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Research Article

Determining the optimal time for artificial propagation in Hilsa shad (*Tenualosa ilisha*) based on thyroid and steroid hormones levels

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Abstract

Steroid hormones in teleost have an important role in gonadal development and sexual maturity. Hilsa shad (*Tenualosa ilisha*) is important for fisheries in the South of Iran. Any information on steroid hormones of Hilsa shad brooders and their relationship with the spawning period provides a vital step for studies on the propagation and culture of this species. Fish samples were captured from four location-time stations: September-fresh water (Salinity: 5.2±0.35 ppt, Temperature: 27±0.17 ºC, DO: 7.9±0.23 mg/L; Karoon river, Khorramshahr; 1st station), September-marine water (Salinity: 44.01±0.5 ppt, Temperature: 26.5±0.41 ºC, DO: 7.45±0.52 mg/L; Bahrakan Port, Persian Gulf; 2nd station), October-marine water (Salinity: 41.77±0.25 ppt, Temperature: 15.27±0.32 ºC, DO: 9.36±0.10 mg/L; Bahrakan Port, Persian Gulf; 3rd station), and November-marine water (Salinity: 40.01±0.28 ppt, Temperature: 14.91±0.37 ºC, DO: 9.45±0.18 mg/L; Bahrakan Port, Persian Gulf; 4th station). Blood samples were immediately collected by a 5 ml syringe from the caudal vein, and serum was separated after centrifuging (7640 *g*,10 min). Also, gonad developmental stages and Gastro-somatic index (GSI) were determined after necropsy of euthanized fish. The enzymatic Immunological Assay method is used for hormone measurements, including thyroxin (T4), triiodothyronine (T3), 17-β estradiol (E2), and testosterone (T). More samples at the 1st station were on the 5th gonadal development stage with the highest GSI index. The maximum and minimum serum E2 levels were recorded at the 1st and 2nd stations, respectively. The serum testosterone concentration at the 1st station was significantly higher than the other ones and the maximum T3 and T4 concentrations were recorded at the 4th station. The results of this study proposed that Hilsa shad females are ready for spawning in September and this time is proposed for collecting broodstocks from the Karoon river (Khorramshahr, Khuzestan) for artificial propagation.

Keywords: Persian Gulf, Karoon River, Thyroxin, Triiodothyronine, 17-β estradiol, Testosterone, *Tenualosa ilisha*

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Introduction

Marine fishes are the main sources of n-3 long-chain polyunsaturated fatty acids such as eicosapentaenoic and docosahexaenoic acids. In recent years, their economic importance and the higher interest in marine fishes have directed attention to their mariculture. Therefore, having comprehensive knowledge about the physiology and biology of marine fishes is vital for the fisheries and it is necessary for the mariculture of the selected species in the future. Hilsa shad (*Tenualosa ilisha*, Hamilton-Buchanan, 1822) belongs to the Clupeidae family. Iranian shad consists of five species from the genus of Alosa, including four species from the genus of Clupeonella and one species from Tenualosa. *Tenualosa ilisha* is a euryhaline pelagic and anadromous species distributed in the sea and river. It usually migrates for more than 100 km during the spawning season (Satari, 2003). During the first days of March, Hilsa shad migrates from the Persian Gulf to the Arvand River. In this location, the frequency of males is more than females in the initial stages of migration. But over time, the frequency of females will grow more than the males (Pillay, 1958; Ahsan *et al.*, 2014). There are two gonad development peaks reported in March and July in this species and based on that it has been proposed that the Hilsa shad propagates twice per year. The eggs are seen in the subsurface layers of water (Pillay and Rosa, 1963; Ahsan *et al.*, 2014). Growth and development of Hilsa shad juveniles take place in the river and surface layers of water, while brooders are seen in the sea and deep-sea water (Pillay, 1957; Ahsan *et al.*, 2014; Bladon *et al.*, 2019). Hilsa shad have the greatest distribution among the five species of this genus and they have been reported from the Persian Gulf, Gulf of Oman, and the South of China Sea (Al-Nasiri and Al-Mukhtar, 1988; Bladon *et al.*, 2019). This species is essential for the fisheries in the South of Iran, Iraq, and the United Arab Emirates. Hormonal assessment is necessary to obtain the physiological status for optimizing fish culture. Asadi Eidivand *et al.* (2017) studied some biological parameters of *T. ilisha* and gonad histological characteristics during the spawning and post-spawning period. Mohindra *et al.* (2019) presented the first study to demonstrate genetic divergence in the highly migratory *T. ilisha* and the presence of population structure, as well as speculated migratory routes among the natural hilsa populations of India river systems in comparison with marine samples. Shafiei Sabet *et al.* (2016), reported the variations in plasma sex steroid hormones of the wild Caspian cyprinid fish, Kutum (*Rutilus frisii Kutum*) which is an anadromous species. Ghobeishavi *et al.* (2016) studied the innate immunity changes of the female anadromous hilsa shad during spawning and post-spawning season. In this study, an increase in the concentration of plasma E2 coincided with the increase of gonadosomatic index (GSI) during the spawning season. Research by Kulkarni

