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## Original Article

# Ovarian development of Caspian roach, *Rutilus caspicus*, in southern Caspian Sea: A histological and ultrastructural study

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**Abstract:** The histology and ultrastructure of the ovarian maturation process in Caspian roach, *Rutilus caspicus*, was studied. A total 170 female specimens were collected from the Gharasoo River, Bandar Turkmen, the southern Caspian Sea to evaluate its maturation cycle. Based on the results, its ovarian follicle's development could be classified into six stages by distinct characteristics. Minimum and maximum diameter of oocytes were recorded in the chromatin-nucleolus and maturation stages as  $56.34 \pm 3.74$  and  $918.83 \pm 14.82$   $\mu\text{m}$ , respectively. The zona radiata was observed from the cortical alveoli stage and its maximum diameter measured in the secondary vitellogenesis stage as  $93.11 \pm 23.0$   $\mu\text{m}$ . Gonadosomatic index (GSI) reached its peak in mid-March and its sharp drop in the late April showed its spawning period from late March or early April till the end of April. A positive correlation was found between the GSI and HSI in the vitellogenesis stage. The results also revealed Caspian roach as iteroparous synchronous spawner.

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## Introduction

Reproductive cycle involves sequential changes of the germ cells based on a typical pattern of changes for each species and this cycle is effected by environmental factors (Shimizu, 2003; Norberg et al., 2004; Howell et al., 2003). Histological study of the seasonal development of gonads is a useful tool to obtain information on the reproductive mechanism of fishes. This type of information, is not only important in the aquaculture industry, but also can apply to preserve fish stocks in wild.

Although, little information about the reproduction cycle of Caspian roach, *Rutilus caspicus*, is available, but there are investigations on other species of this genus (Jafri and Ensor, 1979; Vøllestad, 1987; Vøllestad and L'Abée-Lund, 1987; Jobling et al., 1998; Minier et al., 2000; Sivakumaran et al., 2003; Tyler et al., 2006; Tempero et al., 2006; Shafiei Sabet et al., 2010; Ghomi et al., 2011). *Rutilus caspicus* is a migratory fish found in the southern Caspian sea, in the coastal waters of Iran

and Turkmenistan (Halimi et al., 2011). They enter Iranian rivers e.g. Atrak, Gharasoo and Gorgan Rivers for spawning. The reproduction migration in the southern Caspian Sea starts from January-February to April, frequently when water temperature reaches 10-12°C (Golpour et al., 2013). This species is economically valuable in the Caspian Sea and is also considered as an important food source for sturgeon and other carnivorous fishes (Kiabi et al., 1999).

In recent years, the natural stock of Caspian roach has drastically reduced due to anthropological activities such as water pollution, rivers degradation and overfishing. Therefore, it has been considered as a threatened species of the Caspian Sea (Kiabi et al., 1999). Hence, the present study aimed to describe the reproductive cycle of the female Caspian Roach using histological and ultrastructural assessment. Gonado-somatic index (GSI), Hepato-somatic index (HSI), and relative frequencies of the different type of follicles were other studied parameters.

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## Materials and Methods

**Sampling:** A total of 78 mature female specimens were collected from the Gharasoo River (36°49'N, 54°02'E), 8 times (7-10 fish each time) from February 2012 to December 2013 (on 9 February, 1 March, 19 March, 4 April, 19 April, 12 October, 11 November and 12 December; during their presence in the Gharasoo River), using gill and seine nets with a mesh size of 15 mm. Fish were anesthetized using a solution of 100 ppm of tricaine methane sulfonate, (Sigma, Deisenhofen, Germany), then samples of the ovarian follicles were taken from the anterior, middle and posterior parts of their gonads.

