Research Article

Variations in Plasma Sex Steroid Hormones of the Wild Caspian Cyprinid Fish, Kutum (*Rutilus frisii Kutum*)

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Received 28 February 2015 Accepted 3 April 2015

Abstract

Steroid hormones in plasma play an important role in reproductive cycles of animals especially during the final maturation stages. Steroid hormones synchronize gonad developments depending to fish species reproductive strategies. The wild Cyprinid fish, Kutum (*Rutilus frisii kutum*) is an ecologically and economically important fish species which inhabit in southern coastline of the Caspian Sea in Iran. Over the past few decades, natural reproduction of this species dramatically impaired due to the urbanization, civilization close to the land and shallow water in south western of the Caspian Sea. Therefore annual sex steroid hormones and gonads development were measured to assess the annual reproductive biology of female Kutum. In this study for the first time, our aims were to determine the annual variations in sex steroid hormones; 17 β Estradiol (E2), Progesterone (P) and testosterone (T) and gonad development of female Kutum. Our results showed that plasma steroid levels in females manifested in two phases in annual reproductive cycle; the resting phase (June - February) being characterized with the lowest level of steroid hormones and the peak reproduction activity phase (March–May) with simultaneously a significant increase in level of E2, P and T in plasma. Interestingly, comparing with other Teleost fish species the baseline level of E2 in plasma of Kutum during the resting phase to some extent was also huge. Increase in concentration of plasma E2 was in accordance with an increase of gonadosomatic index during spawning season. Our results contribute to our knowledge about the reproductive biology of Kutum and calls further long-term investigation.

***Keywords*:** Sex steroid profile, Gonad development, Female Kutum

Introduction

Maturation and gonad development in animals are long processes that involving complex physiological and biochemical changes (Barannikova et al., 2002; Patiño and Sullivan, 2002) .Steroid hormones in vertebrate animals have important role in gonad development and subsequently sexual maturity during annual reproduction cycles (Carragher and Pankhurst, 1993; Lubzens et al., 2010; Mojazi Amiri et al., 1996; Mylonas et al., 2010; Pankhurst and Carragher, 1992).

Sexual maturity and steroid hormones during reproductive cycles may change due to species characteristics such as age, sex and personality. Moreover, environmental stimuli can also play with role as drivers to change species steroid hormones levels in plasma. Many studies have shown seasonal changes of steroid hormone profiles and gonad developments in different fish species during spawning seasons (Matsuyama et al., 1988; Scott et al., 1984; Scott et al., 1983) .

To explore spawning pattern in fish species it is crucial to understand annual reproductive cycle of plasma sex steroid hormones and gonad maturity (Lee and Yang, 2002; Manosroi et al., 2003). To our knowledge, there are few studies have been focused on annual sex steroid fluctuations in fish species (Chang and Yueh, 1990; Johnson et al., 1998; Sehafii, 2014). Mormeover, yet there is only anecdotal and no well-detailed data of the annual fluctuations of sex steroid hormones in Caspian Sea Kutum.

Several studies on teleost fish species have investigated ovarian development, gametogenesis process and steroids hormone profiles such as white suckers, *Catostomus commersoni*; Cypriniformes, Teleosteii (Scott et al., 1984; Stacey et al., 1984); rainbow trout, *Salmo gairdneri* (Fostier and Jalabert, 1986; Scott et al., 1983); goldfish, *Carassius auratus* (Kobayashi et al., 1989); orange roughy, *Hoplostethus atlanticus* (Pankhurst and Conroy,

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1988); gudgeon *Gobio gobio* (Rinchard and Kestemont, 1996); brown bullhead catfish, *Ictalurus nebulosus* (Rosenblum et al., 1987); Cyprinid fish Kutum, (Taghizadeh et al., 2013); vocal plainfin midshipman (Sisneros et al., 2004) and river catfish *Hemibagrus nemurus* (Adebiyi et al., 2013). However, despite the importance of species reproductive biology during reproductive seasons surprisingly yet little is known about annual fluctuation of steroid hormones and gonad developments of teleost fishes in Caspian sea habitat. To our knowledge, there are a few studies focused on annual fluctuations and long term investigations on freshwater fish species (Chang and Yueh, 1990; Di Cosmo et al., 2001; Pavlidis et al., 2000; Scott et al., 1984) but not on Kutum in the Caspian sea habitat.

