

Seasonal Variation of Biochemical Components in Clam (*Saxidomus purpuratus* Sowerby 1852) in Relation to Its Reproductive Cycle and the Environmental Condition of Sanggou Bay, China

BI Jinhong^{1, 2)}, LI Qi¹⁾,*, ZHANG Xinjun²⁾, ZHANG Zhixin²⁾, TIAN Jinling²⁾, XU Yushan²⁾, and LIU Wenguang³⁾

1) Key Laboratory of Mariculture of Ministry of Education, Ocean University of China, Qingdao 266003, P. R. China

2) Rongcheng Fishery Technical Extension Station, Rongcheng 264300, P. R. China

3) Key Laboratory of Tropical Marine Bio-Resources and Ecology, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou 510301, P. R. China

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Abstract Seasonal variation of biochemical components in clam (*Saxidomus purpuratus* Sowerby 1852) was investigated from March 2012 to February 2013 in relation to environmental condition of Sanggou Bay and the reproductive cycle of clam. According to the histological analysis, the reproductive cycle of *S. purpuratus* includes two distinctive phases: a total spent and inactive stage from November to January, and a gametogenesis stage, including ripeness and spawning, during the rest of the year. Gametes were generated at a low temperature (2.1 °C) in February. Spawning took place once a year from June to October. The massive spawning occurred in August when the highest water temperature and chlorophyll *a* level could be observed. The key biochemical components (glycogen, protein and lipid) in five tissues (gonad, foot, mantle, siphon and adductor muscle) were analyzed. The glycogen content was high before gametogenesis, and decreased significantly during the gonad development in the gonad, mantle and foot of both females and males, suggesting that glycogen was an important energy source for gonad development. The protein and lipid contents increased in the ovary during the gonad development, demonstrating that they are the major organic components of oocytes. The lipid and protein contents decreased in the testis, implying that they can provide energy and material for spermatogenesis. The results also showed that protein stored in the mantle and foot could support the reproduction after the glycogen was depleted.

Key words *Saxidomus purpuratus*; gametogenesis; biochemical composition; environmental factor

1 Introduction

Clam (*Saxidomus purpuratus* Sowerby 1852), belonging to the family Veneridae, is a stenohaline and cold-water species, and widely distributes in north China, Korea and Japan (Wei *et al.*, 1982). Because of its high nutritive and economic values, *S. purpuratus* is a commercially important bivalve in China. However, due to the coastal pollution and overharvesting, the wild stocks reduced substantially. Accordingly, detailed knowledge of the reproductive cycle of *S. purpuratus* is vital for establishing aquaculture techniques of seeding production. Furthermore, understanding the relationship between the reproductive cycle and environmental factors is crucial to increase the wild population (Hahn, 1994; Peterson, 2002; Lee *et al.*, 2014). Many studies have been carried out on

its ecology (Lee *et al.*, 2005; Selin, 2014), morphological characterization (Kim *et al.*, 2006), gonad development, embryonic development and larval growth (Wei *et al.*, 1982; Choi *et al.*, 2003; Kim *et al.*, 2005), resource enhancement (Wei *et al.*, 1994), and genetics (Yoon and Park, 2006; Cho *et al.*, 2013). However, little is offered to the change in biochemical constituent in relation to its reproductive cycle.

The reproductive activity of marine mollusc generally depends on both abiotic (water temperature, salinity and food availability) and biotic factors (nutrition and hormones) (Chung *et al.*, 2002; Arellano-Martínez *et al.*, 2004; Park *et al.*, 2011). In general, when the food is abundant, energy is stored prior to gametogenesis in the form of glycogen, lipid and protein (Dridi *et al.*, 2007). The particular importance of these substrates they stored and the timing of their use, vary among species, or even among populations of the same species (Giese, 1969; Bayne, 1976). The carbohydrate of mollusc is mainly

* Corresponding author. Tel: 0086-532-82031622

E-mail: qili66@ouc.edu.cn

composed of glycogen (Ansari *et al.*, 1981), and several studies have indicated that glycogen plays an important role in the physiology of mollusc particularly during their reproductive process (Lambert and Dehnel, 1974; Ojea *et al.*, 2004; Yan *et al.*, 2010a). Lipids have two fundamental roles in metabolism: storing energy in the form of triacylglycerol, and serving as membrane components in the form of phospholipids in bivalve (Delgado *et al.*, 2004; Dridi *et al.*, 2007; Liu *et al.*, 2013). Protein is the most abundant biochemical component in tissues, and it also serves as an energy reserve supporting the gametogenesis (Barber and Blake, 1981; Beninger and Lucas, 1984; Ruiz *et al.*, 1992; Berthelin *et al.*, 2000).

The present investigation was undertaken with the objective of elucidating the gametogenesis, the spawning season, and the seasonal variation in biochemical constituents of different organs in relation to the reproductive cycle and environmental factors in *S. purpuratus* from

Sanggou Bay of China.