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and Pruthviraj (2016), presented comparative studies on thyroid hormones and sex steroids hormone levels in four freshwater carp fishes (*Labeo rohita, Catla catla, Cirrhana mrigala, Labeo fimbriatus*) before the breeding period. Thyroid hormones play important roles in the regulation of many biological and physiological activities in teleost including growth performance, metabolism, morphogenesis, and reproduction. Thyroid hormones play their roles at different levels of the hypothalamic-pituitary-gonadal (HPG) axis and a positive correlation has been shown between thyroid hormones and the fish reproduction system. The physiological and molecular mechanisms have also revealed a link between the thyroid hormones and the reproduction system, suggesting a possible co-evolution and interdependence of thyroid hormones and steroid hormones (Tovo-Neto *et al.*, 2018). Based on the review literature and the relationship between thyroid hormones and steroid hormones in some fish species, the study of thyroid and steroid hormones and their relationship with GSI and gonad development for anadromous fishes such as Hilsa shad is vital for determining the optimal time for artificial propagation of this species.

Materials and methods

Fish samples were captured from four location-time stations: September-fresh water (Salinity: 5.2±0.35 ppt, Temperature: 27±0.17ºC, DO: 7.9±0.23 mg/L; Karoon river, Khorramshahr; 1st station), September-marine water (Salinity: 44.01±0.5 ppt, Temperature: 26.5±0.41 ºC, DO: 7.45±0.52 mg/L; Bahrakan Port, Persian Gulf; 2nd station), October-marine water (Salinity: 41.77±0.25 ppt, Temperature: 15.27±0.32 ºC, DO: 9.36±0.10 mg/L; Bahrakan Port, Persian Gulf; 3rd station), and November-marine water (Salinity: 40.01±0.28 ppt, Temperature: 14.91±0.37ºC, DO: 9.45±0.18 mg/L; Bahrakan Port, Persian Gulf; 4th station) (Fig. 1). The geographical locations of sampling stations and water physicochemical properties of the said sampling locations are presented in Table 1. The samplings were done for three months from September to November 2011. The fish samples were captured between 5 am and 10 am by a trawl net. Once captured, the fish blood samples were immediately collected from the caudal vein by a 5-ml syringe on the boat. The blood samples and fish samples were kept close to the ice and transferred to the laboratory. The samples were centrifuged (7640 g for 10 min) and their sera were separated. The enzymatic immunological assay (EIA) method was used for the steroid hormone measurements. The levels of serum thyroxin (T4) and triiodothyronine (T3) were performed by ELISA method and using an ELISA reader at 450 nm and 630 nm, respectively with commercial kits (DRG company, Germany) (Karir *et al.*, 2005). To measure 17-β estradiol (E2) and testosterone, the commercial diagnostic kits (DRG Company, Germany) were utilized. Fish were

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eviscerated and their sexuality was determined by visual examination of the gonads. Note that gonad development staging was performed based on previously established criteria (Table 2) (Almukhtar *et al.*, 2016). The entire gonads were removed, weighed, and the GSI was determined by the formula: GSI = [gonad weight/body weight] × 100, as described by Biswas (1993).



Figure 1: Sample collection stations (Geographical stations), specified with yellow pin on the map (Khorramshahr and Karoon rivers as fresh water station and Hendijan and Bahrakan port as marine water station).

Table 1: Geographical locations of sampling stations (Marine and Fresh water sampling locations), number of collected samples and water physicochemical properties.