Cyprinid fish, Kutum, *Rutilus frisii Kutum* Kamenski 1091 is one of the endemic Teleost fish species which inhabit in the Caspian Sea and belongs to Cyprinidae family (Abdolhay et al., 2012; Abdoli, 1999; Bani and Vayghan, 2011). This species has economically and ecologically important values for biodiversity enrichment in the Caspian sea habitat. Kutum is an anadromous fish species and migrates from Caspian sea to adjacent rivers during spawning seasons (Abdolhay et al., 2011; Bani and Vayghan, 2011; Heidari et al., 2010). Males normally mature and are ready for spawning during third and fourth year and females mature and complete gonad development approximately in fourth year (Abdolhay et al., 2011). The spawning season of this species is from early March to late April with a peak spawning period in early April (Abdolhay et al., 2011; Keivany et al., 2012).

Since few decades ago, potential problems of pollution due to man-made activities have increased in shallow waters, estuaries which are relatively close to land. These locations are prone to human activities as well as attractive habitats to aquatic species. Due to population decline of this ecologically and economically important species, the Iranian fisheries organization (Shilat) started the artificial reproduction program in fisheries facilities (Abdolhay et al., 2011). To attain commercial fish species stock management, it is crucial to know more about annual sex steroid profile changes and gonad developments of bloodstock management for policy makers and fisheries companies to make more appropriate decisions for protecting riverside migratory routs managements and artificial reproduction procedures. This fact would be more pronounced especially if their population dynamics in natural environments are encountered with ecological and survival threat (Abdolhay et al., 2011; Sehafii, 2014). It is important for stakeholders, fisheries policy makers and reproduction facility managers to have more insights about annual reproductive cycle of this economically key fish species

In the current study our aims were to (a) quantify annual variations of the bioactive steroid hormones and (b) identify seasonal variations of gonad developments in Caspian Sea Kutum. We measured three main sex steroid hormone profiles, 17 β Estradiol (e2), Progesterone and Testosterone (T) which are closely related to female Kutum maturity and gonad development. Gonad development stages have been determined monthly according to the histological standard methods (Genten et al., 2009; Lubzens et al., 2010). We expected that temporal changes vary for sex steroid hormone profiles and are directly related to gonad developmental stages.

Materials and Methods

Experimental animals

Cyprinid fish individuals, *Rutilus frisii Kutum* were collected monthly from Sefid-Roud River estuary located in the south-west coast of the Caspian Sea (20 females per each month (20×12=240, with total length : ~30–57 cm), (See figure 1). This range of size class is laid in the most female brood stocks which are used for artificial reproduction purposes in Iranian fisheries companies. All individuals were collected as much as possible in the same location. All fishes were packed in ice and transported to the laboratory facility center for further gonad development investigations. Age, body weight, gonad weight and sex were determined for each fish individual. biometrical data of all individuals such as total length (TL) and gonad weight were measured with accuracy of ±5mm. Quantitative indices with precision 1 mm, 0.01 g and 1 g, evaluation, registration forms and booklet were recorded. Scale samples were kept in the specific small-scale compartment for laboratory studies.

Steroid hormone assay

Plasma containing steroid hormones was collected to determine hormone concentration in female Kutum. Every first week of the month blood samplings were done for all individuals in the morning (approximately 8:00-11:00 AM). Five ml of blood was collected from the caudal vasculature using heparinized needle and samples carried in Eppendorf tubes were kept on ice boxes until centrifugation. Centrifugation was done at 6,000g for 5 min and then was transferred into 0.5 ml

Eppendorf tubes. Plasma samples were stored in the freezer at -80 ^{0}C until steroid assay was performed.