2 Materials and Methods

2.1 Sample Preparation

The sampling site (37°03'N and 122°33'E) is located in Sanggou Bay, Rongcheng, Shandong Province, China (Fig.1). Wild clam (80–100 individuals, 65.4 to 101.4 mm in shell length, 31.3 to 51.3 mm in shell height and 19.6 to 69.8 g in wet weight) were collected monthly from March 2012 to February 2013. At the sampling site, the temperature and salinity of seawater were measured using a mercury-in-glass thermometer and a portable refractometer, respectively. Chlorophyll *a* concentration was determined in the laboratory according to the CSBTS standard method (CSBTS, 2007). To monitor the variation of biochemical constituents monthly, the flesh was carefully dissected into five tissues: gonad, foot, mantle, siphon

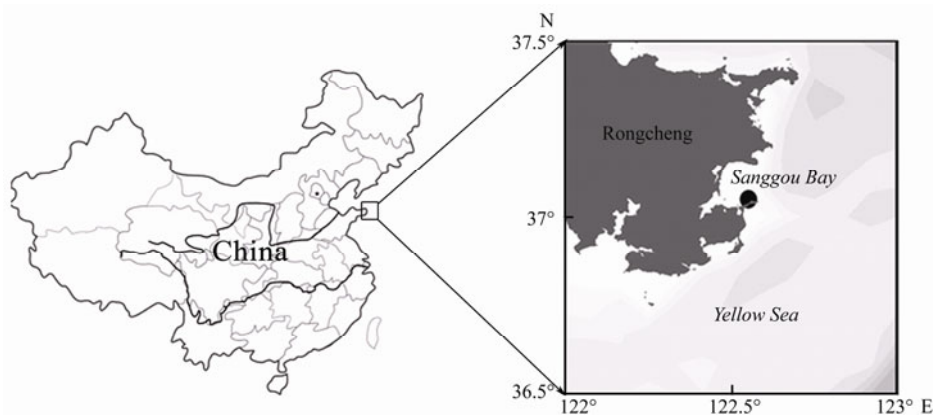


Fig.1 Location of collection site for *S. purpuratus* in Sanggou Bay of China.

and adductor muscle. All tissues were immediately frozen and stored at -80°C .

2.2 Histology

Histological analysis was conducted to determine the sex and gametogenic stage each clam. A 5-mm thick section of gonad each clam was fixed in Bouin's solution for 24 h, dehydrated with serial dilutions of alcohol, and embedded in paraffin wax. Sections of $6\ \mu\text{m}$ thickness were cut on a microtome and stained with hematoxylin and eosin. The specimens were examined with microscopy to develop a profile of gametogenesis. The diameter of 1000 oocytes from 10 specimens (100 oocytes each) was measured to determine the ripeness degree each month. The reproductive maturity was categorized into five stages (Kim *et al.*, 2005): early active (stage I), late active (stage II), ripe (stage III), partially spawned (stage IV), spent and inactive (stage V) (Fig.2).

2.3 Biochemical Analysis

Glycogen, protein and lipid content were analyzed in five tissues of gonad, foot, mantle, siphon and adductor muscle. Tissues of the same gender were pooled each

month to determine the average biochemical composition (Giese, 1969). Three replicates were used to generate means with 10–15 clams pooled in each replicate. The glycogen content was analyzed with a minor modification of the anthrone, sulfuric acid method described by Horikoshi (1958). The 50 mg of powdered, freeze-dried tissues were suspended in 60 volumes of KOH ($0.3\ \text{g mL}^{-1}$), and saponified by heating at 100°C for 30 min. After cooling, a portion of the saponified mixture was treated with 5 mL cold 0.2% anthrone-sulphuric acid solution for 10 min, absorbance of the resulting colored complex was measured at the wavelength of 620 nm. Soluble protein level in tissues was determined using the method of Bradford (1976), with bovine serum albumin as the reference. 100 mg of fresh tissue was homogenized in 2 mL of saline solution. After being centrifuged and diluted, the crude extract was mixed with Brilliant Blue G, and then the absorbance value at 595 nm was measured. The lipid content was analyzed using the gravimetric method. Lipid extraction was made in diethyl ether by using an automatic Buchi extraction system (B-811; Buchi Co., Sweden). The dry tissue powders were re-weighed after extraction and the lipid loss was calculated as the lipid content.

