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# Histological and histochemical studies on the ovarian development of the grass carp, *Ctenopharyngodon idella* with special references to atretic phenomena.

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## ABSTRACT

The grass carp, *Ctenopharyngodon idella*, is an exotic fish in the Egyptian fauna. It is used as a protein source and for biological control of aquatic weeds grown in the water of both advanced and developing countries. Spawning was done by injection with hormone or pituitary extract of another common carp due to the lack of spawning in captivity farms. In May, the gonadosomatic index (GSI) increased and reached the highest value in May (17.645±0.381) which decreased steadily and reached the minimal value in October (0.161±0.034). The water temperature during this time is ideal and ranged from 25 to 28 C$^{o}$. The annual ovarian cycle is subjected to successive developmental periods. This involves the primary and secondary growth process of ova formation changes such as yolk deposition and cytoplasmic vacuolization contributing to its maturation. Owing to the lack of spawning, several studies have been carried out on follicular atresia that needs to be explained. This study was undertaken to explain the aspects of this process by analyzing the environmental factors in correlation with investigating the ovarian wall at a light and ultrastructural level.

## INTRODUCTION

Due to the economic significance of the grass carp as a high protein source and biological control of weeds (Opuszynski and Shireman, 1995; Mitchell and Kelly, 2006), it is reared in rivers and captivity farms of many countries (Fish Base,2004) . The iability of fish to breed in the captivity farms is one of the major problem of fish culture. The cycle change of gonadal development (Sivakumaran *et al*.,2003), and maturation (Tingaud-Sequeira *et al*. ,2008) were carried out to illustrate the aspects of oogenesis and predicted the time suitable for artificial spawning.

Follicular atresia is the failure of ovulation process and resulted in degraded mature ova from ovary during the spawning period (Guraya,1994). It is a common feature in the ovaries of teleosts species and can result from alterations in the environmental condition (Leino and Maccormik, 1997). Follicular atresia also occurred

as a result of early ovarian maturation (Simonsen and Gundersen, 2005) or during postvitellogenesis process as in *Syn. C. gariepinus* (Sheha,2011). Many authors included Ramadan and El-halfawy (2006), Krysko *et al*. (2008). khalafallah and Shehata (2011) recorded this phenomena at the end of the spawning season.Senarat *et al*. (2016) studied *Rastrelliger brachysoma* and categorized atretic process into previtellogenic and vitellogenic stages.

Therefore, the study was designed to predict the ovarian maturation of the grass carp ovary of grass carp and factors involved in follicular atresia and failure of fish in Egypt.

## MATERIALS AND METHODS

Water temperatures were measured three times a week throughout the period of collecting samples. At the end of each month the minimum and maximum of temperatures were measured.

**Fish collection:**

During the time extended from January 2018 to December 2018, Fishes were captured from Damiatta River Nile branch near the Faraskor city. During the study period , about 50 living females were collected . Total fish capture lengths and body weights of catch fishes ranged from 30 to 67 cm in length and 0.8- 4 kg body weight were recorded. The specimens were caught throughout the period of maturation cycle and the sex of each fish was determined depending on examination of the pectoral fin appearance, since, it was soft in females and rough in males. The Females were sacrificed and dissected. The ovary is dissected, weighed and gonadosomatic index is determined. The ovarian specimens were fixed in phosphate buffered formalin (pH 7.4) and processed for histological investigations. The gonadosomatic index (GSI) measured (Yoneda *et al*., 2001; El-Gamal, 2001; El-Gharabawy *et al*., 2003 and Ahmet *et al*., 2004) according to the following equation:

$$GSI = \frac{\text{Gonad weight (g)}}{\text{Gutted weight (g)}} \times 100$$

**Histological and histochemical techniques:**

For histological techniques, the ovarian tissue was separated and fixed in 10 percent in phosphate buffered formalin, dehydrated in ascending concentration of ethyl alcohol, cleared in xylene and mounted in molten paraplast paraffin wax (56-58°C). Serial cross-sections 4-6 µm thick were carried out and stained with Harris's hematoxylin & eosin (Harris,1900) for illustrating histological picture and Mallory triple stains for connective tissues Mallory (1944). Also, periodic acid Schiff's (PAS) was carried out according to the method ofMcMannus (1948) for glycogen and mercury bromophenol blue method for determination of protein (Mazia,1953).

**Scanning electron microscopy:**

Ripening ova specimens were fixed 2% phosphate buffered glutaraldehyde followed by dehydration in ascending grades of ethyl alcohol and absolute acetone. The speciemens were then allowed to critically drying in HCP-2 critical point dryer, coating with gold and visualized on a Hitachi S-2400 scanning electron microscope for observation.

## RESULTS

During the study period, the water temperature ranged from 12.5 to 14.66 C in the winter season and gradually increased in summer season reaching 27.16 C in August. The water temperature declined gradually in the autumn season and reached to 22 C at the end of November (Fig,.1).

