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Reproduction and gametogenesis features of *Anodonta anatina* in Anzali Wetland, Southwest Caspian Sea

Mohaddeseh Ahmadnezhad[1]*, Hossein Khara[2], Mohammad Sayad Bourani[1], Homayoun Hosseinzadeh Sahafi[3]

1. Inland Waters Aquaculture Research Centre, Iranian Fisheries Sciences Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Bandar Anzali, Iran
2. Department of Fisheries, Lahijan Branch, Islamic Azad University, Lahijan, Iran
3. Iranian Fisheries Sciences Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Tehran, Iran

* *Corresponding author's E-mail: m_ahmadnezhad@yahoo.com*

ABSTRACT

Duck Mussel, *Anodonta anatina*, is the most important native mussel in Anzali Wetland, Southwest Caspian Sea. In the last two decades, its population has been threatened by manipulation and pollution of habitat. Therefore, more studies on biological characteristics are necessary for future conservation. Gametogenesis and temporal changes of the gonads throughout the reproductive cycle were investigated through standard histology. All specimens were gonochoristic with a sex ratio of 1.5 F: 1 M. Gametogenesis has been continuous throughout the year. Three stages, developing (spring and summer), spawning (autumn, September), and post-spawning (winter), were detected in both sexes during a maturation period. Glochidia were present in the gills in January. Five continuous stages were observed in female gonad acini including oogonia; previtellogenic oocytes; pedunculated oocytes; oocytes and mature oocytes. Spermatogonia, spermatocyte, spermatid and spermatozoa were found in male gonad acini. The sexual pattern was asynchronous due to the simultaneous presence of different stages of oogenesis and spermatogenesis in gonadal acini. According to the results, in order to protect the Anodont of Anzali Wetland, especially in the period of September to February of each year, due to the critical time for reproduction of this species, conservation plans should be performed by the Environmental Protection Organization.

Keywords: Swan Mussel, Anzali Wetland, Oogenesis, Sex ratio, Spermatogenesis.
Article type: Research Article.

INTRODUCTION

Freshwater bivalves are threatened by manipulation and pollution of habitat in the world (Simberloff 2012). *Anodonta anatina*, is a native mussel (Bivalvia, Unionoida) in Anzali Wetland (37˚_28′N, 49˚ _25′E; Pourang *et al.* 2009) located in the southwest coast of the Caspian Sea and listed in Ramsar Convention since 1975 because of its unique ecological and international importance. However, it was damaged by human activities contamination and was listed on the Montreux Record in 1993 as a wetland in need of restoration (Mousazadeh *et al.* 2015; Navabian *et al.* 2020). All of the problems caused the aquatic life of this wetland to be endangered and so have led to a decrease in the population of *Anodonta*. This mollusk belonging to the Unionidae is filter feeder organisms and important consumer of early phytoplankton production. It plays an important ecological role in aquatic ecosystem functioning such as reducing water turbidity, controlling the amount and composition of suspended particles, transferring biomass from the water column refers to the benthos and rotation of nutrients (Vaughn 2010; Sousa *et al.* 2012). The consequences of the decline of these mussels, in addition to species loss, are the loss of their benefits in freshwater ecosystems, such as trophic and non-trophic functions (Vaughn 2010; Allen & Vaughn 2011). Therefore, in order to take protective measures, it is necessary to have comprehensive and sufficient information on their ecological and biological status. To date, *Anodonta* individuals inhabiting

North Iran have been called as *Anodonta cygnea*. Due to the phenotypic similarities, it was very difficult to distinguish between the two species *A. cygnea* and *A. anatina*. However, recent molecular studies showed that the mussels living in Anzali Wetland are *A. anatina* (Lopes-Lima *et al.* 2021; Mohamadzadeh *et al.* 2022). It's distribution and biodegradation characteristics, homocyte morphology, and the impact of some contaminants on its organs were studied in Iran (Pourang *et al.* 2009; Salimi *et al.* 2009; Eskandari *et al.* 2012; Sarikhani & Javanshir, 2010). However, understanding the reproductive processes is necessary to help it conserve or restock. Reproduction of *A. anatina* is similar to other freshwater mussels (Galhano & Silva 1983). In European regions, there are hermaphroditic populations of this species (Bloomer, 1930; Lima *et al.* 2012). Their gametogenesis occurs in summer, spawning in early autumn, and the production and release of glochidia in winter (Galhano & Silva's 1983). Their spermatogenesis was described by Rocha & Azevedo (1990) and the oogenesis of hermaphroditic specimens by Lima *et al.* (2012, 2017). Although the type of habitat and its changes may affect the reproductive pattern of animals, no studies have been carried out on the reproductive characteristics of this species in Anzali Wetland. The continuous and stable presence of this native species in the wetland depends on natural reproduction and preservation strategies that lead to the continuation of its generation. There are many ambiguities about the structure of reproductive organs and the cycle of reproduction in *Anodonta* of Anzali Wetland so it is necessary to increase the basic biological knowledge for the planning of conservation and restoration management of their species reserves in the wetland ecosystem. The present study aimed to characterize the histology of gonadal maturation stages in *A. anatina* to determine the time of their natural reproduction and the release time of glochidia to obtain basic information for future management in the field of sustainable conservation, propagation, and restocking programs of this species in Anzali Wetland.

