

Macroscopic and microscopic studies of annual ovarian maturation cycle of Shirbot *Barbus grypus* in Karoon river of Iran

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(Received 1 Nov 2008; revised version 29 Dec 2008; accepted 2 Feb 2009)

Summary

The aim of this investigation was to study macroscopic and microscopic structures of ovaries in *Barbus grypus* of Karoon river and their changes during annual maturation cycle. For this purpose, 120 adult *B. grypus* with a mean weight of 835.0 to 1012.0 g, were caught from Karoon river and transferred alive to the laboratory. After biometrical studies, the weights and morphological appearances of gonads were recorded. Then, tissue samples from the anterior, middle and posterior portions of the gonad were excised and fixed in Bouin's solution. For microscopical studies, 5 μ m paraffin sections were prepared and stained with haematoxylin and eosin and periodic acid Schiff (PAS). Gonado-somatic indices, macroscopical and microscopical changes of ovaries were studied during different months of the year. The results showed that gonado-somatic index ranged from $0.35 \pm 0.080\%$ in September to $2.25 \pm 0.321\%$ in June, and the maximum values were seen in June. Cyclic changes of ovarian maturation were divided into seven stages histologically. Ovarian maturation cycle in *B. grypus* began in late summer. Spawning period of *B. grypus* continued approximately from late April to early August, which indicated that *B. grypus* has a prolonged spawning season. Ovarian type of *B. grypus* was group synchronous with a capacity for multiple ovulations within a reproductive season.

Key words: *Barbus grypus*, Annual cycle, Ovary, Fish reproduction

Introduction

Study on reproductive activities of fish is of particular importance in maintenance of healthy populations of fish in the water systems and optimization of appropriate broodstock management strategies. Different species of fish have various reproductive strategies from strict gonochorism to simultaneous functional hermaphroditism. Most species spawn once a year in a demarcated period, but others spawn recurrently in a season (Devlin and Nagahama, 2002). Seasonal cyclic changes were observed in the reproduction of fish synchronized by environmental cues. A successful reproduction is adopted by

fluctuations of aquatic medium results from annual seasonal variations such as photoperiod, water temperature and physico-chemical features, because of optimal environmental conditions for feeding and development of egg and larva (Baggerman, 1990; Munro, 1990; Dufour, 1994; Van Der Kraak and Pankhurst, 1997; Bromage *et al.*, 2001; Glasser *et al.*, 2004).

One of the main fishes of Karoon river is Shirbot, *Barbus grypus*, of Cyprinidae family that widely distributed in the river systems of the West and Southwest of Iran, especially in Khouzestan province; they also found in Iraq and Turkey. *B. grypus* lives in fresh and brakish waters, tolerates a wide range of salinity and temperature, and is a

euryphage fish migrates from down to upstream of Karoon river for spawning from the beginning of May (Nikpei, 1996). *B. grypus* have economical importance among local people and is an interesting species in commercial aquaculture in Iran. There are some reports that show the decline in *B. grypus* populations in river systems of Khouzestan (Nikpei, 1996). Therefore, it seems necessary to study about reproduction of *B. grypus* in order to provide knowledge for development of aquaculture.

Moreover, studies on the reproductive behaviour of fishes require knowledge of the stage of gonadal maturation in each individual fish. There are a few methods to determine the stage of gonadal development such as measurement of oocyte size, staging based on the appearance of whole oocytes, staging based on the external appearance of the ovary, gonad indices and histology. However, despite the fact that histology is time-consuming and expensive, it is the most accurate method used for assessing gonadal development (Hibiya, 1982; West, 1990). Therefore, the present study was performed to determine the macroscopical and microscopical changes of *B. grypus* ovary during its annual maturation cycle.