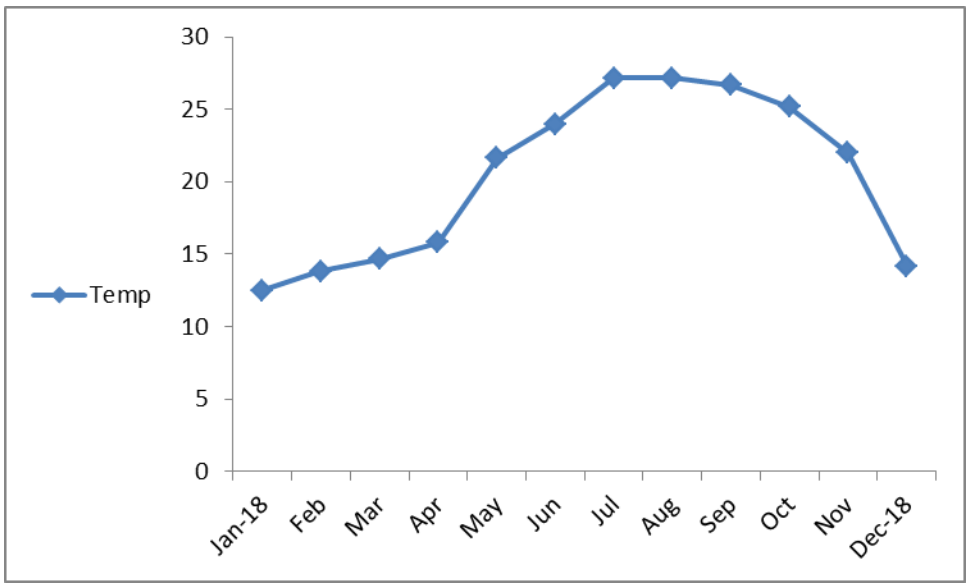


Fig. (1): Monthly fluctuation in water temperature during the study period.

**Gonadosomatic index (GSI) of female:**

The findings showed that the weight of ovaries varies in comparison to the overall body weight in relation to the developmental processes. The GSI value started in January (0.3664±0.025) , increased gradually in March (0.4785±0.101) and continued in April (8.874±5.4668). The increase of GSI reached its maximum peak in May (17.645±0.381) , gradually decreased in June (8.289±0.253) and attained highest reduction in July (3.152±0.22) and October (0.161±0.034) (Table,1 and Fig.,2).

Table (1): Monthly variation of the gonadosomatic index (GSI) of females grass carp, *C. idella* during the study period.

| Months | Min. | Max. | Average± SD |
|---|---|---|---|
| Jan.2018 | 0.345 | 0.624 | 0.3664±0.025 |
| Feb. | 0.436 | 0.728 | 0.5663±0.091 |
| Mar. | 0.345 | 0.624 | 0.4785±0.101 |
| Apr. | 2.812 | 16.467 | 8.874±0.4668 |
| May | 16.228 | 19.542 | 17.645±0.381 |
| Jun. | 8.025 | 8.803 | 8.289±0..253 |
| Jul. | 2.861 | 3.49 | 3.152±0.22 |
| Aug. | 0.827 | 0.92 | 0.868±0.033 |
| Sep. | 0.207 | 0.3 | 0.24±0.039 |
| Oct. | 0.129 | 0.22 | 0.161±0.034 |
| Nov. | 0.156 | 0.22 | 0.184±0.023 |
| Dec.2018 | 0.16 | 0.257 | 0.208±0.036 |

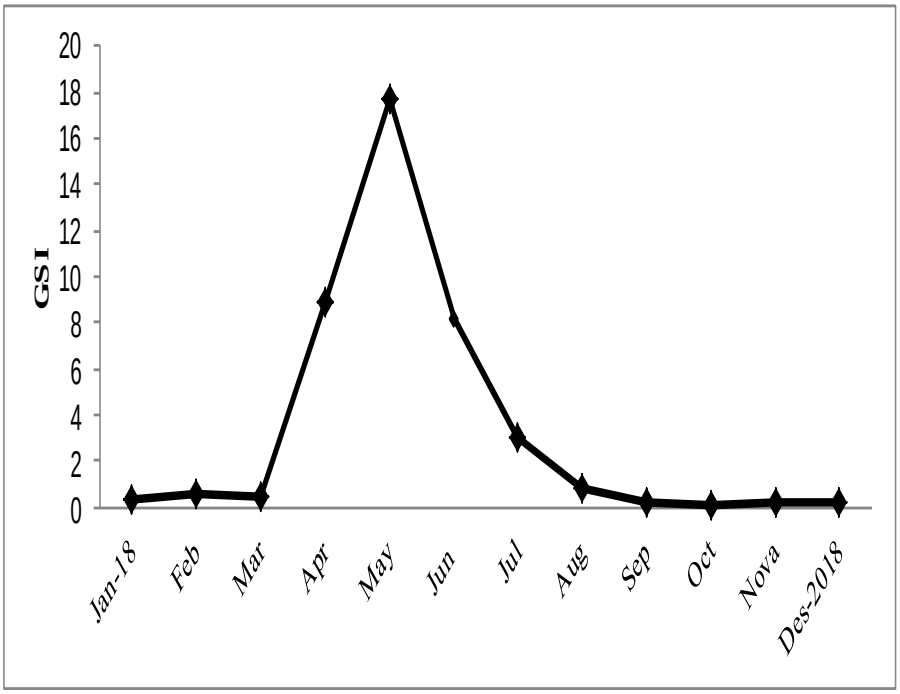


Fig. (2): Monthly variation of the gonadosomatic index (GSI) of females grass carp, *C. idella* during the study period.

