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ORIGINAL ARTICLE

Seasonal modulation of reproductive hormones and related biomarkers in coldwater cyprinid *Barilius bendelisis* (Hamilton, 1807)

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Abstract

Barilius bendelisis is a valuable ornamental and food fish from India in which the endocrine control of its reproduction activity has not been widely reported. The present study was aimed to elucidate changes in serum endocrine hormones: 17β-estradiol (E_2), testosterone (T), progesterone (P_4), 17α, 20β-dihydroxyprogesterone (17α, 20β-P), cortisol (C), triiodothyronine (T_3), and thyroxine (T_4); aromatase activity (ARO), vitellogenin (VTG), and total antioxidant capacity (TAC) during different seasons of the year 2014. All studied parameters showed marked seasonal variations. The highest E_2 level in males and females were recorded in winter (January) and rainy (September) seasons respectively. The T level exhibited dual peaks in both sexes, and the highest values were observed during the winter (January) and rainy (September) seasons. The serum levels of P_4 and 17α, 20β-P during spring (March) and rainy (September) seasons were significantly higher and found associated with final sperm and oocyte maturation. The serum levels of ARO and VTG also showed bimodal pattern during the spring (March) and rainy (September) season and were well correlated with T and E_2. Serum C level was detectable throughout the year in both sexes which were related to glucose metabolism and spawning events. Fluctuations in T_3 and T_4 levels were associated with somatic growth and reproduction events during different seasons in *B. bendelisis*. Serum TAC level was highest during summer (May and July) and autumn (November) seasons suggesting better antioxidant potential during these seasons. The study confirms that *B. bendelisis* is a multiple spawner with two spawning seasons.

Keywords *Barilius bendelisis* · Season · Sex steroid hormones · Aromatase · Vitellogenin · Multiple spawner

Introduction

Barilius bendelisis is a teleost that belongs to order Cypriniformes and family Cyprinidae. It is distributed widely along the Indian Himalayas (mainly in Ganga, Indus, and Brahmaputra river basins) including Bangladesh, Bhutan, Nepal, Pakistan, Myanmar, and Sri Lanka (Talwar and Jhingran 1991; Oo 2002). *B. bendelisis* is an intermittent/asynchronous spawner; first spawning takes place in March–April and second during August–September months with GSI peaks during March and August respectively (Dobriyal and Singh 1987). *B. bendelisis* attained the first maturity at a size of 7.0–7.5 cm and 1+ age (Nautiyal and Negi 2004). *B. bendelisis* is a food fish consumed by the populace of Himalayan region and fetches a reasonable market price of Rs 160–200 per kilogram due to its richness in micro minerals and protein (Sharma et al. 2017). Besides food value, *B. bendelisis* has a good demand in the aquarium trade under the name of "Hamilton's Baril" or "Indian Hill Trout" in India and is exported to various countries as ornamental fish (Sharma et al. 2017). To the best of knowledge, there are no published literatures on reproductive aspects like serum sex steroids, cortisol, thyroid hormones, vitellogenin (VTG), aromatase (ARO) enzyme, and total antioxidant capacity (TAC) that creates vacuity in the literature of *B. bendelisis*, which needs to be addressed for understanding the seasonality of reproduction with seasonal variables and for devising its captive breeding programmes.

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Seasonal fluctuations in the levels of circulating sex steroids and their importance in the reproduction have been reported earlier for abundant species of teleost (Barannikova et al. 2002). The reproductive cycle of fish is characterized by several reproductive processes that include spermatogenesis and oogenesis and are related to gonadal maturation and sex steroid hormones (Lubzens et al. 2010; Schulz et al. 2010; Adebiyi et al. 2013). Studies of reproductive hormones and how the environmental variables (e.g. temperature, season, photoperiod, or resource availability) influence these hormones are of central importance in fishery studies.

The roles of sex steroid hormones like testosterone (T), 17 β-Estradiol (E_2), progesterone (P_4), and 17α, 20β-dihydroxyprogesterone (17α, 20β-P) in fish reproduction or gonad maturation are well known (Lubzens et al. 2010; Schulz et al. 2010; Adebiyi et al. 2013; Young et al. 2005). In females, E_2 plays a vital role in hepatic vitellogenesis by controlling vitellogenin (VTG) synthesis in the liver during different phases of the reproductive cycle (Miura et al. 2007). Vitellogenins (VTG) is egg-yolk lipoproteins transported to the developing oocytes and incorporated into the yolk during final oocyte maturation and assist in assessing the reproductive state in female fishes (Lubzens et al. 2010). Previous studies on teleost fishes reported higher E_2 level and VTG during pre-spawning periods of reproductive cycle (Barcellos et al. 2001). VTG level was also reported in male fishes following exogenous E_2 exposure and therefore used as an indicator of estrogenic contamination in the aquatic environment (Larsson et al. 2002). Recent studies have suggested that E_2 regulates spermatogonial proliferation and Sertoli cell physiology in male teleost (Chaves-Pozo et al. 2008). Increased T level is known to stimulate spermatogenesis and testicular development in male and corresponds with estradiol level in female fish suggesting that T is a precursor to E_2 synthesis (Young et al. 2005). Although T is male-specific androgen, it is also present in blood serum/plasma of female fish (Rinchard et al. 1993).

17α, 20β-dihydroxyprogesterone (17α, 20β-P) and progesterone (P_4) play a significant role in final oocyte maturation and ovulation in cyprinid fishes (Nagahama 1994; Vazirzadeh et al. 2014; Scott et al. 2010). P_4 itself is not considered as a biologically active fish steroid, but it functions as a precursor for the biosynthesis of numerous active fish steroids (like T, 11KT, E_2, and 17α, 20β-P) and plays a vital role in suppressing hepatic VTG production at the time of ovulation (Nagahama 1994; Mull et al. 2010). Besides their role in female fish, both are also present in males playing an essential part in the sperm motility acquisition (17α, 20β-P) and regulation of spermiogenesis and spermiation in several teleost fishes (Nagahama 1994). Aromatase, a CYP19 encoded steroidogenic enzyme mediates the conversion of T to E_2 that further promotes hepatic VTG synthesis in fishes (Adebiyi et al. 2013). ARO activity is low in the testis, maintaining a higher serum concentration of T rather than E_2 in males (Folmar et al. 2001).

Triiodothyronine (T_3) and thyroxine (T_4), the key thyroid hormones (THs) secreted by the hypothalamic–pituitary–thyroid (HPT) axis elicit a diversity of physiological actions in fishes and serum concentrations are linked with changes in ambient temperature, photoperiod, nutritional status, activity level, and reproductive events that can vary across seasons in a species-specific manner (Peter 2011). These seasonal variations may act to support growth, seasonal activity, and reproductive development in fish (Blanton and Specker 2007). Cortisol is a principal glucocorticoid and an essential element of the stress response in fishes, released from the hypothalamic–pituitary–interrenal (HPI) axis playing a significant role in growth, osmoregulation and reproduction (Mommsen et al. 1999). Reactive oxygen species (ROS) production in fishes is widespread in the aquatic environment which is balanced by antioxidant defence system that protects from cellular injuries (Lesser 2006; Liu et al. 2015). Assessment of total antioxidant capacity (TAC) against ROS in fishes provides relevant biological information attributable to the ability to measure the combined effect of all antioxidants present in the plasma/serum (Erel 2004; Słowińska et al. 2013).

It is worth noting that so far the endocrine aspect of the seasonality of reproduction and related biomarkers has not been widely reported in the serum of *B. bendelisis*. Thus, the objective of this work was to investigate the seasonal changes in thyroid hormones (T_3 and T_4) and sex steroids (testosterone (T), estradiol (E_2), progesterone (P_4) and 16a, 20β-dihydroxyprogesterone (17,20β-P)) in male and female *B. bendelisis*, also to examine serum cortisol, VTG, ARO, and TAC in male and female *B. bendelisis*.