Geographical parameters				
Location station name	**Longitude**	**Latitude**	**No. of males**	**No. of females**
Karoon river (as fresh water)	48'10' 03.82″ E	30'25'40.99″ N	0	15
Bahrakan Port (as marine water)	49'26' 38.43″ E	30'4' 46.71″N	7	38
Water physicochemical properties				
	1st station	2nd station	3rd station	4 station
Salinity (ppt)	5.2±0.35	44.01±0.5	41.77±0.25	40.01±0.28
Temperature (ºC)	27±0.17	26.5±0.41	15.27±0.32	14.91±0.37
Dissolved Oxygen (mg/L)	7.9±0.23	7.45±0.52	9.36±0.10	9.45±0.18
pH	7.4±0.3	7.82±0.08	8.29±0.01	8.32±0.15

Data representation and statistics

The data were expressed as Mean±Standard Error. Data normality was analyzed using the Shapiro-Wilk test, and variance homogeneity was performed with the Leven test. One-way and Two-way ANOVA analysis was used to determine the presence or absence of significant differences between the values of each index. Duncan's multiple range's test was used at 95% confidence level to compare the means.

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Table 2: Macroscopic and description of gonad maturation stages of *T. ilisha* females and males (Almukhtar *et al*., 2016).

Sexual Stage	Sex	Brief description of gonad
Stage1: Immature	F	Ovary cord like, waxy translucent, ova invisible to the necked eye, thick membrane. Found during all the study months. GSI range 0.7-2.1%, mean 1.3±0.5%.
	M	Testes thread-like, elongated, translucent to grey in color. Laid in the corner, about half length of body cavity. GSI range 0.06-0.7%, mean 0.65±0.06%.
Stage II: Maturing	F	Pale yellow, more than tree quarter of body cavity, with visible opaque ova, granular appearance, lobular. Found during all the study months except November. GSI range 3.2-16.9%, mean 9.3±4.3
	M	Testes darker than former stage, appearing pale reddish white. Wider and less translucent, about half to three quarters the length of the body cavity, with visible vascularization. GSI range 0.35-2.3%, mean 1.7±1.4%.
Stage III: Running	F	Yellow to orange yellow, large occupies all the body cavity, lobular with translucent yellow tiny ova, delicate ovary membrane. Ova flow out with gentle pressure. Some ova out the genital opening, found in February-November. GSI range 7.5-18.2%, mean 12.9±3.2%.
	M	Swollen Creamy white with homogeneous texture, more than three quarters the length of the body cavity, Sperms flow out with gentle pressure. GSI range 2.1-3.2%, Mean 2.6±0.5
Stage IV: Partial spent	F	Flaccid, bloody brown with some tiny opaque yellow residual ova. Found during May-November. GSI range 5.6-7.4%, mean 6.5±0.9%.
	M	Not turgid flaccid, not homogeneous texture, sperms still flow out with pressure. Not similar coloration with some bloody areas. GSI range 0.2-1.3, mean 0.65±0.2%.
Stage V: Spent	F	Flaccid empty sac, reddish bloody with many tiny opaque yellow residual ova, found during May-Nov. GSI range 2-4, Mean 3±0.9
	M	Bloody white, flaccid, not bright coloration, long slender and delicate. GSI range 0.1-0.5%, mean of 0.2±0.09%.
Stage VI: Resting (recovery)	F	Fleshy color, semi translucent, not turgid and not firm texture. GSI range 3-10.5%, mean 9.2±0.8% found during may-November.
	M	Greyish white, about one third body cavity length, nearly flaccid, homogenized coloration. GSI range 0.6-1.3%, mean 1.0±0.27%.

F: Female, M: Male

Results

The results of the fish biometry indicated that the maximum total length (32.60±1.33 cm) and total weight (295±52.41 g) were recorded at the 1st station (September-fresh water), while minimum total length (29.97±1.04 cm) and total weight (253±26.81 g) were seen at the 3rd station (October-marine water) (Table 3), but there is not any significant difference for these parameters among samples ($p>0.05$). All data related to gonadal developmental stages are reported in Table 4. Based on the results, the samples collected from the 1st station (spawning station) were mainly in the 5th sexual developmental stage. Figure 2 displays the GSI index of Hilsa shad at different time-location stations.

