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Macroscopic and Microscopic Examination of Seasonal Gonad Change in *Alburnus istanbulensis* (Battalgil, 1941) (Teleostei:Cyprinidae)

Ezgi Hamzaoğlu[1], Müfit Özuluğ[2], Yasemin Tunali[1], Melike Erkan[1,*]

[1] Istanbul University, Faculty of Science, Department of Biology, Division of Zoology, 34134, Vezneciler, Istanbul Turkey.
[2] Istanbul University, Faculty of Science, Department of Biology, Division of Hydrobiology, 34134, Vezneciler, Istanbul Turkey.

* Corresponding Author: Tel.: +90.212 4555849;
E-mail: merkan@istanbul.edu.tr

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Abstract

Alburnus istanbulensis (Battalgil, 1941) is a commercially valuable endemic species which exists in the coastal streams of Marmara Region's northern sectors. 317 individuals, which were collected from Darlık Dam, were examined during December 2011- December 2012. The highest GSI values were 13.07±1.63 for females and 6.95±1.28 for males in the beginning of June. The lowest GSI values were observed in August for females (1±0.33) and in September for males (1.05±0.21). By comparing the histological findings with macroscopic observations and GSI values, five developmental stages were determined for females, four developmental stages for males. These findings revealed that *A. istanbulensis* spawn in June.

Keywords: *Alburnus istanbulensis*, spermatogenesis, oogenesis, fish reproduction.

Alburnus istanbulensis (Battalgil, 1941) (Teleostei:Cyprinidae)' in Mevsimsel Gonad Değişiminin Makroskopik ve Mikroskopik Incelenmesi

Özet

Alburnus istanbulensis (Battalgil, 1941) Marmara Bölgesi'nin kuzey kesimlerindeki kıyısal akarsularda bulunan ekonomik açıdan değerli endemik bir türdür. Aralık 2011-Aralık 2012 sürecinde Darlık Barajı'ndan toplanan 317 birey incelenmiştir. En yüksek GSI değerleri, Haziran ayının başında, dişilerde 13,07±1,63 erkeklerde ise 6,95±1,28 olarak görülmüştür. En düşük GSI değeri dişilerde Ağustos ayında (1±0,33) görülürken, erkeklerde ise Eylül ayında (1,05±0,21) gözlenmiştir. Histolojik bulgular, makroskopik gözlemler ve GSI değerleri ile karşılaştırılarak dişilerde beş erkeklerde dört gelişim evresi saptanmıştır. Bu bulgular *A.istanbulensis*'in Haziran ayında yumurta döktüğünü ortaya çıkarmıştır.

Anahtar Kelimeler: *Alburnus istanbulensis*, spermatogenez, oogenez, balık üremesi.

Introduction

One of the most appropriate methods for determining the gonad development cycle in fishes is to examine the seasonal development of gonads (Sivakumaran *et al*., 2003). Histological studies give the most reliable and objective data in gonad staging and they are also significant in determining the maturation cycle (Sivakumaran *et al*., 2003; Tingaud-Sequeira *et al*., 2008). Histological, anatomical and physiological studies have been performed in order to expose the reproductive strategies of many fish species.

Alburnus, which is one of the genera in Cyprinidae family, is distributed through Europe and Western Asia. Turkey is obviously the diversity center of the genus *Alburnus* with 20 species (Özuluğ and Freyhof, 2007a). *Alburnus istanbulensis* (Battalgil, 1941) is an endemic species which exists in coastal streams of Thrace from Papuçdere to Karasu and Lake Sapanca. This species, which is regarded as *Alburnus chalcoides*'s synonym, has been recognized as a valid species after *A. chalcoides* was removed from the fauna of Turkey (Özuluğ and Freyhof, 2007b). There are limited studies about gonad histology and development in *Alburnus* species (Ünver and Yıldırım, 2011; Ünal *et al*., 2005), which has been performed in Ömerli Dam about the reproduction and growth of *Alburnus istanbulensis*.

The aim of this study is to obtain more detailed information about the reproductive biology of *A. istanbulensis* from Darlık Dam and also to examine

the seasonal gonad differentiation.

