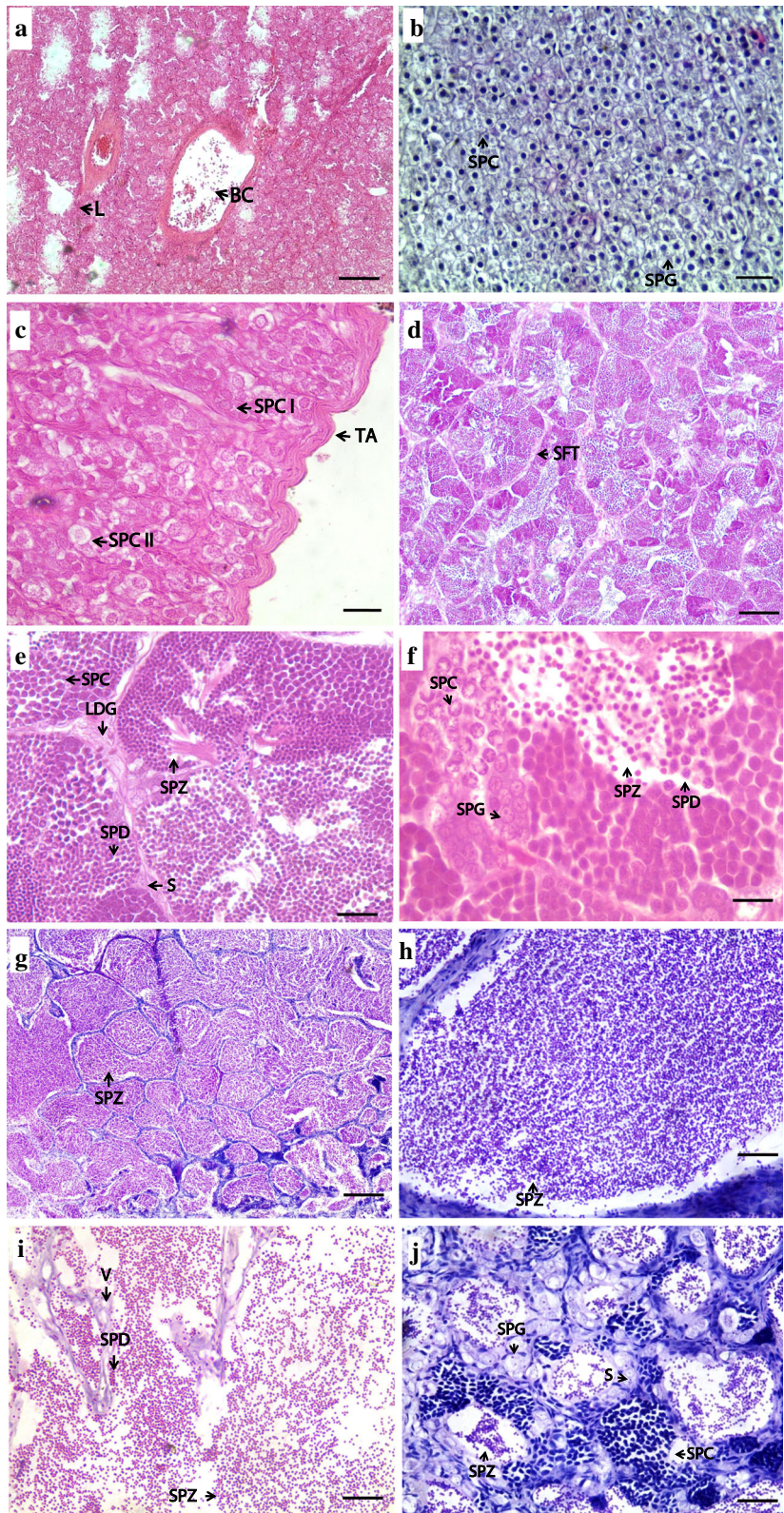
In the present work, we describe for the first time the spermatogenic cycle of wild *T. putitora* in terms of testicular growth and development, in correspondence with androgen (11-KT and T) and progestin (17,20 $\beta$ -P) levels in blood plasma. This study was carried out from February to September 2014, during the breeding season reported for this species (Nautiyal 1984) in its