MATERIALS AND METHODS

Sampling and Biometry

A total of 69 specimens of *A. anatina* were collected monthly (9-10 per month) for 7 months from May 2017 to March 2018 from Anzali Wetland that is located on the southern coast of the Caspian Sea, Iran (37˚_28′N, 49˚-25′E). The mussels were kept in water and transported to the Laboratory of Aquatic Physiology at Inland Waters Aquaculture Research Centre in Anzali, Iran. They were measured (total length, total width, and total height to the nearest 0.1 mm by Vernier calliper) and weighed (total mussel weight to the nearest 0.01 g). Age was determined by counting the annual rings on the surface of the bivalve shells. Sex was determined using three criteria: presence of eggs or glochidia within the marsupial gill; microscopic observation of fresh smear of the gonad tissue with a light microscope and confirmed by microscopic observation of histological sections of gonadal tissues.

Histology

The mussels were dissected and sections of the foot were cut for histological examination. The sections were fixed for a week in Bouin's fluid and then preserved in 70% ethanol. Samples were then dehydrated in graded ethanol, embedded in paraffin, sectioned at 5–7 µm, and stained with haematoxylin and eosin for histological examination (Lima *et al.* 2012; Hinzmann *et al.* 2013). The slides were observed by a Light microscope (BEL, BIO2, ITALY) and the level of gonad maturation and developmental stages of female and male gamete cells were identified according to the descriptions given by Hinzmann *et al.* (2013) and Hliwa *et al.* (2015). Histological quantitative analyses in gonadal cross-sections were performed using a Computer-Based Image Analyzer (Image J 1.46r). Counting of the number of empty acini (acini in which there are no mature oocytes), the number of acini containing mature oocytes, and the number of pedunculated, free, and mature oocytes in 100 mm^2 of the gonadal area of each female were performed in 3 slides and 3 sections per slide and 3 pictures per section (Mohammad Karami *et al.* 2014; Hliwa *et al.* 2015). The size of gamete cells [diameter (µm) and area (μm^2) in each developmental stage, range; mean ± standard error] were measured for both sexes. At least 100 gamete cells were measured for each male or female individual. All statistical analyses were carried out using SPSS 22. Monthly differences between gonadal index measurements were tested by means of the one-way analysis of variance (ANOVA). Tukey test with 95% confidence limits was applied to compare the means.

RESULTS

All of the analyzed duck mussels were mature with a mean total shell length of 10.2 ± 0.2 cm, shell width of 6.0 ± 0.1 cm, and shell height of 4.0 ± 0.1 cm. The average total weight of mussels was 127.1 ± 8.3 g. The mean age

was above 5 years, ranging from 2 to 9 years. The relationship mode between the shell length and width of the males was similar to the females (Fig 1). No sexual dimorphism of *A. anatina* was found. All of the examined *A. anatina* in Anzali Wetland were gonochoristic; 61.2% were female and 38.8% male.

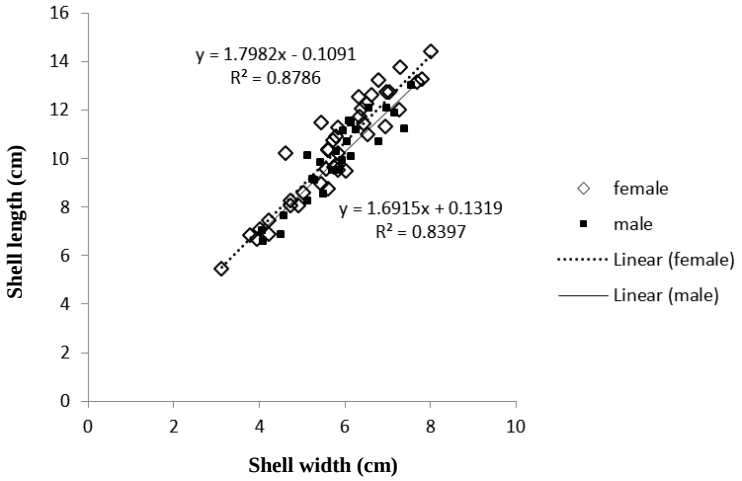

Fig. 1. Relationships between shell length and width in males and females of *Anodonta anatina* from Anzali Wetland.

Gonadal tissue analysis

The gonad of *A. anatina* was interspersed throughout the visceral mass surrounding the glandular digestive tissue and the gut. The fibrous connective tissues and muscle fibres were located between the gonadal acini. Determination of sex was hard by macroscopic observation of gonads in March, May, June, July, and August. However, female tissue was detectable with an orange appearance against the white male tissue in September. Also, the females were identified through the presence of mature glochidia inside their marsupial gills in January. However, male and female could be indicated by microscopic observation of fresh smears of the gonad in all months. The gonadal smear of females contained small or big round oocytes, while the male gonadal smear contained male gamete cells in different stages of spermatogenesis. Male and female mussels were confirmed after a histological study. Both male and female tissue consisted of highly branched follicles surrounded by connective tissue and hemocoel spaces. Different gonad acini from the same organism may exhibit different degrees of development. So, it was difficult to separate the gonad development stages. The gonad development stages are described according to temporal changes in gonadal tissue and the developmental stage table described by Hinzmann *et al.* (2013). In both sexes, germinal epithelium in the early stages of development of gametes had been always present, so gametogenesis was continuous.