Materials and methods

Fish sampling, sampling site, and environmental parameters

Adult *B. bendelisis* males and females ($n = 144$; total catch = 520) were sampled seasonally from a tributary of River Kosi (at Ratighat area) situated in Kumaun hills of Uttarakhand, India (latitude of 29° 27.35′ N and 29° 27.26′ N and longitude of 79° 28.43′ E and 79° 28.48′ E) using cast net (9 m length, 9 m width, and 1/2 cm mesh size). Immediately after collection, fishes were taken to the Fish Nutrition and Physiology Laboratory of ICAR – Directorate of Coldwater Fisheries Research using 250 L plastic tanks having water from the sampling station with constant aeration. As the sex ratio of male and female *Barilius bendelisis* in the wild habitat is near to 1:1 as reported by Dobriyal and Singh (Dobriyal and Singh 1987) from Garhwal Himalaya India, no separate method is used for male and female collection. A total of 520 live

specimens were captured during five seasons, out of which 268 were females and 252 were males. In the laboratory during each season, 12 males and 12 females were randomly chosen from the total fish caught (average ± SE; male 22.19 ± 1.58 g; 12.74 ± 0.31 cm and female 9.84 ± 0.78 g; length 10.41 ± 0.22 cm) for blood collection and dissected after blood sampling for further sex confirmation. The size of all selected males and females was above the length of first maturation reported (7.0–7.5 cm) for this species in India (Nautiyal and Negi 2004). The studies were conducted during all the seasons; these were winter (January), spring (March), summer (May and July), rainy (September), and autumn (November) of the year 2014. The winter season lasted from mid-November to February, spring from March to April, summer from May to mid-July, rainy from mid-July to September, and the autumn lasted from October to mid-November (Sharma et al. 2017). Water quality parameters like temperature, pH, dissolved oxygen, alkalinity, ammonia, nitrate and nitrite of the sampling site were measured during each season. Temperature, pH, and dissolved oxygen were measured with a multi-parameter probe from Hanna (Hanna, 9828). Other water quality parameters like alkalinity, ammonia, nitrite and nitrate concentrations were estimated colourimetrically using Spectroquant® Test Kits (Spectroquant Multy colourimeter, Merk Millipore) following manufactures protocols (Sharma et al. 2017). The concentrations were expressed as parts per million.

Blood sampling

The body surface of the male and female *B. bendelisis* were cleaned with blotting paper to avoid any contamination. Blood was collected from the caudal vein of alive fish (without any anaesthesia) with a 26-gauge syringe without any anticoagulant and then transferred immediately to a dried 1.5 ml eppendorf tube. The blood in tubes was allowed to stand in tilting position to clot at room temperature for 30 min for serum separation. After clotting the blood, the yellow straw colour serum was carefully collected after centrifugation at 3000g for 5 min. Collected serum samples from 12 males and 12 females were then pooled into six samples ($n = 6$; each replicate had pooled samples of two fishes of either sex) of each sex in every season and then immediately frozen at − 20 °C deep freezer (Remi Electrotechnik limited, Vasai India) until further analysis (Sharma et al. 2017). All fish were handled and sacrificed following ethical guidelines on the care and management of laboratory animals and local regulations.

Hormone immunoassay, vitellogenin, and total antioxidant capacity estimation

Assessment of different hormones in the serum samples (17β-estradiol (E_2), progesterone (P_4), testosterone (T) and cortisol (C)) was carried out using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Cayman Chemical Company, Michigan, USA) and expressed as pg/ml. Serum total antioxidant capacity (TAC) was quantified using antioxidant assay kit (Cayman Chemical Company, MI, USA) and expressed as mM Trolox equivalents. Serum T_3 and T_4 were quantified using commercial ELISA kits from Diagnostic Automation/Cortez Diagnostics, Inc. (Calabasas, USA) and expressed as ng/ml and μg/dl respectively. Serum 17α, 20β-dihydroxyprogesterone (17α, 20β-P) and vitellogenin (VTG) were estimated using commercial ELISA Kit from MyBioSource, Inc. (San Diego, USA) and expressed as ng/ml and ng/ml. All the assays were performed following the assay kit manufactures procedures and methods described by earlier authors (Rice-Evans and Miller 1994; Wisdom 1976; Nash et al. 2000; Ismail et al. 2011; Jarvis et al. 2014). A standard curve was run for each ELISA plate, and all the samples ($n = 6$) and standards were analysed in duplicates. All the absorbance was measured by using Eon-C multimode microplate spectrophotometer (Biotek, USA). The detection limit/sensitivities of the assay was 20 pg/ml for E_2, 10 pg/ml for P_4, 6 pg/ml for T, 35 pg/ml for C, 0.06 ng/ml for 17α,20β-P, 0.2 ng/ml for T_3, 0.5 μg/dl for T_4, 5.0 ng/ml for VTG, 1.0 IU/L for ARO, and 0.044 mM for TAC. The intra-assay coefficients of variance for all assays were < 10%. The intra-assay coefficients of variance for all assays were < 10%. All the steroid concentrations presented in the text and in the figures are expressed as mean ± standard error of mean.

Enzyme assay

Serum aromatase (ARO) activity was estimated using fish-specific ELISA kit from MyBioSource, Inc. (San Diego, USA) following manufacturer's instructions. A standard curve was run for ELISA plate, and all the samples ($n = 6$) and standards ($n = 2$) were analysed. The absorbance was read by using Eon-C multimode microplate spectrophotometer (Biotek, USA). Serum aromatase activity was expressed as IU/L.

Statistical analysis

Data in the present study are presented as the mean ± standard error of the mean values (SEM). The differences for the entire hormone and other parameters data between different seasons for male and female were analysed using one-way analysis of variance (ANOVA) by Duncan's Multiple Range Test (DMRT). The correlation between studied indices was analysed by the Pearson coefficient for linear correlation at $P < 0.05$ and T test was used for determining differences in means between sexes. Ordinary least squares algorithm and Fisher's test were used to evaluate the linear regression between reproductive hormones and related biomarkers. Study

results were considered statistically significant at $P < 0.05$. Statistical analyses were performed using SPSS 20.0, and GraphPad Prism 6.0 Software was used to create graphs.

Results

Physico-chemical variables of water

The values of physico-chemical variables observed during the present study period are provided in Table 1. There was a significant difference in water temperature (one way ANOVA $F = 143{,}388$, $P < 0.0001$), dissolved oxygen (one way ANOVA $F = 333.9$, $P < 0.0001$), ammonia (one way ANOVA $F = 9.603$, $P = 0.0019$), nitrate (one way ANOVA $F = 4.516$, $P = 0.0242$) and pH (one way ANOVA $F = 11{,}759$, $P < 0.0001$) between seasons. Nitrite and alkalinity showed no significant difference (one way ANOVA $P > 0.05$) between seasons.

Seasonal visual observations in *B. bendelisis*

There was a marked sexual dimorphism observed between mature male and female *B. bendelisis* during the spring and rainy season in the present study. Males were larger than females in size. During spring and rainy season, males showed the following combinations of morphological characters: brightly coloured skin, fins brighter yellow in colour, enlarged fan-like pectoral and pelvic fins, pectoral fins with three outward rays thickened, expanded dorsal and anal fins, pectoral spine prominent and stiff and both lips including snout and lower jaw with prominent thick spiny tubercles (Fig. 1a). Furthermore, the males had rough surface due to spine-like miniature tubercles on head region and dorsolateral scales of the body. Females lack all these above mentioned prominent morphological characteristics (Fig. 1b). Moreover, spermiating males and ovulating females were observed with slight pressure on belly during spring and rainy seasons which was not evident in winter, summer and autumn season.

Linear regression assessment

Linear regression assessment between studied serum parameters in male and female *B. bendelisis* during different seasons showed significant regressions and shown in Table 2.

Estradiol and vitellogenin

Serum E_2 levels showed significant difference between seasons (overall one way ANOVA $F_{male} = 7.902$, $P = 0.0003$; $F_{female} = 337.9$, $P < 0.0001$). Both male and female showed a significant bimodal peaks (yet relatively low concentration of E_2 in males as compared with females) in E_2 during winter (January) (male 25. 06 ± 0.83; female 371.18 ± 11.76 pg/ml) and rainy (September) seasons (male 20.64 ± 4.07; female 230.61 ± 15.66 pg/ml) respectively. E_2 mean level in male and female declined to its lowest value during spring (March) (male 11.88 ± 0.56 pg/ml) and autumn (November) (female 5.55 ± 0.33 pg/ml; Fig. 2a) seasons. There was a significant difference in serum E_2 levels between male and female *B. bendelisis* across all seasons (*T* test, $P < 0.05$) except in the spring season (*T* test, $t = -1.317$, $P = 0.217$; Fig. 2a). In males, serum E_2 level is significantly positively correlated (Pearson's $P < 0.05$) with T, ARO, and VTG (Table 3). Similarly, in females positively correlated with VTG, ARO, T, and T_3, respectively (Table 4).