Materials and Methods

Field Sampling

The fish samples were collected from Darlık Dam. Darlık Dam is located (41°5'3"N 29°34'52"E) in İstanbul, which was built onto Darlık Stream in 1988. Three hundred seventeen specimens were collected monthly in the period from December 2011 to December 2012. Various gill nets with 12-25 mm mesh sizes and electroshock units were used for sampling. Nets were left in the water for 12 hours nightlong and picked up in the morning. In the months May-June sampling was performed in Darlık Stream which enters the lake with electroshock unit in order to collect small sized samples.

Laboratory Processing

Samples were brought to laboratory with cooling boxes. Total Length (L_T), Standard length (L_S) were measured with 1 mm sensitive caliper and Total weight (W_T) with 0.01 g sensitive scale. The gonads were dissected and measured.

Gonadosomatic index (GSI) was calculated via the formula $GSI=W_G x100/W_T$. Scales were used for determining the ages of samples by read via microfiche reader.

Macroscopic and Histological Processing and Staging

Six adult female and six adult male gonads were randomly selected each month from II-III and IV age groups. Gonads were fixed in Bouin for 12 hours, then they were passed through graded alcohols and paraffin blocks were prepared. Tissues were sectioned into 4-5µm thickness and then stained with Hematoxylin and Eosin (H&E). Three slides were prepared from each individual for cell counting and for staging by light microscope. 10 random areas were selected from each slide by using Nikon Eclipse E200 microscope. 10x10 grid was placed onto these areas, then cell counting was performed.

Ovaries were examined according to color, structure and clarity of the oocytes while testes were examined only according to color and structure (West, 1990). Female gonads were divided according to most dominant oocyte type and presence of mature oocyte and atretic follicles (West, 1990; Smith and Walker, 2004), whereas staging process of male individuals was performed according to spermatogonium, spermatocyte, spermatid and spermatozoa numbers (Smith and Walker, 2004; Schulz *et al*., 2010). Immature individuals were ignored in all evaluations.

100 oocytes with different oogenesis stages, which have been selected from random individuals, were measured laterally with ocular micrometer under 10x and 40x magnification. Only cells with nuclear sections were counted. Cells with inappropriate shape and features were ignored.

Results

Morphometric Data

Total and standard size ranges of 317 individuals which were caught in Darlık Dam were shown in Table 1. Male-female ratio of the examined samples is 1:1.56 (123 male, 194 female). Age range is between 0-IV age groups. Size ranges and averages according to the age groups are given in Table 2.

Monthly Changes in Gonadosomatic Index

Three hundred five specimens were investigated for GSI values. The highest GSI value is in the first half of June with 13.07±1.63 for females and 6.95±1.28 for males. In the second half of June GSI values were decreased with 2.44±1.68 for females and 2.48±1.72 for males. The lowest GSI values were observed in August for females (1±0.33) and

Table 1. The monthly total length (mm) distribution of *A. istanbulensis*

Date	Female				Male			
	n	Min-max	mean	SD	n	Min-max	mean	SD
14.12.11	6	147-191	162	13.4	3	148-170	161	7.5
19.01.12	5	155-170	164	6.8	2	155-155	155	-
26.02.12	6	132-191	157	16.8	11	139-189	149	13.9
19.03.12	21	137-200	155	15.7	16	135-177	145	11.5
23.04.12	1	151	-	-	4	109-148	136	18.3
13.05.12	5	116-16	136	13.8	3	132-139	135	12.6
01.06.12	5	111-131	122	9.9	6	101-114	108	5.0
20.06.12	15	53-163	131	35.4	28	102-163	130	38.2
15.07.12	5	71-160	134	41.8	13	130-164	147	9.6
12.08.12	21	140-195	159	11.9	8	132-166	152	13.4
30.09.12	22	155-225	176	15.4	6	141-165	154	13.2
05.11.12	35	146-219	174	16.0	7	146-177	159	16.0
02.12.12	27	154-203	178	15.8	9	147-189	160	15.2
23.12.12	20	160-255	179	23.6	7	129-165	152	12.5

Table 2. Specimen numbers and total lengths (mm) of *A. istanbulensis* according to age groups

Age Groups	Sample size	Total Lenght Min-Max (mean±SD)
O	42	37-133 (75±30)
I	175	105-203 (152±17)
II	71	130-225 (166±17)
III	23	160-211 (184±14)
IV	2	219-255 (237±25)

September for males (1.05±0.21). These results show that *A. istanbulensis* spawn in June. The GSI values of female and male individuals are stable between November and February (Figure 1, Table 3).