Ovary

The ovarian acini contained different stages of oocytes in all months except March. Several acini were connected to a single germinal duct which was lined by a ciliated epithelium. Mature oocytes were found in the germinal duct lumen in August and September and some atretic oocytes were observed in the lumen during the other month of the study. According to histological studies, the oogenesis (Fig. 2) was mainly divided into five continuous stages that occur consecutively: (i) oogonia; (ii) previtellogenic oocytes; (iii) pedunculated oocytes; (iv) oocytes; and (v) mature oocytes. All of these developmental stages with the difference in their number were found in the examined months other than March. In March, most of the acini were free of oocytes, and only a small number of oogonia were clinging to the wall of the acini. It seems that the less active period occurs in March. The maturation process started in the spring and became intense in the summer and at the beginning of the autumn. Spawning occurred in September and the glochidia were released in January: (i) Oogonia were present throughout the annual reproductive cycle. They were small round cells with an acidophilic cytoplasm, basophilic nucleus, and dispersed chromatin. They were located among the stroma. The mean diameter of them was 8.2 ± 0.1 µm and the mean area was 65.0 ± 1.3 µm^2 (Table 1). (ii) The oogonia grew up and developed into the previtellogenic oocytes that were generally located at the periphery of the acinus wall. They possessed larger nuclei; their mean diameter was 15.5 ± 0.2 µm and their mean area was 227.6 ± 6.5 µm^2. (iii) The previtellogenic oocytes increased in size with a mean diameter of 36.6 ± 1.2 µm and a mean area of 1416.5 ± 84.7 µm^2 (Table 1). These pedunculated oocytes were attached by a stalk to the walls of the oogenic acinus. As they grew, their cytoplasm became more acidophilic.

They kept their connection to the germinal epithelium until more developed and moved to the lumen before spawning. (iv) The vitellogenic oocytes continued to enlarge. Their size was not significantly different from the previous stage. They were located in the centre of the acinus lumens. There was a big nucleus in the centre or close to the centre of the very eosinophilic cell with big distinct nucleoli in the centre of the nucleus. The vitellogenic oocytes' diameter was 38.3 ± 0.5 µm and their mean area was 1376.5 ± 40.6 µm^2. This stage was mainly observable in May, June, and July. (v) The mature oocytes were very large in size with a mean diameter of 59.6 ± 0.8 µm and a mean area of 3171.5 ± 79.4 µm^2 (Table 1). The nucleus became completely close to the oocyte membrane. It was smaller in size and its shape was irregular. Its membrane was ridged. Some projections were sent from the nucleus into the ooplasm. Some very small nucleoli were on the edge of the nuclear membrane. Mature oocytes were present for most of the month except March and these were scattered in the follicular lumen.

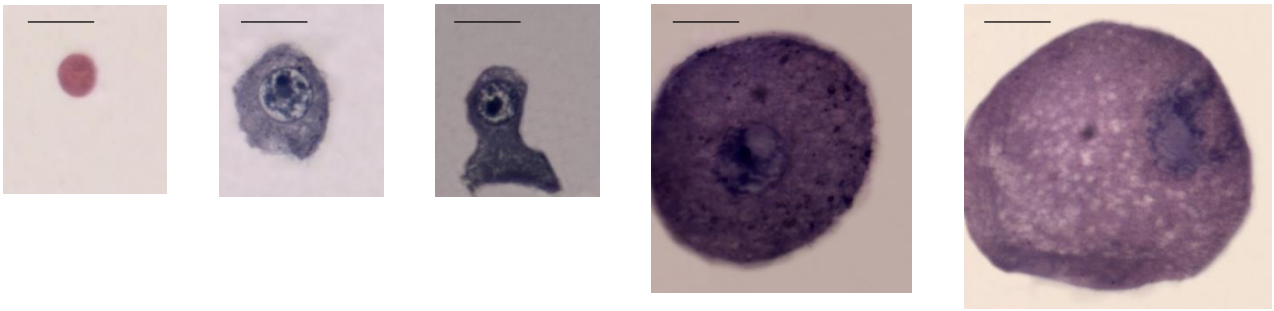

Fig. 2. Oocyte maturation stages in *A. anatina* from Anzali Wetland (Iran). H & E staining; Scale bar = 20 µm.

Table 1. Mean diameter (µm) and mean area (µm^2) of developmental stages of oocyte and spermatozoa in *A. anatina* from Anzali Wetland (IRAN) at (mean ± standard error).

Development stage		Diameter (µm)	Area (µm^2)
Oogenesis	Oogonia	8.2 ± 0.1^a	65.0 ± 1.3^a
	Previtellogenic oocytes	15.5 ± 0.2^b	227.6 ± 6.5^b
	Pedunculated oocytes	36.6 ± 1.2^c	1416.5 ± 84.7^c
	Oocytes	38.3 ± 0.5^d	1376.5 ± 40.6^c
	Mature oocytes	59.6 ± 0.8^e	3171.5 ± 79.4^d
Spermatogenesis	Spermatogonia	8.2 ± 0.2^a	52.8 ± 2.5^a
	Spermatocytes	5.1 ± 0.0^b	21.5 ± 0.3^b
	Spermatid	4.2 ± 0.1^c	13.7 ± 0.4^c
	Spermatozoa	2.2 ± 0.0^d	3.9 ± 0.6^d

Note: Different letters show significant differences in column ($p < 0.05$).