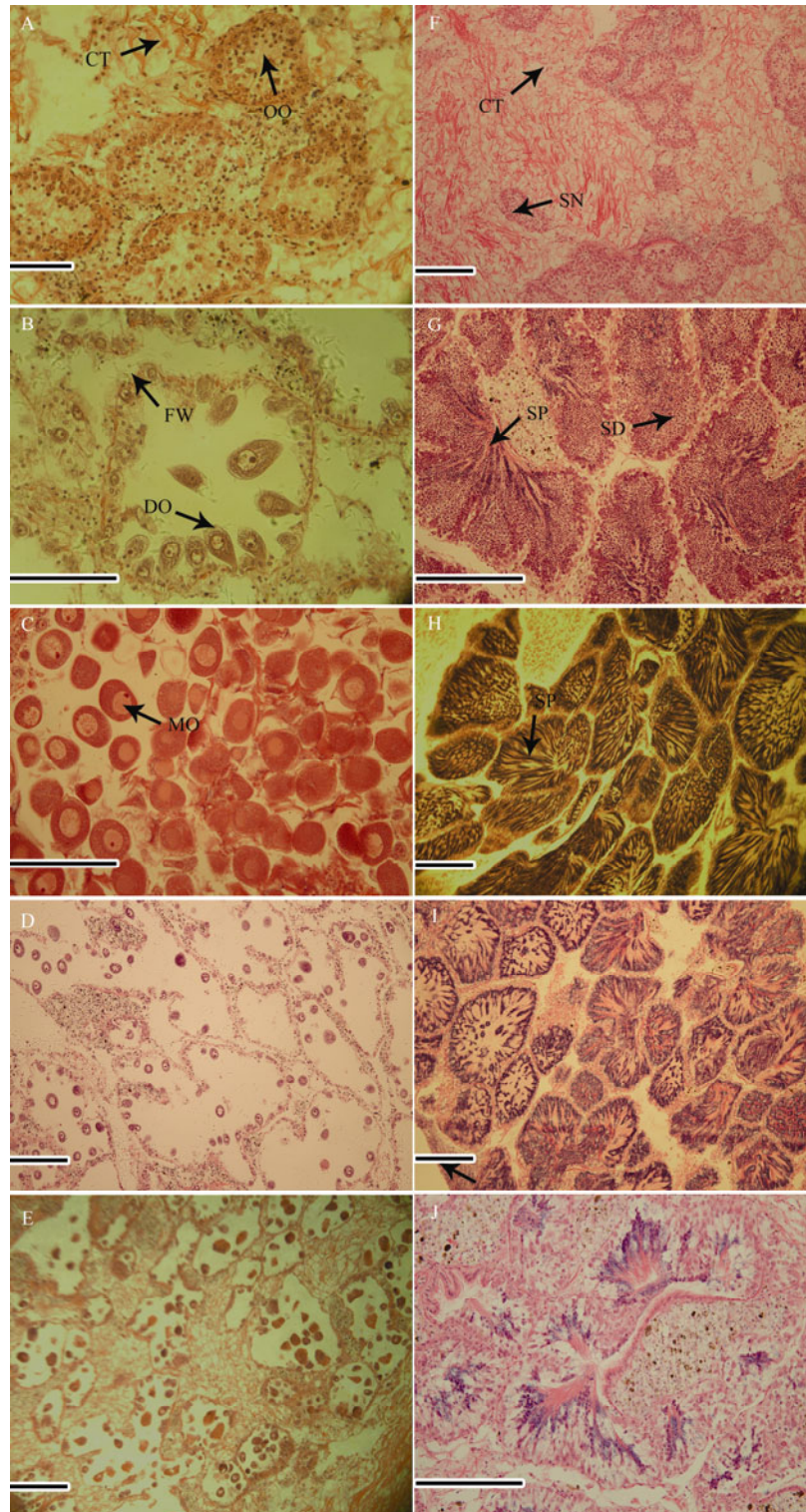


Fig.2 Gonad developmental stages of female *S. purpuratus*: early active (A), late active (B), ripe (C), partially spawned (D), and spent and inactive (E); and male *S. purpuratus*: early active (F), late active (G), mature (H), partially spawned (I), spent and inactive (J). CT, connective tissue; FW, follicle wall; OO, oogonia; DO, developing oocyte; MO, mature oocyte; SN, spermatogonia; SD, spermatid; SP, sperm. All bars=100 μ m.

2.4 Statistical Analysis

All statistical analyses were performed using SPSS software (version 19.0). One-way ANOVA was used to assess the monthly difference in oocyte diameter followed by Duncan's multiple comparison tests. Two-factor ANOVAs were employed to compare the biochemical

parameter on each separate tissue using sex and month as the factors. When the values of each biochemical parameter were significantly different between sexes, the values of biochemical composition of every separate tissue were compared separately between sexes using one-way ANOVA followed by post-hoc comparisons using

Duncan's test ($\alpha=0.05$).

3 Results

3.1 Environmental Parameter

Monthly environment parameters including seawater temperature, salinity and chlorophyll *a* values in Sanggou Bay were shown in Fig.3. Temperature varied between 1.8°C (in January) and 25.8°C (in August). From March, the water temperature continued to increase and maximized in August synchronically with chlorophyll *a* (6.17 $\mu\text{g L}^{-1}$). After September, the temperature and the chlorophyll *a* concentration began to decrease, reaching the lowest (0.40 $\mu\text{g L}^{-1}$) in January. Salinity remained relatively stable throughout the year, slightly varying between 30.6 and 32.1. The chlorophyll *a* concentration was significantly related to the seawater temperature (Pearson correlation, $r=0.975$, $P<0.05$).