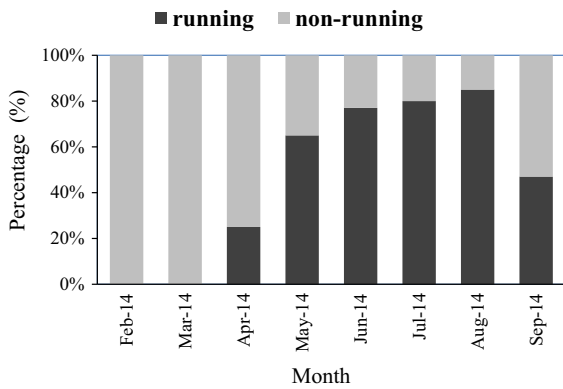
natural habitat in Himalayan regions of northern India. Spermatogenesis was divided into five developmental stages based on macroscopic appearance and relative abundance of germ cell types present in testes of this fish. During the study period, distinct variations in  $I_G$ ,  $K$  and plasma level of sex steroids were observed, in association with different phases of testicular development.

In February and March, the testes of *T. putitora* were at stage I (Immature) and stage II (early spermatogenesis), respectively. It indicates that the spermatogenesis was initiated in few sampled fish during the start of summer (March). All sampled fish had testes at early and late spermatogenic stages (stage II and stage III) in April. In May, testes were at spermatogenic (stage II and stage III) and spermiation stage (stage IV). From June to July, mahseer had fully

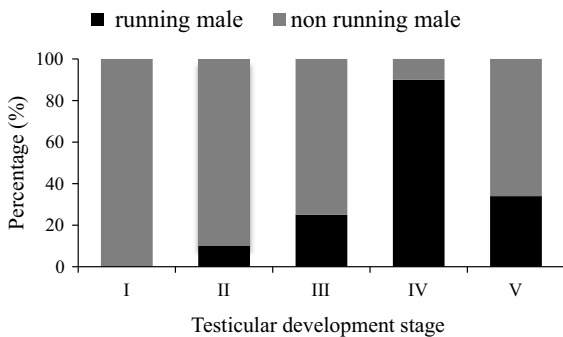


**Fig. 1** Photomicrographs of cross section (4–5  $\mu\text{m}$ ) of golden mahseer, *Tor paitora*, testes at different maturity stages. **a**, **b** Immature spermatogonia (SPG) (stage I); **c** early spermatogenic spermatocytes (SPC I and SPC II) (stage II); **d**, **e**, **f** late spermatogenic SPC and spermatids (SPD), (stage III); **g**, **h** functional maturation, spermatozoa (SPZ) (stage IV); **i**, **j** recovery, SPG and SPD (stage V). TA, *Tunica albuginea*; S, sertoli cell; BC, blood cell; L, lumen; V, vacuoles; LDG, Leydig cell; SFT, seminiferous tubule; *scale bars* 100  $\mu\text{m}$  (**a**, **d**, **g**); 50  $\mu\text{m}$  (**b**, **c**, **j**); 30  $\mu\text{m}$  (**e**, **h**, **i**); 15  $\mu\text{m}$  (**f**). Sections were stained with haematoxylin and eosin (H&E)