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Table 3: Some biometric parameters of *T. ilisha* on different time-location stations (including September-fresh water (1st station), September-marine water (2nd station), October-marine water (3rd station) and November-marine water (4th station))

Biometric parameters	Sample collection station (time-location stations)			
	1st station	2nd station	3rd station	4th station
Total length (cm)	32.60±1.33	31.30±1.51	29.97±1.04	31.02±1.01
Total weight (g)	295±52.41	320±45.27	253±26.81	261±13.45
Gonad weight (g)	30.35±6.64[a]	5.05±1.31 [b]	3.14±1.06 [c]	1.89±0.57 [d]

* Different letters show significant differences between different samples (p<0.05).

Table 4: Determination of gonad developmental stages of *Tenaulosa ilisha* on different time-location stations (n = 60).

	Sample collection station (time-location stations)			
	1st station	2nd station	3rd station	4th station
gonadal developmental stages	IV	III	II	II
	V	IV	II	III
	V	III	V	IV
	II	Male	II	Male
	IV	II	Male	II
	IV	V	III	II
	V	III	II	III
	V	Male	II	II
	IV	IV	Male	II
	V	III	III	IV
	IV	II	II	II
	V	II	II	III
	IV	IV	IV	II
	V	II	Male	II
	V	Male	II	II

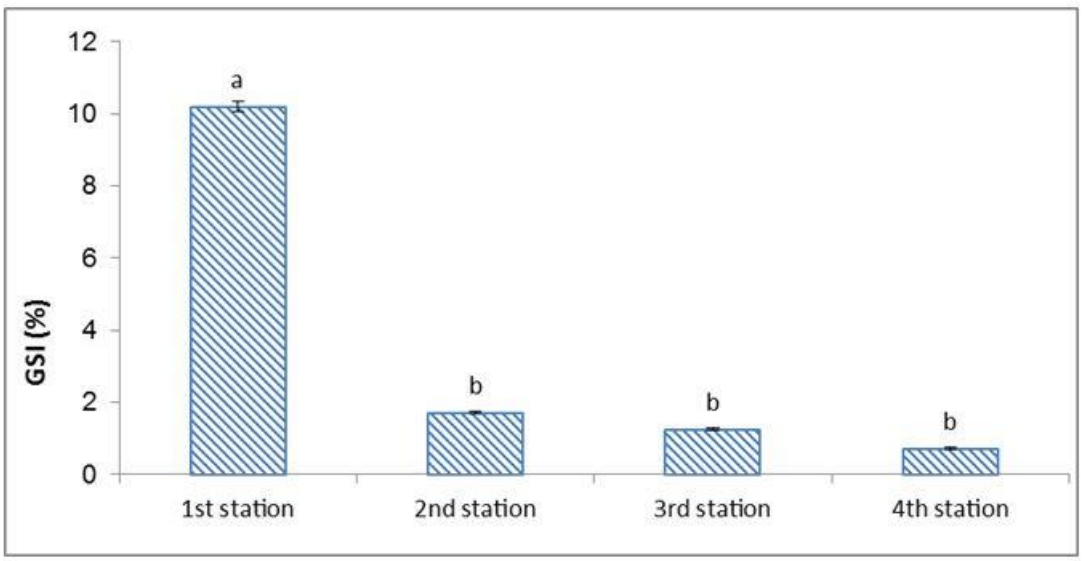


Figure 2: GSI index of both males and females of *T. ilisha* on different time-location stations (including September-fresh water (1st station), September-marine water (2nd station), October-marine water (3rd station) and November-marine water (4th station)), different letter shows significant differences between different samples (p<0.05, n = 60).

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Based on the results, the maximum and minimum GSI were observed at the 1st and 4th stations, respectively. Statistical analysis of E2 revealed a significant difference between the 1st station and the other ones ($p<0.05$). Maximum and minimum serum E2 levels were measured at the 1st station and 2nd stations, respectively (Fig. 3), although, there was not any significant difference among the 2nd, 3rd, and 4th time-location stations ($p>0.05$). According to Figure 4, the maximum serum testosterone concentration was recorded at the 1st station. The maximum serum T3 and T4 levels were recorded at the 4th station, while their minimum levels were observed at the 1st station (Figs. 5 and 6).