Morphology and Histology of Ovary and Testis

Ovaries were divided into five stages morphologically and histologically which are developing, maturating, spawning, spent and re-developing stage (Table 4, Figure 2). Testis morphology and histology was divided into four stages, which are early spermatogenesis, mid spermatogenesis, spawning and spent stage (Table 5 and Table 6, Figure 3). The developmental stages of oocytes and relevant explanations were given in Figure 4. According to the cell counting that were performed in the light of these data, cell ratios are similar at the developmental stage of ovary (January, February and March) and early nucleolar cell is the most abundant (Figure 2A). At the stage of maturation (April and May) an increase in the number of late vitellogenic and mature cells were observed (Figure 2B). At the stage of spawning (June) once again all kinds of cells were present, while atretic follicles were observed in addition (Figure 2C). At the stage of spent (July and August) numerous early and late perinucleolar cells were found (Figure 2D, Figure 5). As a result of cell counting performed on male individuals, the most abundant cell type was primary spermatocyte in the testis at the stage of early spermatogenesis (October, November, December and January) and apart from some spermatogonia A and B cells (Figure 3A-B) were found. Secondary spermatocytes and spermatids were observed at the stage of mid spermatogenesis (February, March, April and May) (Figure 3C-D). The spermatocyst were found, which is full of the spermatozoa in the testis, at the spawning stage (June and July) (Figure 3E-F). Only spermatogonia A and B cells and very few primary spermatocytes were observed at the spent stage (August and September) in the testis (Figure 3G-H, Figure 6).

Reproduction Period

Reproductive period of *A. istanbulensis* was revealed with macroscopic and microscopic observations. According to these findings, spawning starts in June and ends in the beginning of July. Male and female individuals reach to sexual maturity at the first age. The most significant indicator of the spawning time is to observe atretic follicles in females in June (Figure 6).

Gonad development of both sexes is synchronic in Darlık *A. istanbulensis* population. Histological and macroscopic findings of monthly collected female and male samples and the results of gonadosomatic index values show that *A. istanbulensis* spawning occurs once a year.

Discussion

Alburnus istanbulensis is a commercially valuable endemic species which exists in Marmara Region (Özuluğ and Freyhof, 2007b). In this study, annual gonadal development of *A. istanbulensis* was determined monthly by macroscopic, microscopic examinations and GSI values were calculated.

Although there are studies about the reproductive biology of fishes from the genus *Alburnus* (Guerriero *et al*. 1998; Ünal *et al*., 2005; Ünver and Yıldırım, 2011), there is only one study about *A. istanbulensis* (Tarkan *et al*., 2005). The gonad histology of *A. istanbulensis* has been examined for the first time in this study.

GSI is an ideal way to show the seasonal gonad change for the fishes that are spawning once a year (Barros and Regidor, 2002). The monthly GSI analysis and histological findings showed that ovarium type of *A. istanbulensis* is group synchronous and this species spawns once in a breeding season for females and males as *Alburnus* sp. collected from Lake Tödürge (Ünver and Saraydin, 2004).

Four development stages were observed in histological analyses in males. Mid spermatogenesis stage was observed between February and May, following that spawning stage was occurred in June and at the beginning of July. The spawning stage that had started in June continued until July and spent stage was observed in the samples that had been taken in August. Ovary that develops between January and May reaches to the maximum weight and mature oocyte number in June. Numerous atretic follicles were found in the samples that were taken in July. An obvious decrement in GSI values of the samples which were taken in the second half of June was also seen. According to these findings, it can be said that the reproductive period of *A. istanbulensis* in the Darlık Dam is between the second half of June and