**Biometric and histometric study:** For each specimen, the age was estimated using scale method according to Perlmutter (1954). The total length (to the nearest mm) and total body, gonad and liver weights (to the nearest 0.01 g) were recorded. For morphological analyses, we selected undamaged oocytes without retraction and with cuts crossing the nucleus. Gonado somatic indices (GSI) was calculates as follow (Nikolski, 1963):

$$\text{GSI} = (\text{gonads weight} / \text{total weight}) \times 100$$

The hepato-somatic indices was estimated according to following formula (Biswas, 1993):

$$\text{HSI} = (\text{liver weight} / \text{total weight}) \times 100$$

**Gonad histology and ultrastructural study:** The ovarian follicles were fixed into Bouin's solution and the histological slides in 5  $\mu\text{m}$  thickness were prepared based on Patki (1987) and Eagderi et al. (2013). The prepared slides were stained with hematoxylin-eosine based on Clark (1981). To study the zona radiata and yolk granules of the ovarian follicles, the gonad sections were also stained using Periodic Acid-Schife (PAS) based on Banning (1959). The histological sections were studied using the Nikon light microscopy. Photomicrographs were taken using Dinolit 2.00 software.

For transmission electron microscopy (TEM), 0.1 mm pieces of the ovarian tissue were kept at 4°C, in 2.5% glutarealdehyd-phosphate with 0.2 M sodium caccodilate (PH 7.4) and post-fixed in 1% osmium tetroxide in the same buffer. Then embedded in Apon-Araldite resin. Ultra-thin sections were double contrasted with uranyl acetate and lead citrate. Examination and electrography were made on a HITACHI- S4160 electron microscope.

The description of each oocyte development phase was made according to terminology proposed by Geraudie et al. (2010) and West (1990).

**Statistical Analysis:** Significant differences between groups were performed using one-way analysis of variance (ANOVA), and student *t*-test after normality test using Shapiro-Wilk test. The  $P < 0.05$  was used as significance level for data evaluation. All data were analyzed using SPSS software (version 18).

## Results

Mean water temperature and daylight time (light intensity above 100  $\text{W m}^{-2}$ ) during spawning period of Caspian roach were presented in Table 1.

**Histological and ultrastructural study:** The follicles in different developmental stages were observed in ovaries of the Caspian roach during their development. Based on the results, no significant difference was observed in the oocyte frequency between three anterior, middle and posterior parts of ovaries ( $P > 0.01$ ). Based on the cell size, morphology and vitellus accumulation rate, the follicle's development can be classified into six stages as follow:

**1-Chromatin-nucleolus follicle,** with relatively small size (Table 2), resulted from mitotic divisions of germ cells. The oocytes were mostly spherical in shape and its ooplasm was thin and intensely stained while the nucleus was not (Fig. 1A, B). The

Table 1. The average of natural temperature and photoperiod in Gharasoo River from February to April, during spawning period.

Months	9 Feb.	1 March	19 March	4 April	19 April
Water Tempature (°C)	8	10.7	11.1	13.5	13.7
L/D (hour)	10.5/13.5	11.5/12.5	12/12	12.5/11.5	13/11

Table 2. The average of follicles, nucleus and zona radiata diameter of *Rutilus caspicus* in different stage of development.

Development stage	Mean of follicle diameter (µm)	Mean of nucleus diameter (µm)	Mean of Zona radiata diameter (µm)
Chromatin Nucleolus	56.34±3.74	23.97±3.16	-
Perinucleolus	78.55±5.03	49.21±5.15	-
Cortical Alveolus	320.89±43.33	58.61±5.25	6.32±0.36
Primary Vitellogenic	597.12±30.51	122.31±11.27	9.37±1.14
Secondary Vitellogenic	883.73±53.20	127.86±14.52	11.93±0.23
Maturation	918.83±14.82	-	10.1±0.86

