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The Reproductive Cycle of *Potomida littoralis* (Cuvier, 1798) (Bivalvia: Unionidae) in Lake Gölbaşi, Turkey

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Abstract.- In this work, reproductive cycle of *Potomida littoralis*, including histological description and indices of gonad maruration were investigated. There is no data previously available on the species. A total of 300 individuals were monthly collected from September 2009 to August 2010 in Gölbaşı Lake, Turkey. *P. littoralis* is dioecious and although males tended to predominate, the sex ratio was not significantly different from the expected 1:1 ratio ($p>0.05$). Calculation of the condition index (CI), gonadosomatic index (GSI) and histological examination of the gonads showed that gametogenesis began in January. Spawning occurred in June. Annual maximum GSI value peaked in May. A decrease of CI was observed during gametogenesis. When mussels had the highest GSI value, CI was the lowest value. This results suggest that gametogenesis and reproductive cycle are the key factor in mussels physiology and important for captive breeding/artificial mussels production and conservation status (e.g. When to obtain gravid females, when to relocate, when to restock, when to introduce fish host, when not to allow harvesting).

Key Words: Reproductive cycle, gonadostomatic index, condition index, freshwater mussel, sex ratio.

INTRODUCTION

Freshwater mussels (Unionoidea) are among the most endangered invertebrates in the world (Machordom *et al.* 2003; Nagel, 2004; Strayer *et al.*, 2004; Lydeard *et al.*, 2004; Gómez and Araujo, 2008). Their decline is the result of increasing human alterations regarding freshwater habitats, such as water transfer, water abstraction, river channelization, dredging, impoundment, collecting of mussel, pollution, lack of host fish etc (Vaughn and Taylor, 1999). A total of 200 unionoid species are on the Red List of the IUCN (The International Union for Conservation of Nature) (IUCN, 2012). Although *Potomida littoralis* is not yet protected under European legislation and it has also not been listed in the Red List of the Invertebrates of Turkey. But it has been recently listed in the Red List of the Invertebrates of Spain (Araujo, 2006). Given the limited resources for species-by species approaches to conservation, it has been suggested that conservation biologists examine and identify reproductive biology. Reproduction biology and ecology of juvenile and mature mussels are key factors that determine the survival abilities of freshwater mussel populations.

Turkish *P. littoralis* belongs to the family Unionidae, order Unionoida. There are three subspecies of *Potomida* native to the Gölbaşı Lake; *Potomida littoralis delesserti, Potomida littoralis semirugata and Potomida littoralis homsensis* (Schütt, 1982). The generic name of this species *Psilunio*, *Potomida* and *Unio*, is still under discussion (Araujo, 2008). This author suggested using *Potomida littoralis* as a valid name for the West European populations.

There have been several studies on unionoids in Turkey. Çek and Şereflişan (2006) studied on *Unio terminalis delicatus*. They concluded that this species has great potential for aquaculture use. The glochidium of *P. littoralis* as a hookless larvae has been first described by both Guisti (1973) and Araujo *et al*. (2009). Later Şereflişan *et al*. (2009a) studied on three species of freshwater mussels including *Potomida littoralis*. According to that study, the glochidia of *P. littoralis* which were parasitic on the gills of freshwater fish, have no hooks. Following these studies, Şereflişan *et al*. (2009b) examined gametogenesis hermaphroditism and gametogenic cycle of *Anodonta gabilotia pseudodopsis*. Nagel (2004) studied first the reproductive biology of *P. littoralis*. He stated that this species was most likely a tachytictic consecutive brooder. But, Nagel (2004) used only

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macroscopic features in *P. littoralis* from different European countries. However, without examining testes and ovaries under light microscopy above conclusion remain doubtful. Since research by Çek and Şereflişan (2011) on a unionoid is comprehensive. They demonstrated that CI and macroscopic observation of the gonads are inadequate measure of reproductive activity. Their study also provides the first quantified histological analysis of temporal changes in spermatogenesis and egg production in an Unionid.