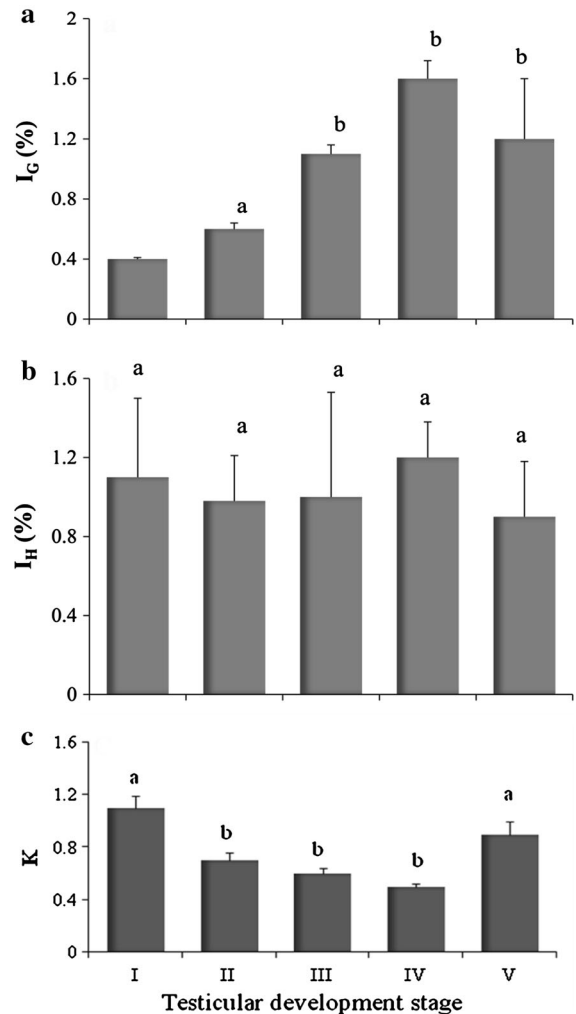
**Fig. 2** Monthly changes in proportion of running and non-running *T. putitora* males



**Fig. 3** Proportion of running and non-running males of golden mahseer at different stages of testicular development. Sample size is given in Table 1

matured testes, with oozing of milt (spermiating stage, stage IV), which is in correspondence with the peak spawning seasons reported for this species in nature (Nautiyal 1984; Dinesh et al. 2010). Spawning is synchronised with heavy rainfall (June–August), a condition that ensures the influx of oxygen-rich water and dropping in water temperature, which stimulates the maturation of testes, and finally spermiation (Ingram et al. 2005; Islam and Tanaka 2007). In September, the sampled fish were at post-spawning (stage V) phase, which suggest the end of the breeding season for this fish. Therefore, this study indicates that spawning of *T. putitora* in the northern Himalayan region extends from May to August, with a peak in August, which is in agreement with the earlier observations (Bhatt et al. 2000; Dinesh et al. 2010).

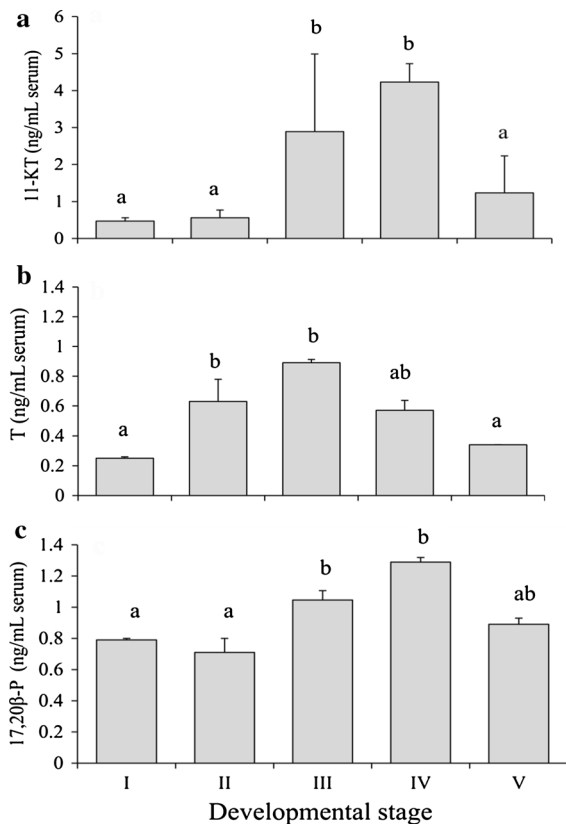
Also, histologically five testicular development stages were distinguished in *T. putitora*, and



**Fig. 4** Variation in (mean  $\pm$  SEM) **a** gonadosomatic index ( $I_G$ ), **b** hepatosomatic index ( $I_H$ ), **c** condition factor ( $K$ ) of male golden mahseer, *Tor putitora*, at five gonadal development stage. Different letters indicate statistical differences ( $p < 0.05$ ). Sample size is given in Table 1

spermatogenesis appears to be cystic type, similar to that reported in many other fishes, like winter flounder, *Pseudopleuronectes americanus* (Harmin et al. 1995), English sole, *Parophrys vetulus* (Sol et al. 1998), *H. hippoglossus* (Weltzien et al. 2002) and *V. variegates* (Koya et al. 2003). It occurs within individual cysts, which line the tubule walls (Santana and Quagio-Grassiotto 2014), and during and immediately preceding the spawning season (stage IV), the central lumen receives the SPZ released from cysts, which remain stationary along the lobule during spermatogenesis. Although *T. putitora* belongs to cyprinid