Materials and Methods

One hundred and twenty fish were collected monthly from September 2007 to August 2008, from Karoon river in Ahvaz city which is located at 31°N and 48°S. The samples were caught by commercial trawler and transferred alive to the laboratory. After anesthesia, their body weight and length were measured. Then, body cavity of each specimen was opened and macroscopical appearance of ovary and gonad weight were recorded. For each fish, gonado-somatic index (GSI) was calculated using the following formula:

$$\text{Gonado-somatic index} = \frac{\text{gonad weight (g)}}{\text{body weight (g)}} \times 100$$

For microscopical studies, tissue samples were taken from the anterior, middle and posterior parts of the right and left ovaries of each fish, and then immersed in Bouin's solution for 24 h before

processing for routine paraffin embedding. Sections of 5 µm thickness were stained with haematoxylin and eosin and periodic acid Schiff (PAS). For analysis of the ovarian cycle, 100 oocytes were staged according to their size and histological features in each individual female fish. Oocytes diameter and surface area were measured by Axiovision 4.5 LE software on digital images. Histological classification of the ovaries was based on the developmental stages of the most advanced oocyte present in the sections (West, 1990).

The maturity stages of Shirbot ovary were determined using the modified gonad maturity scale developed by Ganias *et al.* (2004) and Smith and Walker (2004) (Table 1).

Statistical analysis

All data were analyzed using SPSS software (Ver.0.9) for windows. Pearson's correlation coefficients were calculated between mean gonado-somatic indices and mean oocyte diameters. The p-value less than 0.05 was considered as statistically significant.

Results

Mean body lengths and weights of the collected fish were between 36.5 to 43.5 cm and 835.0 to 1012.0 g throughout the year, respectively (Table 2). Three maturity periods: previtellogenic, vitellogenic and maturation, were observed seasonally.

The weights of the ovaries increased gradually from September, reaching a peak in June and remained unchanged up to July and then began to decrease until September. Mean ± SD values of GSIs were maximum in June (2.25 ± 0.321%) and minimum in September (0.35 ± 0.080%) (Table 2). Mean GSIs had significant correlation with the mean diameter of oocytes during different months (P<0.01, r = 0.765).

Each ovary was enclosed by a thick tunica albuginea and composed of numerous thin walled lobules. The oocytes were present in various stages of oogenesis within the lobules.

Primary germ cells occurred in the interstitial tissue as well as in the wall of the resting lobules. They occurred in small

Table 1: Ovarian periods, maturational stages and corresponding histological features of *B. grypus*. Modified by Ganias *et al.* (2004) and Smith and Walker (2004)

Ovarian period	Developmental stages	Histological features
Previtellogenic	Primary oocyte	A small quantity cytoplasm densely stained with haematoxylin surrounding a proportionally large central nucleus. With progressing oocyte development, the nucleo-cytoplasmic ratio decreases, number of nucleoli increases and migrates to periphery of the nucleus.
	Yolk vesicle	Small vesicles appear in the cytoplasm and then migrate to the periphery of the cytoplasm. The cortical vesicles increase in size and number to form several peripheral rows. Cytoplasm stains lighter and zona radiata appears.
Vitellogenic	Primary yolk globule	Eosinophilic protein yolk globules form among yolk vesicles and fill about a third of the cytoplasm, begin to appear in perinuclear zone. Nucleus is irregular in shape.
	Secondary yolk globule	Yolk globules increase in number and size, fill a more extent portion, about two third at the periphery of cytoplasm.
	Tertiary yolk globule	Yolk globules increase in number, fill two third of cytoplasm. The oocytes increase considerably in size.
Maturation	Migratory nucleus	Nucleus begins to leaves central position and migrates toward periphery. Yolk globules fill more than two third of cytoplasm. Oocyte size nearly remains stable.
	Hydration	Yolk globules fill entire of cytoplasm. Nucleus is observed at animal pole.

Table 2: Mean \pm SD body length, body weight, gonado-somatic index and oocyte diameter in maturation stages during annual ovarian cycle of *Barbus grypus*