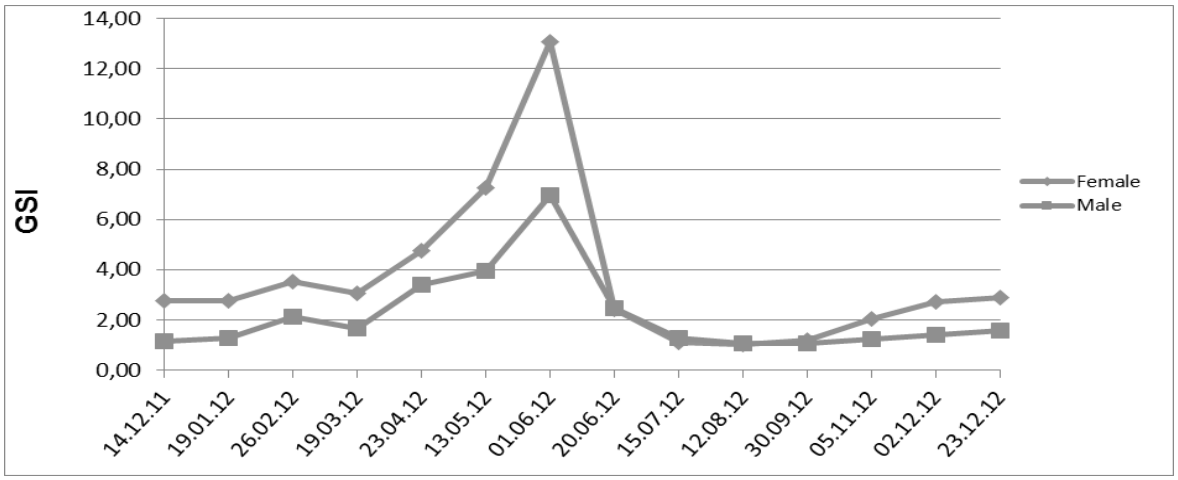

Figure 1. Monthly variations of the GSI values of *A. istanbulensis.*

Table 3. The monthly GSI changes of *A. istanbulensis*

Date	Female				Male			
	n	Min-Max	Mean	SD	n	Min-Max	Mean	SD
14.12.11	6	1.58-4.46	2.76	0.96	3	1.02-1.32	1.13	0.16
19.01.12	5	2.12-3.15	2.76	0.42	2	1.11-1.44	1.27	0.23
26.02.12	6	2.44-2.47	3.52	0.73	11	0.56-2.92	2.13	0.70
19.03.12	21	1.14-5.08	3.07	0.86	16	1.13-3.26	1.65	0.54
23.04.12	1	4.76-4.76	4.76	-	4	0.71-5.73	3.39	2.13
13.05.12	5	4.50-10.34	7.25	2.47	3	1.01-6.01	3.95	2.62
01.06.12	5	11.34-16.05	13.07	1.63	6	5.51-8.63	6.95	1.28
20.06.12	3	0.86-7.07	2.44	1.68	28	0.73-7.22	2.48	1.72
15.07.12	5	0.19-2.11	1.09	0.79	13	0.32-5.34	1.27	1.24
12.08.12	21	0.47-1.63	1.00	0.33	8	0.31-2.01	1.06	0.67
30.09.12	22	0.87-1.59	1.19	0.22	6	0.64-1.22	1.05	0.21
05.11.12	35	1.29-3.11	2.04	0.51	7	0.69-1.78	1.25	0.44
02.12.12	27	2.00-3.96	2.70	0.48	9	0.57-2.19	1.40	0.49
23.12.12	20	2.34-3.96	2.87	0.45	7	0.25-2.64	1.58	0.77

Table 4. Macroscopic and histological characteristics of ovaries at five developmental stages together with the average gonadosomatic index values between January-December 2012

Stage	Macroscopic	Histological	GSI
Developing	Ovary has an average diameter of 3.5 mm and blue-gray color. Oocytes aren't distinctive.	Pre-vitellogenic oocytes (early and late perinucleolar) are dominant. Early vitellogenic and vitellogenic cells are also observed.	GSI=3.12 n=32
Maturation	Ovary has an average diameter of 4.4 mm. Oocytes usually have yellow color and are easily distinguished by naked eye.	Vitellogenic and late vitellogenic oocytes are dominant. Also mature oocytes are observed, though in small amounts. Although advanced oocytes are dominant, it is possible to observe cells at each stage.	GSI=6.01 n=6
Spawning	Ovaries at this stage are quite thickened and have a diameter of 4.9 mm approximately. Ovary has blood vessels with red-pink color and its oocytes are obvious.	Oocytes at late vitellogenic and mature stages are dominant. At this stage atretic follicles are frequently observed so that it indicates the spawning.	GSI=7.75 n=8
Spent	Ovaries have a thread-like appearance. Ovaries have orangish white color and have a diameter of 2.1 mm approximately. Blood vessels are distinctive.	Early and late perinucleolar oocytes are mostly observed at this stage. It is possible to see cortical alveoli and a very small quantity of early vitellogenic oocytes.	GSI=1.04 n=26
Re-Developing	Ovaries with different colors including yellow-orange and green are observed and their average diameter is 3.2 mm.	All stages of vitellogenic oocytes are observed. Early vitellogenic and vitellogenic cells are dominant. Late vitellogenic and mature cells are present even if just a bit.	GSI=2.00 n=104