In March, only one specimen was female and its ovary contained acini with a few or no developmental stages of oocyte; the epithelial wall was degenerating and oogenesis was not active (Fig. 5H). Because of the low sample, the data for this month could not be calculated so it was not shown in graphs. In May, most acini have a small to medium lumen. The wall of acinus was almost thick and degenerate in some places. The ciliated gonoducts contained no mature oocytes. Some Atretic oocytes were present in some acinus (Fig. 5A). The empty acini constituted 80 and 90% of the quantitative share, at this time and in January respectively which was much higher than in the other months (Fig. 3). The number of oocytes was higher than previtellogenic and pedunculated oocytes in this month as well as in other months except in January and March. Also, the number of oocytes was higher than in January, but less than in the other months (Fig. 4). In June and July, the acini containing oocytes increased (Fig. 3). Although it fell in August, most of the acini of the ovarian tissue were full of grown oocytes in September that were moving to gonoducts. The size of the acinus was bigger than in May. The follicular wall was thin but the germinal epithelium containing oogonia and previtellogenic oocytes was thickened. In July presence of pedunculated oocytes attached to the germinal epithelium was higher than in June (Figs. 5B and 5C). In August, two groups of mussels were found. One group had the acini with irregular and discontinuous walls. It seems that the number of previtellogenic oocytes were higher than oogonia in their epithelium. The other group was the mussels with regular and continuous walls in the round of follicular lumens. Their germinal epithelium was full of oogonia and previtellogenic oocytes. Still, pedunculated oocytes and grown oocytes were low which caused to

decline in the number of developmental oocytes rather than in July (Figs. 4 and 5D). In September, there were large acini with a vast lumen. The epithelium sometimes was discontinuous. A significant increase was also observed in the proportion of the acini containing the ripe oocytes compared to the other months (Fig. 3). Thickness of germinal epithelium was low and a decrease was also observed in the proportion of oogonia attached to the wall. In comparison to January, May, June, and August a significant increase was detected in the number of pedunculated, free, and mature oocytes in the early autumn (Fig. 4). Free oocytes in the lumen reached the highest level of maturation, since the nucleus was completely close to the cell wall and the nucleus membrane decomposed and degenerated. The gonoducts were full of gametes that appeared to be the symptom of spawning capability (Figs. 5E and 5F). In January, the small ovarian follicles were evident but empty. Empty spaces and a lower density of vitellogenin and free mature oocytes were observed in the acini lumen. Also, we observed loose stroma within the lumen of the acinus, a skinny epithelium wall, and a disconnection in the oogonia and previtellogenic oocytes in the germinal epithelium. The gonoducts were completely free of oocytes. The marsupial gills were filled with glochidia (Fig. 5G).

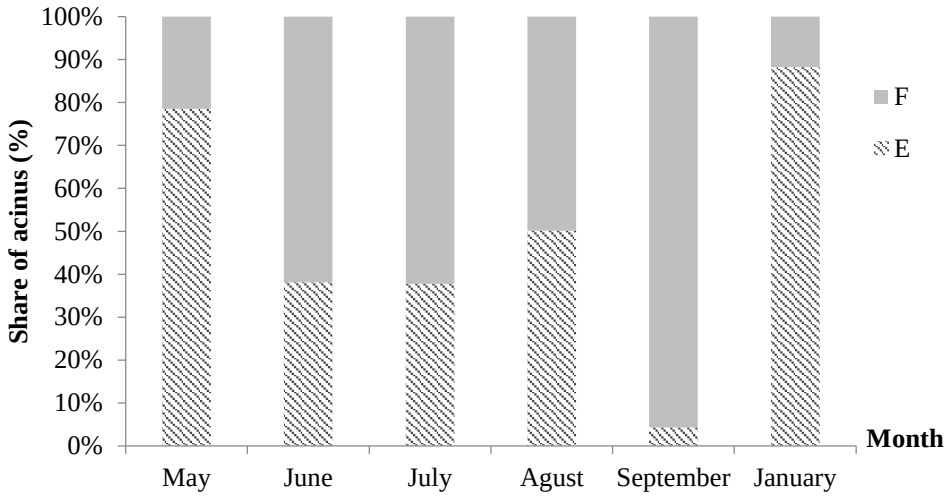

Fig. 3. Quantitative share of acini with (F) and without (E) mature oocytes in female gonads of *A. anatina* from Anzali Wetland in different months. F: full of oocyte, E: empty.

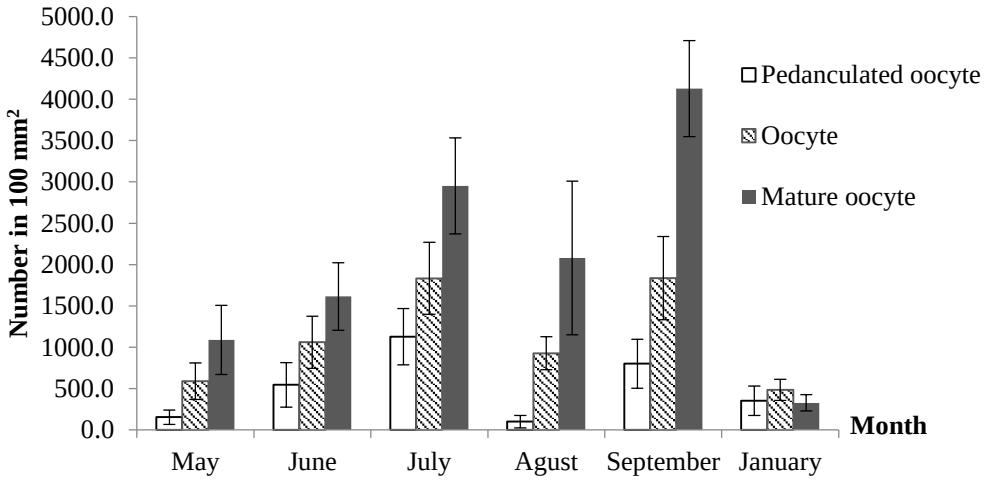

Fig. 4. Comparisons of the number (mean ± standard error) of ovarian germ cells in *A. anatina* from Anzali Wetland in different months.