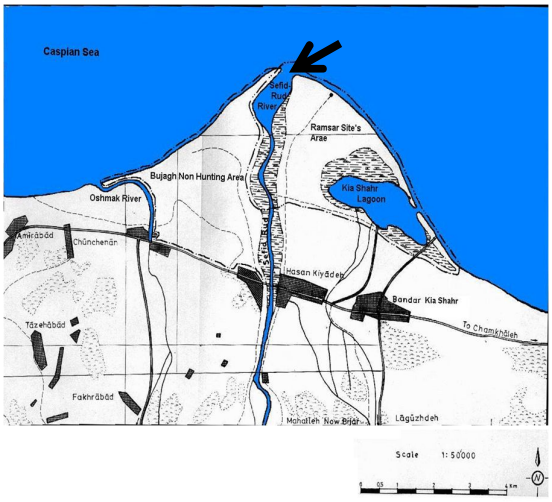

Figure1. The location of individual sampling in Sefid-roud river connected to Caspian sea, IRAN. The black arrow shows the place of sampling.

Plasma levels of the steroid hormones, E2, P and T were measured by means of the radioimmunoassay (RIA) method after plasma extraction using the procedure described by (Kagawa et al., 1982; Rinchard et al., 1993).

Briefly, 50-100 µl of standards, controls and sample plasma was added into tubes coated by antibody (polyclonal rabbit antibodies were used). Thereafter, 500 µl of 125I-labelled E2 (radioactivity 170 kBq, Orion Diagnostica, Finland), 125I-labelled Testosterone (Radioactivity 200 kBq, Orion Diagnostica, Finland), and 1 ml of 125I-labelled Progesterone (Radioactivity 185 kBq, Immunotech, France) tracer was added to all tubes and incubated in a water bath (incubation times varied between steroid assays).

Following washes in phosphate buffer saline, radioactivity was counted using a gamma counter (Wallac/LKB gamma counter). The standard concentrations were ranged from 0-300 ng/ml for E2, 0-14.4 ng/ml for T and 0-50 ng/ml for Progesterone.

Histological study

To evaluate the gonad development stages and changes among months, gonads has been dissected from fish body and kept in physiological serum solution. Gonads were embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined by light microscopy (Poosti et al., 1996). Ovaries were classified by developmental stage according to the relative abundance of the most advanced type of oocytes (Biswas, 1993; Kesteven, 1960; Rinchard and Kestemont, 1996). Five maturity stages were defined for females: stage I, ovogonial stage (presence of ovogonia, small oocytes and a few growing oocytes); stage II, primary oocytes (oocytes with homogenous cytoplasm, circular nucleus, and numerous nucleoli); stage III, cortical alveoli (oocytes with peripheral cortical alveoli, located nucleus, and several nucleoli); stage IV, vitellogenesis (increasing number and size of yolk granules); and stage V, final maturation (large hydrated oocytes with germinal vesicle near the cell membrane). Environmental factors, water temperature and salinity were also measured monthly. To evaluate the gonadosomatic index (GSI) gonads were dissected and weighted.

The gonadosomatic index was calculated as ovary weight divided by body weight multiplied by 100. The following formula was applied: GSI= (GW/BW)*100; Where GW= gonad weight (g) and BW= body weight (g),

Statistical analysis

One way analysis of variance (ANOVA) was applied for statistical analysis. To check for significant changes, Estradiol, Progesterone and Testosterone selected as dependent variables and temporal variation of month as an independent variable. When the ANOVA test was significant, analysis followed by Duncan's multiple range test (DMRT) to estimate the mean differences of different months per each hormone monthly variation and gonadosomatic index.

Spearman correlation test was done to check the correlation between sex steroid hormones with each other. Mean values were used to compare numerical data. Values are presented as mean ± standard errors. . Differences of *P*\0.05 were considered statistically significant. All data were analyzed by using SPSS v. 21.05.

Results

Sex steroid measurements

Results showed that there is a significant effect of temporal variations on 17-beta estradiol levels (ng/ml) among months ($F_{11, 96}$:22.99, P<0.001) (See figure 2a). There was a significant increase for E2 with onset of spawning season from February and the highest level of E_2 was observed in March. Post-hoc test, Duncan Multi comparison test results showed in the table 1. We did find a significant effect of months on level of plasma progesterone $F_{11, 96}$:14.73, P<0. (See figure 2b).