This paper describes in detail the gametogenic cycle of *P. littoralis* in Golbaşı Lake, South Eastern Turkey. The gametogenic cycle, oocyte growth and sex ratio are described using histological analysis. These data will be essential to improve understanding of unionid biology (*i.e.* reproductive season and population sex ratio) and hence to make this information relevant for promoting a future extensive culture for this species, and to design action plans aimed at conserving the population.

MATERIALS AND METHODS

Sampling

Specimens of *P. littoralis* were collected by scuba diving (1-6 m depth), in one area of the Gölbaşı Lake, a major water storage located c. 50 km east of Antakya. The site was deep lake (Fig.1); this site was classified as first-degree clean water by Şereflişan *et al*. (2009a). Total area of Gölbaşı Lake is 1200 ha which consists of 400 ha with marshy area. The lake is fed by underground water and used for agricultural irrigation and recreational facilities. Sampling was carried out between September 2009 and August 2010. The condition index (CI) and gonodosomatic index (GSI) were estimated from 25 specimens, measuring between 4.70±0.52 and 12.45±01.18 cm in shell length, sampled every 30 days. The bivalves were washed in the lake water, were transported over ice in a cooler box and were processed within 24 hours. In the laboratory, shell length (SL), height (SH), and width (SW) were recorded to the nearest 0.01mm. The shell was opened and the soft body removed and weighed for the total weight of wet tissue (TWW) to the nearest 0.01g.

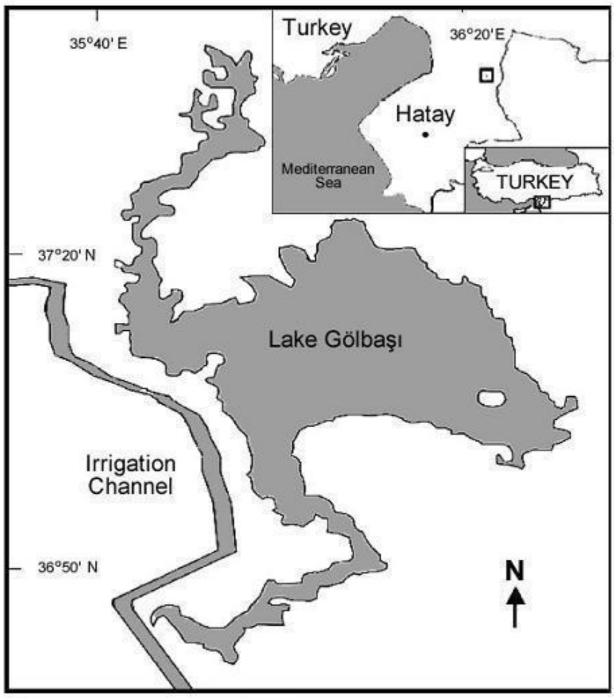

Fig. 1. Map of the Gölbaşı Lake showing the study site, Hatay city-Turkey.

Where

$$\mathrm{CI} = \frac{\mathrm{TWW}}{\mathrm{SL} \times \mathrm{SH} \times \mathrm{SW}} \, 100$$

The condition index was calculated using the equation adopted by Kang *et al*. (2007).

The GSI was calculated using the equation adopted by Wolff (1988):

$$GSI = \frac{GW}{TWW} 100$$

where GSI, gonadosomatic index; GW, gonad wet weight; TWW, total wet weight.

The gonads from 25 specimens used in the calculation of the condition, gonadosomatic index and sex ratio. Ten specimens were then randomly sampled each month for histological analysis of the gonads.