**Fig. 5** Plasma levels of sex steroids (mean  $\pm$  SEM) in male golden mahseer, *Tor putitora*, at different testicular development stages. **a** 11-ketotestosterone, 11-KT; **b** testosterone, T; **c** 17,20 $\beta$ -dihydroxy-4-pregnen-3-one, 17,20 $\beta$ -P. Different letters above histograms indicate significant difference ( $p < 0.05$ ). Sample size is given in Table 1

**Table 3** T, 11-KT and 17,20 $\beta$ -P intra-assay and inter-assay coefficient of variation for male *Tor putitora*

Hormones	T	11-KT	17,20 $\beta$ -P
Inter-assay coefficient of variation	6.56	9.34	7.30
Intra-assay coefficient of variation	9.32	8.50	8.00

T testosterone, 11-KT 11-ketotestosterone, 17,20 $\beta$ -P 17,20 $\beta$ -dihydroxy-4-pregnen-3-one

group of fishes, its spermatogenetic pattern is different from all other cyprinids (mainly tropical cyprinids such as carps and few other *Tor* spp.) (Ingram et al. 2005). In other cyprinids, SPG and SPZ were present throughout the year in the testes, allowing continuous

production of matured SPZ (Neetu Shahi, unpublished data), whereas in *T. putitora* matured spermatozoa is produced during certain month of the year (May–September). Histological examination of testes of this fish indicates that both SPD and SPZ were present at certain stage of testicular development. This may be the reason for the release of sperm during a certain month of year, mainly at the peak of gonad development and endocrinological activity. At late spermatogenesis, in the testes of *T. putitora*, meiotic activity leads to the accumulation of SPD and SPC in the cortical seminiferous lobules for a short period of time, since they get converted into SPZ heavily, a phenomenon which indicates synchronous spermatocyte development and complete maturation. Since spermiogenesis occurs simultaneously, at the final phase of gonad development, the testis accumulates large quantity of sperm (4–5 mL, personal observation), which can easily be extracted with a gentle pressure on the lower part of the abdomen. It has been recognised that in cystic spermatogenesis, synchronous germ cell development produces a large number of simultaneously mature SPZ, compared with semi-cystic spermatogenesis (García-López et al. 2006). Except for *T. tambroides* (Ismail et al. 2011), so far, there are no reports describing previously the testicular development of this species or any other *Tor* species. However, the study conducted by Ismail et al. (2011) in *T. tambroides* did not mention the type of spermatogenesis; rather, they described the annual sex hormone profile and germ cell development. Unlike *T. putitora*, which is a temperate water species and a seasonal spawner (Nautiyal 1984), the *T. tambroides* is a tropical species, which spawns throughout the year (non-seasonal spawner). Therefore, further study in detail is essential to clarify the relationship between cystic type spermatogenesis and the dynamics of the testicular development in *T. putitora* and any other *Tor* species as well.

Spermiating fish were present over a relatively long period of time from May to August, and almost all male fish examined during July to August had matured testes. The proportion of RM was low during the winter (February–March), increased in summer (April–May) and reaching its maximum at monsoon (July–August). Subsequently, as winter approaches the RM declined. This indicates that this fish breeds during rainy months, as most important spawning cues for breeding are flooding (Ingram et al. 2005). Within



the five stages of testicular development, the highest and lowest proportion of RM was found in fish with testes at stage IV and II, respectively. Approximately 90 % of fish with testes at stage IV released sperm. However, RM was not found in fishes with stage I testes, which indicates that this species is a seasonal spawner and spawns only for few months during a year, which is in contrast to the observation of Ismail et al. (2011) in *T. tambroides*.