Month	No. of Fish	Body length (cm)	Body weight (g)	GSI (%)	Oocyte diameter (μ m)
January	11	42.0 \pm 6.70	901.0 \pm 208.8	0.40 \pm 0.139	355.07 \pm 88.42
February	9	39.3 \pm 4.30	835.0 \pm 119.2	0.57 \pm 0.225	460.65 \pm 192.53
March	10	40.5 \pm 6.20	912.0 \pm 202.5	0.70 \pm 0.256	604.74 \pm 228.35
April	7	38.5 \pm 5.35	952.0 \pm 220.4	0.75 \pm 0.317	729.18 \pm 193.63
May	15	40.6 \pm 5.41	1012.0 \pm 266.2	1.30 \pm 0.412	933.56 \pm 146.24
June	8	39.8 \pm 4.26	971.0 \pm 201.1	2.25 \pm 0.321	1012.80 \pm 106.15
July	13	43.5 \pm 6.80	964.0 \pm 235.7	1.82 \pm 0.441	505.75 \pm 440.47
August	9	41.0 \pm 5.33	910.0 \pm 273.4	0.71 \pm 0.202	286.49 \pm 302.75
September	8	37.2 \pm 4.21	925.0 \pm 216.2	0.35 \pm 0.080	177.711 \pm 62.23
October	10	42.0 \pm 5.36	948.0 \pm 233.7	0.41 \pm 0.111	228.67 \pm 98.61
November	5	36.5 \pm 4.22	892.0 \pm 114.6	0.38 \pm 0.101	262.65 \pm 103.48
December	15	38.0 \pm 4.45	885.0 \pm 130.9	0.42 \pm 0.105	293.97 \pm 106.41

SD: Standard deviation

number throughout the year, but were most prominent after the breeding season when they divided mitotically to form nests of oogonia. These cells have a single and large nucleus with peripheral chromatin. The nucleoli were located along the peripheral part of the nucleus. The cytoplasm of the oogonium was stained with haematoxylin. Each oogonium was surrounded by 2-3 follicular cells (Fig. 1A).

During the previtellogenic period, right and left ovaries were more or less equal in length and size and were thin and transparent. At this period, primary oocyte and yolk vesicle stages were distinguished. In primary oocyte stage, the number of nucleoli increased and arranged in the periphery of the nuclei. The oocytes were surrounded by squamous follicle cells. The ooplasm was also stained densely with

haematoxylin (Figs. 1B and C). Mean \pm SD diameter of oocytes and surface area at this stage were $151.70 \pm 45.40 \mu\text{m}$ and $18.446 \pm 4.53 \text{mm}^2$, respectively.

In yolk vesicle stages, small vesicles were appeared, migrated to the periphery of the cytoplasm and increased in size and number to form several cortical vesicles. Cytoplasm was stained lighter and zona radiata appeared (Fig. 1D). The ooplasm stained densely with haematoxylin. At this stage, mean \pm SD diameter of oocytes and surface area were $325.11 \pm 54.22 \mu\text{m}$ and $90.862 \pm 8.47 \text{mm}^2$, respectively. The frequency of previtellogenic oocytes reached its maximum in September and minimum in May and June (Fig. 2). Previtellogenic oocytes were the only oocytes seen in ovaries from July to September.

During the vitellogenic period, ovaries were considerably larger, white to yellowish and opaque with apparent small blood

capillaries. At this period, primary yolk globule, secondary yolk globule and tertiary yolk globule stages were distinguished. In primary yolk globule stage, eosinophilic protein yolk globules formed among yolk vesicles and filled about a third of the cytoplasm and began to appear in perinuclear zone. Nucleus was irregular in shape (Fig. 1E). At this stage, mean \pm SD diameter of oocytes and surface area were $387.43 \pm 51.32 \mu\text{m}$ and $119.935 \pm 11.65 \text{mm}^2$, respectively.

In secondary yolk globule stage, yolk globules increased in number and size and filled about two third at the periphery of cytoplasm (Fig. 1F). At this stage, mean \pm SD diameter of oocytes and surface area were $472.92 \pm 62.78 \mu\text{m}$ and $177.218 \pm 16.72 \text{mm}^2$, respectively.

In tertiary yolk globule stage, yolk globules increased in number and filled two third of cytoplasm. The oocytes increased