The sex of each specimen was identified by examination of the visceral mass and the gills were examined for the presence of embryos. Since *P.*

littoralis incubates the embryos in the four gills. In the middle portion of the inner demibranchs of the females the interlamellar septa were distinctly thickened to provide structural support for the developing young (Jupiter and Byrne, 1997). This was a permanent feature and in non-brooding specimens facilitated identification of females. Determination of sex was also made macroscopically. Since the visceral mass of the *P. littoralis* was thick and gametes were often evident. To test whether the sex ratios observed were significantly different from the expected sex ratios of the mussels-based on secondary sex characteristics and on gonad histology the Chi-Square (χ^2) test was used (Zar, 1996).

Histological procedures

Ovaries and testes samples from specimens for each month were directly fixed in 10 % neutral buffered formalin. After being, preserved in formalin for about one week, transverse sections of the central portion of the gonad samples were dehydrated in graded ethanol, embedded in paraffin, sectioned at 5µm and stained with haematoxylin and eosin (Merck) for histological examination (Çek and Şereflişan, 2006). After histological work, all slides were examined under a light microscope (CH-2 Olympus-Japan). Developmental stages of female and male gamete cells were identified according to descriptions given by Çek and Şereflişan (2006) and Şereflişan *et al*. (2009a). The stages of oocytes and spermatozoa development were classified on the basis of observations of changes in the nucleus, nucleoli and cytoplasm.

RESULTS

Reproductive cycle

The gonads surrounded the glandular digestive tissue and the gut. They were diffused organs consisting of highly branched follicles surrounded by connective tissue and haemocoel spaces. In *P. littoralis* male and female gametes were separately organized in follicles (Figs. 2C, D; 3C, D). The oogenesis of the *P. littoralis* was divided into five stages: oogonia, early vitellogenic oocytes, vitellogenic oocytes, late vitellogenic oocytes and mature oocytes (Fig. 2A, B, C). Spermatogenesis was also divided into five stages: spermatogonia, primary spermatocyte, secondary spermatocyte, spermatids and spermatozoa.

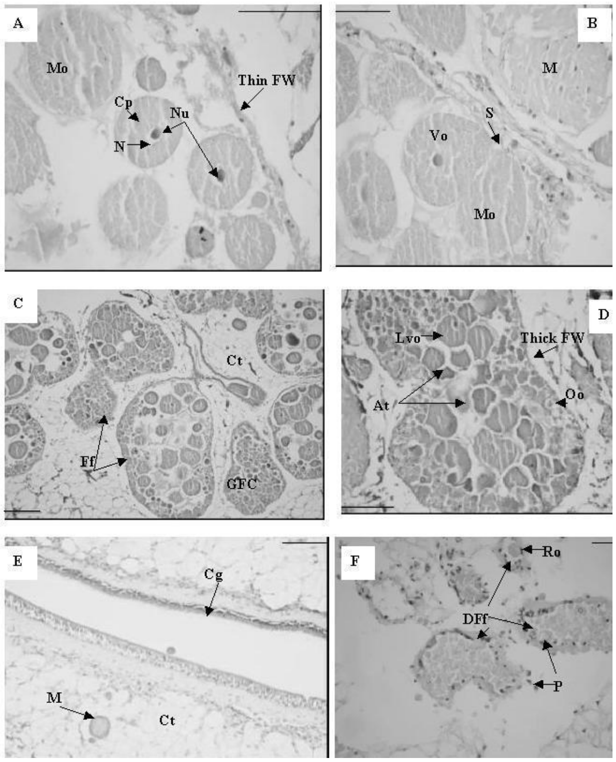

Fig. 2. A portion of the *Potomida littoralis* ovary, showing the ovary at various stages of development. A, Section of an ovary showing different developmental stages in June. Scale bar= 175µm; B, Oocytes connected to the gonad wall by a stalk (S) Scale bar = 175µm; C, Partly spawned follicle, Scale bar = 150µm; D, Late vitellogenic oocytes, (Lvo) and oogonia is shown, Scale bar = 125µm; E, Mature oocytes and ciliated gonoduct is shown in August. Scale bar = 125µm; F, Degenerated follicle is shown, note presence of phagocytes (p) along follicular wall. Scale bar = 125µm. M, muscle; Oo, oogonia; Ct, connective tissue; Vo, vitellogenic oocytes; Mo, mature oocytes; N, nucleus; Nu, nucleoli; ThickFW, thick follicle wall; ThinFW, thinner follicle wall; Ff, female follicle; Ro, resorbing oocytes; Cg, ciliated gonoduct; DFf, degenerating female follicle; At, atretic oocytes. Haematoxylin and Eosin (H&E).