The  $I_G$  in *T. putitora* increased from 0.43 % at the beginning of the gonadal development (stage I) to 1.68 % at maturity (stage IV). The same evolution in  $I_G$  has been reported in *C. pallasii* (Koya et al. 2002), where  $I_G$  increased from 0.38 % at the beginning of the testicular development to 17.20 % at maturity. In *C. gariepinus* (Cavaco et al. 1997a, b), the  $I_G$  varied from approximately 0.1 % in immature male to 9 % in matured male. Similarly, Adebisi et al. (2013) found higher  $I_G$  (7.06 %) in mature river catfish, *Hemibagrus nemurus*. Such conspicuous variation in  $I_G$  during spermatogenic cycle was also observed in *H. hippoglossus* L. (Weltzien et al. 2002), *C. pallasii* (Koya et al. 2002), *V. variegates* (Koya et al. 2003) and Japanese sardine *Sardinops melanostictus* (Matsuyama et al. 1991). In contrast, *S. senegalensis* (García-López et al. 2006) and giraffe nosed catfish, *Auchenoglanis occidentalis* (Shinkafi et al. 2011) showed slight changes in  $I_G$  throughout the spermatogenic cycle. In *T. putitora*, the  $I_G$  increased a little from stage I to stage II, probably caused by cellular proliferation due to initiation of meiosis. From stage II to III, the  $I_G$  showed a significant increase, probably in association with further accumulation of germ cells of various types. At stage IV, the  $I_G$  increased considerably, which may be due to the excessive secretion of seminal fluid (observed mainly at anterior part of the gonad) in the testis of the fish after spermiogenesis. This was further supported by the observation that  $I_G$  was significantly higher in RM in comparison with non-RM. At post-spawning phase, when the sperm was released, the  $I_G$  gradually declined.

During spermatogenesis, the energy is mobilised from liver to gonad for development. In our study, the  $I_H$  did not varied significantly throughout the spermatogenic cycle. However, the values obtained for  $K$  suggest an inverse relationship between this factor and testis development, which confirms the influence of spermatogenesis on the physiological condition of the male fish. Similar results were obtained in

*Astyanax fasciatus* (Gurgel 2004), which indicates a reduction in  $K$  during the reproductive cycle. This may be due to the use of energy reserves at the time of gonad development and reduction in feeding activity.

In teleosts, 11-KT, T and 17,20 $\beta$ -P are the main sex steroids known to control the initiation and progression of spermatogenesis (Koya et al. 2003). 11-KT stimulates the development of secondary sexual characteristics, spermatogonial proliferation and spermiation (Lintelmann et al. 2003), whereas T, a biosynthetic precursor of 11-KT, stimulates spermatogenesis (Fostier et al. 1983). 17,20 $\beta$ -P induces sperm motility (Miura et al. 1992). In the present study, plasma levels of 11-KT were being quantitatively higher than the T, as previously reported in few other fishes (Liu et al. 1991; Sol et al. 1998; Weltzien et al. 2002; García-López et al. 2006). During our study, increase in plasma titre of 11-KT was detected at late spermatogenesis (stage III), which indicates the onset of spermatozoa maturation and release of milt. Plasma 11-KT progressively increased and reached peak in association with the appearance of spermiating/running male fish (stage IV testes of *T. putitora*), which suggests the physiological role of 11-KT in spermiation rather than to spermatogenesis. Thereafter, the level of this androgen declined concomitantly with the appearance of post-spawning phase of spermatogenesis (stage V). The similar progression in 11-KT has also been observed in *H. Hippoglossus* (Weltzien et al. 2002) and in *C. gariepinus* (Cavaco et al. 1997a, b). The peak level of plasma 11-KT in this study (4.23 ng/mL) was quantitatively higher than the plasma level of 11-KT in river catfish *Hemibagrus nemurus* (0.017 ng/mL, Adebisi et al. 2013) and *T. tambroides* (0.7 ng/mL, Ismail et al. 2011). However, the serum level of 11-KT in *T. putitora* was comparable with other fishes such as *V. variegates* (2.8 ng/mL, Koya et al. 2003), *C. pallasii* (6.58 ng/mL, Koya et al. 2002) and *H. hippoglossus* (4.0 ng/mL, Weltzien et al. 2002).