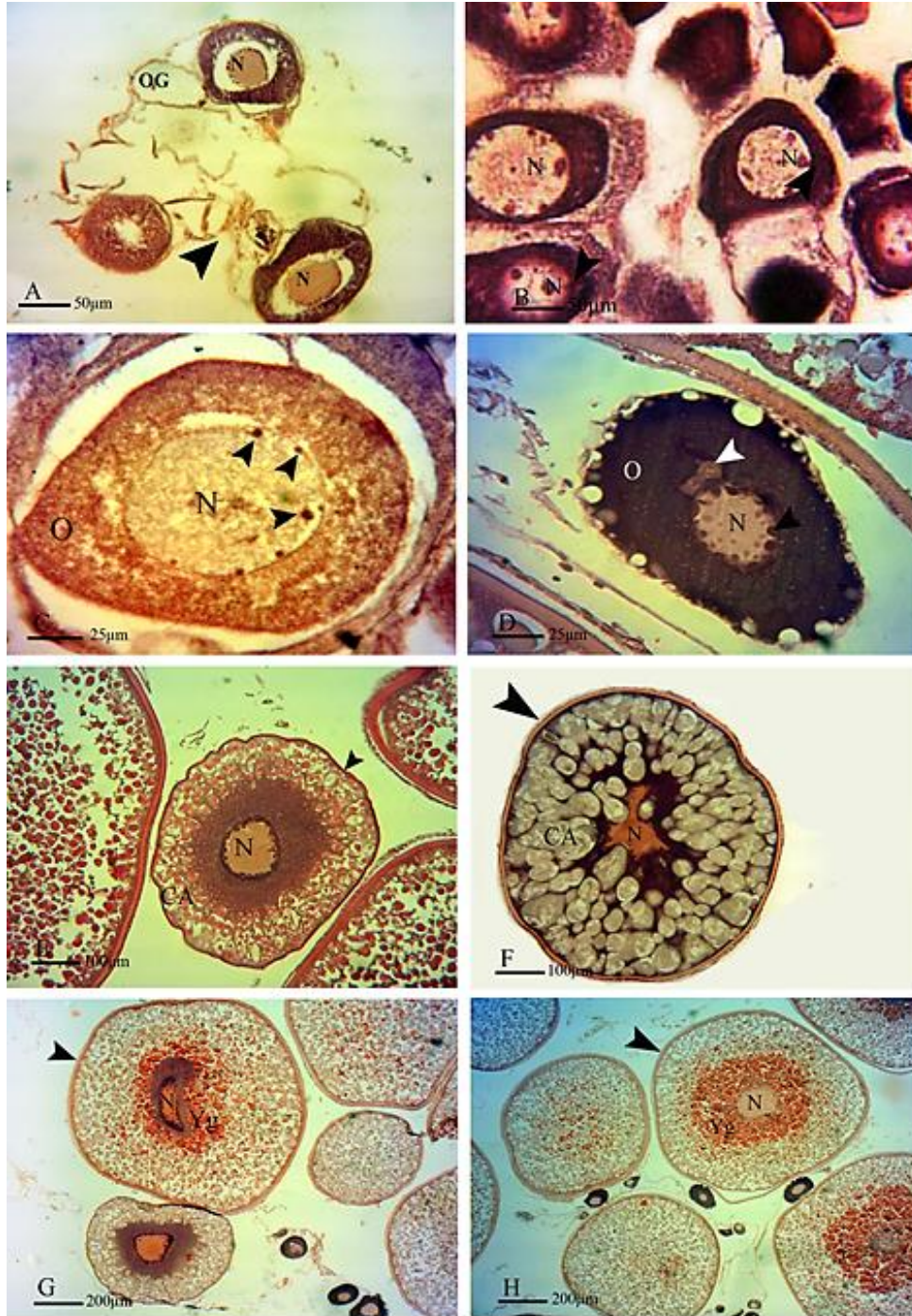




Figure 1. The sections of ovary in *Rutilus caspicus*. (A) Early chromatin, nucleus stage (black arrows: germinal cells) (H&E), (B) late chromatin, nucleus stage (black arrows: scattered nucleolies) (H&E), (C and D) perinucleus stage, several nucleolies (black arrows) appear beneath the nuclear membrane, juxta nuclear complex of organelles (balbiani body) appear near the nucleus (white arrow), ooplasm (O) is intensively basophilic, (E) Early Cortical alveoli stage (H&E) cortical alveolies appear at the peripheral zone of ooplasm (CA), (F) alveolies increase in number gradually at late stage (PAS), (G) primary vitellogenic stage (H&E); yolk granules (Yg) can be seen at the central zone, follicle layers can be seen easily (black arrow), and (H) secondary vitellogenic stage (H&E), the yolk granules (Yg) gradually extend to out and fill whole ooplasm (N: nucleus and OG: oogonia cells).