TESTIS

The male gonad contained acini with different developmental stages of gamete cells that were generally found in the same organism or acinus simultaneously and spermatogenesis had the same continuous pattern seen for the female. Four main stages of development were determined (Figs. 6-7): (i) spermatogonia; (ii) spermatocytes; and (iii) spermatids that may be organized in clusters forming spermatid morulae; and (iv) spermatozoa which are the

mature gametes, developing only under suitable environmental conditions. All the germ cells were round to oval shape, except spermatozoa that had a rod shape and flagellum. The last stage was mostly visible in tissue and fresh samples of September but the early stages and spermatid morulae were observed throughout the year (Fig. 7). Spermatogonia were present in the male acini throughout the reproductive cycle and were mostly located near the acinus walls. They were the largest cells and oval (Fig. 7A) and contained relatively little cytoplasm with a big nucleus. Their diameter was approximately 6-10 µm (mean: 8.2 ± 0.2 µm) and the area was around 30-79 µm^2 (mean: 52.8 ± 2.5 µm^2; Table 1).

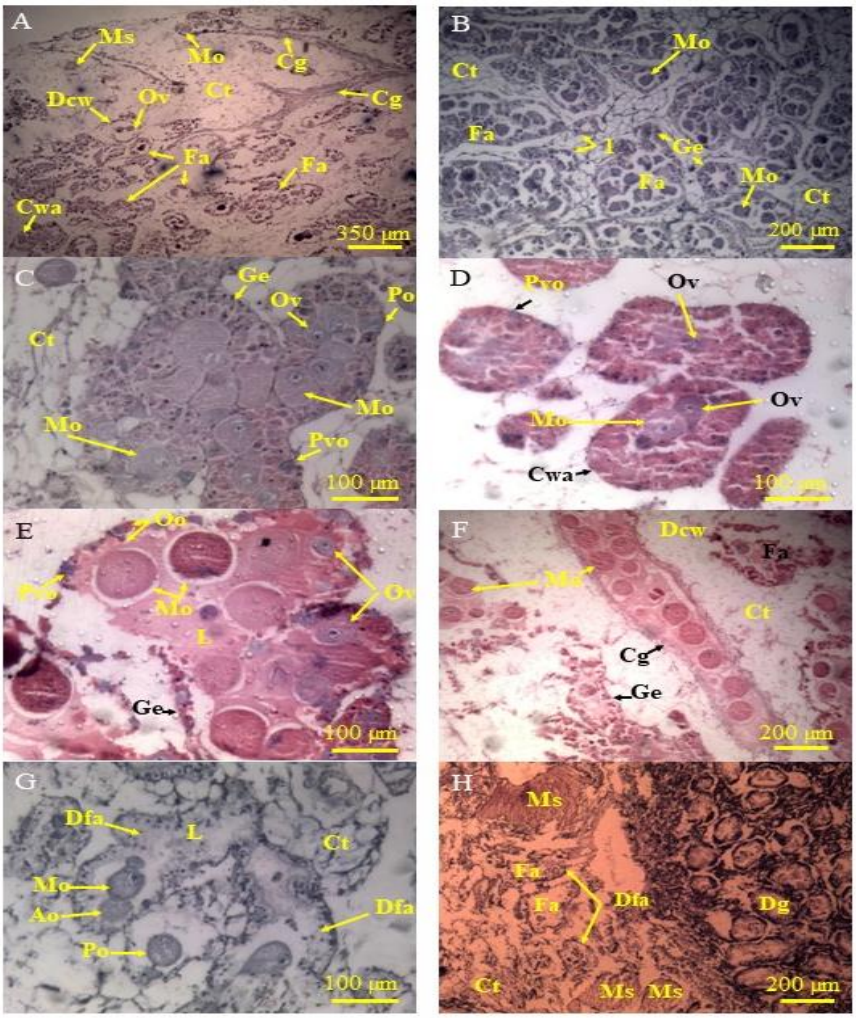

Fig. 5. Histological sections of *A. anatina* ovaries from Anzali Wetland in: May (A), June (B), July (C), August (D), September (E), September (F), January (G) and March (H). Ao: Atretic oocyte, Cg: Ciliated Gonoduct, Ct: Connective tissue, Cwa: Continues wall acinus, Dcw: Discontinues wall acinus, Dfa: Degenerative female acini, Dg: Digestive glands, Fa: Female acinus, Ge: Germinal epithelium, L: Lumen, Mo: Mature oocyte, Ms: Muscle, Oo: Oogonia, Ov: Oocyte, Po: Pedunculated oocyte, Pvo: Previtellogenic oocytes, 1: acinus with free lumen.