**A-Morphological observations on the ovary:**

The morphology of the ovary differed throughout the annual period. The color, size and blood supply of the ovary differ with the success of maturation. In immature stage, the colour of the ovary has changed from transparent to yellow color in the ripe stage. Atretic follicles acquired reddish to yellowish colour (Figs., 3 d). According to the morphological criteria of ovary, the developmental stages can be classified into five stages.

**Stage 1 (Immature stage):**

In this stage, the ovary was a colourless and appeared as a thin thread, adhering in the abdominal cavity's upper wall above the kidney. During the immature stage , the ovary cannot be separated from the testis . The blood supply cannot be detected with the naked eye (Fig., 3 a).

**Stage 2 (Maturing stage):**

The ovary seemed to be reddish in colour at this point, and the size of the ovary increased in thickness. It was difficult to observe the actual oocyte with the naked eyes and the blood supply was still not detected. (Fig. 3 b).

**Stage 3 (Ripe stage):**

The ovary occupied large region in the body cavity. It was large, opaque and had a yellow appearance. The eggs loaded with yolk and become clearly visible with the naked eyes. The blood supply of the ovary can be detected and the blood capillary can be extended as a network on the outer surface of the ovarian wall. (Fig. 3c).

**Stage 4 (Spawning stage):** Also, the ovary is enlarged and enclosed by fully mature ova, the spawning period ha not been completed.

**Stage 5 (Atretic stage):**

The scale of the ovary was gradually decreased and its colour become flaccid, flabby and yellowish in colour. Some of the ovaries visible to the naked eye from translucent to opaque containing residual eggs. While in the others eggs, they possessed cracked egg surface and disintegrated. Compared to ripe stages, the ovary showed increased vascularity (Figs. 3 d).

**B-Histological observations:**

The ovary consists of dendritic ovigerous lamellae that projected from the ovarian wall and take a direction toward the lumen of the ovary that lined with epithelial cells. The oocytes appeared as bead-like in arrangement instead of haphazard fashion. The oogenesis process, began with oogonia which fromed from primordial germ cells (Fig. 4 a). This oogonia undergoing growth and formation of primary oocyte , followed by the formation of yolk vesicle and consequently proceeded vitellogenesis leading to maturation of the ova (Fig. 4d).

The oogenesis process is divided into many stages according to the size, quantity and distribution of yolk inclusions, as well as nuclear changes, differentiation of oocyte

membrane into zona radiata, follicles and theca cells. The oogenesis stages are chromatin nucleolus, perinucleolus ( Fig. 4 a), yolk vesicle ( Fig. 4c), yolk globule (Fig. 4d,e), ripe ( Fig. 4d,g) and atretic stages ( Fig. 6c).

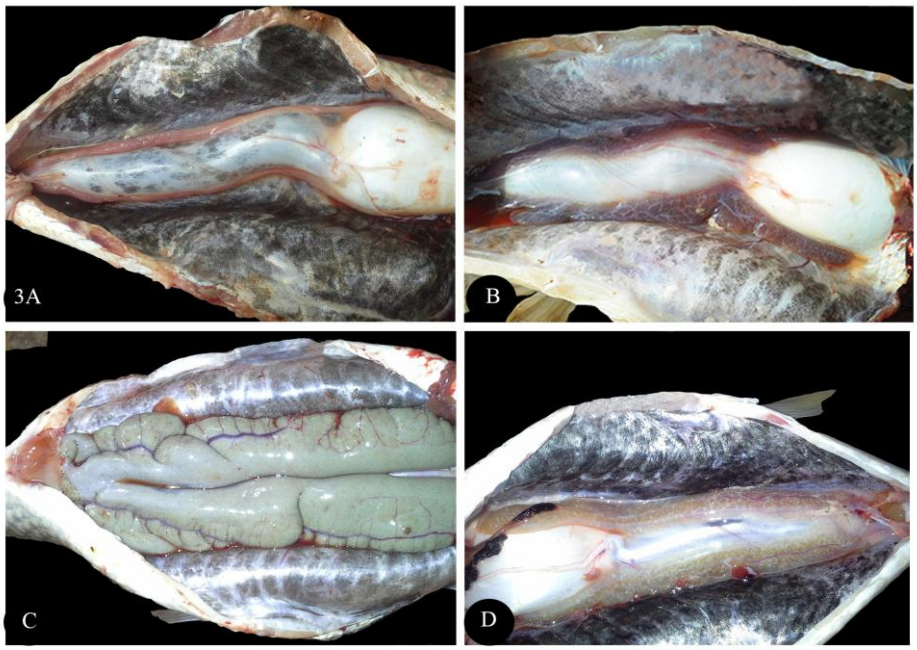


**Fig. 3 (A-D): Photomacrographic of ovaries during development. (X5).**
A. Showing transparent thin thread immature ovary.
B. Showing maturing elongated ovary appeared orange in colour.
C. Showing yellow ovary with ripe stage..
D. Showing ovary with atretic stage.

The oocytes become surrounded by a thin membrane in the chromatin nucleolus stage . A single layer of follicle cells was completely ensheathed the oocyte (Fig.,4a). During the early yolk vesicle, the oocyte wall structure was established and its thickness attained 5µm and formed of two layers follicular cells located above the granulose cells ( Figs. 4b).