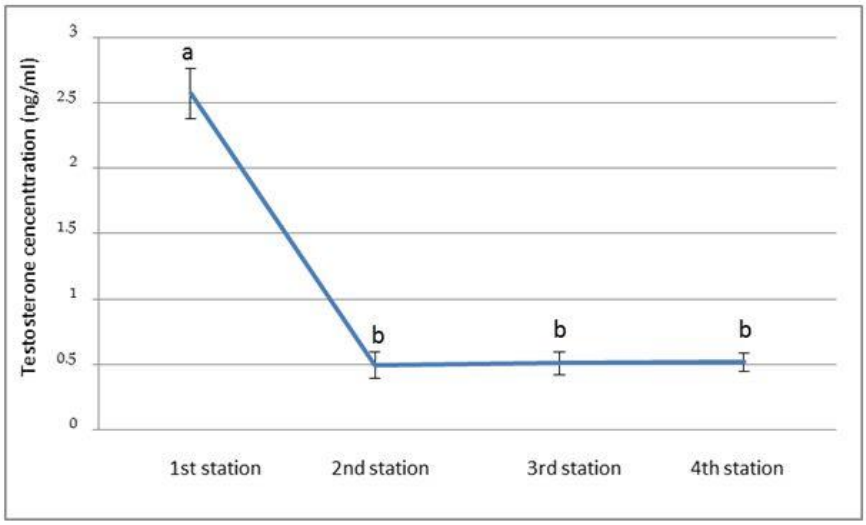


Figure 4: Serum testosterone levels changes in males of *Tenaulosa ilisha* on different time-location stations (including September-fresh water (1st station), September-marine water (2nd station), October-marine water (3rd station) and November-marine water (4th station)), different letter shows significant differences between different samples ($p<0.05$, n=7).

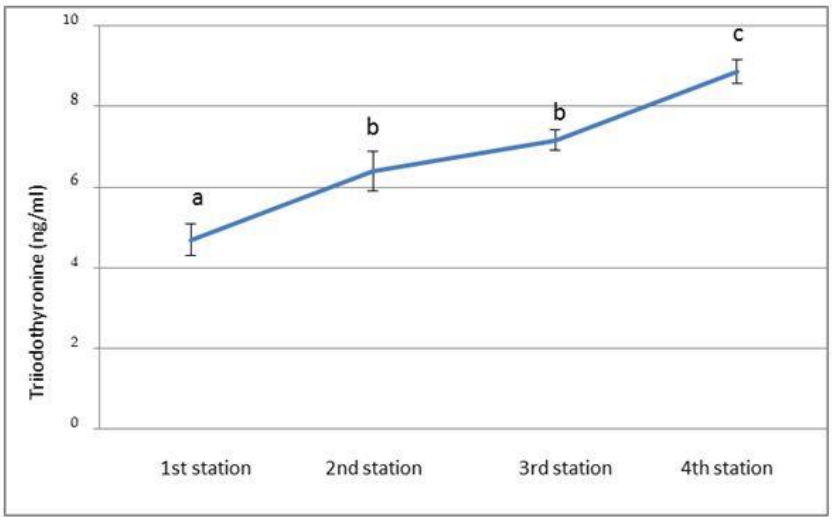


Figure 5: Serum triiodothyronine levels changes in both males and females of *Tenaulosa ilisha* on different time-location stations (including September-fresh water (1st station), September-marine water (2nd station), October-marine water (3rd station) and November-marine water (4th station)), different letter shows significant differences between different samples ($p<0.05$, n=60).