Plasma T levels gradually increased concomitantly in association with the progression of spermatogenesis (stage II and III) and peaked in late spermatogenic males. This situation differs from pleuronectiformes, in which peak levels of T were detected, when the testes accumulate a large quantity of SPZ (Harmin et al. 1995; Sol et al. 1998; Weltzien et al. 2002). Though such differences are not well understood, it may be related to the type of spermatogenesis and its sex steroid control. In *T. putitora*, the plasma T level

gradually declined at the onset of spermiation and also in post-spawning phase. This suggests that in male golden mahseer, the physiological role of T may be related to spermatogenesis rather than to spermiation. This is in concurrence with earlier reports, where T is elevated during spermatogenesis, and falls on the spermiation stage (Fostier et al. 1983). The fall in T at spermiation may be due to the conversion of T into  $17\alpha,20\beta$ -P (Baynes and Scott 1985) or the synthesis of downstream metabolites of T (Matsuyama et al. 1991). Several previous information from other teleosts (Matsuyama et al. 1991) supports this concept that T possibly plays a role in the initiation and maintenance of spermatogenesis. Moreover, in golden mahseer, it remains to be investigated whether the androgens (T and 11-KT) are mainly produced by the testes, or also whether non-testicular tissues such as adrenal gland and inter-renal tissue can be a source of androgens (Mayer et al. 1990; Vermeulen et al. 1995; Cavaco et al. 1997a, b) and have the capacity of androgen production and conversion. In addition, the role of androgens in development of secondary sexual characteristic in maturing *T. putitora* also needs to be investigated.

Generally,  $17,20\beta$ -P is considered as a maturation-inducing steroid in a number of fishes (Canario and Scott 1990; Canario 1991; Scott and Canario 1992) and involved in maturation of germ cells and spermiation (Nagahama 1994). Administration of this steroid induces spermiation and sperm mobility in salmonids (Miura et al. 1992) and black porgy, *Acanthopagrus schlegelii* (Yueh and Chang 1997). Peak in  $17,20\beta$ -P during the periods of spermiation was also reported in sole (Canario 1991; García-López et al. 2006). The serum levels of  $17,20\beta$ -P were considerably high (0.7–1.3 ng/mL) in our study and comparable to the observations in *S. senegalensis* (García-López et al. 2006). Its peak in concentration (1.3 ng/mL) was observed at spermiation stage, suggesting that this steroid may be the maturation-inducing steroid in *T. putitora* as suggested for *S. senegalensis* (García-López et al. 2006) and *S. solea* (Canario 1991). Moreover, in our study plasma levels of this steroid show significant variations and interestingly remained at relatively high concentrations throughout the testicular developmental stages. In contrast, study conducted by Degani et al. (1998) in male carp, *Cyprinus carpio*, reported that no significant changes was recorded for blood plasma of

$17,20\beta$ -P during the breeding season. Although the exact cause of high  $17,20\beta$ -P in the blood plasma of *T. putitora* could not be understood, it could possibly be related to the transformation of SPD into SPZ into successive batches (a pulsatile hormonal control may be acting) and also could be related to the short duration of  $17,20\beta$ -P in the bloodstream of this fish (Rinchard et al. 1997), due to metabolism (Scott and Canario 1992). Unfortunately, there are no reports describing previously the level and dynamics of this steroid in *Tor* species or other cyprinids to verify this hypothesis, and therefore, more precise and detailed studies must be carried out to determine the role and evolution of  $17,20\beta$ -P in golden mahseer.

## Conclusion

The current study describes testicular development stages of male *T. putitora* in terms of spermatogenic cycle in relation to changes in plasma levels of 11-KT, T and  $17,20\beta$ -P. Dynamics of plasma level of sex steroid reflected changes in  $I_G$  and testicular histology, suggesting their significant role in regulation of spermatogenic cycle of this fish. *T. putitora* males were also found to exhibit ‘associated reproductive pattern’ (Scott et al. 1980; Billard et al. 1982), which means that spawning occurs at the peak of gonadal activity. However, additional studies targeting on identifying the physiological effects and mechanism of sex steroid are urgently required, which will help to develop a captive breeding programme for this endangered species. To our knowledge, this is the first study relating the testicular development by gonad histology and sex hormone levels in *T. putitora*.

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