At the late yolk vesicle stage, the wall of the oocytes differentiated and consisted of three layers from inside to outside are zona radiata, granulosa and follicular cells (Fig. 4 c). Cytoplasmic vacuolization started with one row of small vacuoles aligned close to the oocyte wall (Fig. 4a).

During yolk globule stage, the oocytes wall was highly differentiated but was still composed of three cell layers (Figs. 4 c,e,f).

During maturation stage, the nucleus migrated toward the animal pole (Fig. 4 d). Small funnel-shaped depression (micropyle were detected in the animal pole (Fig. 4 g). The micropyles were considered the site of sperm penetration (Fig. 4g). Scanning electron microscopic investigation revealed only one micropyle structure in the ova wall of grass carp. The micropyle formed from three distinguished structures , centrical micropyle channel and micropyle hole (Fig. 4 h).

**Atresia action**

At the ending of the spawning season, the grass carp failed to be ovulate due to varying environmental condition. Destruction and absorption of the ova were carried out. The mature fish exhibited two generations (batches) of eggs which lacked to spawn. During follicular atresia, the oocyte shrinked , associated with cracks of their outer wall. The damaged of oocyte showed abnormal irregular structure, folding and degraded zona radiata, hypertrophied follicle cells and disintegrated yolk granules were considered as the main feature of enhancing the atretic action (Fig. 6c).

During progress of follicular atresia, the zona radiata lost their striated structure and achieved considerable hypertrophy (Fig. 5 a). Also there was a considerable hypertrophy of granulosa cell associated with disintegration and resorption of yolk granules (Fig. 6 a) and (Figs.5 c and d). There have been some remarks of infiltrated yolk materials through the pores of zona radiata (Figs. 5 d and e). The yolk materials was detected in in the interstitial space between zona radiata and follicle cells through the pores of zona radiata (Figs. 5 b, d and e).

The inner margin of hypertrophied zona radiata revealed the growth of phagosomes as a finger-like extension (Figs. 5b ,c and f) . The scale and structure of hypertrophied zona radiata decreased and retarded with proceeding development (Fig. 5f) and eventually disintegrated (Figs. 5 g and h).This was accompanied by pronounced hypertrophy of granulosa cells with characteristic phagocytic activity for the liquefied yolk. The atrial space was formed by the remnant of liquefied yolk material (Fig. 5h). The aggregation of granulosa cells predicted the final step of follicular atresia (Fig. 6c).

During the atretic process , zona radiata, thecal cells and granulose cells showed intensely stained, with PAS, while yolk granules exhibited faintly PAS staining (Figs. 6a, d and e). The residual active granulosa cells were intensely stained with a PAS reaction when the yolk content in the ooplasm was absorbed (Fig. 6 c).

**C-Histochemical observations:**

When PAS reaction was applied , the cytoplasm of the mature ova showed a fainly staining, while zona radiata, thecal cells and granulose cells were deeply stained with PAS reaction (Fig. 6 F). On the other hand, the mature ova possessed intensely PAS reaction compared to faintly staining in the immature stage (Fig. 6 F).

During post-spawning, the ovary shrinked and ova underwent atreasia. Zona radiata, thecal cells and granulose cells were intensely stained with PAS , while yolk granules were faintly PAS staining (Fig. 6E).

After bromophenol blue staining, both the wall and cytoplasm of the mature ova were moderately stained in blue colour (Fig. 6 g and h). The cortical alveoli of the outer egg layer was negatively stained with bromophenol blue. Zona radiata, thecal cells and granulose cells showed intense protein staining following the oocyte entered the follicular atresia (Figs. 6g and h).