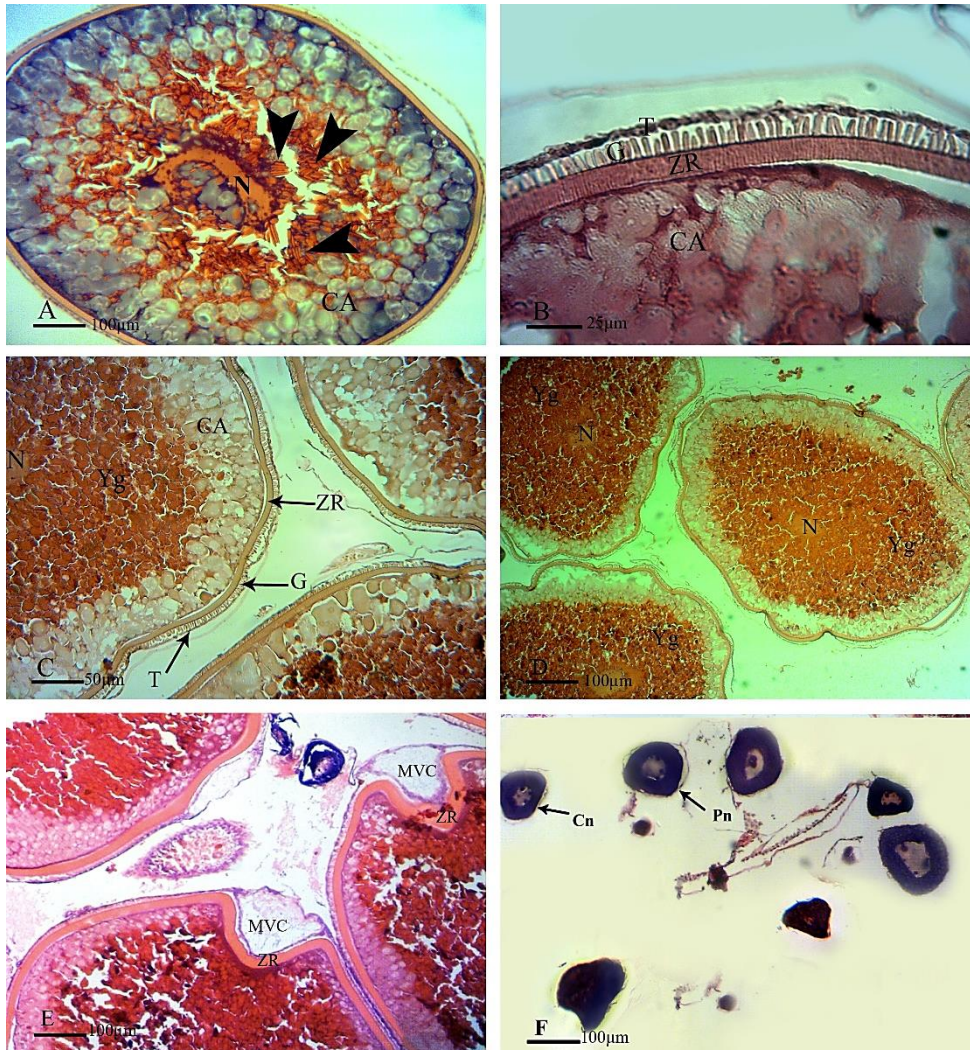


Figure 2. The sections of ovary in *Rutilus caspicus*. (A) Yolk granules corresponded to small elongated structures (black arrow), in secondary vitellogenic oocyte (PAS), (B) follicular layers of oocyte at vitellogenic stage, (C) early maturation stage of oocyte; zona radiata (ZR) is thick and granulosa (G) and theca (T) cells are easily identified, (D) early mature stage of oocyte, the nucleus (N) moved toward the animal pole of cell, yolk granules (Yg) are combined together and fill ooplasm totally, (E) later oocyte maturation stage, microvillous corridor (MvC) is appeared in animal pole and (F) discharged oocyte; perinucleolus oocytes (Pn) and chromatin-nucleolus oocytes (CN) can be seen (N: nucleus, Yg: yolk granules, and ZR: Zona radiata).

synaptonemal complex was ultrastructurally observed during meiosis was (Fig. 3A).