Oogenesis was intense in January, February and March. In March, the ovarian follicles were well-ordered, situated radially around distal genital ducts. Oogonia cells were present in the follicle of the *P. littoralis* throughout the reproductive cycle (Fig. 2D). This stage was mostly detectable in August and September.

At the beginning of cytoplasmic growth, each oocyte had an egg stalk and was attached to the follicle walls of the oogenic follicle (Fig. 2B). Most of these oocytes retained their attachment to the germinal epithelium, by this basal stalk until an advanced stage of development when they moved to the lumen in preparation for spawning (Fig.2A, D).

Previtellogenic and early vitellogenic oocytes were present for most of the year and these were scattered along the follicular wall. Throughout the year some females contained a large store of unspawned eggs while others had fewer loosely arrayed oocytes. In February, March and April, many small early vitellogenic oocytes were attached to the thickened follicle walls (Fig. 2D). In July the follicle wall became thinner relative to the early developmental stages (Fig. 2A). The ovaries reached their maximum gravid stage in May. At this time they were filled with fully-grown eggs and the inter-follicle space was minimal. After the onset of spawning June/September the eggs were loosely arrayed and the follicles were less crowded (Fig. 2C). This condition was maintained through the spawning season, during the autumn month. In the spawning season some post-ovulatory follicles had an opening to the ciliated gonoduct through which mature oocytes had been released (Fig. 2E). At the end of the breeding season in November all the mussels had spent gonads containing few or no oocytes and gonads were almost completely replaced by connective tissue (Fig. 2E, F). Ripe mussels of both sexes were dominant in May. *P. littoralis* with no gametogenic activity predominated in November and December. These gonad sections contained no follicles at all or only a very few contracted follicles between connective tissue, and showed resorbtion of the undischarged eggs in follicle of the females (Fig. 2F). In some females, very thin follicle cells occasionally still surrounded the oocytes. In November follicles were occasionally disturbed. Such follicles suggested degenerating ovarian follicles with granular follicle cells (Fig. 2F). Degenerating female follicle and Atretic oocytes were clearly detected in post-spawned *P. littoralis* in December (Fig. 2D, F).

Gametogenesis in male *P. littoralis* had the similar continuous pattern seen for the females (Fig.3A-F). The male follicle also contained sperm at various stages of development with clusters of spermatocytes and spermatids along the male follicular wall. The testicular follicles were neat and regularly arranged in spring months (Fig. 3A). Spermatogonia cells were also present in the male follicle of the *P. littoralis* throughout the reproductive cycle. Spermatids were polyhedral and the nucleus was completely homogeneous. The spermatids developed into spermatozoa (Fig. 3B). Their diameter was recorded, as 3.4µm. Spermatozoa were smaller than the spermatids and were strongly basophilic. Its diameter was 2 µm. Yellow-brown granules were a common feature of the follicles in September and December. Minute Yellow-brown granules were also often seen in the epithelia of the genital ducts (Fig. 3F). In June the follicles contained a quantity of sperm, which in many males was flooding into the genital ducts (Fig.3D). It was clear that mature spermatozoa exited a male follicle through a ciliated gonoduct (Fig. 3E). Sperm morulae were detected in May, June and July and in most study specimens. Sperm morulae, multi-nucleated aggregations of sperm cells were also common (Fig. 3A, B). Males partly spawned in April. In June, July and August male gonad was almost identical to that observed in April (Fig. 3). In October, the follicle contained mature spermatozoa, many of which were degenerating. Male follicle was also degenerating. (Fig. 3E, F). By December, the male follicle was almost empty and spermatogonia were at the male follicle periphery (Fig. 3E). The entire male gamete cell line from the spermatogonia to the spermatozoa was present in spring and summer months.