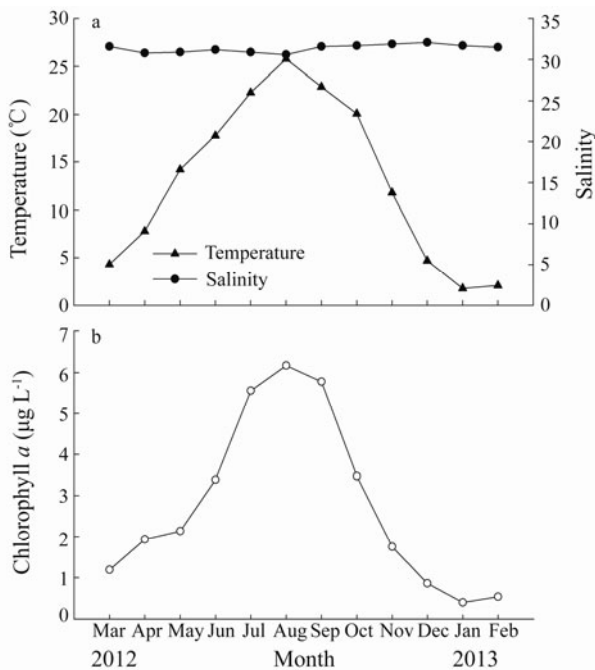


Fig.3 Seasonal variation of seawater temperature, salinity and chlorophyll *a* concentration in Sanggou Bay.

3.2 Gametogenic Activity

The mean oocyte diameter showed significant difference among months ($P<0.05$). It increased from 12.7 μm in March to a maximum of 44.1 μm in July, and then decreased. From November to January, the mean oocyte diameter was not estimated because the gonad of clam was totally at spent and inactive stage (Fig.4). Based on histological observation of the gonads, the sex was clearly identified and no hermaphrodite was found. Both female and male had a synchronically gonad development with gametogenesis initiated in February (Fig.5). Most individuals were at the early active stage (82.4% of females and 83.3% of males) in March. The late active stage was found from March to May. The gonads became ripe in May, and 75% of females and 78.6% of males were ripe

in June. The spawning activity was observed from June to October with the spawning peaked in August (80% of females and 70.6% of males). The clam were totally at spent and inactive stage between November and January.

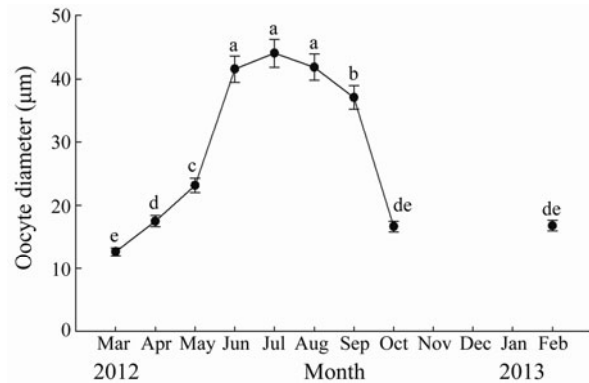


Fig.4 Seasonal variation of oocyte diameter of *S. purpuratus*. Different small letters indicate significant monthly difference ($P<0.05$).

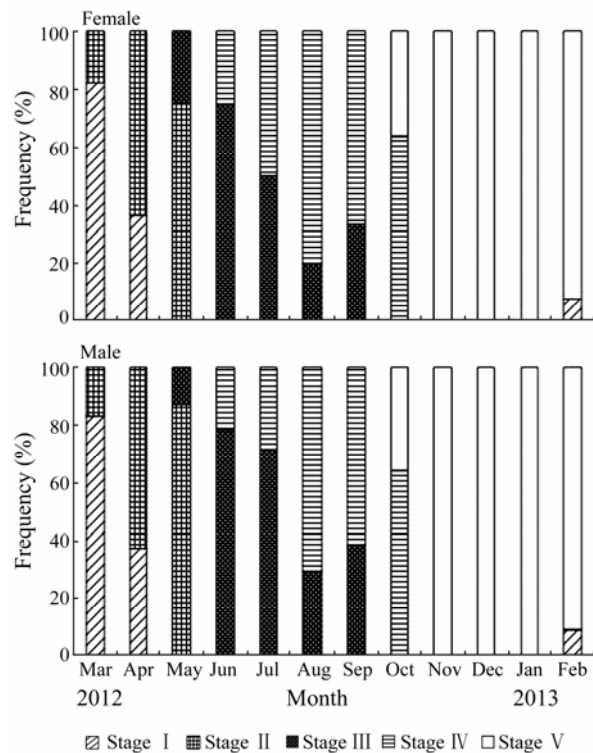


Fig.5 Seasonal distribution of *S. purpuratus* at different stages of gonad development.

3.3 Biochemical Composition

The general trend in glycogen content of three tissues (gonad, foot and mantle) was similar, which decreased from March to the minimum in September. In siphon and adductor muscle, the glycogen content of both sexes increased from March, peaking in May and June respectively, and then declined dramatically, bottoming in September. The glycogen content of five tissues all recovered in autumn and winter (Fig.6). Two-factor ANOVA analysis indicated that there was no significant difference between gender in glycogen content of foot, gonad, siphon and

mantle ($P > 0.05$), though the glycogen content changed yearly significantly ($P < 0.05$). The glycogen content in

adductor muscle showed significant monthly variation, as well as significant difference between gender ($P < 0.05$).