**2- Perinucleolus follicle**, was larger in size (Table 2). This stage characterised by presence of several nucleoli oriented peripherally beneath the nuclear membrane. The ooplasm contained the juxta nuclear complex (Balbiani body) (Fig. 1D). The follicular epithelium was observed in late perinucleus follicles,

particularly in large ones; however, differentiation of these two epithelial layer was not possible. The ooplasm was intensively basophilic due to abundant free ribosomes in cytoplasm that make granular appearance (Fig. 1C). Just few structures, such as endoplasmic reticulum and mitochondria could be distinguished in TEM pictures (Fig. 3B).

**3-Cortical alveolus follicles** ranged from  $320.89 \pm$

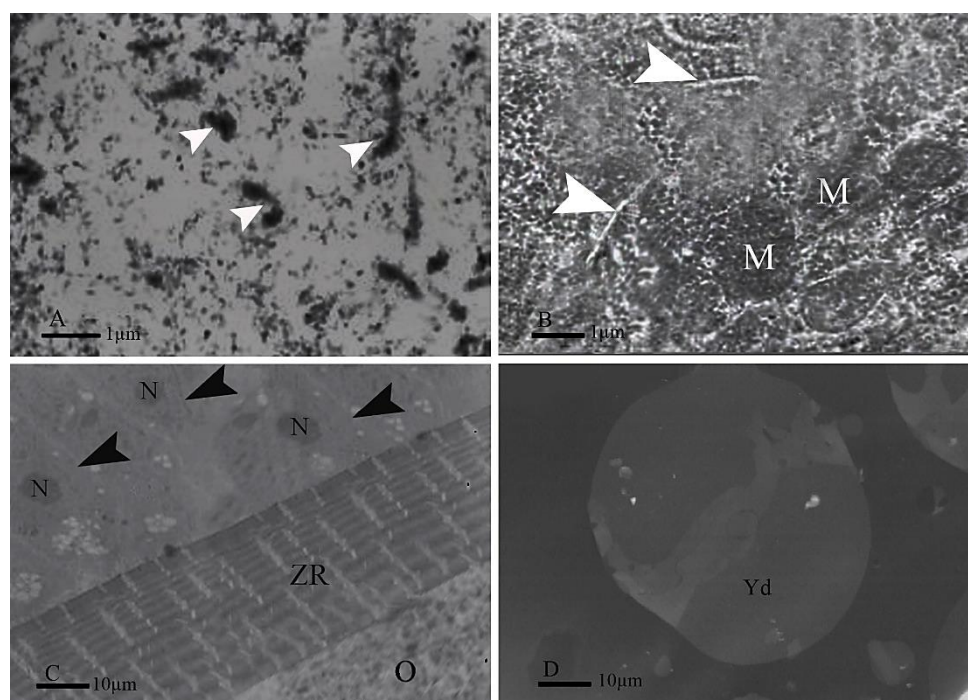


Figure 3. (A) Synaptonemal complex in the chromatin-nucleolus oocytes of *Rutilus caspius* (TEM), (B) perinucleolus oocytes, (C) radial striae occur in the zona radiata at late vitellogenic stage and (D) a yolk drop (Yd) in the animal hemisphere of mature oocyte (TEM) (M: mitochondria, N: nucleus, ZR: zona radiata, white arrows: rough reticulum endoplasmic, black arrows: follicle cell).