They were divided by mitotic division and formed spermatocytes. Spermatocytes were spherical cells with a large homogeneous nucleus. However, their nuclear membrane was not visible and so the nucleolus was not clearly detected (Figs. 7A-B). The mean diameter and area of spermatocytes were 5.1 ± 0.04 µm (5-5.5 µm) and 21.5 ± 0.34 µm^2 (18-25 µm^2), respectively (Table 1). Thus, they were smaller than the spermatogonia and developed into spermatids, which were darkly stained with haematoxylin and distributed in the centre of lumina of the acini (Fig. 7A). Spermatids were polyhedral and the nucleus was completely homogeneous. Their mean diameter and area were recorded as 4.2± 0.1 µm (3.5-4.5 µm) and 13.7 ± 0.0 µm^2 (8-18 µm^2), respectively. The spermatids developed into spermatozoa whose mean diameter was 2.2 ± 0.0 µm (1.5-2.5 µm) and the mean area was 3.9 ± 0.6 µm^2 (2.5-5 µm^2; Table 1). In May-June, the acini had an irregular shape with a large lumen and a very thin wall membrane. These acini contained spermatogonia attached to the wall, while cluster of spermatocytes and spermatids located along the wall and free of them within the lumen, in addition to a quantity of sperm, which in many males was

flooding into the genital ducts. Several acini were generally connected to a sperm duct. The columnar ciliated epithelial cells were arranged in the wall of the male genital ducts (Figs. 6A-B). Spermatogenesis continued in July and August (Fig. 6C, 6D, and 6E). In September, the acini were full of spermatozoa but still spermatogenesis continued on the sidelines of the acini at low rates. The mature spermatozoa exit a male acinus through a ciliated gonoduct. It can be shown that male spawning begins in September (Fig. 6F). In January, the acini and gonoducts still contained some mature spermatozoa but were mainly in a degenerative stage. Also, the epitheliums of the gonoduct and acini walls were very thin and discontinuous at some points (Fig. 6G). In March, the male acini and gonoduct were almost empty and spermatogonia were located at the male acinus periphery. Degeneration was observed in some acini walls (Fig. 6H). The entire male gamete cell line from the spermatogonia to the spermatozoa was present in January and March.

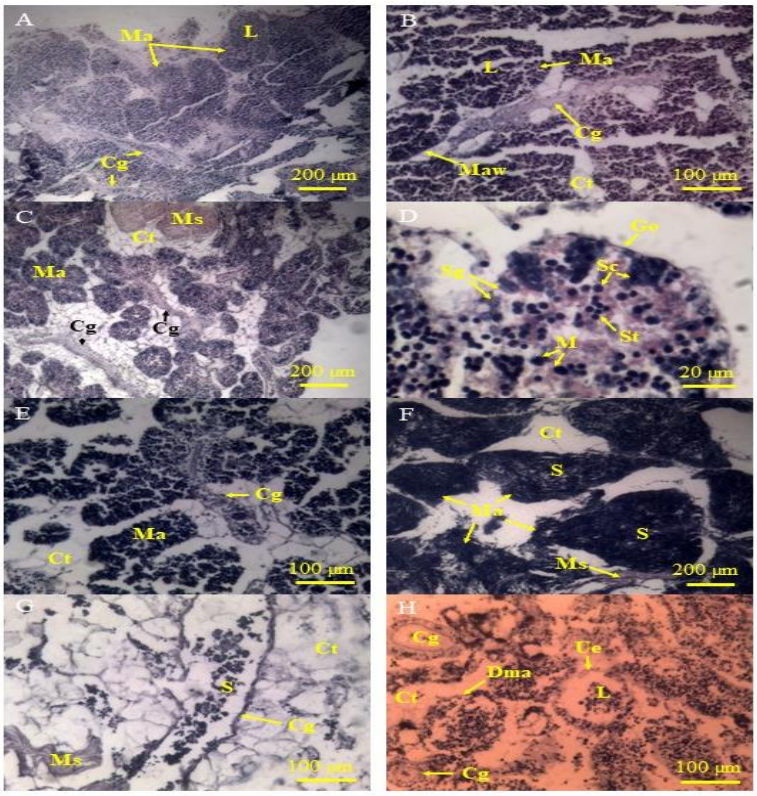

Fig. 6. Histological sections of *A. anatina* testes from Anzali wetland in: May (A), June (B), July (C), July (D), August (E), September (F), January (G) and March (H). Cg: Ciliated gonoduct, Ct: Connective tissue, Dma: Degenerative male acinus, Ge: germinal epithelium, L: Lumen, M: Spermatid Morulae, Ma: Male gonad acinus, Maw: Male acinus wall, Ms: Muscle, Sg: Spermatogonia, Sc: Spermatocyte, St: Spermatid, S: Spermatozoa, Ue: Undifferentiated epithelium.