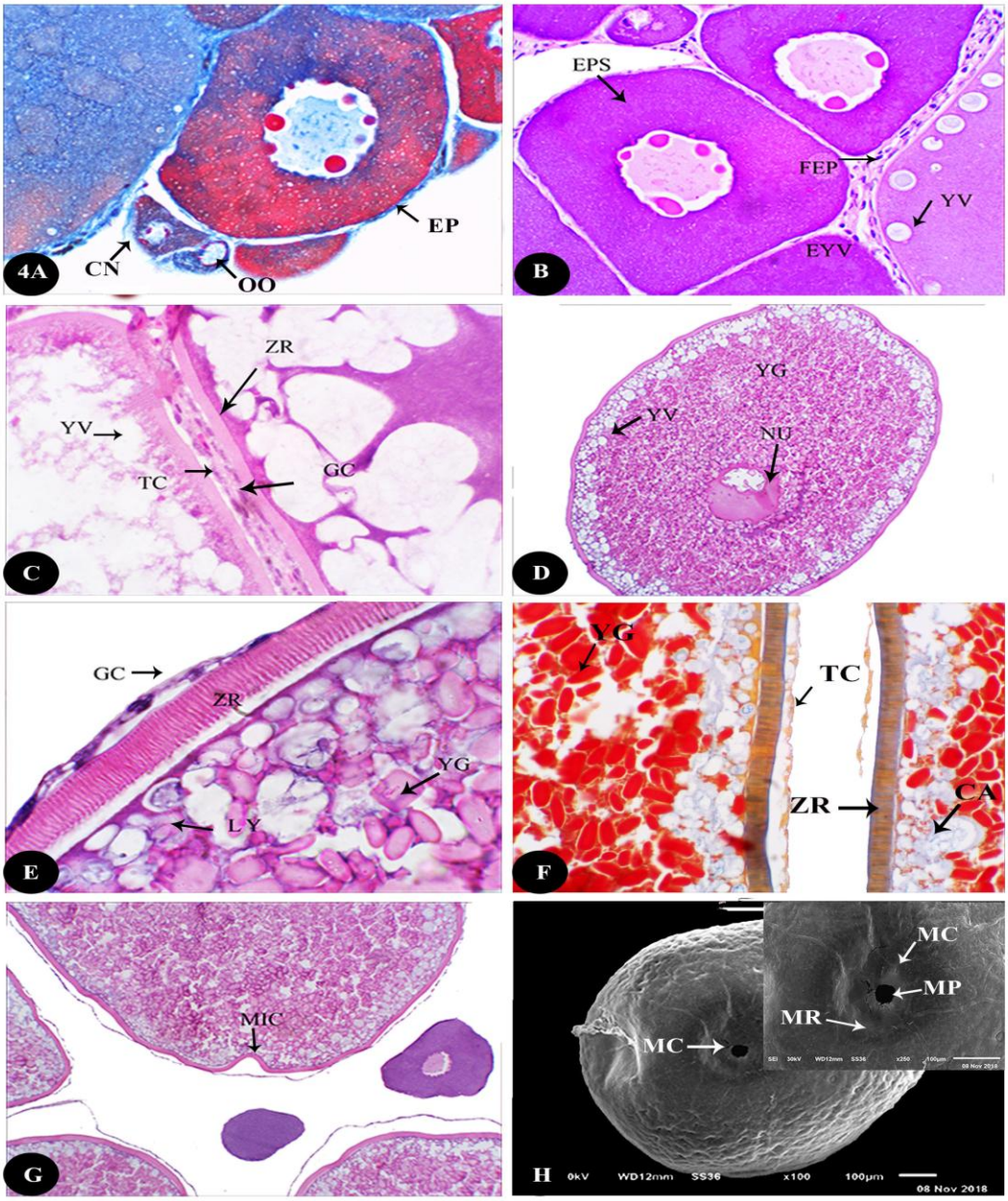


**Fig. 4(A-H): Photomicrographs of histological sections in the ovary of grass carp.**

A. Showing oogonia (OO) with chromatin nucleolus stage (CN) and early perinucleolus (EP) stage. (Mallory, X400)

B. Showing cytoplasmic yolk vesicle (YV) predicting early yolk vesicle stage (EYV) and follicular epithelium cells (FEP). (H & E, X400)

C. High magnification of oocyte during late yolk vesicle showing well developed granulose cells (GC), zona radiate (ZR) and outer layer of thecal cells (TC). (H. & E., X1000)

D. High magnification of ripe oocytes, showing cytoplasm filled with yolk granules (YG) and several rows of yolk vesicle (YV) aligned near oocyte membrane and nucleus (NU). (H. & E., X100)

E. High magnification of ripe oocyte, showing liquefied yolk (LY), granulose cells (GC), zona radiate (ZR) and yolk globules (YG). (H. and E., X400).

F. Showing ripe ova with aggregation of cortical alveoli (CA).(Mallory stain, X400)

G. High magnification of the ripe ova showing micropyle (MC). (H. and E., X250).

H- Scanning electron micrograph of the egg surface showing micropyle region (MR), oval micropylar canal (MC) and micropyle pit (MP). (X5000)