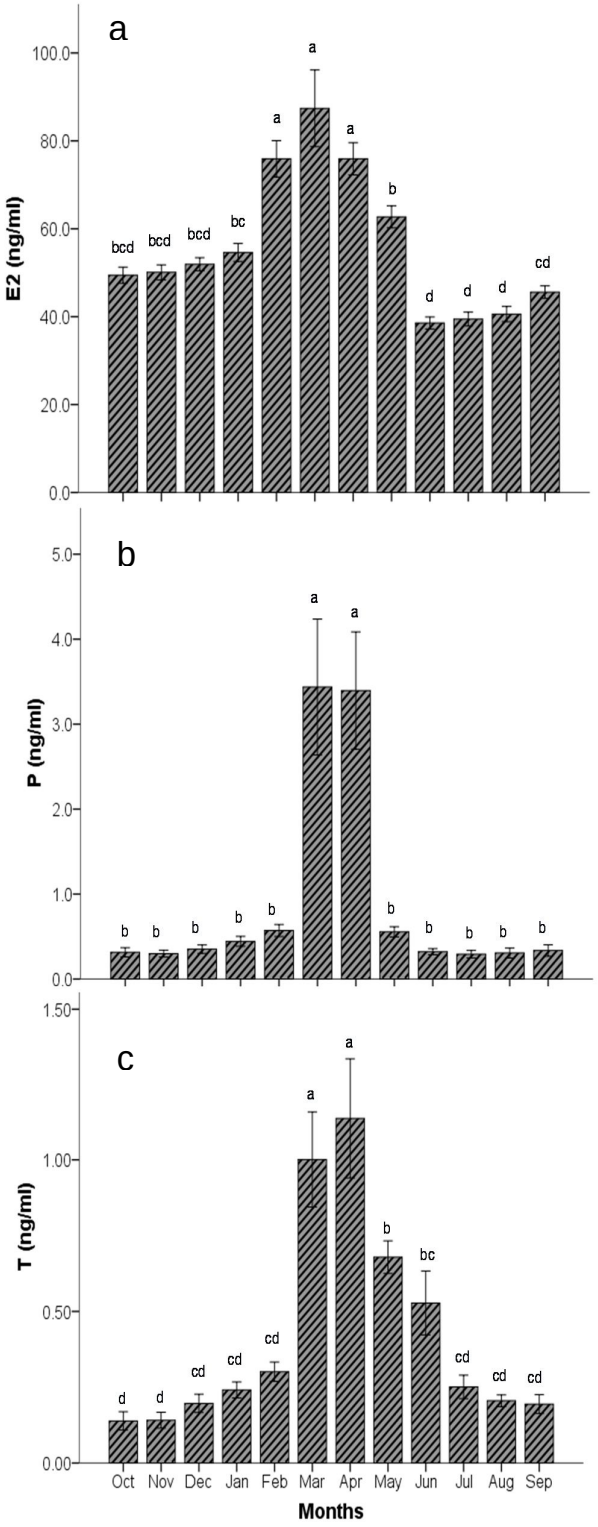

Figure 2. Effect of the temporal variations on sex steroid hormones in plasma content (ng/ml) from October to September; a) Estradiol, b) Progesterone and c) Testosterone. Mean annual plasma steroid hormone concentrations in *Rutilus frisii Kutum* from October to September. Different letters reveal significant differences (n= 106).

There was only slightly increase from January till February and then suddenly increased significantly during February and March. Post-hoc test, Duncan Multi comparison test results showed in table 1.

Table 1. Annual absolute numbers (Mean ±S.E.M) of Gonadosomatic index (GSI) and plasma sex steroid hormones (ng/ml) of Estradiol, Progesterone and Testosterone in Caspian Sea Kutum (n=106).

Months	E2 (ng/ml)	T (ng/ml)	P (ng/ml)	GSI (%)
October	49.45±5.44	0.13±0.09	0.31±0.16	18.06±0.35
November	50.08±5.11	0.14±0.07	0.29±0.12	17.50±0.60
December	51.93±4.42	0.19±0.09	0.35±0.14	21.50±1.31
January	54.62±6.15	0.24±0.07	0.44±0.17	24.43±0.87
February	75.91±12.40	0.30±0.09	0.57±0.21	23.40±0.75
March	87.40±26.19	1.00±0.47	3.43±2.39	23.66±1.96
April	75.935±10.99	1.13±0.58	3.39±2.07	30.40±1.15
May	62.70±7.52	0.67±0.16	0.55±0.17	22.73±0.80
June	38.55±4.09	0.52±0.31	0.32±0.10	19.36±0.61
July	39.44±4.72	0.25±0.11	0.29±0.13	17.13±0.35
August	40.591±5.22	0.20±0.05	0.30±0.17	17.56±0.47
September	45.59±4.27	0.19±0.09	0.33±0.20	17.86±0.40