In two of the tree hundred specimens, the demarcation of the two advanced oocytes were easily discernible microscopically in the male gonads. But no female follicle was detected. Male follicles were arranged in brown clusters, while mature oocytes were located in the male gonoduct (Fig. 4A-C).

Sex ratio

P. littoralis possessed gonadal tissue all year

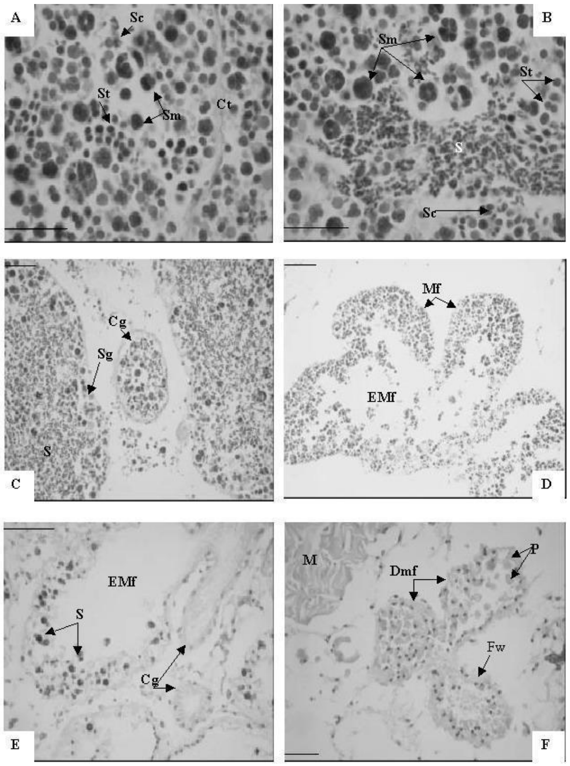

Fig. 3. Histological sections of male *Potomida littoralis* gonad; A, A portion of the *P. littoralis* testis, showing germ cells at various stages of development in June. Scale bar = 150µm; B, Showing sperm morulae and free spermatozoa inside the lumen. Scale bar = 150µm; C, Spawned male with spermatogonia at the male follicle periphery, Scale bar = 100µm; D, Empty male follicle is shown, Scale bar = 100µm; E, Completely degenerated male follicle with ciliated gonoduct Scale bar = 125µm; F, Phagocyte is shown within degenerated male follicle. Scale bar = 125µm. Sg, spermatogonia; Sc, spermatocytes; St, spermatids; S, spermatozoa; Sm, sperm morulae; Ct, connective tissue; Cg, ciliated gonoduct; Emf, Empty male follicle; Mf, male follicle; M, muscle; P, phagocyte; Fw, follicle wall, (Stained with H&E).

Table I.- Ratios of absolute and relative (%) frequencies of male (M) and female (F) *Potomida littoralis* from Lake Gölbaşı between September 2009 and November 2010 with results of the Chi-Square (χ^2) test for a significant difference from 1:1 in the sex ratio.

Year	Sex distribution (Female: Male) F : M	Sex ratio (%) F : M	χ^2
2009-2010	137:161	45.97:54.03	1.93, d.f.= 1,n.s.

d.f: degrees of freedom, n.s: not significant, (P>0.05) in, n= 298.