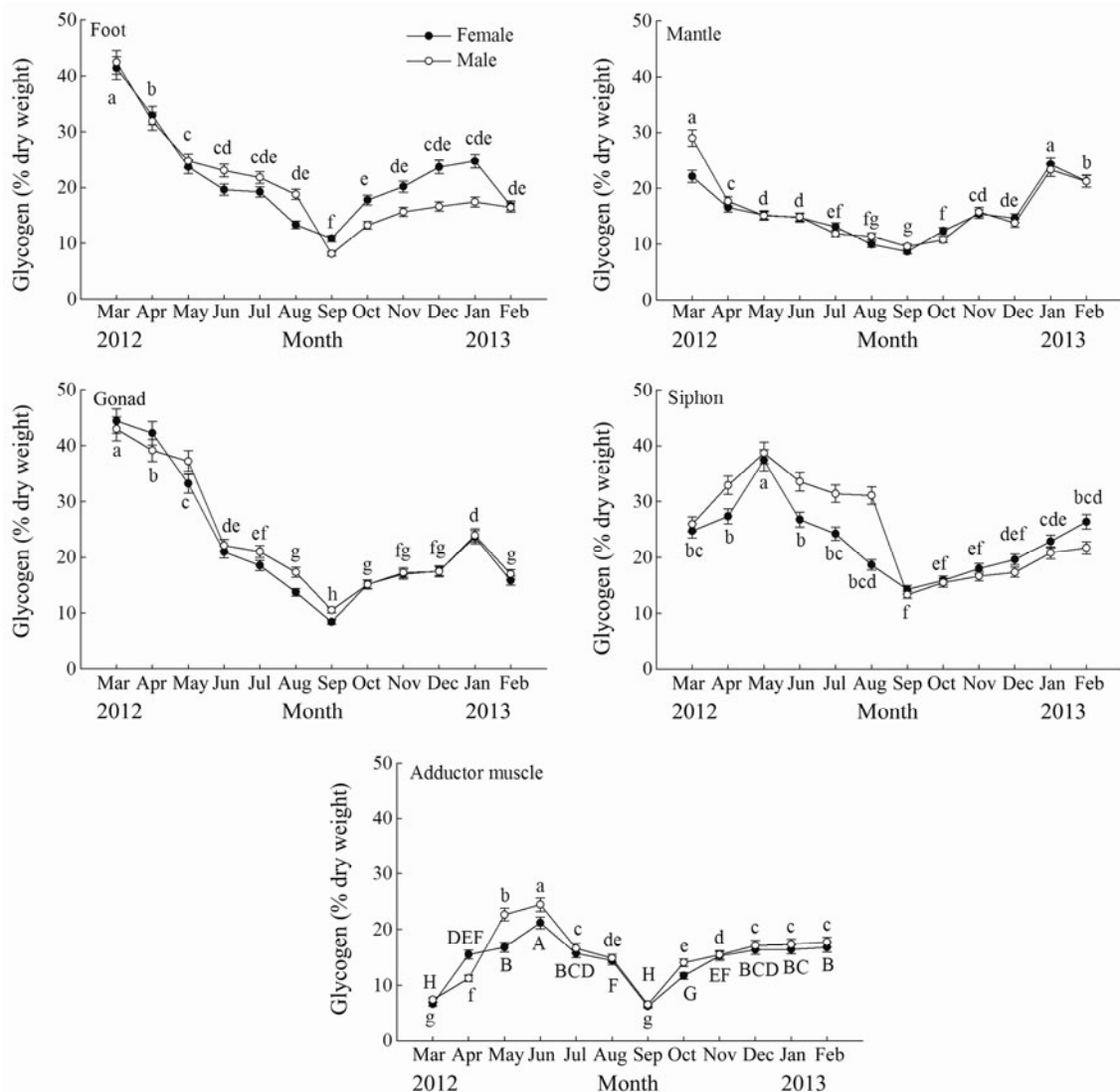


Fig.6 Seasonal variation in glycogen content (percentage of dry weight) of gonad, mantle, siphon, adductor muscle and foot of *S. purpuratus* (mean±SD, n=3). Different small letters indicate significant monthly difference in foot, gonad, mantle and siphon ($P < 0.05$); no significant difference between sexes was found. Different capital and small letters indicate significant difference between months in female and male adductor muscle, respectively ($P < 0.05$).

The lipid content in gonad and mantle of both sexes showed marked variation throughout the reproductive cycle, but did not show significant variation in foot, siphon and adductor muscle (Fig.7). In ovary, the lipid increased from March to a maximum in June, followed by an apparent decrease, bottomed in October, and then recovered in winter. However, the lipid content in testis had a converse tendency, which decreased gradually from March to the minimum in August, and then recovered in autumn and winter. In mantle of both sexes, there was an obvious decrease in lipid content from March to July, and a slight increase from July to February. Lipid content in foot, siphon and adductor muscle of both sexes remained at a stable level below 4%. Two-factor ANOVA analysis indicated the lipid content did not change over the year and between the sexes in foot, siphon and adductor muscle ($P > 0.05$). However, significant sexual and temporal

variation was found in the gonad ($P < 0.05$), and a significant temporal variation was found in the mantle ($P < 0.05$).

