Histological and histometrical study of common carp ovarian development during breeding season in Khouzestan province in Iran

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Abstract:

This survey was aimed at detecting histological and histometrical changes of Common Carp ovaries in Khouzestan Province climate conditions. The goal was to determine the ovarian status in the breeding period of the reproductive cycle. Ovaries of 49 Common Carp brooders were collected from April to October, 2010. The samples were taken from the anterior, median and posterior portions. 5-6µ sections were made by the paraffin embedding method and stained by H&E and PAS. The results showed that the breeding season in Common Carp in this area takes at least 7 months and spawning continues from April to October. It seems that the previtellogenesis and vitellogenesis stages are short in the Khouzestan climate conditions and this species rapidly enters into the maturation phase. It seems that Common Carp have the ability to spawn three times during an annual cycle in the Khouzestan climate conditions. Histological and histometrical results showed that in the three stages, from anterior to posterior, the mean percentage of vitellogenic follicles increased significantly ($p<0.001$). The minimum diameters of follicles were observed in the chromatin nucleolus and the maximum diameters were observed in the secondary vitellogenic follicles. Zona pellucida appeared in the cortical alveolus follicles and reached to a maximum thickness in the secondary vitellogenic follicles. The diameter of the nucleus increased gradually and it reached to a maximum diameter in the primary vitellogenic follicles. There was a significant difference in parenchyma percentages between the three stages of the reproductive cycle from the previtellogenesis stage to the vitellogenesis stage, and then the maturation stage. This ratio increased significantly ($p<0.05$).

Introduction

Common Carp is one of the most important fresh water fish species in aquaculture (Kucharczyk et al., 2008). Common Carp and other closely related *Cyprinidae* species provide over 30% aquaculture production in the world (Xu et al., 2011). It is well known that the ovarian structure of the *cyprinidae* family has experienced changes in the annual reproductive cycle (Galas et al., 1999; Sivakumaran et al., 2003; Mabudi

et al., 2010). The most suitable method for determining the reproductive cycle in female fish is to observe seasonal developmental changes in the gonads (Sivakumaran et al., 2003). Histological studies yield the most reliable, objective information on spawning season and are essential for detecting details within the maturation cycle (Sivakumaran et al., 2003; Tingaud -Sequeira et al., 2009). Common Carp have an annual reproductive cycle and exhibit asynchronous ovarian follicle development (Sivakumaran et al., 2003). Also, it is known that the photoperiod and temperature has profound effects on reproductive performance of fish (Arabaci et al., 2001; Quintana et al., 2004; Gabillard et al., 2006; Takashima et al., 2008; Bapary and Fainulelei., 2009; Ghomi et al., 2011). Bapary et al (2009) showed that the photoperiod and temperature are involved in the regulation of gonadal development and a long photoperiod within a suitable range of water temperatures is required for continuity of reproductive activities. Miranda et al (2009) reported that female fish that were kept under short photoperiod had low GSIs and their ovaries contained only previtellogenic oocytes. In contrast, females exposed to the long photoperiod had high GSIs and ovaries with vitellogenic oocytes. Quintana et al (2004) showed that high environmental temperature is enough to trigger sexual maturity in fish from a temperate climate. Tempero et al (2006) reported that the spawning period in Common Carp is typically the time when the water temperature is between 18-28^{o}C. Sivakumaran et al (2003) reported that during the peak spawning months the daily average of water temperature was 16.5- 22.6 ^{o}C, with a maximum of 20-25^{o}C. Khouzestan Province is located in southwest of Iran with long photoperiods and high temperature. It is a major centre for the culture and export of freshwater fish (Maktabi et al., 2011). Several studies were conducted on reproductive biology and histology of Common Carp in several regions (Guha and Mukherjee, 1991; Galas et al., 1999; Davis et al., 2003; Sivakumaran et al., 2003; Tempero et al., 2006), but only limited information is available on the reproductive biology of Common Carp in Khouzestan Province. The aim of this survey was the determination of the Common Carp ovarian status and structure in the breeding season and in climate conditions of Khouzestan Province, Iran.