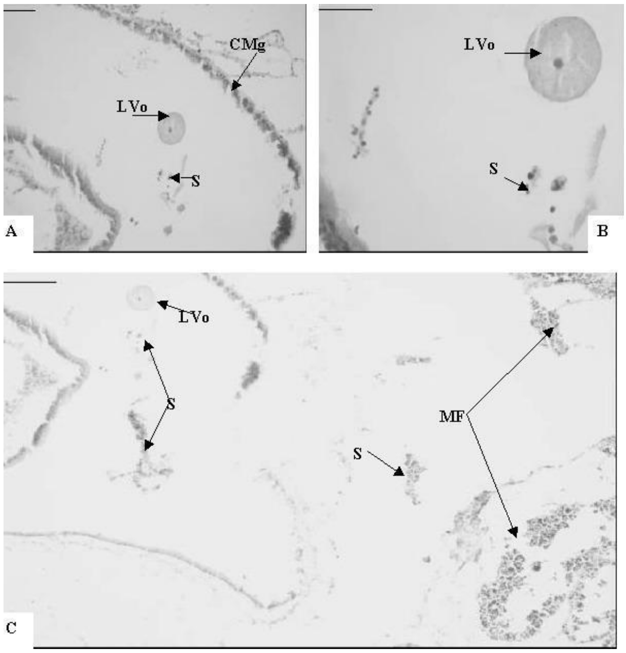

Fig. 4. Two oocytes in *P littoralis* testis duct; A, B, Viewed at high magnification, Scale bar for A, 75µm; for B, 50µm; and C, Viewed at low magnification, Scale bar for C, 120µm. Lvo, late vitellogenic oocytes; S, spermatozoa; Mf, male follicle; CMg, ciliated male gonoduct.

and therefore their sex was readily identified. Of the 300 Gölbaşı *P. littoralis* examined, 161 (53.66%) were males, 137 (45.66%) were females. The male: female sex ratio (1.17M: 1F, n=300) did not differ significantly (p > 0.05) from the expected ratio of 1M: 1F. Two micro hermaphrodite *P. littoralis* was observed at the lake (Table I).

Condition index

Results of the condition index calculations for *P. littoralis*, from September 2009 to August 2010 are represented in Figure 5. In the 2009-2010 breeding season, a gradual increase of CI from May (9.75±0.35) to August (13.99±0.92 g) did coincide with spawning. Condition index dropped dramatically from September (13.25±0.49) to November (11.56±0.94) when most of *P. littoralis* released their gonadal mass through spawning (Fig.5). During the 2009 to 2010 season, the mean CI ranged from 9.75±0.35 to 13.99±0.92 g. The peak CI was recorded in August (13.99±0.92 g). CI varied seasonally which was expected since reproductive development typically drives a seasonal physiological cycle.

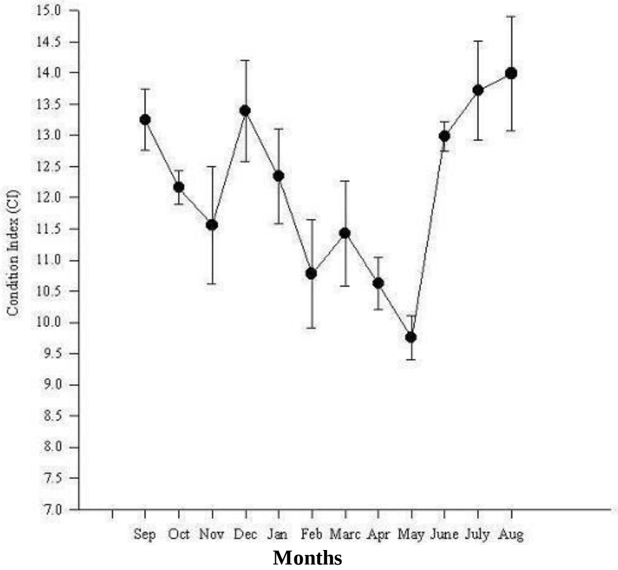

Fig.5. Seasonal variation in the condition index of *Potomida littoralis* from September 2009 to August 2010, (Mean +/-sd, n=25).