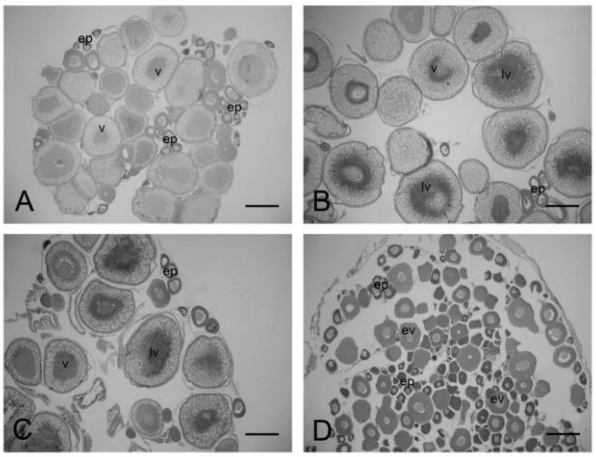

Figure 2. The histological structure of ovarium in *A. istanbulensis* (A) Developing, (B) Maturating, (C) Spawning and (D) Spent stages. ep: early perinucleolar, v: vitellogenic, lv: late vitellogenic, ev: early vitellogenic (x4, scale bar: 300µ; H&E).

Table 5. Macroscopic characteristics of testes at four developmental stages and gonadosomatic index values of *A. istanbulensis*

Stage	Macroscopic Appearance	GSI
Early spermatogenesis	Testes are usually slightly transparent and gray-pink colored and they have short and thick appearance. Since the blood vessels are not so distinctive, appearance is transparent. The average diameter of testis is 2.1 mm at this stage	1.39 n=25
Mid spermatogenesis	Since blood vessels are more distinctive, testes appear to have pink color. Testis has an average diameter of 2.7 mm.	2.77 n=34
Spawning	Testes of fishes at this stage have reached to an average diameter of 3.4 mm by getting quite thick. Blood vessels are so distinctive and usually have white-gray color.	3.58 n=47
Spent	The testes of the individuals have emptied therefore became thinner. Their colors are usually pink-white and diameters are 1.1 mm averagely.	1.05 n=14

Table 6. Histological characteristics of testes at four developmental stages of *A. istanbulensis*

Stage	Histology
Early spermatogenesis	Especially 3 types of cell are seen at this stage. Spermatogonia A and B are abundant. Spermatocysts of primary spermatocytes are quite dominant and encircle the lobules.
Mid spermatogenesis	Primary and secondary spermatocytes are dominant and developed cells migrate towards the lumen. Spermatogonia A and B type cells are also observed but they are fewer compared to spermatocytes in terms of number.
Spawning	Lumens are broadened that testis appears to have lost its unity. At these stage spermatids can be found in addition to primary and secondary spermatocytes. Numerous mature sperms are observed as a pack or dispersed in the lumen inside the spermatocysts. Mature sperms can be easily distinguished by their large, basophilic head and their long eosinophilic tail.
Spent	Lobules are narrowed because the sperms are shed. Interstitial tissue and blood vessels are distinctive. At this stage, spermatogonia A and B are the dominant cell types. Spermatocyte can be seen rarely.

the beginning of July.

It has been stated that the species of *Alburnus albidus* from the pond of the Alento River spawn between May and June (Guerriero *et al*.,1998). Ünver and Yıldırım (2011) stated that spawning of *Alburnus* sp. collected from Lake Tödürge, was occured between May and June. In another study, which has been carried out the ovary of *Alburnus* sp. from Lake Tödürge, it was stated that spawning begins at the end of May and ends at the beginning of July (Ünver and Saraydin, 2004). In a study that examined the species *Alburnus tarichi* collected from Lake Van and Karasu Stream, reproduction occurs between May and June (Ünal *et al*., 1999). In the study which was carried out with the species *A. istanbulensis* caught from Ömerli Dam, the GSI values of the individuals were examined and it was detected that this species reproduce May and June