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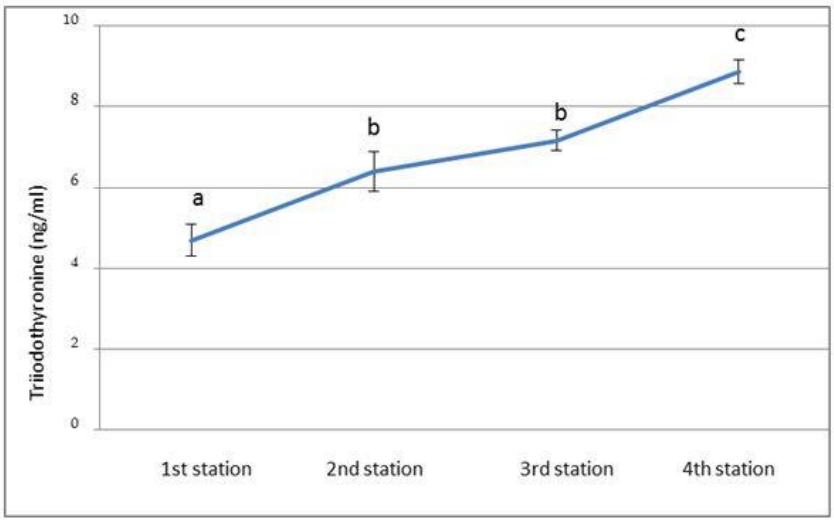


Figure 6: Serum thyroxin levels Serum 17-β estradiol levels changes in both males and females of *Tenaulosa ilisha* on different time-location stations (including September-fresh water (1st station), September-marine water (2nd station), October-marine water (3rd station) and November-marine water (4th station)), different letter shows significant differences between different samples (p<0.05, n=60).

Also, 17-β estradiol (E2) had no significant correlation with GSI, but testosterone revealed a direct correlation with GSI (r=0.81, sig= 0.00) and elevated by increasing GSI. Both serum E2 and testosterone decreased after the spawning period (Fig. 7). In this study, E2 and testosterone showed a direct significant correlation with each other (r=0.57, sig=0.00). At the end of the spawning period in September, serum E2 and testosterone were high, but both of them diminished after the spawning period (Fig. 8). There were no significant correlations among thyroid hormones and biological factors as well as thyroid hormones and sex hormones.

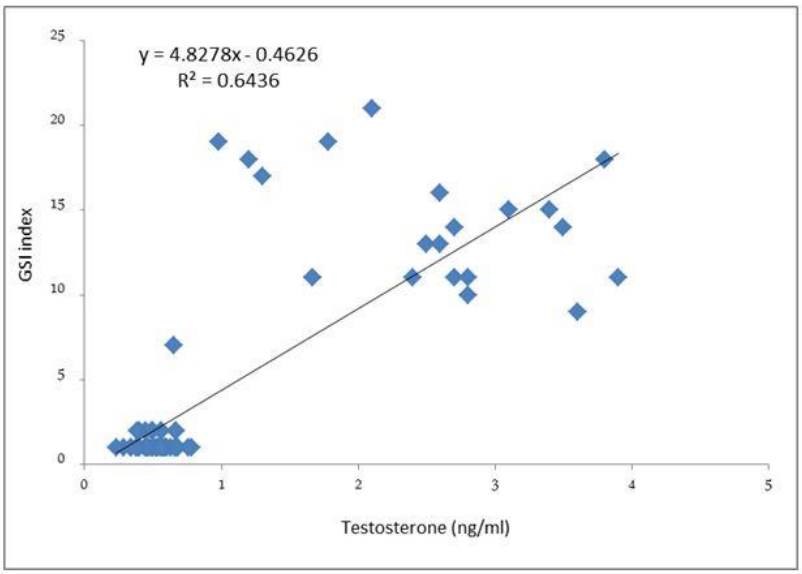


Figure 7: Correlation between GSI index and serum testosterone concentration in males of *T. ilisha.*

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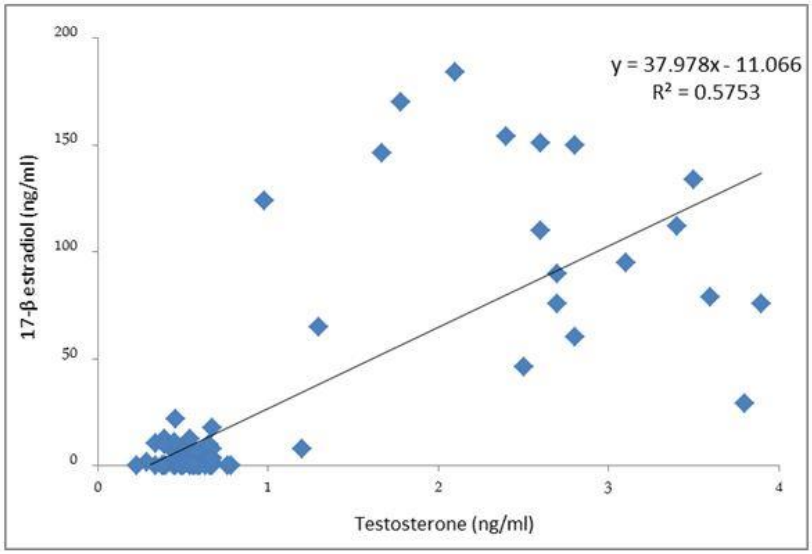