Materials and Methods

Seven female Common Carp were obtained in each month of April to October, from culture ponds of Shehid Maleki and Shush, Khouzestan, Iran. These fish were anesthetized by MS222 at concentration of 250 ppm. The fish were weighed. Then, ovaries were collected, weighed and samples taken from the anterior, median and posterior portions of the ovaries. These samples were fixed in Buoins solution for 2 weeks, then dehydrated in ethanol and were passaged in autotechnicon and blocked in paraffin wax. The 5-6µ sections were made by microtome and were put on microscopic slides and stained by H&E. Also, for study of zona pellucida and yolk granules, they were PAS stained. The oocytes growth and histometrical details were studied. The numbers of previtellogenic and vitellogenic follicles were counted in three portions of the ovaries in each stage of the reproductive cycle and their mean percentages were calculated. Follicles were considered as the parenchyma, vessels, and connective tissues of the ovary were considered as a stroma. Three slides from each portion, and three fields per slide, were counted. Zona pellucida formation was studied. Oocytes diameter, zona pellucida thickness and nucleus diameter were measured during the oocyte growth by digital microscope and Dino-lite capture 1. The status of nucleolus was studied. The parenchyma-stroma ratio was measured by a calibrated ocular lens in three portions of the ovaries. The Gonadosomatic Index (GSI) was calculated (GSI=Weight of gonad/ total weight of fish × 100). The results were analyzed by one-way ANOVA, two ways ANOVA, and the least significantly difference (LSD) multiple comparison test.

Results

Histological and histometrical studies of dissected ovaries indicated that 7 different growing follicles were present in each stage of the reproductive cycle as below:

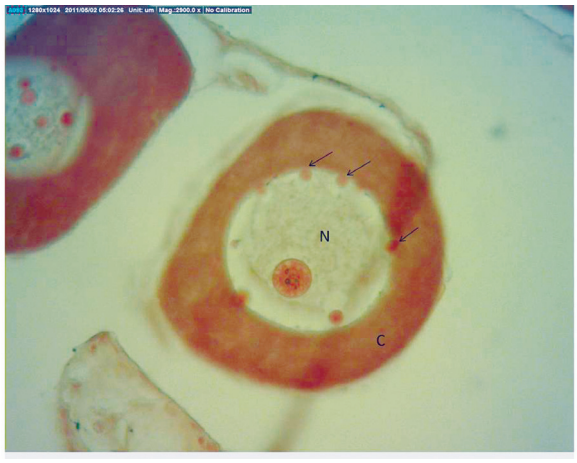

Figure 1: Humoral immune responses in *S. iniae* vaccinated rainbow trout streptococcosis survivors and previously non-exposed fish.

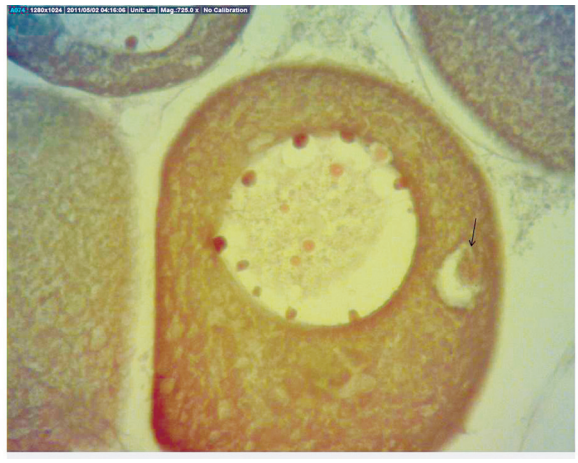

Figure 2: Balbiani body (arrow) in the ooplasm of a perinucleolus follicle (H&E ×40).