Serum vitellogenin (VTG) levels in females showed similar patterns of variation with E_2 along different seasons (overall one way ANOVA $F = 951.0$, $P < 0.0001$), peaked significantly during winter (January) (female 294.08 ± 2.13 ng/ml) and rainy (September) seasons (female 250.42 ± 2.66 ng/ml), reaching minimum levels in spring (March) season (136.08 ± 2.34 ng/ml) (Fig. 2b). Interestingly, VTG was also detected in male fish (relatively in low concentration as compared with females) throughout all seasons and vary significantly (overall one way ANOVA $F = 118.1$, $P < 0.0001$). Male VTG showed two small peaks during winter (January) (male 92.75 ± 1.02 ng/ml) and rainy (September) (male 68.50 ± 2.86 ng/ml) seasons similar to females but reached minimum levels in summer season (May and July) (20.21 ± 2.07 ng/ml) (Fig. 2b). A

Table 1 Water quality parameters of sampling site in different seasons during the study period

Seasons	Water temperature (°C)	Dissolved oxygen (mg L^{-1})	Ammonia–N (ppm)	Nitrite–N (ppm)	Nitrate–N (ppm)	Alkalinity (ppm)	pH
Winter	10.64 ± 0.017^{a}	10.62 ± 0.06^{e}	0.03 ± 0.003^{b}	0.003 ± 0.0006^{a}	0.60 ± 0.06^{b}	60.70 ± 0.30^{a}	8.81 ± 0.003^{e}
Spring	16.25 ± 0.012^{b}	9.75 ± 0.03^{d}	0.02 ± 0.000^{a}	0.002 ± 0.0007^{a}	0.53 ± 0.07^{b}	60.67 ± 0.33^{a}	8.75 ± 0.003^{d}
Summer	22.11 ± 0.003^{e}	8.28 ± 0.04^{a}	0.02 ± 0.002^{a}	0.004 ± 0.0003^{a}	0.38 ± 0.04^{ab}	60.55 ± 0.00^{a}	7.78 ± 0.007^{a}
Rainy	20.87 ± 0.012^{d}	8.90 ± 0.06^{b}	0.02 ± 0.000^{a}	0.004 ± 0.0007^{a}	0.40 ± 0.12^{ab}	60.93 ± 0.12^{a}	7.84 ± 0.003^{b}
Autumn	17.77 ± 0.012^{c}	9.55 ± 0.05^{c}	0.03 ± 0.000^{b}	0.002 ± 0.0006^{a}	0.20 ± 0.06^{a}	61.00 ± 0.06^{a}	8.66 ± 0.006^{c}

Values expressed as mean ± SE, $n = 3$. Different superscript letters (a–e) in the same column indicate significant difference (one way ANOVA, Duncan's test, $P < 0.05$)

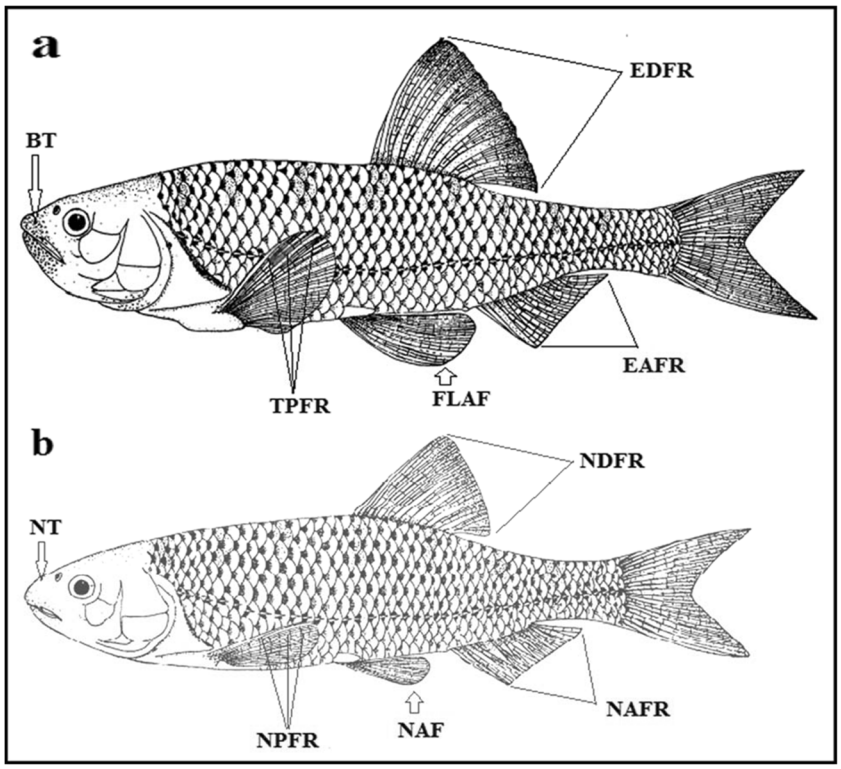

Fig. 1 Sexual dimorphism in male (**a**) and female (**b**) *Barilius bendelisis* (Figure modified from original source: Tilak et al. 1984) Abbreviations: BG breeding tubercles, TPFR thickened pectoral fin rays, EDFR expanded dorsal fin rays, FLAF fan like anal fin, EAFR expanded anal fin rays, NT no tubercles, NPFR normal pectoral fin rays, NDFR normal dorsal fin rays, NLAF normal anal fin, NAFR normal anal fin rays

significant difference in serum VTG levels between male and female *B. bendelisis* across all seasons studied (*T* test, $P = 0.000$). Vitellogenin level both in males and females displayed significant correlation (Pearson's $P < 0.05$) with E_2, T, ARO, and T_3 (Tables 3 and 4).

Testosterone and aromatase

Serum T levels and ARO activity showed similar trends with respect to E_2 and VTG and mean levels vary significantly between seasons (overall one way ANOVA Testosterone: $F_{male} =$

Table 2 Linear regression assessment between studied serum parameters in male and female *B. bendelisis*

Regression parameters	Sex	Equation	Relationship significance and correlation: positive/negative*
ARO - E_2	Male	$Y = 0.5998x + 1.3529$, $R^2 = 0.880$, $P = 0.018$	S; Positive
	Female	$Y = 6.5333x - 326.98$, $R^2 = 0.913$, $P = 0.011$	S; Positive
C - E_2	Male	$Y = -0.0116x + 51.123$, $R^2 = 0.947$, $P = 0.005$	S; Negative
	Female	$Y = -0.3822x + 1173.9$, $R^2 = 0.894$, $P = 0.015$	S; Negative
17α, 20β-P – P_4	Male	$Y = 213.11x + 288.05$, $R^2 = 0.971$, $P = 0.002$	S; Positive
	Female	$Y = 600.83x - 18.151$, $R^2 = 0.793$, $P = 0.042$	S; Positive
ARO - C	Male	$Y = -50.133x + 4254.5$, $R^2 = 0.869$, $P = 0.021$	S; Negative
	Female	$Y = -16.511x + 3886.1$, $R^2 = 0.952$, $P = 0.005$	S; Negative
T - VTG	Male	$Y = 0.2548x + 11.268$, $R^2 = 0.906$, $P = 0.013$	S; Positive
ARO - VTG	Female	$Y = 2.839x - 1.1419$, $R^2 = 0.935$, $P = 0.007$	S; Positive
ARO - T4	Female	$Y = -0.0224x + 13.167$, $R^2 = 0.919$, 0.010	S; Negative
C - VTG	Female	$Y = -0.1686x + 657.89$, $R^2 = 0.944$, $P = 0.006$	S; Negative
E_2 - T	Female	$Y = 0.6001x + 33.813$, $R^2 = 0.850$, $P = 0.026$	S; Positive
E_2 - VTG	Female	$Y = 0.4242x + 142.3$ $R^2 = 0.977$, $P = 0.001$	S; Positive
T_4 - VTG	Female	$Y = -115.79x + 1540.5$, $R^2 = 0.850$, $P = 0.026$	S; Negative
TAC - T	Muscle	$Y = -869.95x + 475.66$, $R^2 = 0.924$, $P = 0.009$	S; Negative