Moreover, Pearson linear correlation test revealed that there is a positive correlation between plasma estradiol levels (E2) and progesterone levels (P). Pearson linear relationship: 0.78. T= 13.11, df= 106, $P<0.001$. (See figure 3a). Measurement of Testosterone content of plasma showed that there is a significant effect of temporal variations on T among months $F_{11, 96}$:16.91, P<0.001. (See figure 2c). Plasma level of T increased to some extent from december till february and after that reached to the highest peak levels in march and april. Positive correlation was observed between level of estradiol (E2) and testosterone (T).
Pearson linear relationship: 0.69. T=9.83, df=106, P<0.001. (See figure 3b).

Maturity stages and gonad developments

According to the results of the present study, tissue sections in three areas, posterior, middle and end ovarian Kutum, there was no significant difference in the frequency of oocytes. Investigations with regard to the stages of growth and independence ovarian Kutum can be divided into six stages (see figure 4 and 6) including: first stage (stage nucleolus chromatin), the second stage (stage nucleolus side), the third stage (stage vesicles yolk), fourth step (step seeds yolk), the fifth stage (maturity stage) and stage six (oocytes stage). In all stages, follicular layer, cell wall, vacuoles, nuclear and nucleolus stage were then investigated.

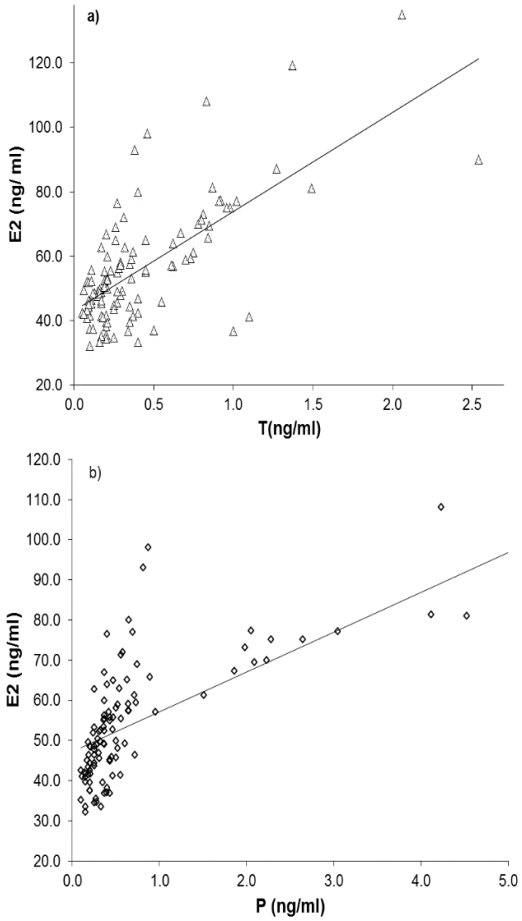

Figure 3. Positive correlation between plasma concentrations of E2 and P in Kutum and E2 and T in Kutum (n=106).

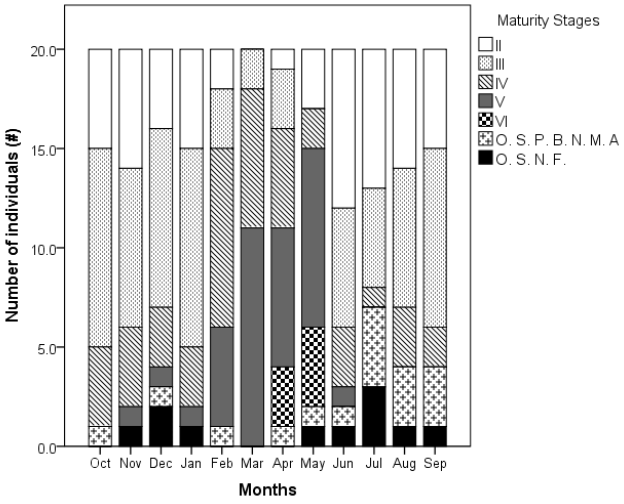

Figure 4. Stages of gonadogenesis and gonad development (II-VI) in Caspian Sea Kutum (n=20 per each month) from October to September.