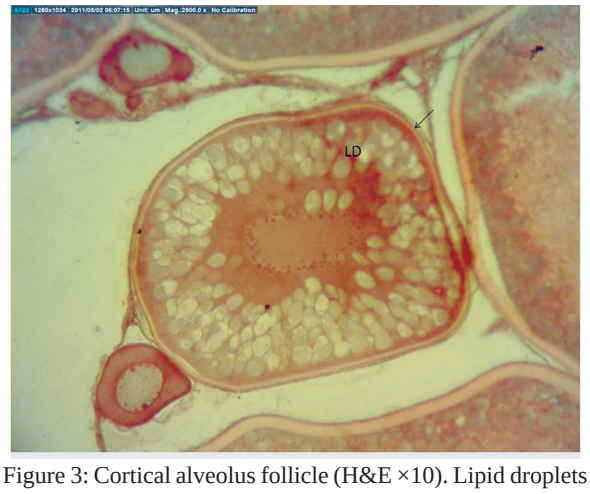

Figure 3: Cortical alveolus follicle (H&E ×10). Lipid droplets (LD) and thin zona pellucida (arrow).

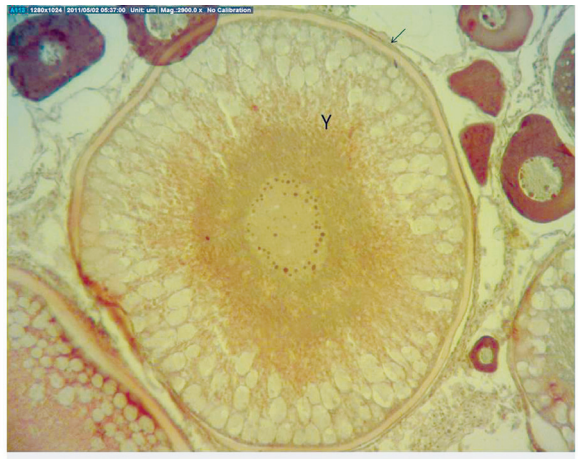

Figure 4: Primary vitellogenic follicle (H&E ×10). Thicker zona pellucida (arrow), yolk spheres (Y).

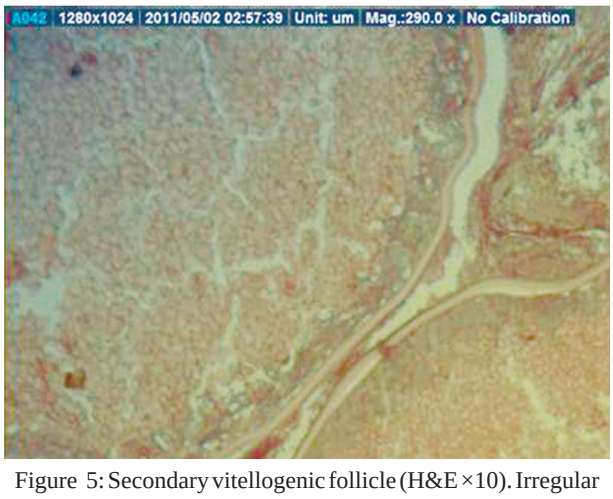

Figure 5: Secondary vitellogenic follicle (H&E ×10). Irregular nucleus membrane (arrow). The yolk spheres were moved toward the center and cortical alveoli and lipid droplets moved to the periphery of the ooplasm.

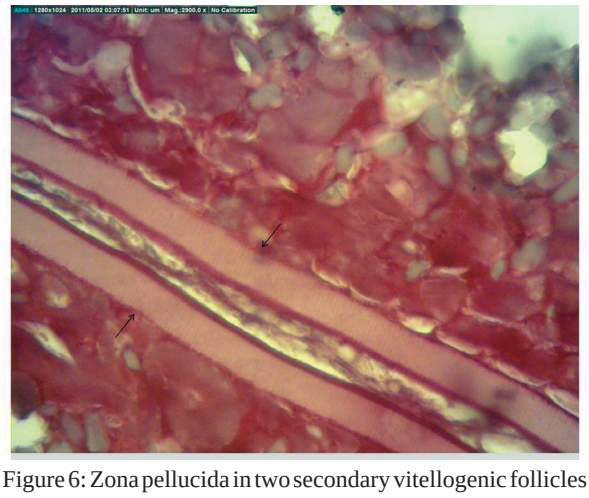

Figure 6: Zona pellucida in two secondary vitellogenic follicles (PAS ×40).There is considerable thickness of the zona pellucida (arrows).