ARO aromatase; E_2 17β-estradiol; *C* cortisol; *17α, 20β-P* 17α, 20β-dihydroxyprogesterone; P_4 progesterone; *T* testosterone; *VTG* vitellogenin; T_4 Thyroxine; *TAC* total antioxidant capacity; *S* significant correlation

*Only significant regression is given; $P < 0.05$

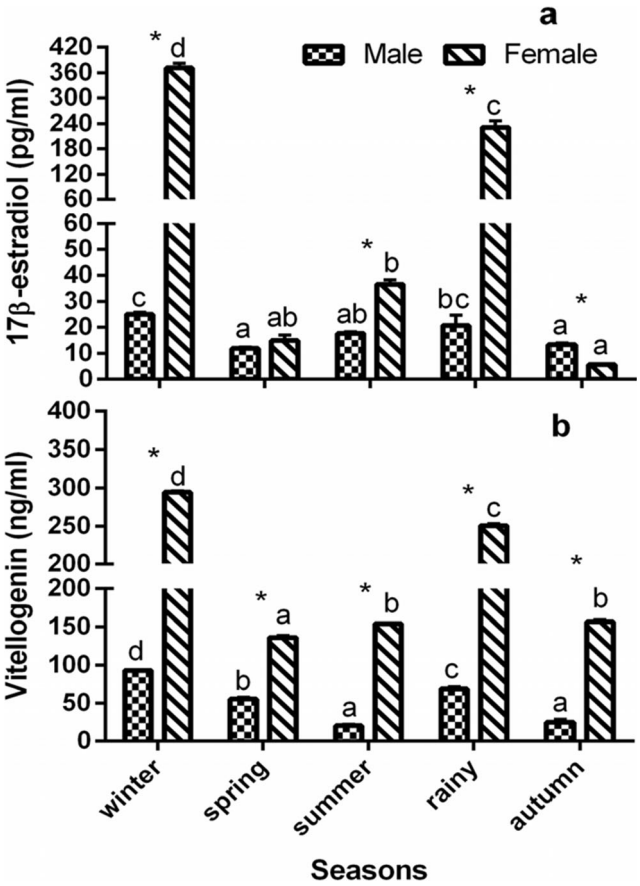

Fig. 2 Seasonal changes in serum 17β–estradiol (**a**) and vitellogenin (**b**) levels in male and female *Barilius bendelisis*. Different superscripts (a, b, c, d) in each series under each panel signify statistical differences (one way ANOVA, Duncan's test, $P < 0.05$). Values expressed as mean ± SE; $n = 6$. Asterisk in the series (if any) in each panel implies sex-specific significant difference (T test, $P < 0.05$)

145.1, $P < 0.0001$; $F_{female} = 373.6$, $P < 0.0001$; Fig. 3a, b). Peaked serum T level was observed in winter (January) (male 313.60 ± 17.89; female 278.62 ± 8.62 pg/ml) followed by rainy season (September) (male 224.21 ± 8.72; female 136.63 ± 7.97 pg/ml), and declined significantly (one way ANOVA $P < 0.05$) in spring (March) (male 152.11 ± 6.98; female 104.23 ± 2.92 pg/ml) and summer (May and July) (male 96.40 ± 0.75; female 35.54 ± 1.32 pg/ml) reaching lowest in autumn (November) (male 18.94 ± 0.46; female 9.43 ± 0.84 pg/ml) (Fig. 3a). Sexes showed significant difference (T test, $P = 0.000$) in all seasons except in winter (T test $t = 1.759$, $P = 0.109$). Both in male and female, T presented significant positive correlation (Pearson's $P < 0.01$) with VTG, ARO, and E_2. Furthermore, female T was positively correlated with T_3 (Tables 3 and 4).

Males and females showed similar seasonal trend in ARO activity (Fig. 3b) but relatively low concentration of ARO activity in males as compared with females. Among male and female ARO activity, the present study also found significant differences in mean values by seasons (overall one way ANOVA $F_{male} = 65.31$, $P < 0.0001$; $F_{female} = 134.1$, $P < 0.0001$ Fig. 3b). ARO activity was significantly (one way ANOVA $P < 0.05$) higher in winter (January) (male 36.86 ± 0.63; female 98.85 ± 1.30 IU/L) and rainy (September) season (male 34.73 ± 2.16 P; female 91.18 ± 4.30 IU/L) reaching its lowest level in spring (March) (male 16.48 ± 0.32 IU/L; female 45.88 ± 0.75 IU/L). Serum ARO activity levels between male and female showed significant difference across all seasons (T test, $P = 0.000$). ARO correlation was significant (Pearson's $P < 0.01$) with T, E_2, and VTG in both sexes (Tables 3 and 4). Additionally, females also showed positive correlation with T_3.

17α, 20β dihydroxy progesterone, and progesterone

Serum levels of 17α, 20β-P also fluctuate significantly among different seasons (overall one way ANOVA $F_{male} = 763.8$, $P < 0.0001$; $F_{female} = 935.4$, $P < 0.0001$; Fig. 4a, b). Mean

Table 3 Pearson's correlation analyzed between reproductive hormones and related biomarkers in male *B. bendelisis*

Parameters	E_2	P	T	17α, 20β-P	ARO	VTG	C	T3	T4	TAC
E_2	1									
P	− 0.076	1								
T	**.611****	0.224	1							
17α, 20β-P	− 0.112	**.898****	0.332	1						
ARO	**.607****	− 0.234	**.665****	− 0.082	1					
VTG	**.457***	0.202	**.908****	0.347	**.595****	1				
C	**− .673****	0.299	**− .730****	0.189	**− .819****	**− .648****	1			
T3	− 0.201	**− .742****	**− .537****	**− .862****	− 0.346	**− .570****	0.163	1		
T4	− 0.354	− 0.184	− 0.214	− 0.258	**− .597****	− 0.163	0.349	**.601****	1	
TAC	− 0.341	− 0.036	**− .764****	− 0.152	− 0.301	**− .838****	**.494****	0.233	− 0.255	1

E_2 17β-estradiol; *P* progesterone; *17α, 20β-P* 17α, 20β-dihydroxyprogesterone; *T* testosterone; *ARO* aromatase; *VTG* vitellogenin; *C* cortisol; *TAC* total antioxidant capacity

** Correlation is significant at the 0.01 level (2-tailed); * Correlation is significant at the 0.05 level (2-tailed)

Table 4 Pearson's correlation analyzed between reproductive hormones and related biomarkers in female *B. bendelisis*

Parameters	E_2	P	T	17α, 20β-P	ARO	VTG	C	T3	T4	TAC
E_2	1									
P	0.019	1								
T	**.914****	0.272	1							
17α, 20β-P	0.155	**.880****	0.241	1						
ARO	**.915****	−0.082	**.760****	0.16	1					
VTG	**.981****	−0.059	**.851****	0.13	**.941****	1				
C	**−.859****	−0.016	**−.717****	−0.259	**−.875****	**−.885****	1			
T3	**.587****	−0.028	**.509****	0.067	**.492****	**.653****	**−.553****	1		
T4	**−.823****	0.127	**−.608****	−0.189	**−.907****	**−.879****	**.896****	**−.554****	1	
TAC	**−.782****	−0.306	**−.936****	−0.172	**−.571****	**−.713****	**.540****	**−.471****	**.389***	1

E_2: 17β-estradiol; P: progesterone; 17α, 20β-P - 17α, 20β-dihydroxyprogesterone; T: testosterone; ARO – aromatase; VTG: vitellogenin; C: cortisol; TAC: total antioxidant capacity

**Correlation is significant at the 0.01 level (2-tailed); *correlation is significant at the 0.05 level (2-tailed)

17α, 20β-P levels in both the sexes remained relatively low for autumn (November) (male 0.25 ± 0.02; female 0.31 ± 0.03 pg/ml) but abruptly peaked in rainy (September) (male 1.72 ± 0.04; female 1.92 ± 0.02 pg/ml) and spring (March) (male 1.66 ± 0.03; female 1.70 ± 0.03 pg/ml) seasons exhibiting dual peak pattern. Significant difference (*T* test