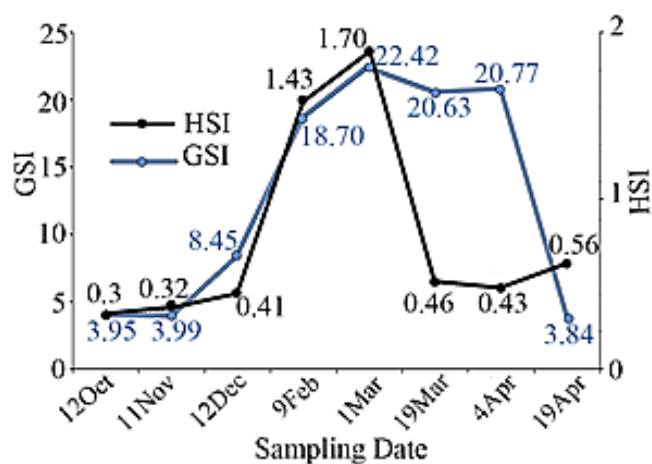


Figure 4. Gonado-somatic indices (GSI) and Hepato-somatic indices (HSI) during reproductive cycle of *Rutilus caspius*.

34.33  $\mu\text{m}$  in diameter (Table 2). In this stage, cortical alveoli were firstly appeared at the peripheral zone of the ooplasm (Fig. 1E) and increased in number to form a peripheral row. Then, the alveolies which enlarged and got denser (Fig. 1F). In addition, the nuclei were irregular in shape. Most of the nucleoli were attached to inner border of nuclear membrane. In the late cortical alveolus follicles, the follicular layers were thickened and zona radiata start to appear. Balbiani body disappeared in this stage (Fig.

1E).

**4-Primary vitellogenic follicles:** the vitellogenesis is occurred in the follicles ranged from  $395.57 \pm 11.27$   $\mu\text{m}$  in diameter (Table 2). The yolk droplets are observed firstly at the central zone of the oocytes (Fig. 1G). The zona radiata was thicker and follicular cells were easily identified (Fig. 2B).

**5-Secondary vitellogenic follicles:** The accumulation of lipoproteic yolk was completed during the secondary vitellogenic stage. Because of the continuous accumulation of yolk sac, the limited area occupied by ooplasm (Fig. 1H). The yolk accumulated as vesicles or granules. The yolk granules were as small elongated structures that were less abundant than yolk vesicles (Fig. 2A). Secondary oocytes reached the maximum size and characterised by irregular nucleus membrane and maximum thickness of zona radiata (Table 2). The secondary oocytes were surrounded by a large acellular envelope exhibiting a porous fibrillar structure. The radial striae was occurred in the zona radiata at the end of this stage (Fig. 3C).

**6-Mature follicles:** Follicular layers were folded irregularly. The nucleus is moved toward the animal



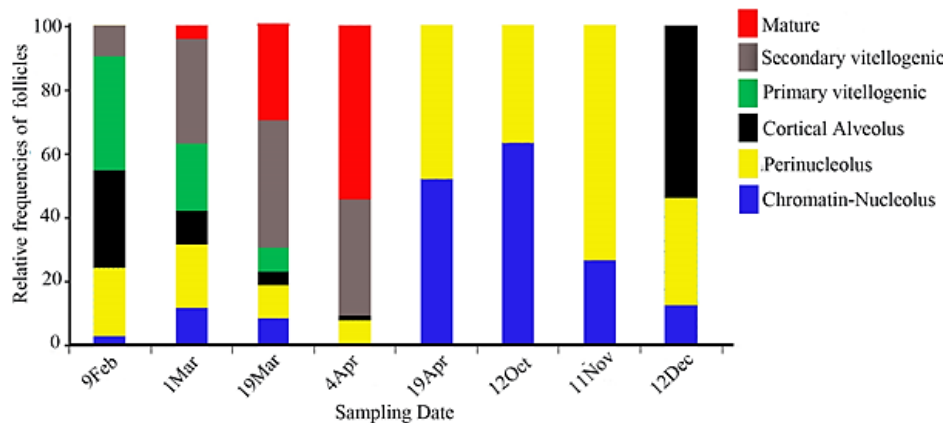


Figure 5. Frequencies of the different stages of follicles in *Rutilus caspicus* during annual reproductive cycle.