Table 1: The mean and SEM of zona pellucida thickness, nucleus diameter and follicle diameter in different stages of growing follicles. SEM= standard error of mean, a, b, c, d and e, below the mean indicated that there are significant difference between these follicles ($p<0.05$).

Type of follicle	Zona pellucida thickness (µm)	Nucleus diameter (µm)	Follicles diameter (µm)
Chromatin-nucleolus [a]	–	20.98±1.69 [b,c,d]	35.96±4.52 [c,d,e]
Peri nucleolus [b]	–	69.26±8.20 [a,c,d]	111.22±17.67 [c,d,e]
Cortical alveolus [c]	1.78±0.29 [d,e]	101.23±5.79 [a,b,d]	200.46±22.57 [a,b,d,e]
Primary vitellogenesis [d]	7.31±0.71 [c]	132.53±4.09 [a,b,c]	413.10±27.53 [a,b,c,e]
Secondary vitellogenesis [e]	11.64±0.70 [c]	iregular	769.50±44.41 [a,b,c,d]

Table 2: The mean and SEM of vitellogenic follicles percentages in different portions of the ovary in each stage.

	Portion		
Stage	Previtellogenesis	Vitellogenesis	Maturation
Anterior [a]	0.049±0.001 [b,c]	0.21±0.008 [b,c]	0.27±0.006 [b,c]
Median [b]	0.080±0.001 [a,c]	0.29±0.015 [a,c]	0.37±0.003
Posterior [c]	0.12±0.003 [a,b]	0.36±0.013 [a,b]	0.37±0.004

Table 3: The mean and SEM of parenchyma percentages in the three stages of the reproductive cycle.

	Portion		
Stage	Anterior	Median	Posterior
Maturation [a]	73.60±1.10 [b,c]	77.72±1.63 [b,c]	75.56±0.35 [b,c]
Previtellogenesis [b]	47.08±0.14 [ac]	48.80±0.30 [a,c]	53.24±0.12 [a,c]
Vitellogenesis [c]	70.92±0.59 [a,b]	73.16±0.50 [a,b]	71.80±0.44 [a,b]

Table4: The mean and SEM of GSI in three stages of reproductive cycle.

Stage	Average± SEM
Maturation [a]	8.4 ± 0.1 [b,c]
Previtellogenesis [b]	1.9 6± 0.18 [a,c]
Vitellogenesis [c]	7 ± 0.18 [a,b]

Chromatin- nucleolus follicles: Oocyte became enclosed by follicular cells forming a definitive follicle. The nucleus was spherical with multiple nucleoli that arranged irregularly; then, they lay at peri-nuclear position. Ooplasm was thin and basophilic (Fig 1). The diameter of these follicles was 35.96 ±4.52µ (Table1).

Perinucleolus follicles: The nucleus enlarged. Numerous large and basophilic nucleoli were at the periphery of the nucleus. The ooplasm was basophilic. The size of these follicles increased with the addition of ooplasm. Ooplasm was contained juxta nuclear complex of organelles (Balbiani body) (Fig. 2). The diameter of these follicles was 111.22 ±17.67µ (Table1).

Cortical alveolus follicles: Cortical alveoli appeared at various depths in the ooplasm. Then, they moved peripherally beneath the oolemma. In this stage there were small lipid droplets around the nucleus. Zona pellucida appeared as a thin band between the oocyte and follicular cells (Fig. 3) and its thickness was 1.78 ±0.29µ. The nucleus enlarged significantly ($p<0.05$) and small nucleoli remained at the periphery of the nucleus. The ooplasm became less basophilic. The balbiani body disappeared. The diameter of these follicles was 200.46 ±22.57µ (Table 1).

Because yolk spheres were not seen in these three types of follicles, we considered chromatin-nucleolus, perinucleolus and cortical alveolus follicles as pre-vitellogenic follicles.