Figure 8: Correlation between serum testosterone and 17-β estradiol concentrations in females of *T. ilisha.*

Discussion

The Hilsa shad have entered freshwater for spawning, so, the 1st station (Karoon River as a freshwater station) was the spawning station of this species, therefore, all samples which were collected from this station were in their spawning period and all of them were mature and maximum weight and length were recorded in these fishes. Maremazi (1995) reported a significant correlation between GSI and spawning season which is in agreement with the results obtained from the current study. Maremazi (1994) reported that the spawning period of *T. ilisha* started in April and continued to September and October in Bahman Shir River. The same results were reported by Almukhtar *et al.* (2016) in Shatt Al Arab River, Roomiani *et al.* (2014) in Northwest of the Persian Gulf, and Bladon *et al.* (2019) in Myanmar's Ayeyarwady Delta. The observed temporal pattern of fish length and weight was probably due to the spawning migration of the species (Mutlak, 2012; Mohamed and Qasim, 2014). The dominance of females of Hilsa shad coincides with some studies in Kuwait (Al-Baz and Grove, 1995), Iran (Roomiani *et al.*, 2014), and Iraq (Hussain *et al.*, 1991; Mohamed and Qasim, 2014). Other reasons suggested for the unequal sex ratios in other studies, include differences in mortality, growth, and longevity (Vicentini and Araujo, 2003; Zhang *et al.*, 2009).

According to the results, the highest mean rate of GSI was observed at the 1st station (September-fresh water) which coincided with the spawning period, then, GSI has been decreased after the spawning period (other time-location stations). The lowest rate of GSI was recorded at the 4th station (November-marine water) which coincided with the post-spawning

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period. The temporal and spatial variation in GSI could be used to determine the spawning season. The results of this study suggest that the spawning season in coastal lines of Iran has extended from April to October and it coincides with the findings of Almukhtar *et al.* (2016) and Mutlak (2012) in the same species. Furthermore, Roomiani *et al*. (2014) suggested that the spawning season of Hilsa shad in the Khuzestan province rivers occurred from May to August. On the other hand, Hussain *et al.* (1991, 1994) found that Hilsa shad in the Shatt al Arab spawn in the period from June to August. These differences in the spawning period of Hilsa shad populations may be due to changes in the environmental factors caused by climate change. Climate change has a clear impact on the annual timing of life-history events of animals and plants (Walther *et al.*, 2002), such as selective pressures on the date of spawning (Crozier *et al.*, 2008) and variations in reproductive characteristics (Barange and Perry, 2009).

The levels of sex hormones (E2 and testosterone) in serum were very low in marine water stations (2nd, 3rd, and 4th time- location stations). As the fish sampled at the 1st station (September-fresh water) are in the spawning period (based on the biometric examination, GSI index, and macroscopic examination of maturation stages), it may be the best reason for increased levels of sex hormones in the 1st station (September-fresh water). In this context, Hellqvist *et al.* (2006) reported that testosterone levels of Stickleback females increased by elevated GSI, but it diminished after spawning. In the current study, serum testosterone hormone levels in Hilsa shad females at the 1st station (September-fresh water) with maximum GSI index were higher than samples at marine water stations, where testosterone levels decreased after spawning. Furthermore, macroscopic examination of fish gonads at the marine stations (2nd, 3rd, and 4th stations) revealed that the most of samples were at primary sexual developmental stages; meanwhile, all samples at the 1st station (September-fresh water) were at IV and V stages indicating fish were on the spawning period. High levels of testosterone at the 1st station (September-fresh water), which is a precursor of the E2, coincided with the spawning period of this species. There are some reports on females of *Capoeta capoeta* (Erdogan *et al.*, 2002), *Tinca tinca* (Pinillos *et al.*, 2003), and (*Ictalurus punctatus*) (MacKenzie *et al.*, 1989), suggesting that testosterone levels diminished after spawning. In females of some teleost fish species, the maximum serum testosterone levels were observed at the end of vitellogenesis and during spawning (Mayer *et al.*, 1990; Sulistyo *et al.*, 1998). This information also showed that serum testosterone concentration grows along with oocyte maturation. Norris and Carr (2006) reported that the primary estrogen in vertebrates is E2 which is differentiated by aromatase from testosterone. Galas and Epler