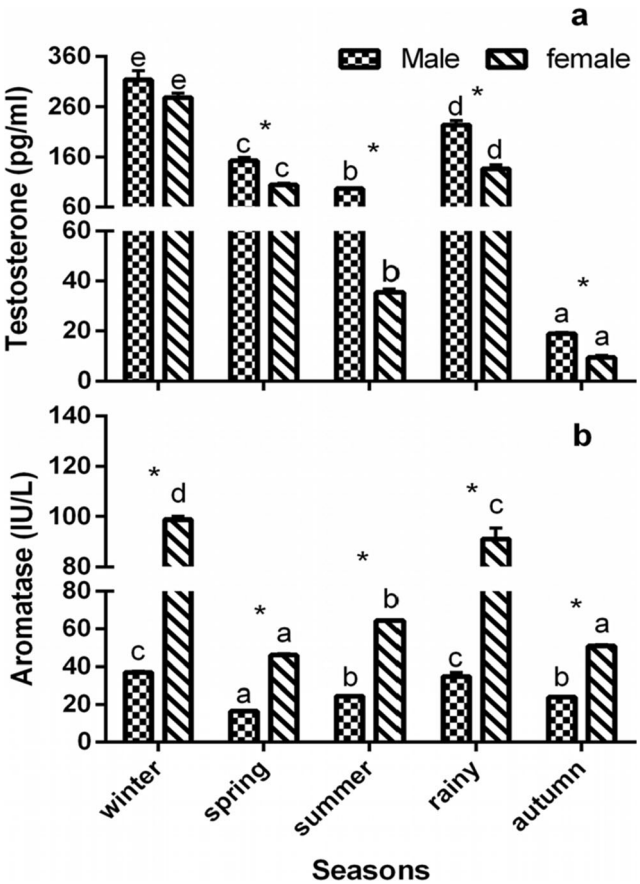

Fig. 3 Seasonal changes in serum testosterone (**a**) level and vitellogenin (**b**) activity in male and female *Barilius bendelisis*. Different superscripts (a, b, c, d, e) in each series under each panel signify statistical differences (one way ANOVA, Duncan's test, $P < 0.05$). Values expressed as mean ± SE; $n = 6$. Asterisk in the series (if any) in each panel implies sex-specific significant difference (*T* test, $P < 0.05$)

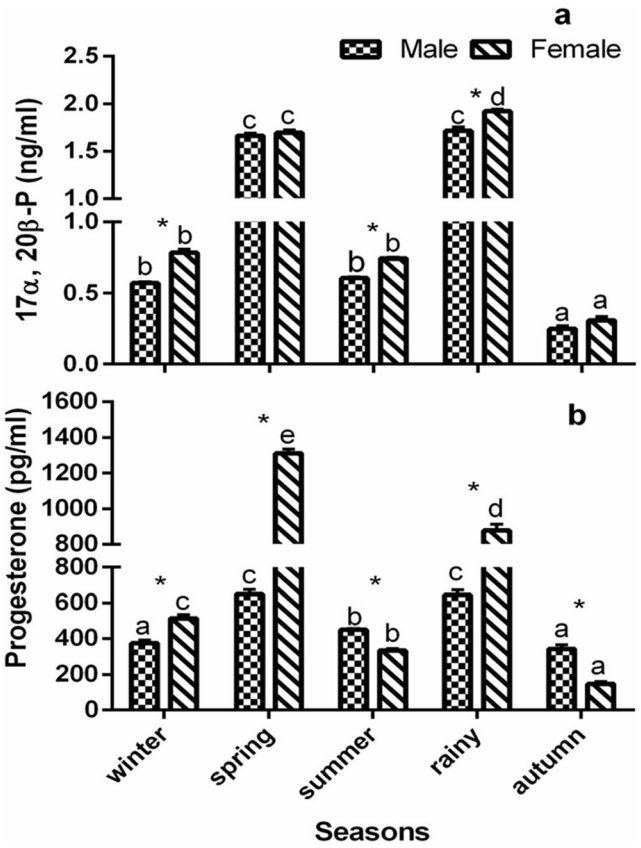

Fig. 4 Seasonal changes in serum 17α, 20β-dihydroxyprogesterone (**a**) and progesterone (**b**) levels in male and female *Barilius bendelisis*. Different superscripts (a, b, c, d, e) in each series under each panel signify statistical differences (one way ANOVA, Duncan's test, $P < 0.05$). Values expressed as mean ± SE; $n = 6$. Asterisk in the series (if any) in each panel implies sex-specific significant difference (*T* test, $P < 0.05$)

$P < 0.05$) between sexes was found during winter ($t = -8.725$, $P = 0.000$), summer ($t = -11.667$, $P = 0.000$) and rainy ($t = -4.418$, $P = 0.001$) seasons (Fig. 4a). A significant positive correlation was observed between 17α, 20β-P and P_4 in both the sexes (male $r = 0.898$, $P < 0.01$; female $r = 0.880$, $P < 0.01$). Males showed significant negative correlation with T_3 (male $r = -0.863$, $P < 0.01$).

The seasonal patterns of P_4 level followed the same trends described above for 17α, 20β-P (Fig. 4a, b) with mean levels significantly varying across the seasons (overall one way ANOVA $F_{male} = 43.78$, $P < 0.0001$; $F_{female} = 407.6$, $P < 0.0001$; Fig. 4b). P_4 level increased significantly (one way ANOVA $P < 0.05$) reaching maximum level during spring (March) (male 651 ± 26.06; female 1310.47 ± 25.29 pg/ml) followed by rainy season (September) (male 644.41 ± 29.97; female 876.71 ± 36.00 pg/ml) displaying two strong peaks in both sexes than those of other seasons (Fig. 4b). The minimum level of P_4 was observed during autumn (November) (male 342.47 ± 23.84; female 148.14 ± 12.24 pg/ml) in both males and females. A sex-specific difference in P_4 level was significant (T test, $P < 0.05$) across all seasons. Serum P_4 levels in both the sexes were significantly positively correlated (Pearson's $P < 0.01$) with 17α, 20β-P. However, in males, P_4 was negatively correlated (Pearson's $P < 0.01$) with T_3 (Tables 3 and 4).

Triiodothyronine and thyroxine

Serum T_3 levels in both sexes differ significantly (overall one way ANOVA $F_{male} = 671.4$, $P < 0.0001$; $F_{female} = 32.72$, $P < 0.0001$) between different seasons (Fig. 5a, b). Higher level of serum T_3 in males was evident in summer (1.36 ± 0.004 ng/ml) followed by autumn (1.35 ± 0.008 ng/ml), whereas in females during winter (1.35 ± 0.014 ng/ ml) and rainy season (1.32 ± 0.009 ng/ml), respectively. Minimum level of T_3 reached during rainy season (0.59 ± 0.015 ng/ml) in male and during summer (0.91 ± 0.011 ng/ml) in females (Fig. 5a). Difference in T_3 levels between sexes was significant (T test $P < 0.05$) in all seasons except in spring season (T test $t = -1.854$ $P = 0.093$). Males displayed significant (Pearson's $P < 0.05$) positive correlation with T_4 ($r = 0.599$, $P < 0.01$), whereas in females, positive correlation of T_4 was noticed with E_2, T, VTG, and VTG.

Level of serum T_4 also vary significantly (overall one way ANOVA $F_{male} = 72.20$, $P < 0.0001$; $F_{female} = 81.15$, $P < 0.0001$; Fig. 5b) among different seasons. Significantly (one way ANOVA $P < 0.05$) higher level of T_4 was found in spring (male 12.34 ± 0.005; female 12.25 ± 0.03 μg/dl), and the lowest level of T_4 was observed during the rainy season (male 8.55 ± 0.299; female 10.93 ± 0.111 μg/dl) in both males and females. Except winter (T test $t = 1.536$, $P = 0.156$), difference in serum T_4 between sexes was evidently significant (T test $P < 0.05$). Significant positive correlation (Pearson's $P < 0.05$) was noticed with T_3 in males, whereas females showed no significant (Pearson's $P > 0.05$) positive correlation with any hormone assayed in the present investigation.