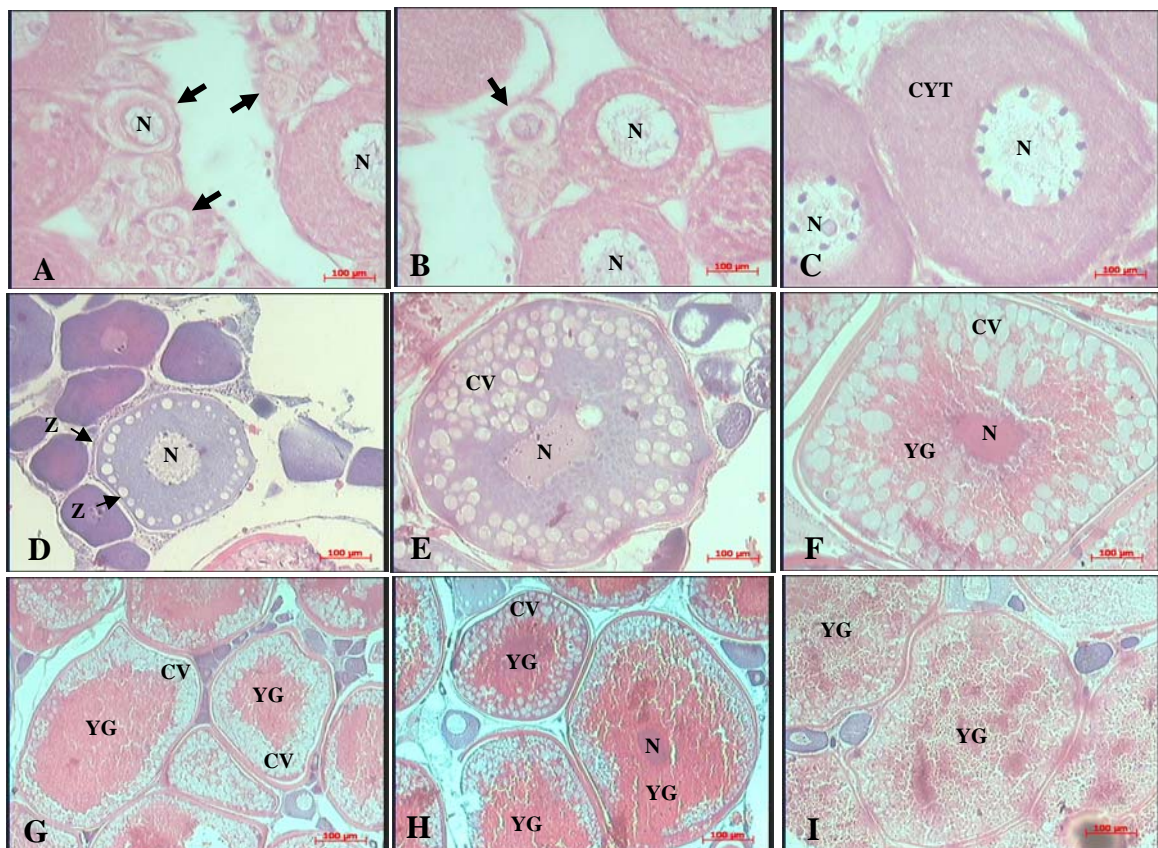


Fig. 1: Stages of oocyte maturation in *B. grypus*. (A) Oogonium (H&E); (B) Early primary oocyte (H&E); (C) Primary oocyte (H&E); (D) Yolk vesicle stage (H&E); (E) Primary yolk globule stage (H&E); (F) Secondary yolk globule stage (PAS); (G) Tertiary yolk globule stage (PAS); (H) Migratory nucleus stage (PAS); (I) Hydrated oocyte (PAS). Arrows: Oogoniums. Scale bar = 100 μm . N: Nucleus, CYT: Cytoplasm, Z: Zona plucida, CV: Cortical vesicle and YG: Yolk globule