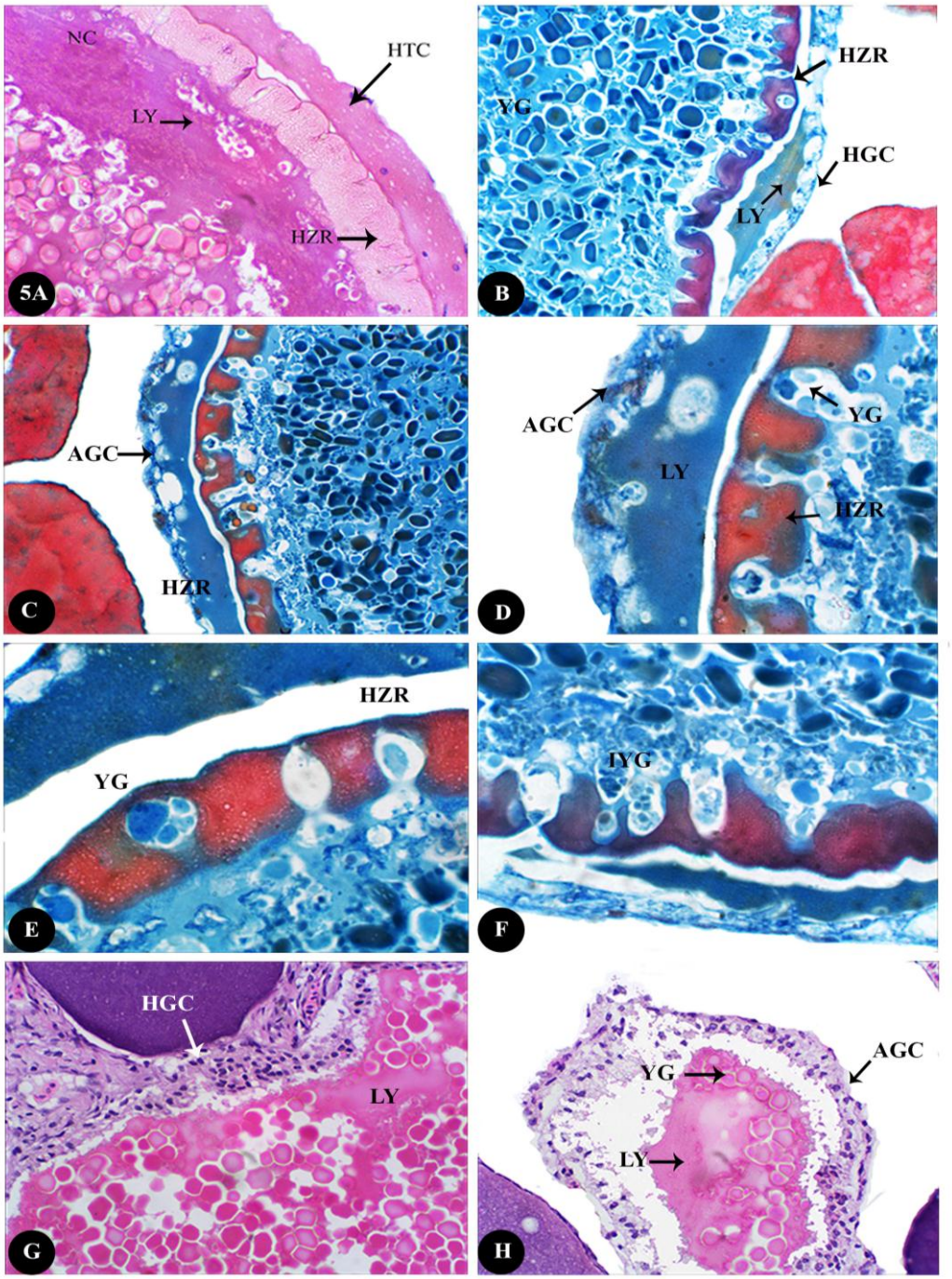


**Fig. 5 (A-H): Photomicrographic sections in the ovary**.

A. Showing hypertrophied zona radiata (HZR) , liquefied yolk (LY) and hypertrophied thecal cell (HTC). (H. and E., X, 500)

B. Showing amoeboid surface of zona radiata (HZR) and accumulation of yolk (LY) enclosed between and hypertrophied thecal cell (HTC). (Mallory stain, X500)

C. Showing disintegration of yolk granules and granulose cell (AGC). (Mallory stain, X1000)

D. Showing phagocytosis of zona radiate (HZR) and absorption of liquid yolk (LY) with active granulose cell, (Mallory stain, X500)

E. Showing phagocytosis of with hypertrophied zona radiata. (Mallory stain X1000)

F. High magnification showing degradation of zona radiata and hyaline degeneration of yolk granules (IYG). (Mallory stain, X1000)

G. High magnification of oocyte showing disappearing of zona radiata and invading active granulosa cell and yolk granules lost the road like structure and take irregular circle. (H. and E., X500)

H. Showing active invading hypertrophied granulose cell and atretic oocytes remnants of liquefied and yolk granules. (H. and E., X500).