Primary vitellogenic follicles: Yolk spheres lied between the cortical alveoli in the ooplasm. The ooplasm was less basophilic (Fig. 4). The zona pellucida

thickened and its thickness was 7.31± 0.71µ. The diameter of the nucleus increased significantly and it reached to a maximum diameter in this stage. The nucleoli were at the periphery of the nucleus. The diameter of these follicles was 413.10± 27.53µ (Table 1).

Secondary vitellogenic follicles: The yolk spheres moved toward the center of ooplasm and cortical alveoli and lipid droplets moved to the periphery. The nucleus membrane became irregular (Fig. 5). The zona pellucida thickness was 11.64± 0.70µ and it reached to maximum diameter in this stage (Fig. 6). The diameter of these follicles was 769.50± 44.41µ. The follicles reached a maximum diameter in this stage (Table 1).

Tertiary vitellogenic follicles: The yolk spheres were increased and combined together and totally filled the ooplasm. The nucleoli gradually moved toward the center of the nucleus (Fig. 7).

Maturation: The nucleus was gradually displaced toward the animal pole. Later, the nuclear membrane disappeared (Fig. 8).

Because yolk spheres were not observed in these four types of follicles, we considered primary, secondary, tertiary vitellogenic and mature follicles as vitellogenic follicles.

The study results showed that the reproductive cycle of Common Carp can be divided into 3 stages according to percentage of different types of ovarian follicles. These stages include: maturation, previtellogenesis and vitellogenesis.

In the previtellogenesis and vitellogenesis stages, the percentage of vitellogenic follicles from the anterior to the posterior portion increased significantly ($p<0.05$). In the maturation stage, the percentage of vitellogenic follicles in the median portion was more than the anterior portion ($p<0.05$), but there was not any significant difference between the median and posterior portion (Table 2). The percentage of parenchyma in the three portions of the ovary from the previtellogenesis stage to vitellogenesis and maturation stage increased significantly ($p<0.05$) (Table 3).

The ovigenous lamellae, which it covered by germinal epithelium, were extended to the ovary center. Three different germ cell types were recognized inside the lamellae: chromatin nucleolus, peri nucleolus and early cortical alveolus follicles (Fig. 9).

There was a significant difference in the gonadosomatic index (GSI) between the three stages of the reproductive cycle from the previtellogenesis to the vitellogenesis and maturation stages showing that it increased significantly ($p<0.05$) (Table 4).

The mature ovaries were seen during April, May and June. In July, 1 fish (14.3%) was in the previtellogenesis stage and 6 fish (85.7%) had mature ovaries. In August, 4 fish (57.1%) were in the previtellogenesis stage, and 3 fish (42.9%) were in the maturation stage. In September, 4 fish (57.1%) were in the vitellogenenisis stage, and 3 fish (42.9%) were in the maturation stage. In October, 3 fish (42.9%) were in late vitellogenesis, and 4 fish (57.1%) were in the maturation stage.

Discussion

It is well known that environmental factors, such as the photoperiod and temperature, can have profound effects on the timing of gametogenesis, vitellogenesis and maturation in fish (Quintana et al., 2004; Takashima et al., 2008; Miranda et al., 2009). Although the exact mechanisms that are involved have not yet been fully explained, the neuroendocrine system is clearly involved (Miranda et al., 2009). Miranda et al., showed that all levels of the brain-pituitary-gonadal axis were stimulated by the increase in day length and it is possible that water temperature has an effect at later stages of oocyte maturation and spawning. It may also be important as a trigger for reproductive activity. The spawning period in Common Carp is typically during the period when the water temperature is between 18 -28^{o}C (Tempero et al., 2006). Sivakumaran et al. (2003) reported that during the peak spawning months the daily average of water temperature was 16.5-22.6 ^{o}C, with maximum of 20-25 ^{o}C, in Australia. Spawning generally peaks during spring and early summer, but also occurs until autumn and can even start in late winter in some sites