Total antioxidant capacity

Serum TAC was significantly (overall one-way ANOVA $F_{male} = 254.5$, $P < 0.0001$; $F_{female} = 241.1$, $P < 0.0001$) influenced by seasonal changes (Fig. 6a). Higher TAC value was observed during summer (male 0.49 ± 0.01; female 0.52 ± 0.01 mM) and autumn (November) (male 0.46 ± 0.005; female 0.51 ± 0.01 mM), whereas the least value was observed in winter (January) season (male 0.28 ± 0.002; female 0.23 ± 0.01 mM). In the rainy season (T test $t = -1.112$ $P = 0.292$), difference in TAC level between male and female was significant (T test $P < 0.05$) when compared to other seasons. TAC in males and females showed significant ($P < 0.05$) positive correlation with C (Tables 3 and 4).

Cortisol

Level of serum cortisol fluctuated significantly (overall one-way ANOVA $F = 32.08$, $P < 0.0001$; $F = 36.06$, $P < 0.0001$) between studied seasons (Fig. 6b). Cortisol level peaked during spring (March) (male 3365.53 ± 100.76; female 3089.91 ± 75.92 pg/ml) and autumn (November) (male 3139.28 ± 86.54; female 3009.45 ± 93.59 pg/ml) in both the sexes followed by a minimum level during winter season (January) (male 2188.16 ± 58.38 pg/ml) in males and rainy season (female 2285.52 ± 65.98 pg/ml) in females. Difference between sexes was significant only during rainy season (T test $t = 4.548$, $P = 0.001$; Fig. 6b). Cortisol level in both sexes showed significant positive correlation (Pearson's $P < 0.05$) with TAC only (Tables 3 and 4).

Discussion

Based on the present study results and considering spermiating males and ovulated females as evidence of spawning, it is possible to suggest that *B. bendelisis* from Indian Himalaya exhibit bimodal pattern of spawning during late spring (late-March and early April) and rainy (late-September) season respectively. It has been previously suggested from different river, Nayar (Indian Himalaya), that *B. bendelisis* presents a major spawning period during April (spring season) and a minor one in September (rainy season) (Dobriyal and Singh 1987) which was confirmed by the present study. No evidence of spermiating males and ovulated females were observed in other seasons. All the hormones and aromatase enzyme activity assessed during the present study showed fluctuations more for reproductive activity (Dobriyal and Singh 1987) rather than physico-chemical

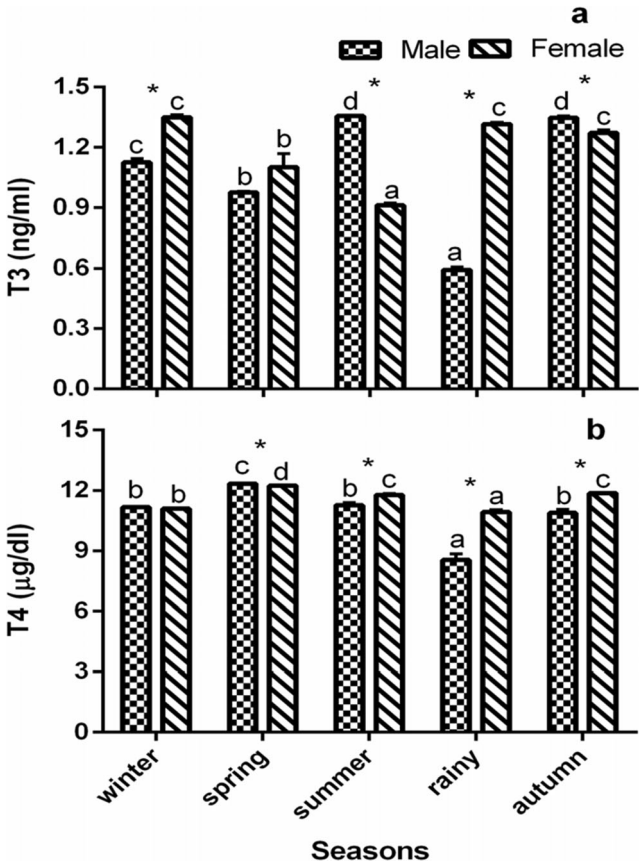

Fig. 5 Seasonal changes in serum triiodothyronine (**a**) and thyroxine (**b**) levels in male and female *Barilius bendelisis*. Different superscripts (a, b, c, d) in each series under each panel signify statistical differences (one way ANOVA, Duncan's test, $P < 0.05$). Values expressed as mean ± SE; $n = 6$. Asterisk in the series (if any) in each panel implies sex-specific significant difference (T test, $P < 0.05$)

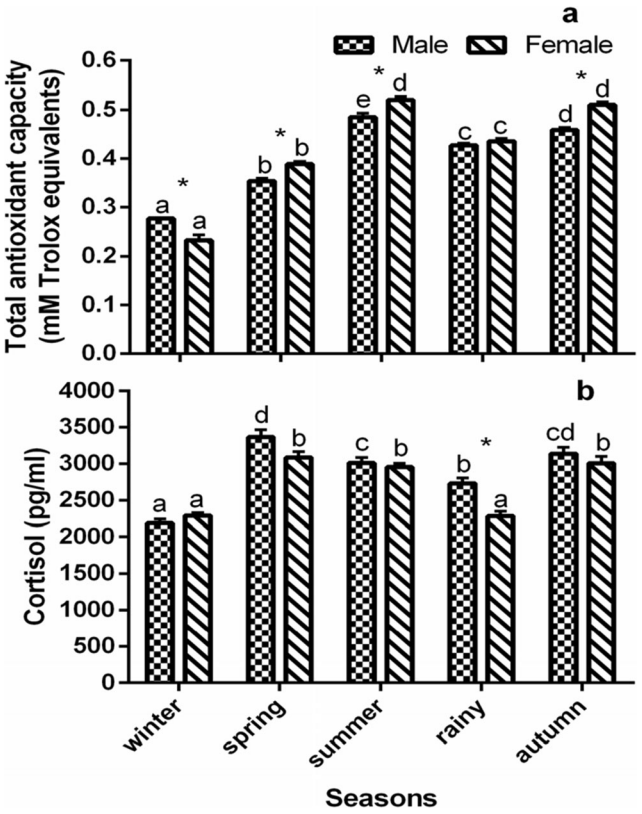

Fig. 6 Seasonal changes in serum total antioxidant capacity (**a**) and cortisol (**b**) levels in male and female *Barilius bendelisis*. Different superscripts (a, b, c, d, e) in each series under each panel signify statistical differences (one way ANOVA, Duncan's test, $P < 0.05$). Values expressed as mean ± SE; $n = 6$. Asterisk in the series (if any) in each panel implies sex-specific significant difference (T test, $P < 0.05$)

variables. The present study provides new data on the seasonal changes in vitellogenin, aromatase, cortisol, reproductive and thyroid hormones in a wild population of *B. bendelisis* from the Indian Himalaya which has not been previously characterized in this cyprinid.

Physico-chemical variables of water

In all seasons, ammonia, dissolved oxygen, alkalinity, nitrite, nitrate, and pH were within the optimum limit and related to respective seasons (Sharma et al. 2017).

Seasonal visual observations in *B. bendelisis*

Marked sexual dimorphism was observed in the present study as per the earlier study of Dobriyal and Singh (1987). As tubercles were observed only in mature and male fishes, therefore, they are considered as breeding tubercles and peculiar to the males of *B. bendelisis* as supported by the recent study of Venkataramanan et al. (2016).