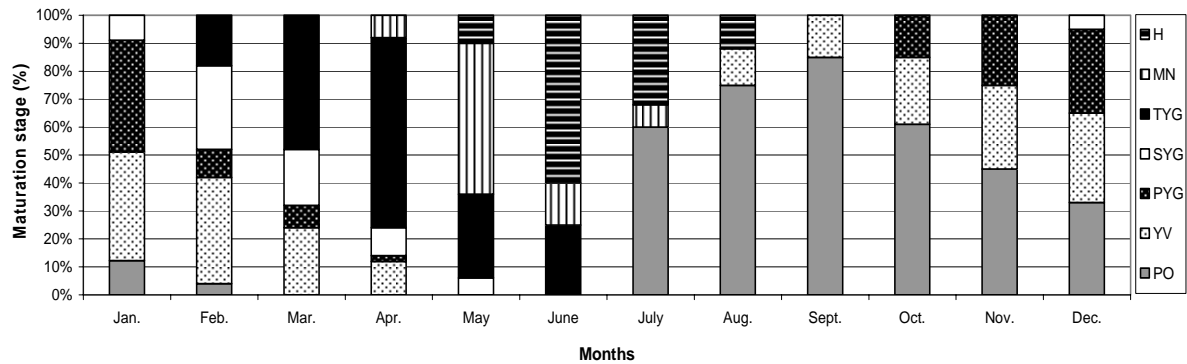


Fig. 2: Monthly changes in frequency (%) of oocyte in maturation stages of *B. grypus* in histological sections. PO: Primary oocyte; YV: Yolk vesicle; PYG: Primary yolk globule; SYG: Secondary yolk globule; TYG: Tertiary yolk globule; MN: Migratory nucleus and H: Hydration

considerably in size (Fig. 1G). At this stage, mean \pm SD diameter of oocytes and surface area were $835.70 \pm 84.61 \mu\text{m}$ and $561.625 \pm 32.24 \text{mm}^2$, respectively.

During the vitellogenic period, deposited inclusions were stained pink by eosin. The frequency of vitellogenic oocytes attained maximum in April and minimum in July, August and September (Fig. 2).

During the maturation period, ovaries were yellowish, distinctly became swollen and lobular in appearance, had abundant large blood capillaries and filled the body cavity. At this period, migratory nucleus and hydration stages were distinguished. In migratory nucleus stage, nucleus began to leave central position and migrated towards periphery. Yolk globules filled more than two third of cytoplasm. Oocyte size remained relatively stable (Fig. 1H). At this stage, mean \pm SD diameter of oocytes and surface area were $1010.68 \pm 124.14 \mu\text{m}$ and $795.756 \pm 41.61 \text{mm}^2$, respectively.

In hydration stage, yolk globules filled entire the cytoplasm. Nucleus observed at animal pole (Fig. 1I). Mean \pm SD diameter of oocytes and surface area at this stage were $1087.13 \pm 152.74 \mu\text{m}$ and $826.445 \pm 54.33 \text{mm}^2$, respectively, which was the maximum size of oocytes during oogenesis. The oocytes of maturation period were seen from April to August, and the highest frequency of these oocytes was in May and June (Fig. 2).

Monthly changes in the mean diameters of oocyte in maturation stages in histological sections are shown in Fig. 3. The maximum of mean diameters of oocyte in maturation stages was in June ($1012.80 \pm 106.15 \mu\text{m}$)

and the minimum was in September ($177.711 \pm 62.23 \mu\text{m}$) (Table 2).

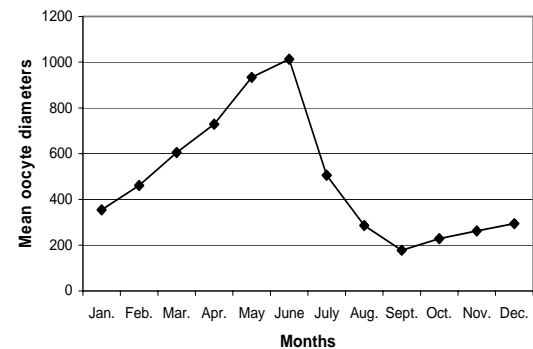


Fig. 3: Monthly changes in mean diameters (μm) of oocyte in maturation stages of *B. grypus* in histological sections

After ovulation, the spent ovaries were small, bloodshot and granular with scattered residual oocytes. These ovaries composed of postovulatory follicles, immature oocytes and mature eggs left unspawned. Postovulatory follicles consisted of follicular and thecal cell layers. Ovulation seems to start in late May and ended at the beginning of July.

Discussion

This study is a comprehensive description of annual ovarian maturation in *B. grypus*. Several scales of gonadal maturation have been proposed for fish. In the majority of teleost fishes, the process of oogenesis may be divided into five, six or even eight stages (Nagahama, 1983; West, 1990; Fishelson *et al.*, 1996; Unal *et al.*, 1999; Poortenaar *et al.*, 2001). In the present

study, all stages of gonadal development in *B. grypus* were identified according to the scales modified by Ganias *et al.* (2004) and Smith and Walker (2004), and divided into three main periods: previtellogenic, vitellogenic and maturation.