pole (Fig. 2D) where microvillus corridor (MVC) (Fig. 2E) was appeared gradually. Lipids that accumulated during vitellogenesis, joined together and formed several distinguishable lipid drops in the animal hemisphere (Fig. 3D). Later, the nuclear membrane disappeared. The microvillus layer continued to grow and rolled around the oocyte.

GSI is gradually increased during the November to early April and reached its peak as  $20.77 \pm 1.1$ . Then, it is significantly decreased. HSI is sharply increased from December to early March and then is suddenly decreased in the mid of March (Fig. 4). Frequency of the different oocyte types during the annual reproductive cycle of Caspian roach is presented in Figure 5. The chromatin-nucleolus and perinucleolus oocytes were present throughout year while mature oocytes were observed only in March and mid of April (Fig. 5).

## Discussion

Oocyte maturation follows a similar pattern in most teleosts (Casadevall et al., 1993; Carrason and Bau, 2003; Brandão et al., 2003). In teleosts, the process of oogenesis may be divided into five, six or eight stages (Nagahama, 1983; Fishelson et al., 1996; Ünal et al., 1999; Gökçe et al., 2003). According to our result, the oocyte development of Caspian roach follows the same pattern as other teleosts (Selman et al., 1993). Based on the histological and ultra-structural characteristics of follicles, this process in *R. caspicus* could be described in 6 stages, including chromatin-nucleolus, perinucleolus, cortical

alveolus, vitellogenic (primary and secondary) and mature follicles. Geraudi et al. (2010) divided oocyte maturation in *Rutilus rutilus* into five stages based on cell size, morphology and extent of vitellus accumulation. Ovaries of *R. rutilus* contain oocytes in synchronous groups which were discharged together once a year (Geraudi et al., 2010). Microscopic analysis indicated a group synchronous oocyte development characterized as iteroparous synchronous spawners.

Early stage of oocyte maturation in fishes is characterized by an active synthesis of RNAs (Cárdenas et al., 2008) and is easily identified through the abundance of rough endoplasmic reticulum and mitochondria in the ooplasm as observed in Caspian roach. Oocytes gradually accumulate energy reservoirs and enlarge. In the present study, the size of oocytes increased gradually during gonad maturation and the appearance of the yolk vesicle within the oocytes was an indicator of maturation. According to Geraudi et al. (2010), the size of oocyte in *R. rutilus* ranged from  $11.8 \mu\text{m}$  (oogonia) to  $639 \mu\text{m}$  (secondary oocyte) during gonadal development. The results of the current study showed the size of oocytes increased sharply during oogenesis (from  $54.55 \mu\text{m}$  in chromatin-nucleolus stage to  $918.83 \mu\text{m}$  in mature oocytes). In fish species with pelagic eggs, water absorption is mainly responsible for increasing oocyte size (Shabanipour and Haidari, 2004); however, in species with benthic eggs, vitellogenin accumulation is main cause of oocytes enlargement (Ravaglia and

Maggese, 2002; Heidari et al., 2009). Based on the results, vitellogenesis could be considered as the main cause of oocyte enlargement in *R. caspicus*; since increasing egg diameter during this stage was significantly high.