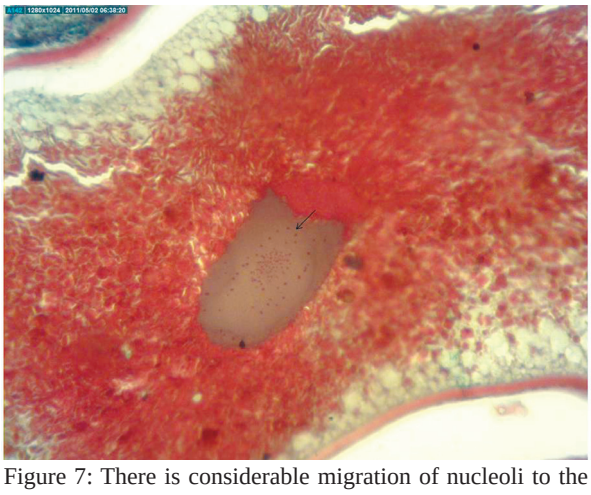

Figure 7: There is considerable migration of nucleoli to the center of the nucleus in the tertiary vitellogenic follicle (arrow) (PAS ×10).

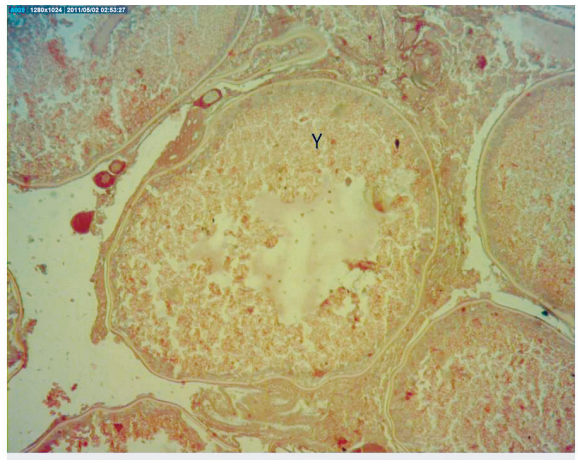

Figure 8: The mature follicle: The ooplasm filled completely by yolk spheres (Y) (H&E ×10).

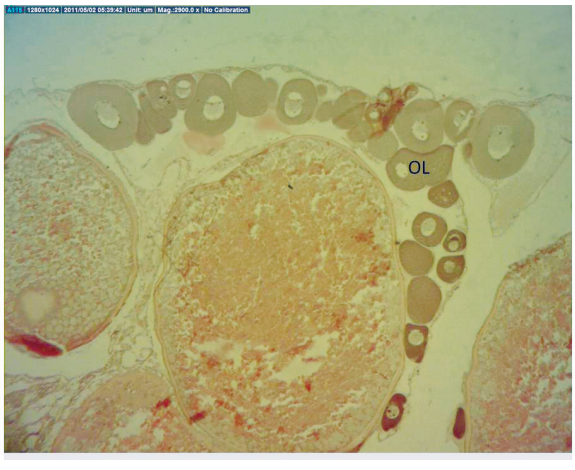

Figure 9: Ovigenous lamellae (OL) (H&E ×10).

(Sivakumaran et al., 2003). The Khouzestan province is located in southwestern Iran with high temperatures and long photoperiods from April to October (Maktabi et al., 2011). The histological results showed that Common Carp in this area had mature ovaries in April. This indicated that these fish were ready for spawning at the onset of the spring season. The histological observation showed that some of the fish are in the maturation stage from April to October. The observations indicated that the Common Carp breeding season in Khouzestan Province take as long as 7 months and that this species spawned from April to October. The present study confirms that Common Carp ovarian maturation correlated with the temperature range in Khouzestan Province. Although, in Japan, an examination showed that the photoperiod is involved in ovarian maturation, even under cool temperatures (Davies et al., 2003). Davis et al. showed that the Common Carp ovulated one month earlier in a long photoperiod. In tropical Brazil and Bangladesh, spawning seasons extended for 5-6 months and included multiple spawning (Sivakumaran et al., 2003). At high latitudes where the water temperature is required for spawning, carp may spawn only once over a 2-4 months period (Tempero et al., 2006). In another survey, Guha and Mukherjee (1991) reported two clear reproductive cycles in one year in Common Carp in West Bengal. These differences were directly related to latitude (Tempero et al., 2006). In our study there was a significant increase in GSI from the previtellogenesis to vitellogenesis and maturation stages. This observation corresponds with Sivakumaran et al. (2003) in Australia and Tempero et al. (2006) in New Zealand. They reported that female GSI varied with the season and was negatively related to water temperature. This index increased as oocytes matured in preparation for spawning during the spring and early summer with the subsequent release of oocytes causing a decrease in GSI. Also, Al Mukhtar et al. (2006) reported that fecundity increases with the size of the fish and the GSI. They showed that the average GSI for female fish increased from December to March (before spawning) and decreased during April (after spawning). Tempero et al. (2006) reported that female Common Carp GSI was at a minimum in January (after spawning in New Zealand) rising to a peak in September when the first spawning activity