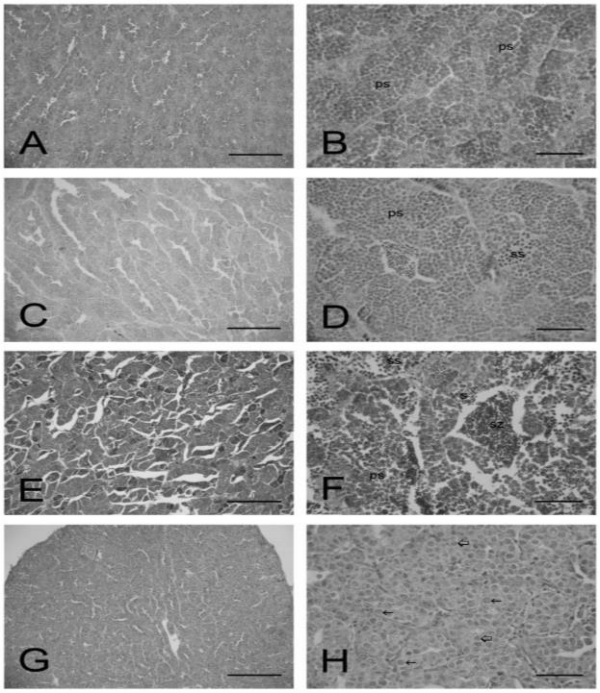

Figure 3. Histological appearance of testes in *A. istanbulensis* (A-B) Developing, (C-D) Maturating, (E-F) Spawning and (G-H) Spent stages. ps: primary spermatocyte, ss: secondary spermatocyte, sz: spermatozoa, s: spermatid, arrow: spermatogonia A, hollow arrow: spermatogonia B (A, C, E and G x10 scale bar: 200µ; B, D, F and H x40 scale bar: 50µ; H&E).

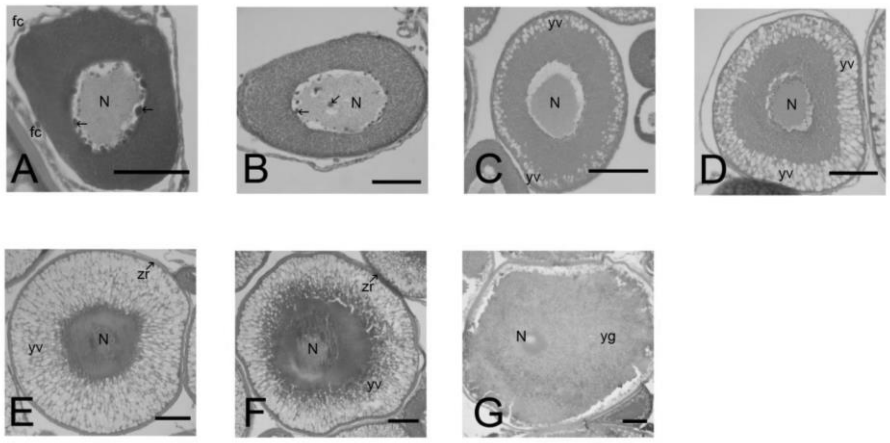

Figure 4. Development stages of oocytes. fc: follicle cell, N: nucleus, yv: yolk vesicle, zr: zona radiate, yg: yolk granule, arrow: nucleoli (A and B x40, scale bar:50µ; C-G x10, scale bar:100µ; H&E).

(A) Early Perinucleolar: This type of oocytes (r=108µ) does not have smooth shape and they have a large nucleus (56µ). Nucleoli are in the periphery of nucleus. Their numbers and sizes vary from cell to cell. They are round and stained dark with hematoxylin. Nucleus is eosinophilic. Oocytes are covered with a thin follicle layer.

(B) Late Perinucleolar: The sizes of oocytes (177µ) and nucleus (78µ) are larger than perinucleolar oocyte, but they are similar in terms of general features. Nucleoli are in the periphery of nucleus and numerically larger. The affinity of cytoplasm for hematoxylin has decreased, Therefore it is stained paler. Nucleus is eosinophilic.

(C) Cortical Alveoli: Small yolk vesicles are arranged as 2-3 lines in the periphery of cytoplasm in these oocytes (230µ). These vesicles are going to fill the whole cytoplasm and form bigger vesicles by merging in the end. The affinity of cytoplasm for hematoxylin decreases and it is stained paler. Nucleus (86µ) is eosinophilic.