The protein content in three tissues of gonad, foot and mantle showed clear seasonal variation, but there was no seasonal variation in siphon and adductor muscle (Fig.8). In ovary, the protein content increased gradually from March to the maximum in June and then decreased. A converse tendency was observed in testis, which decreased from March to the minimum in July and then increased. In foot, the protein content of both sexes increased gradually from March until it peaked in July, and then declined dramatically, bottoming in October. In mantle, similar variation was observed as in foot except for the highest level in May. In siphon and adductor muscle, the protein fluctuated around 25.6–37.3 mg g⁻¹ and 26.1–45.1 mg g⁻¹ respectively. Two-factor ANOVA

analysis indicated the protein content did not change over the year and between sexes in siphon and adductor muscle ($P > 0.05$). However, significant sexual and temporal

variation was found in the gonad ($P < 0.05$), while a significant temporal variation was found in foot and mantle ($P < 0.05$).

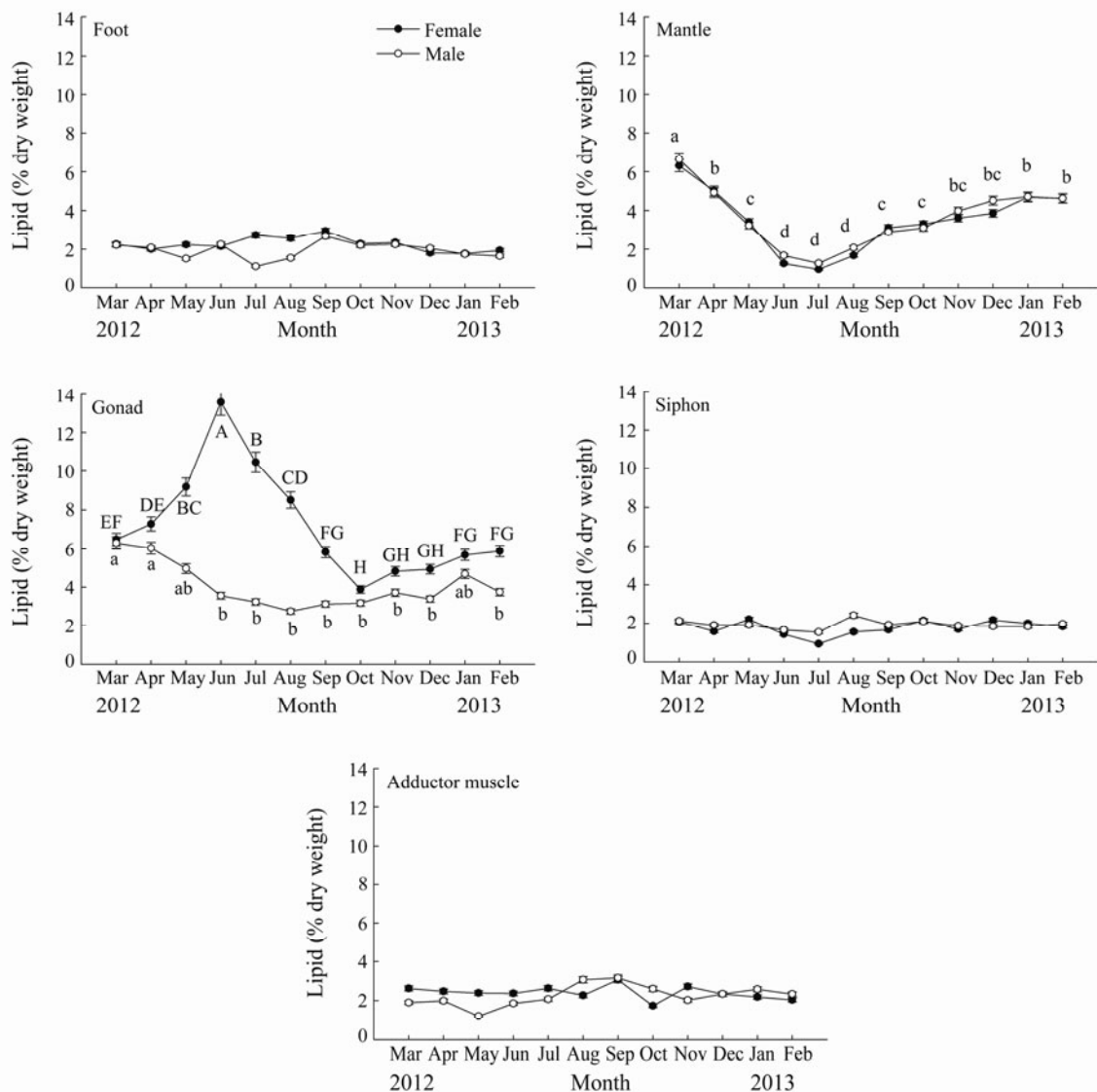


Fig.7 Seasonal variation in the lipid content (percentage of dry weight) of gonad, mantle, siphon, adductor muscle and foot of *S. purpuratus* (mean \pm SD, $n=3$). Different capital and small letters indicate difference between months in the ovary and testis, respectively ($P < 0.05$); different small letters indicate significant monthly difference in the mantle ($P < 0.05$); no significant difference between sexes were found.