was observed. It was also observed that only 20% of the fish were in a maturation stage in September (Tempero et al., 2006) while in the present study 42.9% of the examined fish were in the maturation stage in September. Also, Sivakumaran et al. (2003) reported that seasonal maturation of ovaries begins in August and continues through to March.

This is the first histological study that shows that Common Carp have spawning ability for three times during an annual cycle in the Khuzestan climate conditions. Bapary and Fainuulelei (2009) reported that a long photoperiod and high water temperature resulted in increases in GSI and the induction of vitellogenic oocytes. It seems that the previtellogenesis and the vitellogenesis stages were shorter in the Khuzestan climate conditions and this species entered into a maturation phase rapidly. The study's findings are in agreement with a Bieniarz et al. study (1978) in Poland. They reported that soon after spawning, under a long photoperiod and warm temperatures, Common Carp re-initiate gonadal growth.

Common Carp have asynchronous ovaries and follicles of all sizes were seen at any time, but the stage of the ovary cycle was determined by the percentage of different types of follicles. This observation was in agreement with Sivakumaran et al. (2003). A size increase is the most obvious manifestation of oocyte development, also, the status of nucleus can help to determine the type of follicles. The oocytes grew both by an enlargement of the nucleus and the addition of ooplasm. This growth was accompanied by a decrease in the nuclear/ cytoplasmic ratio. The minimum diameter of oocytes was observed in the chromatin nucleolus stage and the diameter reached the maximum size in the secondary vitellogenesis stage. In the tertiary vitellogenesis stage, the oocyte did not have considerable growth. The difference between secondary and tertiary vitellogenesis was in the combination of yolk spheres and the position of nucleoli. These observations are in agreement with Sivakumaran et al. (2003) and Mabudi et al. (2010).

In spawning, the ovaries released mature oocytes and the connective tissue replaced the ovarian structure after ovulation. Because of this, in the previtellogenesis stage (after spawning), the parenchyma were decreased significantly ($p<0.05$). After this stage, vitellogenesis occurs and immature oocytes enter into the vitellogenesis phase and the number of vitellogenic oocytes increased significantly ($p<0.05$). Because of this, the parenchyma was significantly more than the stroma. This study's results showed that the percentage of maturating oocytes increased from the anterior to posterior portion of ovaries in three stages of the reproductive cycle, It seemed that since in the boney fish spawning moved down from posterior of the ovary (Shafiei Sabet et al., 2011), that this may be a cause for further migration of mature follicles to the posterior portion.

In fact, however, the ovary of Common Carp is asynchronous and different types of follicles can be found in all portions and in all stages of the reproductive cycle. A difference between portions in the ovaries can be observed. From the anterior to posterior portions, the percentage of vitellogenic and mature follicles can increase. Also, the results showed that due to specific climate conditions in Khouzestan Province (high temperature and long photoperiod), and the proven impact of temperature and the photoperiod on reproduction of fish, it seems that the annual reproductive cycle in Common Carp appears to be somewhat different in this area. It seems that the Common Carp ovary, under these conditions, experience the previtellogenesis and vitellogenesis stages quickly and enter the maturation stage. This maturity can increase the frequency of spawning in Common Carp in the Province.

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