(D) Early Vitellogenic: The number of yolk vesicles increases and half of the cytoplasm is filled. Inside of these vesicles begin to be filled with strongly eosinophilic yolk granules and from the periphery of cytoplasm to the nucleus. This is the beginning of real vitellogenesis. The diameter of oocyte (349µ) increases, nucleus (93µ) starts to lose its smooth shape.

(E) Vitellogenic: Yolk granules fill more than the half of cytoplasm. Sizes of oocyte (515µ) and nucleus (105µ) increase. Small yolk granules cause larger yolk vesicles to be formed by merging into large vesicles. Zona raidata forms between oocyte and follicle layer and is distinctive in these types of oocytes.

(F) Late vitellogenic: Yolk granules fill two thirds or more of cytoplasm and are eosinophilic. The sizes of oocyte (620µ) and nucleus (116µ) become larger. Zona radiata is very distinctive and has become thicker.

(G) Mature: Almost the whole cytoplasm is filled with yolk granules. Nucleus begins to migrate from the center of oocyte to its periphery and ovulation occurs as a result. The diameter of oocyte reaches to its maximum value (790µ).

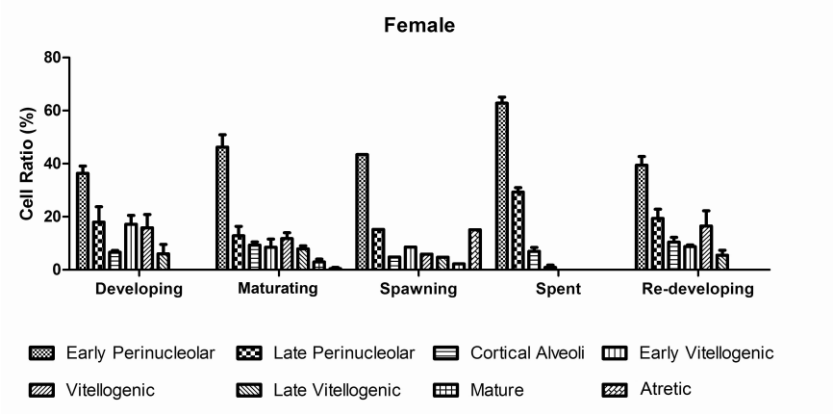

Figure 5. Cell ratios of female individuals of *A. istanbulensis* at each stage of development.

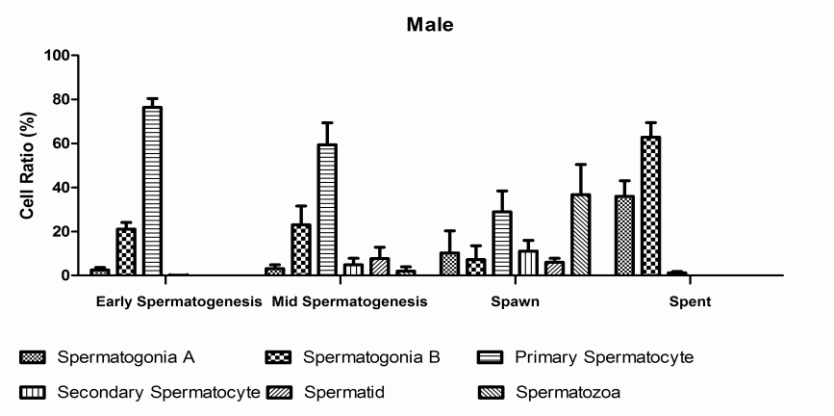

Figure 6. Cell ratios of male individuals of *A. istanbulensis* at each stage of development.

(Tarkan *et al*., 2005). These data showed that the spawning time of *A. istanbulensis* is parallel with the other *Alburnus* species. It is also known that small differences may originate from environmental factors, such as the quantity of precipitation, temperature and photoperiodism (Miranda *et al*., 2009).

In conclusion it was determined that, in accordance with the macroscopic and microscopic methods and GSI analysis, the population of *A. istanbulensis*, which lives in Darlık Dam, spawn June. The data obtained by this study will give information for future studies carried out on *A. istanbulensis.* It will also enable the reproductive cycles of *A. istanbulensis* populations that live in other regions and close species to be compared.

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