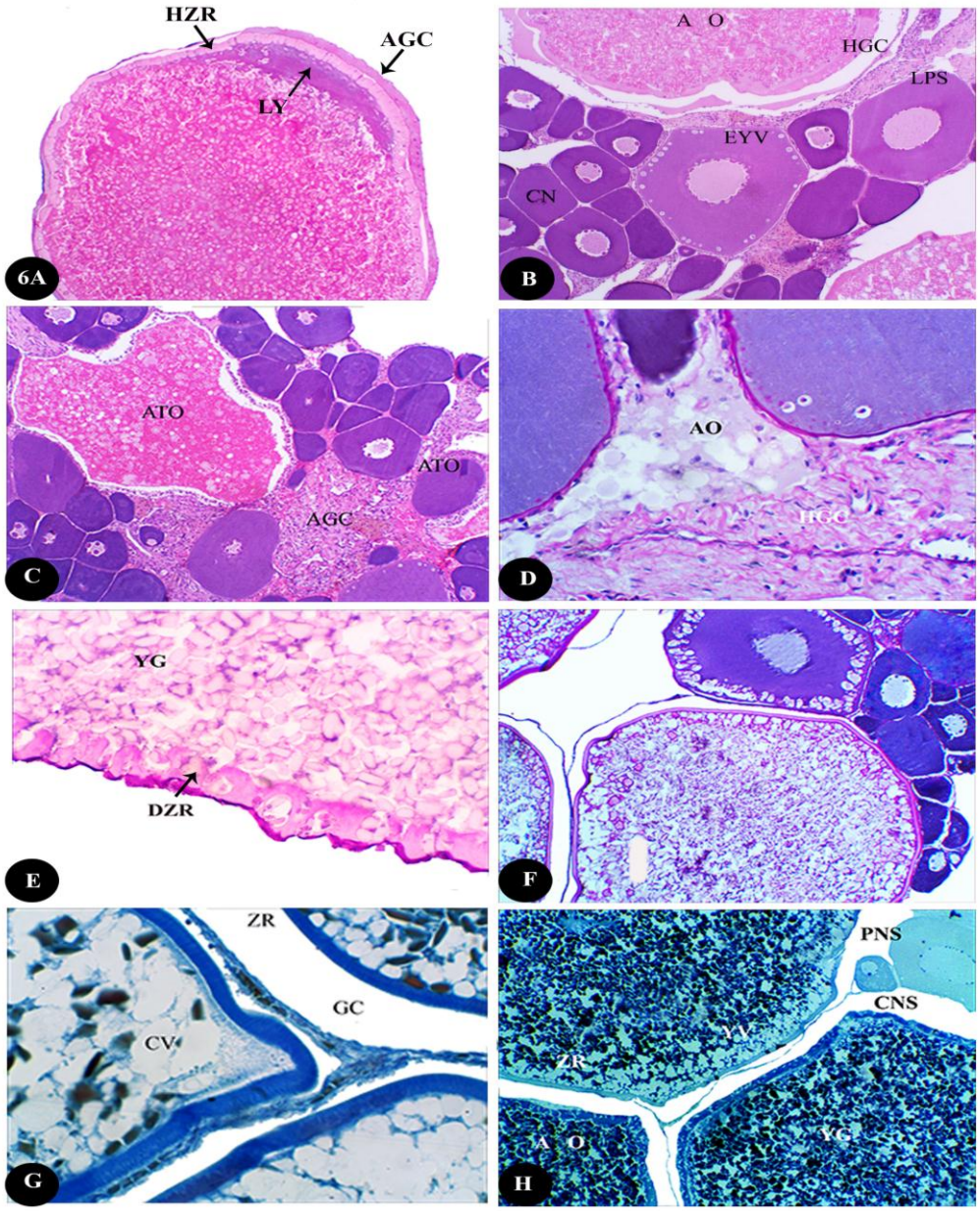


**Fig. 6 (A-H): Photomicrographic sections during follicular atresia.**

A. High magnification of early atretic follicle, showing hypertrophied zona radiata (HZR) ,liquefied yolk (LY) adjacent to animal pale, and hypertrophied granulosa cell.
(H. and E., X200)
B. Showing the presence of chromatin nucleus, and early yolk vesicle stages, hypertrophied granulose cell (HGC) , atretic oocytes (AO) and late perinucleolus stage (LPS).
(H. and E., X100)
C. Showing hypertrophied granulose cell and atretic oocytes. (H. and E., X100)
D. Showing granulose cells and remnant of atretic oocyte. (PAS reaction, X200)
E. Showing zona radiate and granulose cells with dense protein staining, and non-stained cortical alveoli. (Bromophenol blue, X400)
F. Showing PAS positive yolk granules , zona radiata and granulose.
(PAS reaction, X200)
G. Showing dense protein staining within zona radiate and granulose cells while cortical alveoli were not stained. (Bromophenol blue, X400)
H. Showing intense protein staining zona radiata. (Bromophenol blue, X200)

**Monthly variation in the ovary during annual reproductive cycle**:

The monthly distribution of maturity stages in The female of grass carp possessed varied ovarian stages throughout the period from January to December 2018 (Fig.7) and illustrated as follows:

**1-Immature stage**

It was detected through the period from January to the end of February and its percentage reached 100%. Then the average percentage was decreased to 78 % in mid of March, and declined to about 10% in April. The immature stage vanished from the beginning of May to the end of June. as a result of proceeding maturation process. In late the immature process attained 30% in July 30% and 89% in August and was the only observed during the rest of the annual cycle.

**2-Maturing stage**

Mature eggs were observed in March (22%) and its percentage was increased to April (50%) and missing in May.

**3- Ripe stage**

The ripe stage makes their first appearance in April (40%) and reached in May up to 100 %, then declined reached 54% in June.

**4-Spawning stage**

The mature egg failed to ovulate and the spawning season is missoing.

**5-Atretic stage**

This was carried out through June 46%, July 70% and August 11% only of the year.