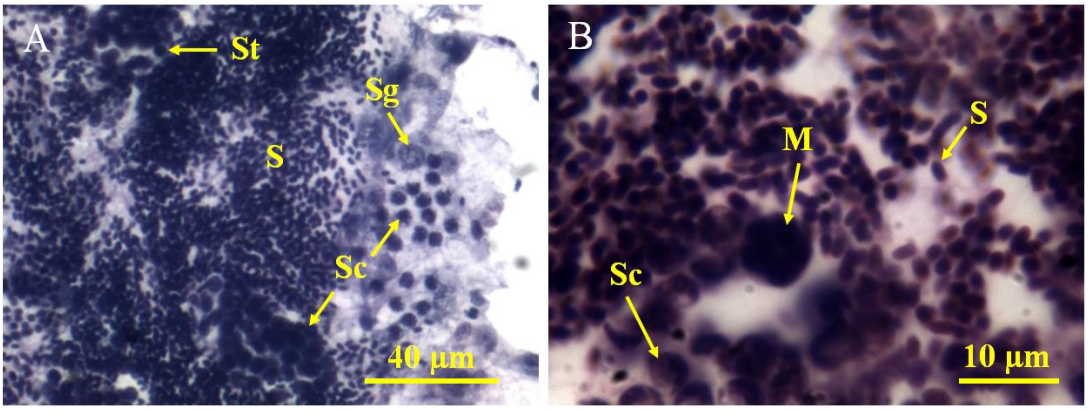

Fig. 7. Different view of male gamete cells developmental stages in *A. anatina* from Anzali wetland in September. Sg: Spermatogonia, Sc: Spermatocyte, St: Spermatid, S: Spermatozoa, M: Spermatid Morulae.

DISCUSSION

In the present study, the biometric results in Anodonta from Anzali Wetland, the Southwest Caspian Sea were almost as same as the mean meristic characters of this species from Cildir in Turkey (length 10.4 ± 0.5 cm, width 3.3 ± 0.2 cm and height 5.4 ± 0.2 cm; Bascinar *et al.* 2009). The mussels of the present study were bigger and older than this species from Lake Dabie in Poland (length 6.6 ± 1.1 cm, width 2.2 ± 0.4 cm, height 4.0 ± 0.8 cm and 5 years; Chojnacki *et al.* 2011), however smaller than the mussels of Mira Lagoon in Portugal (mean length: 12.7 ± 2.5 cm, and mean weight: 256.0 ± 90.5 g; Lima *et al.* 2012). These variabilities in biometric features were reported in other bivalve species as well (Ravera & Sprocati 1997; Weber 2005; Lajtner & Crnean 2011; Hliwa *et al.* 2015). The bivalves are characterized by gonochoristic or hermaphroditic breeding. Variability in sex ratio was also recorded among their populations (Park & Chung 2004; Sereflisan *et al.* 2009; Hliwa *et al.* 2015). In the present study, two types of gonad structure were identified in *A. anatina* according to their histology. Female (Type I) and male (Type II) gonads. The females were dominated in the Anzali Wetland mussel population and were almost 1.5 times the males. The sex ratio of *A. gabillotia pseudodopsis* was recorded as 1.11 F: 1 M (Sereflisan *et al.* 2009), and in *Sinanodonta woodiana* as 1 F:1 M (Hliwa *et al.* 2015). Perhaps these differences in sex ratio reflect environmental changes or species differences. Total mussels were hermaphrodite in the study of Lima *et al.* (2012) in Mira Lagoon in Portugal. Sereflisan *et al.* (2009) have divided the gonad structure of *Anodonta pseudodopsis* into three main types: Type I typically female with a high dominance of female tissue, Type II typically male with a high dominance of male tissue, and Type III hermaphroditic but with a high prevalence of female over male tissue. Hinzmann *et al*. (2013) recorded a fourth type (Type IV) with approximate amounts of female and male acini interspersed in the whole gonad in *A. anatina* in Mira lagoon in Portugal. This fourth type was found in animals only from the stagnant water populations and they demonstrated that it is possibly found in species that use alternative sexual strategies such as in *A. anatina* and also in *A. cygnea*. However, no hermaphroditic characteristics were found in all specimens of the present study. In the oogenesis of the unionids, oogonia turned into early vitellogenic oocytes, which subsequently grew within follicles, formed vitellogenic oocytes, entered late vitellogenesis, underwent maturation, and finally were ovulated. These 5 developing stages of oocytes were also described in *A. anatina* as in other unionids according to the histological analysis in the present study. Various stages of oocyte were different in size, which is reported in other species of *Anodonta* (Sereflisan *et al.* 2009; Lima *et al.* 2012; Hinzmann *et al.* 2013) and in *Hyriopsis bialatus*, as well (Chatchavalvanich *et al.* 2006). Also, during the oogenesis stages, the changes were similar to those previously reported for other unionids (Grande *et al.* 2001; Henley, 2002; Park and Chung 2004; Cek & Sereflisan, 2003; Sereflisan *et al.* 2009). Other authors described 4 phases for the development of oocytes in *A. cygnea* (Lima *et al.* 2012) and *A. anatina* (Hinzmann *et al.* 2013). It has appeared that they unified early and late vitellogenic oocyte stages. One of the characteristics of oocyte development in bivalves is oogenic acinus wall adhesion from early to advanced stages of development shown in *Dreissena polymorpha*, *A. gabillotia pseudodopsis*, *A. cygnea*, *A. anatina*, *Sinanodonta woodian* and so on (Mantecca *et al.* 2006; Sereflisan *et al.* 2009; Lima *et al.* 2012; Hinzmann *et al.* 2013; Hliwa *et al.* 2015). So, the pedunculated oocytes were observed in the ovary sections of the specimens of the present survey. In the present study, it was also found that mature oocytes enter the ciliated germinal ducts. The cilia help to drive the gametes along the duct. These mature oocytes enter the gill cavities through the genital tract. Then fertilized with sperms inserted into the gills of the females, where they develop to become glochidia. These characteristics of *Anodonta* in Anzali Wetland are similar to other mussels studied by other researchers (Chatchavalvanich *et al.* 2006; Sereflisan *et al.* 2009; Lima *et al.* 2012; Hinzmann *et al.* 2013). Atretic oocytes were present in May, June, July, August, September, and January. However, it was more observed in winter and spring. Several reasons can be due to atresia: (i) Limited capacity of acini and control mechanisms of the cell counts; (ii) A spontaneous clearance process at the end of gametogenesis to prepare the gonad for the next cycle; and (iii) Responding to the stress caused by environmental conditions or contamination (Motavkine & Varaksine 1989). All stages of oocyte maturation were in the ovary in different months of the year, but the relative cell density of each of these stages varied in different seasons (Fig. 4). Therefore, according to this reason and so the percentage of empty and full ovarian acini and based on the table of sexual maturity described by Hinzmann *et al.* (2013), three maturation stages were detected in females: (i) Developing (in the months of spring and summer); (ii) spawning (between September and January); and (iii) post-spawning (in January and March). Histological analysis of *A. anatina* spermatogenesis revealed that spermatogonia undergo proliferation, growth, maturation, and division. There was a morulae form and transformation stage at the end. The cell size from spermatogonia to