4 Discussion

Numerous studies have shown the effect of environmental factors on the reproductive process of mollusc (Beltran-Lugo *et al.*, 2006; Serdar and Lök, 2009; Ruan *et al.*, 2014). Environmental factors influence the quantity and quality of available food and the physiological state of mollusc, playing a vital role in reproductive cycle (Laruelle *et al.*, 1994; Carrasco *et al.*, 2006; Gharsallah *et al.*, 2010). The duration of gametogenic cycle of bivalves, particularly that of spawning period, is directly influenced by geographical latitude which determines the temperature of seawater (Laruelle *et al.*, 1994; Chavez-Villalba *et al.*, 2002). In present study, gametogenesis

began at low temperature (2.1°C) in February. From March, with the water temperature increasing, the gametes grew rapidly and ripened between June and July when water temperature was between 17.8 and 22.3°C . The spawning took place once a year from June to October, while the massive spawning occurred in August when water temperature reached the highest. Nevertheless, in the west sea of Taean of Korea (where the latitude is lower than that of Sanggou Bay), the gametogenesis of *S. purpuratus* began in January and the spawning season was from May to December (Kim *et al.*, 2005).

Gametogenesis usually occurs when food is abundant in environment (Serdar and Lök, 2009). As a suspension feeder, *S. purpuratus* predaes algae and organic particles. Chlorophyll *a* was used as a conventional measure to

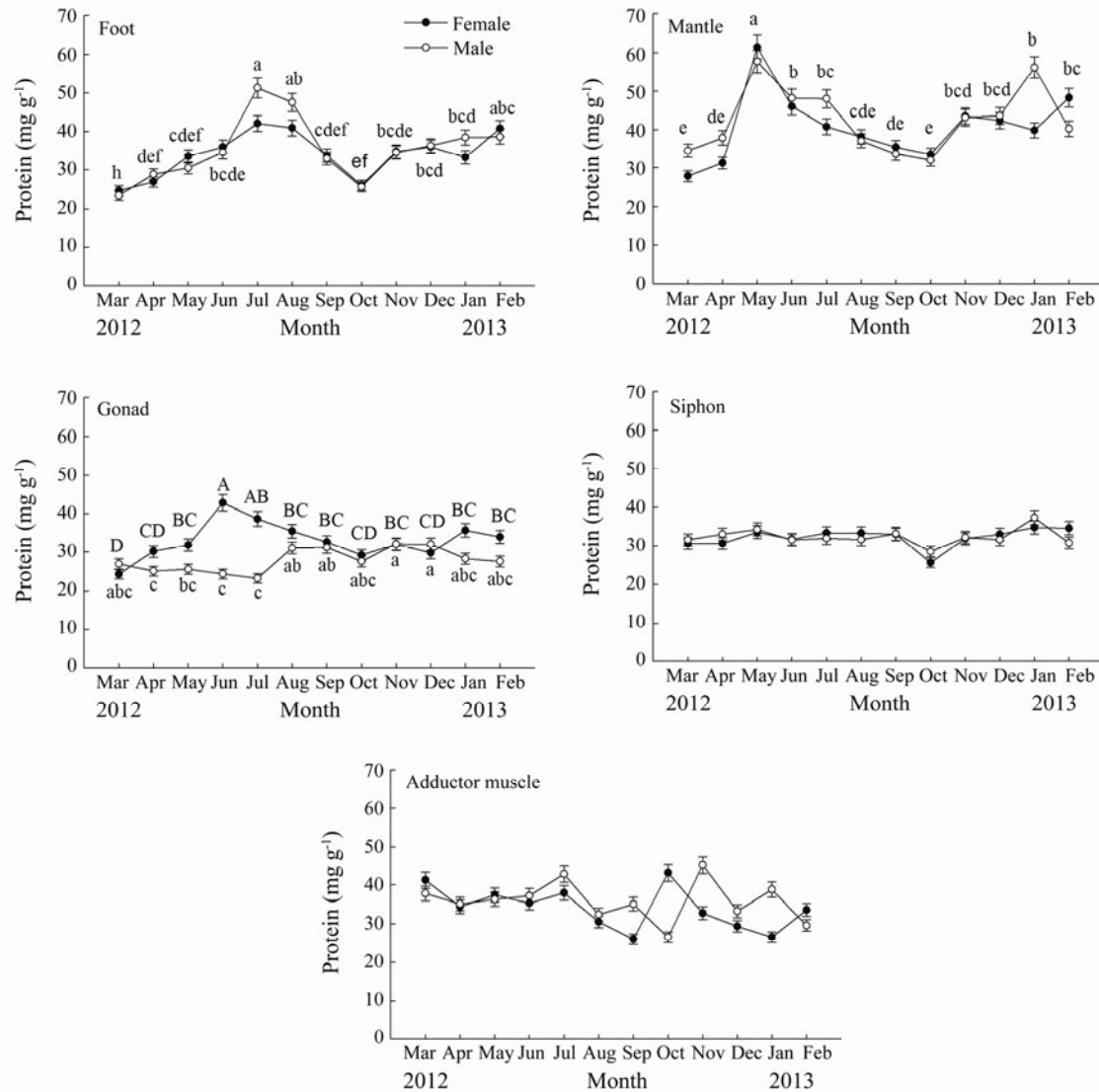


Fig.8 Seasonal variation in the protein content (mg g⁻¹ wet weight) of gonad, mantle, siphon, adductor muscle and foot of *S. purpuratus* (mean±SD, n=3). Different small letters indicate significant monthly difference ($P<0.05$) in the foot and mantle since no significant difference between sexes were found in these tissues; different capital and small letters indicate difference between months in the ovary and testis, respectively ($P<0.05$).