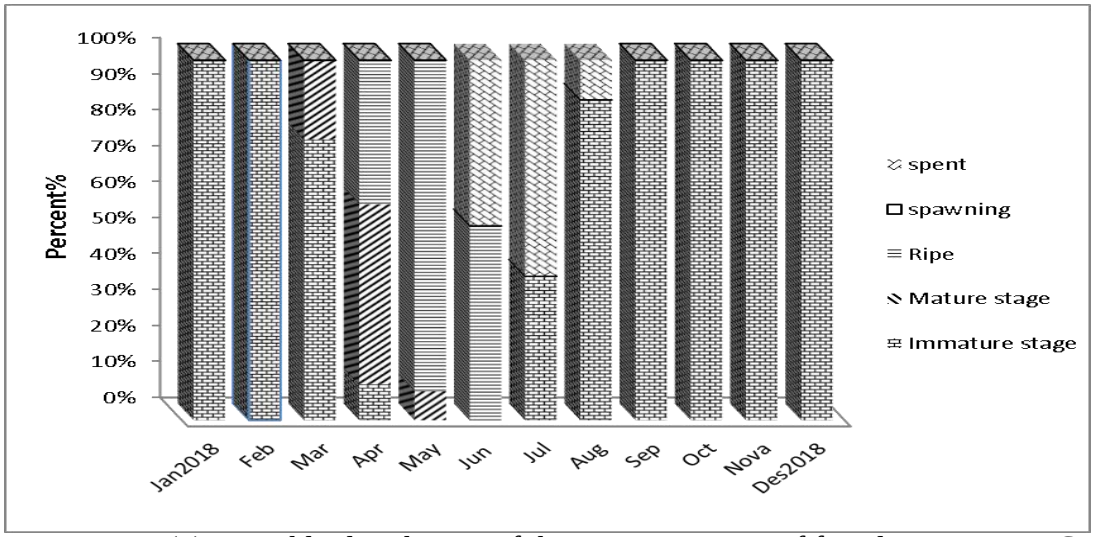


Fig. (7). Monthly distribution of the maturity stages of female grass carp, *C. idella* during the spawning season, 2018.

## DISCUSSION

From the present findings, the GSI of female grass carp increased in April ,reached its maximum peak in May and then steadily decreased in June, taking in consideration that the spawning season extended from March to the late of June. During this this time, the water temperature was adequate for spawning. The present results were consistent with Rodriguez *et al.* (2006) who noted that the pre-breeding season of an adult carp fish,extends from June to September, while the breeding period lasts from February to May and the post-breeding season begins from October to January. Barnabè (1994) reported that the GSI of sea bass reached the highest peak during the spawning season. On the other hand, Shaikh and Prakash Lohar ( 2011) mentioned that the decrease of GSI during the post-spawning season in *Labeo rohita*, *Catla catla*, and *Cirrhinus mrigala* may result from follicular atresia and gonadal arrest. According to Nandikeswari and Anandan, (2013), the sharp reduction of GSI predicted the end of spawning season and progress of follicular atresia.

It is known that the ovarian maturation of teleost is normally graded into primary growth phase, secondary growth phase and maturation phase (Çek and Yalimaz, 2007). The maturation period of the grass carp in the present study was divided into seven stages based on cytological changes. These are oogonia, chromatin nucleolus stage, perinucleolus stage, yolk vesicle stage, yolk gobule stage, ripe stage, and atretic oocyte. However in the other teleost species, the oogenesis stages classified from five to eight stages according to the aspects described by Nagahama (1983), West (1990) and Gökçe *et al*.(2003).

In addition, the oocyte wall has been differentiated into a single follicular epithelial layer during the chromatin nucleus period of the grass carp. The cell wall was divided into two layers, peripheral follicular epithelial cells followed by granulose cells, as the oocyte reaches the yolk vesicle level. These results support the work of West (1990). Also, during vitellogenesis, three layers of oocyte wall are developed, external thecal cell layer, medium granulose cell layer and inner zona radiate(non-cellular structure). Similar findings were observed in Syn. C. gariepinus (Arukwe and Goksyr, 2003; Sheha,2011). However the oocyte wall is differentiated into five in *Carangid trachinotus* ovatus (El-Gharabawy *et al*., 2003).

Ovulation failure of the female grass carp with subsequent reabsorption of ova and follicular atresia appeared to result from hypertrophied zona radiata, degradation of transverse striated structure , and swallowing of yolk granules by phagosomes. Fausto et al. (1994) proposed a defensive function of zona radiata layer. Oppen (1990) and Esmaeili and Gholamifard (2012) reported that zona radiate carried sperm receptors and prevented polyspermy post-fertilization.

In grass carp, zona radiata and hypertrophied granulose cell have played a critical role in the atretic action of matured ova. The present findings supported the work of sheha (2011) who observed hypertrophy of granulosa cells and reabsorption of yolk materials in Syn. C. gariepinus.

Also, during the end of spawning , ovary atrophied associated with degradation of zona radiata and theca and granulose cells as well as reabsorption of yolk granule.Similar findings were reported in captive *M. cephalus* ( Mylonas *et al.* 1997; Assem *et al*.,2015). The mentioned authors reported disintegration in the oocyte nucleus was the first signs of atresia, followed by the fragmentation and hypertrophy of the follicle cells. In multiple spawning atretic oocytes have been recorded in S. undosquamis. However some teleost fishes such as N. japonicus, R.haffara and L. carinata displayed partial follicular atresia between 30 to 35 % of their mature ova (Ramadan and El-halfawy, 2006).

In addition, scanning electron microscope investigations revealed the presence of only one micropyle in the mature ova. It is appeared in the form of conical groove with central pore in the animal pole . Similar findings have been reported in the Cyprinion tenuiradius mature ova (Esmaeili and Gholamifard, 2012). The mature ova had up to 52 micropyles in the other teleosts (Lahnsteiner, 2003).

## CONCLUSION

It can be concluded that grass carp has short spawning season that restricted in April and May. The gonadosomatic index reached to its maximum peak in May with temperature ranged from 21.5 to 25c$^{o}$. The large oocytes accumulate yolk granules, protein, carbohydrate and lipid within their cytoplasm. Due to inhabiting of fishes in non-satisfied environmental condition, the mature ova failed to spawn and undergoing follicular atresia due to alterations of zona radiate and granulosa cells.

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