Histological studies showed that Kutum had the same stages of oocyte development during migration season and this indicated synchronous oocyte development. Gonadosomatic index fluctuated among months and the highest level was observed in April; $F_{11,24}$:56.20, P<0.001. (See figure 5). Duncan multi comparisons test was applied for Post hoc analysis among months.

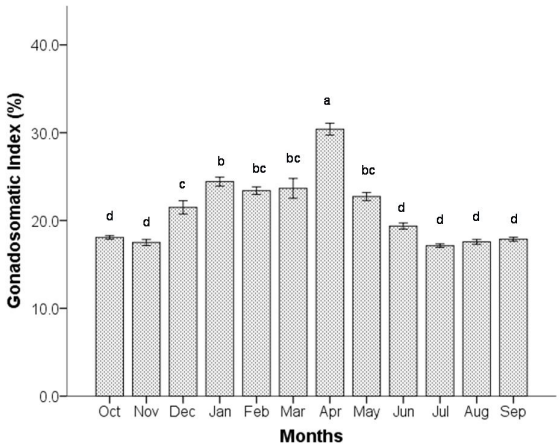

Figure 5. Annual fluctuations of the Gonadosomatic index (GSI) in Caspian Sea Kutum. (n=20 per each month) from October to September.

Discussion

The present study for the first time provides annual fluctuations of sex steroid hormones and gonad developments during reproduction cycle of the migratory Cyprinid Kutum. Our results revealed that there is a significant fluctuation of steroid hormones during annual reproduction cycle in Kutum. The highest peak level of steroid hormones were observed in spawning season; E2 (February – May), P (March - April) and T (march-April). During June- January which is the estivation stage, and also well known as foraging stage, when predominantly the ovary of the Kutum is small and present in stages II AND III, circulatory content of E2 and T were in low levels. Moreover, the level of steroid hormones significantly suppressed after the spawning period. From February concentration of E2 increased and subsequently in March concentration of T and P in plasma began to rise and reached immediately within the month at the maximum levels.

During this period in April the female individuals had reached to the maximum GSI when approximately oocyte maturation took place.

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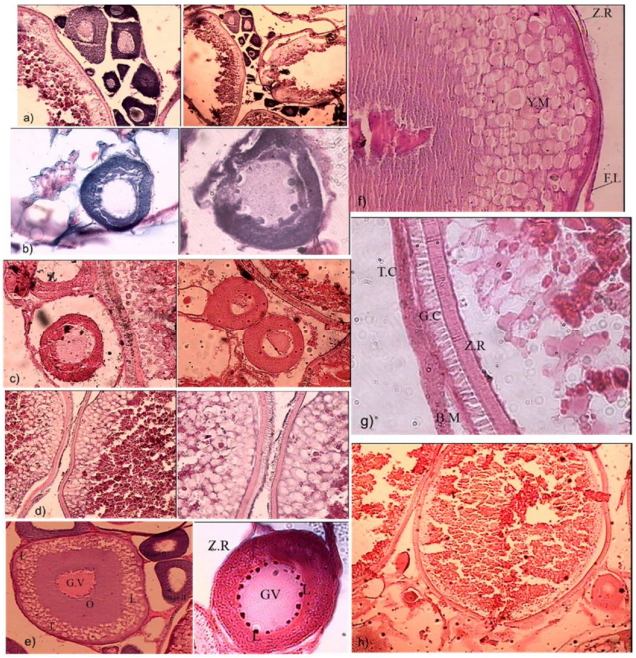