The previtellogenic period was divided into two stages; primary oocyte and yolk vesicle. In this period, immature non-yolky oocytes were present in the ovaries. The occurrence of these oocytes in ovaries started in late summer. So, it seems that this time is the beginning of ovarian cycle in *B. grypus*.

The vitelline membrane appears commonly at the yolk vesicle stage and sometimes at the end of primary oocyte (West, 1990; Unal *et al.*, 1999). This situation may vary from species to species. In the present study, the vitelline membrane began to develop at the end of the yolk vesicle stage.

The vitellogenic period was divided into three stages; primary, secondary and tertiary yolk globule. In this period, synthesis of the yolk substances occurred in oocytes. During late autumn and all over winter the ovaries of *B. grypus* were mostly occupied by these oocytes, but immature, non-yolky oocytes were also seen.

Synthesis of the primary yolk occurs in oocytes, expressed as endogenous vitellogenesis (Khoo, 1979; Baggerman, 1990). The components of secondary and tertiary yolk globules were synthesized in the liver; the process is therefore referred to as exogenous vitellogenesis (Khoo, 1979; Baggerman, 1990). Beginning of the secondary and tertiary yolk accumulation in ovaries of *B. grypus* took place during winter.

There is a close relationship between the amount of vitellogenin and the oocyte size. Concomitant with increases in the accumulation of vitellogenin in the oocytes, the size of oocytes also begins to increase. Therefore, the nutritional situation of the population, especially during the period of vitellogenesis, is the main biological factor affecting the process of vitellogenesis (Hibiya, 1982; West, 1990).

The maturation period was divided in two stages; migratory nucleus and hydration. In this period, ovaries were full of yolk

oocytes. During spring and until the beginning of summer, the ovaries of *B. grypus* were mostly occupied by these oocytes, but vitellogenic oocytes were observed as well. According to Hibiya (1982), after beginning the movement of the nucleus to the animal pole, the first meiotic division occurs and the first polar body is released. Both the movement of nucleus and the breakdown of its membrane is a commonly used indicator of the final maturation (West, 1990).

Therefore, the spawning period of *B. grypus* continued approximately from late April to early August, indicating that *B. grypus* have a prolonged spawning season. Advantages conferred on species with this reproductive strategy include reduction in larval crowding and decreased impact of predation and unfavorable environmental conditions on eggs and larvae (Morse, 1981; McEvoy and McEvoy, 1992). In an aquaculture situation, multiple spawning over a protracted season has major benefits in providing a consistent supply of high quality larval.

At the beginning of the spawning period, adult *B. grypus* usually migrate from down the river to the upstream. The adults return to the previous areas after spawning. However, during the spawning and migration period, the fish are caught by local people, which is the main reason for decline in populations of this species. So, fishing must be prohibited during the spawning period to protect the population of *B. grypus*.

In the ovaries of the observed samples, mainly two or three and occasionally four stages of oocyte development were observed at any time. According to West (1990), group synchronous ovaries are those in which at least two size groups of oocytes could be seen at the same time. Therefore, the ovarian type of *B. grypus* is group synchronous with a capacity for multiple ovulations within a reproductive season.

Water temperature and photoperiod are known to be the main environmental cues, which determine the reproductive cycle in *B. grypus*. The combination of these factors in different seasons regulates sexual maturation during the year. It is appeared that low water temperature in winter is the stimulus for

final maturation in *B. grypus*. In addition, exposure to cold water prior to spawning advances and synchronizes ovulation and spawning and improves egg survival in salmonids (Nakari *et al.*, 1987; Taranger *et al.*, 2000). Rainbow trout (*Oncorhynchus mykiss*) exposed to high temperatures in the vitellogenesis period demonstrate dysfunction of ovaries and disturbances of ovulation (Davies and Bromage, 2002). Obviously, there is a temperature limit for sexual activity and breeding in fish (Chmievsky, 2000; Davies and Bromage, 2002).

The present study was performed in natural environment of *B. grypus* throughout a year to provide a detailed description of the annual ovarian maturation in *B. grypus*. The proposed scale of gonadal maturation will be useful in the histological identification of the oogenesis stages in *B. grypus*.

Acknowledgement

The authors wish to thank the vice chancellor for research of Chamran University for the research grant.

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