spermatozoa is a decreasing trend. Spermatogonia is the largest and the most distinct cell, however spermatozoa is the smallest cell, which is not well visible in tissue sections due to its high density. These features were also observed in a number of studies on the various aspects of spermatogenesis of the Unionoida (Park & Chung 2004; Sereflisan *et al.* 2009; Lima *et al.* 2012, 2017; Hinzmann *et al.* 2013; Hliwa *et al.* 2015; Nichols *et al.* 2021). The morphology of spermatozoa in *Anodonta* of Anzali wetland was similar to the structure described for spermatozoa of freshwater Unionoida. In this family, the spermatozoa have a flagellum that is attached to the end of its bullet-shaped head by the acrosome region. The acrosome is usually visible by electron microscopy (Shepardson *et al.* 2012; Hliwa *et al.* 2015). In the present study, the spermatozoan head and long flagella were visible in the smear and sections of the male gonadal tissue in October. Spermatozoa are small in the species of Unionida. For example, spermatozoan head length has been reported in *Truncilla trincata*, 2.8 μm (Waller and Lasee, 1997), in *Anodonta grandis*, 4 μm (Lynn, 1994), in *Prisodon alatus*, 4.2 μm (Matos *et al.* 1998), in *Anodonta gabillotia pseudodopsis*, 2 μm (Sereflisan *et al.* 2009), in *Anodonta anatina*, 2-5.1 μm (Hinzmann *et al.* 2013) and in *Sinanodonta woodiana*, 3.4 μm (Hliwa *et al.* 2015). The average diameter of spermatozoa in *Anodonta* of Anzali Wetland is closer to *Anodonta gabillotia pseudodopsis*. Morulaes were observed during all sampling months. However, in October it was lower in number. The role of this structure is not yet fully understood. Some researchers attribute this special structure to a specific method of spermatogenesis. They believed that Morulaes were cells that initially contained spermatids (Matos *et al.* 1998). Shepardson *et al.* (2012) described two pathways of spermatogenesis (typical and atypical) in *Venustaconcha ellipsiformis* (Unionidae). Spermatozoa produced by both pathways were not necessarily morphologically distinguishable, but atypical spermatozoa appeared to be produced in a pathway that included spermatozoa morulae and spermatids derived from spermatogonial cells adjacent to Sertoli cells. However, in our study, we did not notice any Sertoli cells in the histological sections. So, the origin of the morulae in *Anodonta* of Anzali Wetland is a subject that needs further research, especially through electron microscopy. In the present study, all the cellular stages of spermatogenesis, including spermatogonia, spermatocytes, spermatid, and spermatozoa, were observed in the gonads of males throughout the year. However, the density and accumulation of sperm in September were higher than in other months. The spermatogenesis was developed in spring and summer and the spermiation occurred in autumn (September). The male gonad entered post spawning stage in winter and renewed development next spring. According to the table of sexual maturity described by Hinzmann *et al.* (2013), three maturation stages were detected in males: (i) Developing; (ii) Spawning; and (iii) Post-spawning. Since all stages of oocyte maturation exist in the ovarian acini simultaneously and all stages of sperm maturation were present in testes acini, the spawning pattern in *Anodonta* of Anzali Wetland is asynchronous. Prolonged gametogenesis and the continuity of gonadal maturation stages in both *Anodonta* males and females in Anzali Wetland and the appearance of glochidia larvae in gills are among the characteristics that have been reported in European *Anodonta* (Hinzmann *et al.* 2013; Hliwa *et al.* 2015). However, the difference in the gonadal discharge time, as well as the time required for larval development in the gills and the time when the larvae leave the gills, can be related to differences in climate.

CONCLUSION

The reproductive cycle and gonadal tissue characteristics in the *Anodonta* males and females in Anzali Wetland were similar to other bivalves of Unionoida. Gametogenesis was present in all seasons and the pattern of sexual maturation was asynchronous. Hermaphroditism was not recorded and it was observed as essentially a dioecious sexual strategy. Spawning occurred synchronously in September and the time of glochidia release was between January and March. In March, the gonad was in the post-spawning and regeneration. The information gathered could be a valuable help in the future *Anodonta* conservation of Anzali Wetland. However, Molecular genetic studies are needed in the future.

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