Figure 6. Histological observation of cyprinid fish, Kutum gonad development. a) Cross-section of ovary, stage I (of nucleus chromatin) 20_X & 40_X b) Cross-section of ovary, stage II (nucleus and side) 20_X & 40_X. c) Cross-section of ovary, stage III (Vesicles yolk) 20_X. d) Cross-section of ovary, stage IV (yolk grains) 20_X. e) Light micrograph of an oocyte in the cortical alveoli phase from 5 µm cross section in *Rutilus frisii kutum*. Lipid droplets (L) are present under the (ZR): zona radiata. GV: germinal vesicle. 70x. f) Light micrograph of an oocyte in the vitellogenic phase from 5 µm cross section in *Rutilus frisii kutum*. YM: yolk material; ZR: zona radiata; FL: follicular layer. 480_X. g) Light micrograph of follicular layers and oocyte in the maturation phase from 5 µm cross section *R. frisii kutum*. ZR: zona radiata; GC: granulosa cell; TL: thecal layer; O: ooplasm. 525x. h) Oocytes in Stage V (mature) and i) Stage VI (eggs found or spawned)

The rapid increase in plasma steroids in Kutum was highly correlated with increases in the gonadosomatic index. After May, although the GSI continued to increase further and reached the highest values until April, plasma concentration of E2 exhibited a sharp decrease in April. There was a positive correlation between plasma level of E2 with P and T concentration. Gonad development had reached to the stage V as a final gonad maturation stage during February-May.

From February concentration of E2 increased and subsequently in March concentration of T and P in plasma began to rise and reached immediately within the month at the maximum levels. This higher level of E2 and T are coinciding with the vitelogenic procedures in ovary. The level of steroid hormones diminished significantly after the spawning period. This part of the result is in line with earlier study on Indian major carp species *Labeo rohita* (Sen et al., 2002). Testosterone has been reported in the blood of a number of female Teleost such as cyprinid fish, Kutum (Heidari et al., 2010; Taghizadeh et al., 2013). Although testosterone and 11-ketotestosterone are male specific androgens, they are also present in blood plasma of female fish (Rinchard et al., 1993). As a precursor of E2 production, T is available in ovary for final female gonad aromatization. There is a relationship between testosterone and 17b-estradiol in female fish. Testosterone leads to production of 17b-estradiol, which in turn leads to vitellogenin production l (Kagawa et al., 1983).

The GSI is an invaluable factor for monitoring the progression of gametogenesis in cyprinid fish, Kutum. High gonadosomatic indices recorded in April implied that gonads of Kutum were matured in this month and the females would likely spawn during these month in the wild (Heidari et al., 2010; Abdolhay et al., 2011; Taghizadeh et al., 2013). Moreover, earlier studies have shown that increments in ovarian gonadosomatic index and oocyte development are associated with changes in E2 levels in plasma (Lee and Yang 2002; Heidari et al., 2010; Adebiyi et al., 2013; Taghizadeh et al., 2013). High gonadosomatic indices in the month April with high levels of E2 in March-April indicated that as maturation of oocytes progressed in the ovary, levels of E 2 also increased in the plasma of Kutum. Thus, confirming the role of E2 in oocyte maturation and vitellogenesis (Scott et al., 1983; Rinchard and Kestemont 1996; Patiño et al., 2003; Adebiyi et al., 2013).

In non-mammalian vertebrates, ovarian follicle cells produce two different steroid hormones, E2 and maturation inducing hormone (P), in response to pituitary gonadotropins, which play important roles in two phases of oogenesis, vitellogenesis and oocyte maturation, respectively. Estradiol-17β (E2) promotes vitellogenesis in members of all non-mammalian vertebrates. In each of these groups, the time of vitellogenin production corresponds to the period of elevated E2 levels. Vitellogenin is the precursor molecule for egg yolk, which is of considerable importance as the source of metabolic energy for the developing embryo. In response to increased levels of plasma E2, the liver synthesizes and secretes vitellogenin, which is carried in the bloodstream to the oocytes (Nagahama 1994). The developing oocytes take up vitellogenin and convert it to egg yolk. On the other hand, a variety of progesterone have shown to be effective in the

initiation of meiotic maturation in fish and amphibian oocytes (Nagahama 1994; Kobayashi et al., 1996).