Gonadosomatic index

Results of the gonadosomatic index calculations for females and males, from September 2009 to August 2010 are represented in Figure 6. In the 2009-2010 breeding season, there was one major period of spawning. In October gonads of males and females were almost empty (Fig. 6). During the 2009 to 2010 season, the index ranged from 15.95±1.13 to 28.37±0.93g. The peak GSI was recorded in May (28.37±0.93g for females, 22.51±0.79 for males). The index then dropped dramatically to 23.27±1.19g in June and 21.21±0.8g in July for females. Similar observations recorded for males. The lowest value of GSI was recorded for females (17.97±1.02) and males (15.95±1.13) in September. In this month gonads of males and females were almost empty.

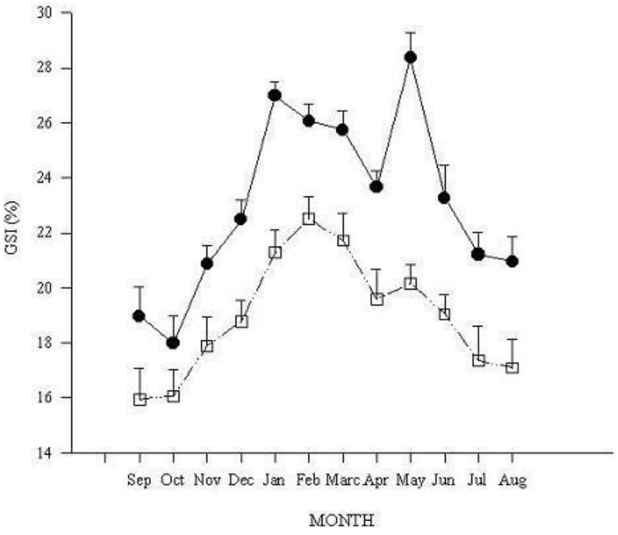

Fig. 6. Variation in the gonadosomatic index of *Potomida littoralis* from September 2009 to August 2010, (Mean +/-sd, n=25).

DISCUSSION AND CONCLUSION

The present study briefly described histological characteristics of developing oocytes that were assigned to 5 stages. In *P. littoralis*, as in other unionids, oogonia turned into early vitellogenic oocytes, which subsequently grew within follicles, formed vitellogenic oocytes, entered late vitellogenesis, underwent maturation, and finally were ovulated into marsupia. During these phases the changes were similar to those previously reported for other unionids (Grande *et al*., 2001; Park and Chung, 2004; Çek and Şereflişan, 2006; Şereflişan *et al*., 2009a). A number of studies exist on various aspects of spermatogenesis in unionids (Park and Chung, 2004; Çek and Şereflişan, 2006, 2011; Şereflişan *et al*., 2009a). As in oogenesis, spermatogonia undergo proliferation, growth and maturation and division. However, unlike oogenesis, the growth stages were not clearly defined. In spermatogenesis there was a transformation stage at the end. In another words, the proliferation of spermatogonia was a gradual but short process in *P. littoralis***.** The proliferation of sperm cells occurred suddenly in contrast to the slower maturation of oocytes.

In *P. littoralis* gonadal tissue intermingles with the digestive cells as in other unionoids in which gonadal tissue occurs among gut loops and even enveloping the digestive gland (Çek and Şereflişan, 2006, 2011; Şereflişan *et al*., 2009a). The footed stalks and microtubules of the oocytes of the Turkish *P. littoralis* were similar to those described in a Finland freshwater mussel species, *Margaritifera margaritifera* (Hanstèn *et al*., 1997). The genital canals of *P. littoralis* had ciliated cells and mucous cells. The mucous cells were mostly basophilic as they were in *M. margaritifera* (Hanstén *et al*., 1997; Byrne, 1998), *Unio terminalus delicatus* (Çek and Şereflişan, 2006), *Anodonta gabillotia pseudodopsis* (Şereflişan *et al*., 2009a) and *Leguminaia whaetleyi* (Çek and Şereflişan 2011). Because the oocytes apices were free even in the ovary studied in October, the flat cells maybe abnormal vestiges of follicle cells. Because all the oocytes were nearly of similar size, the ovaries thus are assumed to be nearly mature.