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(2002) introduced E2 as the main hormone released during vitellogenesis. The same results were reported by MacKenzie *et al.* (1989), Pinillos *et al.* (2003), Mishra and Joy (2006,) and Jerez *et al.* (2006) in different teleost species.

Coinciding with the GSI values, Pramanicka *et al.* (2013) recorded a decline in plasma testosterone and E2 levels from October in Hilsa shad females, reaching their lowest values in January and followed by a rapid rise in March up to April when the ovary contained mostly vitellogenic follicles (previtellogenic stage). Furthermore, in this study Plasma E2 level was detected in March and reached peak value in April during oocyte maturation and after spawning, but all sexual hormones declined to the lowest values in June (Pramanicka *et al.*, 2013) in Hilsa shad which coincide with the results of the current study.

Thyroid hormones are vital for the proper functioning of the female reproductive system since they modulate the metabolism and development of ovarian tissues. Therefore, hypo- and hyperthyroidism may result in subfertility or infertility in animals (Silva *et al.*, 2018). Meanwhile, T4 is the primary active form of thyroid hormone. T4 converts to T3 by deiodinase before attachment to thyroid receptors on target cells confirming that thyroxin is a prohormone (Norris and Carr, 2006). T3 is the most effective biological iodothyronine in fish (Mancera and McCormick, 2007), and is recognized as the active form of thyroid hormone (Cyr and Eales, 1996). Norberg *et al.* (1989) reported that thyroxin hormone levels of *Salmo trutta* decreased during the spawning period. In the current study, thyroxin hormone levels at the 1st station were lower than in other time-location stations (post-spawning). In this sense, Ueda *et al.* (1984) reported that elevation of T4 levels in coastal regions is probably related to the migration of *Oncorhynchus keta* from sea to fresh water, where thyroxin has probably an effective role in the regulation of fish migration behavior. In the current study, serum T4 levels were higher in marine water stations than in freshwater stations caused by the migration of this species to the river and probably reduction of the feed intake during spawning migration. During spawning migration, female hilsa tend to reduce or cease feeding activities (Bhaumik and Sharma, 2012). In addition, Farbridge *et al.* (1992) and Gaylord *et al.* (2001) reported the reduction of feeding led to the reduction of T3 and T4 levels in rainbow trout and channel catfish, respectively. Also, Maremazi (1994) reported that the stomach of 82 samples out of 92 Hilsa shad was empty during spawning migration in the Bahmanshir river.

It has been confirmed that vitellogenin has an important role as a carrier for transmission of T3 in developing oocytes and reduces plasma T3 during propagation season (Monteverdi and Di Giulio, 2000). In this context, it has been reported that

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the increasing trend of serum E2 led to decreased level in serum T3 in Great Lake Salmon (Flett *et al.*, 1994). Indeed, during the production of vitellogenin, T3 is transferred through developing oocytes, and its plasma levels decline. On the other hand, Baldissereto *et al.* (2007) revealed that the Plasma T3 concentration in Salmonids temporarily increases during the migration and smoltification period. In this regard, in the present study, the serum T3 concentration in Hilsa shad at the post-spawning period (marine water stations) was higher than in the 1st station (September-fresh water) which may be related to osmoregulation, as T3 affects Na+/K+-ATPase pump activity by which it controls fish osmoregulation (Baldissereto *et al.*, 2007). Specifically, higher levels of T3 on marine water stations in comparison with a freshwater station may be a result of the following reasons: 1) high level of E2 during the spawning period; 2) off-feeding of Hilsa shad during spawning migration, and 3) osmotic changes during migration to freshwater. Taken altogether, these results are expected to be utilized as important preliminary data for artificial breeding and larval propagation of *Hilsa shad* for mariculture purposes.

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