A sudden drop in the plasma E2 level in cyprinid fish, Kutum from vitellogenic stage to postvitellogenic stage may be explained in terms of swit Fostier off the arkobayomatase (CYP 19) activity as the oocytes progressed to maturation. Almost a similar pattern of fluctuations in E2 profile has been reported during the transition from vitellogenic to maturational stage in rainbow trout (Scott et al., 1983; Scott et al., 1984; Fostier and Jalabert 1986); amago salmon (Young et al., 1983); masu salmon (Yamauchi et al., 1984); coho salmon (Van Der Kraak and Donaldson, 1986); medaka (Sakai et al., 1987) and Indian major carp (Sen et al., 2002). In this context, however, it may be mentioned that in some other teleost such as a gudgeon, (Rinchard et al. 1993); Perciforms, (Prat et al., 1990) and cyprinid fish, goldfish, (Kagawa et al., 1983) there was no decrease of E2 level during oocyte maturation.

Our results showed that in another cyprinid fish species there is a drop of E2 levels during final period of oocyte maturation. Earlier study has shown that at high concentration, plasma level of T might also be involved in hepatic vitellogenin synthesis (Fostier and Jalabert 1986). Moreover, it is clear from our results that concentration of the sex steroids in plasma is dramatically different between spawning season migration and the rest of the reproductive cycle in Kutum. Several studies on structural changes in ovarian morphology of Teleost fishes have been done during oogenesis process (Guiguen et al., 1993; Prat et al., 1990; Scott et al., 1983; Sen et al., 2002; Shabana et al., 2012). Our histological measurements reinforce earlier study on gonad development of this cyprinid fish and indicated that the Kutum, *R. frisii kutum* exhibits synchronous group oocyte development (Heidari et al., 2010; Saeed et al., 2010).

The sexual mechanism of Teleost fish species is a complex aspect including gonad developments, maturation of oocytes, ovulation of mature eggs and subsequently spawning activities, which are regulated by the hypothalamus organ. This area of the brain produces an agent that releases gonadotropins hormones from the pituitary gland and regulates the target organs of the gonadotropins (Mojazi Amiri et al., 1996). Gonad structure of Kutum with the results obtained in this investigation were similar to other Teleost fish with the same spawning strategy (Lee and Yang, 2002; Manosroi et al., 2003). More studies are needed to investigate annual fluctuation of sex steroid hormones and their relation with the gonad development. However, individual morphology and environmental factors seem to play an important role in this context.

In conclusion, , this study has investigated plasma sex steroid levels of the wild female Caspian Sea Kutum and the accompanying changes in gonadal development. from our results we conclude that in the wild cyprinid Kutum, sex steroids biosynthesis have significantly increased and reached to highest level during reproduction cycle at the onset of spawning migration in early spring. These hormonal changes are closely connected to the gonad development, maturity and subsequently gonadosomatic index. Based on information from plasma sex steroid hormonal profile, gonad histology and gonadosomatic index of the present study, it can be inferred that the annual reproductive cycle of Kutum did show a seasonal spawning pattern.

Interestingly our results for the first time revealed that this Cyprinid fish species show hugely high plasma E2 levels and high GSI even in non-reproductive fish individuals during non-reproductive seasons in June-September) with the basal levels of 40 ng/ml E2 (See figure 2a). And we can see a slightly increase in E2 levels during October –January with the basal levels of 55 ng/ml E2. We argue that this high levels of E2 may suggesting either non-migratory Kutum species populations inhabit in the Caspian Sea or Kutum species which migrate to adjacent wetlands not in spring but during autumn for spawning activities (Abdolhay et al., 2012; Rezvani Gilkolaei et al., 2011). The present study add the growing body of reproductive biology of one of the most important Cyprinid fish species, Kutum in Caspian Sea. Moreover, to achieve a successful artificial reproduction of Kutum it is important to explore further the impact of brood stock fish body size on temporal fluctuation of steroid hormones. More longtime period investigations are needed to establish a breeding protocol for Kutum population in captivity for restock management purposes.

Acknowledgements

We would like to give our thanks to Dr Sarpanah, Sharif pour, Shabani, Shabanpoor, sudagar and Mr. Kazemi, Halajyan, Sadeghi, Jalali and other respected colleagues in the laboratory diagnosis for medical staff pathology Sadeghi clinic, Astaneh Ashrafiyeh, respected ichthyology laboratory sufferer and Higher Education Center for Science and Fisheries technology Mirza Koochak Khan Rasht. All experiments and procedures were

approved by the local animal experiment authority in accordance with the laws and regulations controlling experiments and procedures on live animals (no: 8621013105).

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