In the present study, sperm morulae were found in dioecious and microhermaphrodite specimens this finding is contradictory to those of Grande *et al*. (2001). They concluded that sperm morulae are a sing of microhermaproditism in *M. margaritifera*. Coe and Turner (1938) explained cytolysis of sperm morulae in *Mya arenaria* as a possible way of supplying nutrients and according to Kotrla (1989) these structures are evidence of abnormal spermatogenesis in certain bivalves. Heard (1975) suggested that some sperm morulae become mature sperm although their viability is unknown. Heard (1975) found sperm morulae in June in *Anodonta grandis*, but by July all sperm morulae had disappeared and the follicle were full of mature sperm. Our study suggests that sperm morulae are simply clusters of spermatids and become mature sperm when environmental conditions are suitable. Our data concurs with data for *Anadonta grandis* (Heard 1975), *Unio terminalis delicatus* (Çek and Şereflişan, 2006), *Anodonta gabillotia pseudodopsis* (Şereflişan *et al*., 2009a) and *Leguminaia whaetleyi* (Çek and Şereflişan, 2011). Similar to those of Heard (1975) we found sperm morulae in May, June and July in most of the studied specimens of *P. littoralis* but by October all sperm morulae had disappeared and the follicle were full of mature sperm.

In the population studied, *P. littoralis* appeared to be a dioecious species. Nagel (2004) studied on *P. littoralis* and showed that sexes were typically separate. Studies of other freshwater mussels show that sexes are typically separate (Pekkarinen and Valovirta, 1997; Çek and Şereflişan, 2006). However some species may become hermaphrodites capable of self-fertilization when population density is low (Ghiselin, 1969; Grade *et al*., 2000). Moreover some species of bivalvia are recorded as micro hermaphrodite and some true hermaphrodite (Byrne, 1998; Grande *et al*., 2001). Byrne (1998) found that females predominated in samples of Australian freshwater mussel. Recently *Anodonta gabillotia pseudodopsis* (Şereflişan *et al*., 2009a), *Leguminaia whaetleyi* (Çek and Şereflişan, 2011) were found to be hermaphrodite with self-fertilization capacity.

Seasonal variation in the condition index indicates that the *P. littoralis* has one large spawning event with various minor spawnings. Seasonal variation in CI is also associated with seasonal fluctuations in food availability (Lee *et al*., 1999). In our study CI was largely influenced by the gametogenetic cycle. A decrease of CI during gametogenetic period could be explained by the important energetic cost to form reproductive cells. The gametogenetic cycle leads to important variations of body weight due to storage and further use of metabolic reserves and by the production and release of gametes. Kautsky and Wallentinus (1980) showed that over the 90% of the energy goes into gamete production. Thus a positive effect of food supply on mussel growth was covered up by gametogenesis. Lemaire *et al*. (2006) demonstrated a great influence of gametogenesis on the mussel growth and energy reserves. Their study supported our findings. It seemed that when GSI value was higher, the CI value was lower.

The GSI is widely used as an index of gonadal activity and development and has been particularly useful in the study of the molluscs (Lubet, 1983; Wolff, 1988). An increase in the average value of this index is interpreted as the beginning of sexual maturation, while a sudden drop in this index is indicative of a spawning event. Changes in GSI in females and males followed a similar pattern during gametogenesis in winter months. This finding is similar to that reported for other freshwater mussel species (Alfaro *et al*., 2003). On the basis of the results obtained from the GSI, it is suggested that *P. littoralis* posses a single large spawning event in June.

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