Estradiol and vitellogenin

E_2 and T levels in male and female *B. bendelisis* exhibit a very similar seasonal pattern, although the peak of both steroids during the winter (January) is much more pronounced than rainy (September) season. The presence of two peaks of E_2 in the serum of female *B. bendelisis* in the winter (January) and rainy (September) season (Fig. 2a) indicated *B. bendelisis* undergo vitellogenesis during this period. Many studies supported the fact that vitellogenesis is initiated by rising in E_2 levels (Adebiyi et al. 2013; Miura et al. 2007; Barcellos et al. 2001). These findings are consistent with the recognized role of E_2 in stimulating hepatic synthesis of the yolk protein precursor, vitellogenin (Adebiyi et al. 2013; Nagahama 1994), which also show a similar trend for E_2. The same observation was reported earlier by several workers in different fish species (Larsson et al. 2002; Vazirzadeh et al. 2014; Heidari et al. 2010). The E_2 level was also detected in male fish, revealing a pronounced seasonal variation similar to female *B. bendelisis* but in low concentration (Fig. 2a) suggesting its role in spermatogenesis. The similar role of E_2 in spermatogenesis is reported previously by numerous authors in teleost fishes

(Chaves-Pozo et al. 2008; Amer et al. 2001; Miura and Miura 2001).

VTG is a female-specific serum protein and yolk proteins precursor produced in liver in response to circulating E_2 in fishes (Adebiyi et al. 2013; Larsson et al. 2002). The E_2 level increases intensely during the oocytes vitellogenesis and reduces upon completion of vitellogenesis (Lubzens et al. 2010). In the present study, bimodal pattern (winter—January and rainy—September) of VTG level in female was observed (Fig. 2b) that is well correlated with the increase in E_2 level. This suggest that *B. bendelisis* undergo active vitellogenesis during the winter and rainy season since the E_2 was reported to initiate vitellogenesis (Barcellos et al. 2001; Larsson et al. 2002; Ismail et al. 2011; Berg et al. 2004). In teleost fishes, males do not express VTG, although they contain the gene for VTG (Scott et al. 2006). VTG in the male of different fish species have been reported before by several researchers and employed an indicator of estrogen (endocrine disrupting chemicals, EDCs) exposure in the environment/food chain (Scott et al. 2006; Matozzo et al. 2008). Interestingly, the present study also detects VTG level in male *B. bendelisis* during all seasons (Fig. 2b) but in a lower concentration than females displaying two small peaks during winter (January) and rainy (September) seasons. There is no previous evidence that the presence of VTG in the serum of males is a natural part of the life cycle or indeed caused by a direct outcome of exogenous EDCs or indirectly via the endogenous secretion of E_2. In the present study, the presence of VTG in males does not seem to be associated with known point sources of endocrine disruptors, but cumulative and persistent weak EDCs are almost undoubtedly present in the freshwater environment. Their effect would be difficult to establish in an environment dominated by short-lived EDCs as compared to EDCs originating from sewage treatment plants, pulp mills, and factories. It is therefore likely that male *B. bendelisis* pick up estrogenic endocrine disruptors through the food chain due to carnivorous feeding habit (Nautiyal and Negi 2004) that make their way in the aquatic environment through agriculture run-off and non-point sources (Scott et al. 2006; Jobling and Tyler 2003). As will be evident from the above discussions, substantial evidence is still needed that raised VTG level in male *B. bendelisis* is due to endocrine disruption. One obstacle to obtaining this evidence will be that, when sampling in the wild habitat, one cannot be sure that any particular site is normal. In other words, it may substantiate difficult to find a control population of *B. bendelisis* that has not in some way been affected by human activity.

Testosterone and aromatase

In teleosts, T increases gradually as spermatogenesis proceeds and decrease at spermiation (Young et al. 2005). Serum T level observed in the present study peaks during winter (January) and rainy (September) season (Fig. 3a) which indicates that male *B. bendelisis* undergo active spermatogenesis during these seasons as already been reported in different fish species (Dobriyal and Singh 1987; Young et al. 2005). Testosterone, a male-specific androgen, is also present in female fish and roles in ovarian maturation (Young et al. 2005). In females, T level displayed similar fluctuation for male fish and peaked during the winter (January) and rainy (September) season (Fig. 3a), respectively. This pattern of T in females was possibly due to testosterone involvement in oocyte growth and vitellogenin synthesis (Vazirzadeh et al. 2014; Fostier et al. 1983). Fostier et al. (1983) reported that T acts as a precursor of E_2 and is released in serum when no longer required for aromatization. So aromatization of T to E_2 is likely the reason behind the observed pattern during the present study. T and E_2 levels in male and female fish during the spring (March) decrease considerably (Young et al. 2005) whereas in rainy (September) season maintain a higher level. This behavior might be due to the presence of some oocytes undergoing late vitellogenesis in females and final maturation of spermatocytes during late spermatogenesis in males since sampling was done little earlier during the rainy season. This further suggests late spawning in rainy (September) season in *B. bendelisis* (Miura et al. 2007; Vazirzadeh et al. 2014; Heidari et al. 2010). The T and E_2 profile of *B. bendelisis* during different seasons was in parallel with previous research on other cyprinid species with asynchronous breeding pattern such as tench (*Tinca tinca*), river catfish (*Hemibagrus nemurus*) and common carp (*Cyprinus carpio carpio*) (Miura et al. 2007; Vazirzadeh et al. 2014; Heidari et al. 2010).

In teleost fishes, synthesis of E_2 from T by aromatization is performed by aromatase (ARO), a member of the cytochrome P_{450} family responsible for promoting VTG synthesis indirectly through E_2 action (Adebiyi et al. 2013; Nagahama 1994). The present study observed significant fluctuations in ARO activity in both males and females with higher activity during winter (January) and rainy (September) season followed by the lowest activity in spring (March) and also showed significant correlation with T, E_2, and VTG (Fig. 3b). This pattern of serum aromatase is well correlated with the increase in E_2 level that is required for active vitellogenesis and spermatogenesis during winter (January) and rainy (September) seasons in female and male *B. bendelisis* respectively (Adebiyi et al. 2013; Young et al. 2005; Nagahama 1994; Senthilkumaran et al. 2004; Aggarwal et al. 2014). Moreover, decrease in ARO activity during spring (March) is possibly due to influence of higher P_4 and 17,20β-P levels for suppressing the E_2 secretion to attain final oocyte and sperm maturation (Senthilkumaran et al. 2004). On the contrary, in the rainy season, ARO activity showed a stable increase that might be due to E_2 requirement to sustain some oocytes present undergoing late vitellogenesis in females and spermatocytes in final maturation stage of spermatogenesis in

males (Miura et al. 2007; Vazirzadeh et al. 2014). ARO activity in the male was lower than females suggesting their possible role in maintaining a higher serum concentration of testosterone rather than estradiol (Folmar et al. 2001). Results of the present study on ARO, E_2, and VTG also suggested their coordinated pattern with respect to the reproductive activity in *B. bendelisis*. The current study findings are in parallel with previous research on other species such as Japanese eel (*Anguilla japonica*) and gilthead seabream (*Sparus aurata L.*) (Chaves-Pozo et al. 2008; Miura and Miura 2001).

17α, 20β dihydroxy progesterone and progesterone

17α, 20β-P is well known as a maturation-inducing hormone (MIH) in the diversity of teleosts, and spawning is associated with elevated serum concentrations of 17α, 20β-P, which trigger final oocyte maturation (Young et al. 2005; Barcellos et al. 2001; Nagahama 1994). In the present study, there were two peaks of 17α, 20β-P serum levels both in males and females (Fig. 4a). A significant peak was observed in the spring (March), another small peak during rainy (September), suggesting that both seasons are favourable spawning seasons for *B. bendelisis*. Previous studies also reported the elevated level of 17α, 20β-P during the spawning seasons in gudgeon (Rinchard et al. 1993), Caspian kutum (Heidari et al. 2010), and common carp (Folmar et al. 2001). The key role of 17α, 20β-P as maturation-inducing hormone and in meiosis resumption in fishes is well-known as it is shown that peak 17α, 20β-P production occur at and about the time of ovulation and peak sperm production in female and male fishes (Young et al. 2005; Nagahama 1994; Nagahama and Yamashita 2008; Scott et al. 2010).