compare the food availability (Li *et al.*, 2009). In our study, the gametes of *S. purpuratus* developed as the chlorophyll *a* concentration increased, and the spawning peak was in August when food (chlorophyll *a*) reached the highest. The similar phenomenon was reported in *Macrta veneriformis* (Ke and Li, 2013), which suggested that food availability can affect the oocyte diameter, and the abundant food resources could provide the condition for spawning.

Glycogen plays an important role in energetic and metabolic supply of gametogenesis in many bivalves (Bayne *et al.*, 1982; Mathieu and Lubet, 1993; Park *et al.*, 2001). Our research indicated that glycogen level was high before gametogenesis, and then decreased significantly along with the gonad development in the gonad, mantle and foot of both females and males. Glycogen was accumulated between March and May (stage I–II) in siphon, and between March and June (stage I–III) in adductor muscle. The accumulated glycogen can be used in

the rest time of gametogenesis. After spawning, the glycogen content in all tissues began to recover in winter. This characteristic allowed us to conclude that the five tissues were all the reservoir for glycogen and they can supply energy at the different stages of gonad development. The clam can firstly use glycogen in the gonad, mantle and foot. When the glycogen was not enough for gametogenesis, glycogen stored in siphon and adductor muscle could be supplemented. It was reported that glycogen content in all tissues decreased during gametogenesis in other bivalves like *Macrta chinensis* (Li *et al.*, 2011) and *Sinonovacula constricta* (Yan *et al.*, 2010a). However, in *M. veneriformis*, glycogen increased significantly during the entire maturation process, and decreased dramatically in the spawning period (Ke and Li, 2013).

Lipids are usually used as an energy source during gametogenesis (Holland, 1978), and constitute the principal nutritional reserve in eggs and larvae (Helm *et al.*, 1973). In our study, during sexual development, the ovary ac-

cumulated lipid with notable increases. The similar result was documented for *Crassostrea plicatula* (Li et al., 2006) and *Placopecten magellanicus* (Napolitano et al., 1992). Gabbott (1983) pointed out that the gonad development involved the metabolic conversion of glycogen to lipid. In fact, glycogen can be converted to triglycerides which are incorporated into the developing eggs (Barber and Blake, 1985). Our result showed that the lipid content in testis decreased. As detailed previously, spermatozooids do not store lipids. The males mostly accumulate membrane lipid like sterols or phospholipids which are not energetic reserves (Soudant et al., 1996). The lipid content in the mantle of both females and males decreased during gametogenesis, suggesting that they can provide substrates and energy for gametogenesis. There was no seasonal variation of lipid content in foot, siphon and adductor muscle of both sexes, indicating that the lipid located in these tissues did not contribute to the gonad development of *S. purpuratus*.

Protein can serve as an energy reserve in adult bivalve particularly during gametogenesis, and it is also a major organic component of bivalve oocytes (Beninger and Lucas, 1984). In this study, the higher protein content in ovary was observed at near-ripe or ripe stage, and the change of protein level was similar with that of lipid content. It suggested that the protein and lipid in ovary would be accumulated as vitellin in oocytes (Ojea et al., 2004). Similar results occurred in *Crassostrea gigas* (Dridi et al., 2007) and *Cyclina sinensis* (Yan et al., 2010b). The protein content in testis decreased during the gonad development, suggesting that the protein can be an energy and material source for spermatogenesis. The protein was accumulated between March and May (stage I-II) in the mantle, and between March and July (stage I-IV) in the foot. During the rest time of gametogenesis, the accumulated protein could provide nutrient and material when glycogen and lipid reserve was not enough. Berthelin et al. (2000) pointed out that protein contributed to the energetic maintenance in the period of reduced glycogen level. The protein content in siphon and adductor muscle of both females and males exhibited no change throughout the year, suggesting that protein could not be transferred to the gonad as the gametes developed.

In conclusion, this study is for the first time to describe the seasonal variation in biochemical constituents of *S. purpuratus* from Sanggou Bay in relation to its reproductive cycle and environmental factors. Gametogenesis initiated in February when water temperature was 2.1 °C, and most of the individuals ripened between June and July. The spawning peak was in August with the highest water temperature and chlorophyll *a* levels. The five tissues (gonad, foot, mantle, siphon and adductor muscle) were all the storage areas of glycogen. They can supply energy at different stages of gonad development. During the sexual development, the ovary accumulated lipid and protein as the major organic components of oocytes. In testis, lipid and protein could provide energy and material for spermatogenesis. The data gathered in our study provided valuable information for the suitable exploitation

and management of wild stocks as well as for aquaculture of this species.

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