Zona radiata is related to the pore channels that regulates the transportation of yolk substances (Leino et al., 2005). The zona radiata was observed in oocyte developmental stages, including cortical alveolus, vitellogenic and mature oocytes in Caspian roach. Its minimum and maximum diameters were  $5.94 \pm 0.32 \mu\text{m}$  in the cortical alveolus stage and  $11.93 \pm 0.23 \mu\text{m}$  in the vitellogenic oocyte, respectively. Demersal eggs are often subjected to abrasive forces and then generally develop thick envelopes with complex lamellae around eggs (Guraya, 1986; Suzuki et al., 2000; Brandão et al., 2003). Therefore, the differences in zona radiata's thickness is directly related to oocytes resistance (Brandão et al., 2003). A thicker zona radiata can provide mechanic protection against abrasion at the bottom (Nagahama, 1983). According to Suzuki (2000), the thickness of the zona radiata is an adaptation to protect the egg from physical abrasion in the environment.

GSI has been widely used in different fishes as an indicator of spawning period (Santos et al., 2005; Chandrasekhara Rao and Krishnan, 2009; Geraudie et al., 2010). However, using this indices along with other reproductive indicators such as histological assay techniques can be more effective. In the present study, the GSI ranged from  $3.95 \pm 1.22$  (during previtellogenic stage) to  $20.77 \pm 1.11$  (during vitellogenic stages and just before spawning) and seems to be due to increase oocyte size by vitellogenin accumulation during the vitellogenic stages. In mature stage, the GSI reached the highest values when the mature gonads occupied almost the whole coelomic cavity. In fact, following an increase in oocyte size that causes an increase in gonad weight, GSI also increases. The results also showed a significant increase of HSI from November to early March when the yolk granules were occupying most of the oocytes. Liver synthesis the precursors of yolk

granules (vitellogenin) which accumulate in the oocytes during vitellogenesis stages. Hence, an increase in liver weight (which leads to an increase in HSI value) prior to spawning, may be related to vitellogenin synthesis in the liver. Based on the these results, the breeding season in *R. caspicus* population from the Gharasoo River starts from early April and is last till the end of April.

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## چکیده فارسی

### مطالعه توسعه تخمدان کلمه خزری (*Rutilus caspicus*) در جنوب دریای خزر: مطالعه ساختار بافتی و فراساختاری

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#### چکیده:

در این پژوهش بافت‌شناسی و فراساختاری روند بلوغ تخمدانی کلمه خزری *Rutilus caspicus* مورد مطالعه قرار گرفت. تعداد ۱۷۰ قطعه ماهی کلمه ماده از رودخانه قره‌سو، بندر ترکمن، حوضه جنوب دریای خزر از دیدگاه ریخت‌شناسی برای ارزیابی چرخه‌ی رسیدگی جنسی بلوغ این گونه مورد مطالعه قرار گرفت. نتایج نشان داد که مراحل توسعه فولیکول‌های تخمدانی می‌تواند به شش مرحله با مشخصات متمایز تقسیم شود. حداقل و حداکثر قطر اووسیت به ترتیب در مرحله‌ی کروماتین هسته و مرحله بلوغ به مقدار  $56/34 \pm 3/74$  و  $918/83 \pm 14/82$  میکرومتر اندازه‌گیری گردید. لایه‌ی زونا رادیاتا از مرحله‌ی آلوتول قشری در اووسیت‌ها قابل مشاهده بوده و بیشترین میانگین قطر این لایه  $11/93 \pm 0/23$  میکرومتر در مرحله‌ی زرده‌سازی ثانویه اندازه‌گیری شد. مقدار شاخص گنادی (GSI) در اواسط اسفندماه به بالاترین میزان رسید و افت شدید آن در پایان فروردین ماه نشان داد که دوره تخم‌ریزی این گونه، از اواخر اسفندماه تا فروردین ماه می‌باشد. یک همبستگی مثبت بین شاخص گنادی و شاخص کبدی در مرحله‌ی زرده‌سازی یافت شد. نتایج همچنین نشان داد که کلمه خزری یک تخم‌ریز سالیانه همزمان می‌باشد.

**کلمات کلیدی:** چرخه تولیدمثلی، شاخص گنادی، زونا رادیاتا، فصل تخم‌ریزی.