P_4 in teleost fishes remained elevated at or around the time of ovulation/spermiation, peaking during late ovulatory/spermiation phase and then returning to basal levels after ovulation/spermiation (Mull et al. 2010; Tricas et al. 2000). The serum levels of P_4 in both the sexes increased significantly during the spring (March) and rainy season (September) showing the bimodal pattern as compared to other seasons (Fig. 4b). The pronounced surge in serum P_4 levels in the spring (March) and rainy (September) seasons in correlation with significantly enhanced 17α, 20β-P levels suggest their collective role in the final oocyte maturation in females (pre-ovulatory surge) and spermiation (pre-spermiation surge) induction in males of *B. bendelisis* (Scott et al. 2010). The predominance of P_4 levels for a short period may indicate their indirect involvement in final oocyte maturation through 17α, 20β-P during the spring (March) and rainy (September) seasons as it is a precursor of many steroids including 17α, 20β-P (Adebiyi et al. 2013; Nagahama 1994). The changes in P_4 levels indicate their role analogous to that of 17α, 20β-P in *B. bendelisis* following to their conversion because 17α, 20β-P plays a critical role in oocyte meiosis in several teleost's (Adebiyi et al. 2013; Nagahama and Yamashita 2008). P_4 and 17α, 20β-P have been displayed to suppress hepatic VTG production preventing further follicular development and spermatogenesis till P_4 concentrations decline following ovulation (Young et al. 2005; Mull et al. 2010). This role is achieved by switching steroidogenic pathway from the production of predominantly E_2 by decreasing expression of P_{450} aromatase to the production of maturation-inducing steroids (17α, 20β-P) which is prerequisite for the oocyte and sperm to undergo final maturation (Senthilkumaran et al. 2004; Nagahama and Yamashita 2008). Similar results were observed during the present study in which increase in P_4 and 17α, 20β-P levels during spring (March) and rainy (September) season followed by a substantial decrease in serum ARO activity which supports the above hypothesis.

Triiodothyronine and thyroxine

Thyroid hormones exert a plethora of physiologic actions in fishes which extend its effects on growth, development, and reproduction (Blanton and Specker 2007; Raine 2011). As well, seasonal cycles of water temperature, day length, and other seasonal parameters mostly make difficulties in interpreting the thyroid results (Raine 2011; Comeau and Campana 2006). Male and female *B. bendelisis* in the present study presented a different pattern (Fig. 5a) of serum T_3 secretion which exhibited two peaks in male coinciding with summer and autumn (November) and three peaks in females coinciding with winter (January), rainy (September), and autumn (November). T_3 is also known to suppress estrogen synthesis in many fishes by reducing expression of gonadal aromatase (Peter 2011). However, the present study results did not support such previous hypothesis in *B. bendelisis* as T_3 and E_2 showed a similar pattern (in spite of antagonistic pattern) of secretion mainly during winter (January) and rainy (September) seasons when E_2 level peaked. The present study also observed peak level of T_3 during winter (January) and rainy (September) season in females suggesting its possible role in vitellogenesis (Raine 2011; Comeau and Campana 2006). This pattern of T_3 is also seen in fish species with short breeding cycles where thyroid hormones increase during the early stages (vitellogenesis) of reproductive cycle (Raine 2011). Slightly lower level of T_3 during spring (March) and rainy (September) season might be due to its role in final maturation given the fact that falling TH levels are likely required for the final maturation of the ovaries (Comeau and Campana 2006). A higher level of T_3 during the summer and autumn (November) may be related with growth phase of the life cycle, i.e. corresponds with a period of minimal gonadotropic function and maximum somatotropic activities (Peter 2011; Comeau and Campana 2006).

T_4 showed little fluctuations during different seasons in the present study and found to be correlated with T_3 (in males). A

previous study reported an increase in blood T_4 during spawning season in females (Raine 2011; Comeau and Campana 2006). In this study, the highest T_4 level was noticed during the spring (March) suggesting a possible role in the final maturation in both the sexes whereas in the rainy season (September) T_4 level decreased as compared to other seasons (Raine 2011; Comeau and Campana 2006; Fig. 5b). Higher T_4 level during summer and autumn suggests its possible role in somatic growth during the higher temperature months after spawning activity (Peter 2011). There was no uniform pattern in T_3 and T_4 secretion, but these differences may be species-specific or may be attributable to variations in water temperature, nutritional status, developmental stage, photoperiod and the interface with other hormones (Rolland 2000). It was observed that interactions between the thyroidal and reproductive systems in *B. bendelisis* seemed reasonable for maintaining certain phases of the reproductive cycle.

Total antioxidant capacity

In fishes, determining TAC offers an index of the sum of the activities of all antioxidants (Luty-Frackiewicz et al. 2006; Kadam et al. 2010). Regarding TAC, it has been reported previously that deficiencies in any component of the antioxidant defense system can cause a reduction in the TAC of an organism (Erel 2004; Luty-Frackiewicz et al. 2006). In the present study, increasing trend was observed in TAC level from winter to summer and decreased slightly during rainy and again rises in autumn season (Fig. 6a). Moreover, TAC level was strongly correlated with water temperature. This trend suggests that *B. bendelisis* could have the better antioxidant potential to resist oxidation processes during warm seasons, i.e. spring, summer, rainy, and autumn than the colder season, i.e. winter. The lower level of TAC during winter (January), spring (March), and rainy (September) as compared to other seasons may be due to low feeding intensity in winter (January) due to low temperature and less feeding. During summer and autumn seasons (post-spawning seasons for *B. bendelisis*), there was an increase in feeding intensity after exhaustive breeding which may have contributed to higher serum TAC. Also, there is a possibility that lack of some components of antioxidative status in winter season may contribute to lower TAC level (Erel 2004; Luty-Frackiewicz et al. 2006). There is no parallel study on seasonal TAC in the literature to substantiate the present findings.

Cortisol

Cortisol is a multifaceted hormone (principal glucocorticoid) involved in carbohydrates, protein and lipid metabolism, an essential component of the stress response, and osmoregulation (Mommsen et al. 1999; Pankhurst 2011; Ray and Sinha 2014). Maximum cortisol level observed in *B. bendelisis* during the present study was 3365.53 pg/ml for male and 3089.91 pg/ml for female (Fig. 6b), which is less than 10,000 pg/ml (for undisturbed wild teleosts). This level suggests that the pattern observed was a natural part of their life cycle and not due to environmental or sampling stress (Pankhurst 2011). Since cortisol is multifunctional hormone in fishes, linking it with a particular event is somewhat difficult. As glucocorticoid, the pattern of cortisol in the current study is justified by a similar seasonal pattern of glucose level observed in male and female *B. bendelisis* (Sharma et al. 2017) suggesting its direct role in glucose metabolism during different seasons (Mommsen et al. 1999; Pankhurst 2011; Ray and Sinha 2014). The whole process regulates the substrate level (glucose) to produce enough energy according to the demand of fish in different seasons of the year. The role of cortisol in fish reproduction was also reported earlier by several authors (Mommsen et al. 1999; Berg et al. 2004; Sisneros et al. 2004). There was an inverse relationship of cortisol with E_2, VTG, and T in the present study in different seasons as evident by regression and correlation analysis and reported previously by many researchers (Berg et al. 2004). Correspondingly, serum cortisol upregulation in both sexes was observed during the spring (March) season suggesting its role in ovulation and spermiation (Sisneros et al. 2004). Therefore, changes seen in cortisol level in the present study are related to glucose metabolism and reproductive activity during different seasons in *B. bendelisis*.

Conclusion

The present study provides the first analysis of male and female *Barilius bendelisis* seasonal pattern of serum steroid hormones, thyroid hormones, vitellogenin, aromatase activity, and total antioxidant capacity that may have been related to gonadal development. Significant correlation and regression analysis between different steroid hormones, VTG levels including ARO activity in different seasons suggest their coordinating pattern in regulating reproductive activity in *B. bendelisis*. This study confirms that *B. bendelisis* is a multiple spawner, spawning occurs in the late-spring (March) and late-rainy (September) seasons and a substantial direct physiological evidence of a dual ovulation peak was observed. Cycles of steroid hormone levels and ARO activity were associated with important events in the reproductive cycle, such as vitellogenesis and ovulation. Ovulation was restricted to spring and rainy season during the study period, as verified by steroid hormone profiles. Because this population is relatively undisturbed and unpolluted, the concentrations of sex steroids and related biomarkers reported herein can be referred as seasonal baseline measurements for the other ranges where the species exists. These measurements can be used for

comparisons to other populations exposed to the increased individual or population-level disturbances.

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Electronic supplementary material

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval During all stages of our research, all applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Informed consent Not applicable in this type of study.

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