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Oogenesis and Reproductive Cycle in *Neptunea* (*Barbitonia*) *arthritica cumingii* on the West Coast of Korea

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ABSTRACT

The gonadosomatic index (GSI), oogenesis and reproductive cycle in female *Neptunea* (*Barbitonia*) *arthritica cumingii* were investigated by light and electron microscope observations. In the early vitellogenic oocyte, the Golgi complex and mitochondria were involved in the formation of glycogen, lipid droplets and yolk granules. Late in the vitellogenic oocytes, the rough endoplasmic reticulum and multivesicular bodies were involved in the formation of proteid yolk granules in cytoplasm. In particular, compared with the results of other gastropods, it showed a different result that appearances of cortical granules at the cortical layer and microvilli on the vitelline envelope, which is associated with heterosynthetic vitellogenesis, were not observed in vitellogenic oocytes during oogenesis. A mature yolk granule was composed of three components: main body (central core), superficial layer, and the limiting membrane. Monthly changes in the gonadosomatic index in females studied in 2004 and 2005 were closely associated with ovarian developmental phases. Spawning occurred between May and August in 2004 and 2005 and the main spawning occurred between June and July when the seawater temperature rose to approximately 18-23℃. The female reproductive cycle can be classified into five successive stages: early active stage (September to October), late active stage (November to February), ripe stage (February to June), partially spawned stage (May to August), and recovery stage (June to August).

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INTRODUCTION

Neptunea arthritica cumingii is one of the most important edible gastropods in East Asian countries such as Korea, Japan, China and Russia (Yoo, 1976; Kwon *et al.*, 1993). This species is especially found in silty sand of the subtidal zone of the west coast of Korea. Recently, as the standing stock of this species gradually decreased due to extensive reclamation projects and reckless over-harvesting, it has been designated as one of the important organisms in need of natural resources management.

On *Neptunea* spp. in foreign countries, previously there have been some studies on aspects of reproduction including the reproductive cycle (Takahashi *et al.*, 1972; Takamaru and Fuji, 1981; Fujinaga, 1985; Kawai *et al.*, 1994) and spawning (Miyawaki, 1953; Amio, 1963; Son, 2003), on aspects of ecology including distribution (Ito and Tachizawa, 1981; Ito, 1982; Kwon *et al.*, 1993), growth (MacIntosh and Paul, 1977; Fujinaga, 1987; Suzuki *et al.*, 1996) of *N. arthritica*, and feeding (Pearce and Thorson, 1967) of *N. antiqua*. On *N. cumigii*, especially, there has been one study on the spawning season in the East China Sea (Amio, 1963). But, there are still gaps in our knowledge for reproductive biology. So far, little information is available on ultrastructural study on germ cell differentiation and

sexual maturation of *N. arthritica cumingii* in the Korean waters and the Japan Sea (Chung and Kim, 1996). However, there is some information on ultrastructural study of oogenesis in other gastropods (McCann-Coillier, 1977, 1979; Griffond and Gomot, 1979; Griffond, 1980; Hodgson and Eckelbarger, 2000; Pal and Hodgson, 2002). Therefore, the results of ultrastructural studies on germ cell differentiation of this species and other gastropods will provide important information for the reproductive mechanism. The reproductive cycles of the local populations in marine gastropods vary with environmental factors such as water temperature and food availability (Chung *et al.*, 2002). Understanding the reproductive cycle and the spawning period of *N. arthritica cumingii* will provide necessary information for natural spat collections or the recruitment period and age determination of this population. Therefore, the main aim of the present study is to understand germ cell differentiation during oogenesis, the reproductive cycle and first sexual maturity of this species.

MATERIALS AND METHODS

Sampling

Specimens of *Neptunea arthritica cumingii* (Crosse, 1862) were collected monthly at the subtidal zone of Maldo, Gunsan, Korea, from January 2004 to December 2005 (Fig. 1). The snails ranging from 41.0 to 106.8 mm in shell height were used for the present study. After the snails were transported alive to the laboratory, shell heights were immediately measured.

Gonadosomatic index (GSI)

A total of 446 individuals were used for calculation of the GSI. Monthly changes in the mean gonadosomatic index (GSI) were calculated by the following equation (Chung *et al.*, 2002) (Fig. 2):

$$GSI = \frac{\text{The thickness of the gonad x 100}}{\text{Diameter of posterior appendage including the gonad and digestive gland}}$$

Germ Cell Differentiation by Electron Microscopical Observation

For electron microscopical observations, excised pieces of the gonads were cut into small pieces and immediately fixed in 2.5% paraformaldehyde-glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h at 4℃. After initial fixation, the specimens were washed several times with the same buffer and then further fixed in 1 % osmium tetroxide dissolved in 0.2 M phosphate buffer solution (pH 7.4) for 1 h at 4℃.

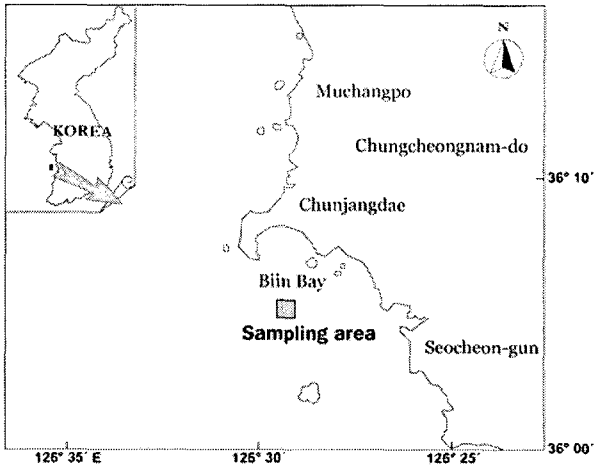

Fig. 1. Map showing the sampling area.

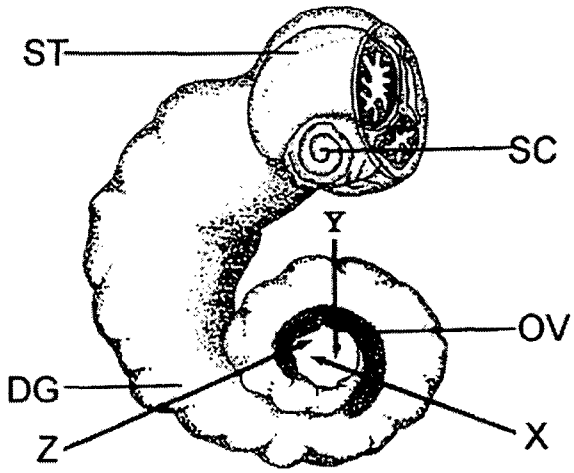

Fig. 2. Anatomy of *Neptunea arthritica cumingii*, removed from its shell. Posterior appendage showing the ovary and digestive gland. X, Y and Z denote the sections for measurement of GSI. Three sections are spaced equally.
Abbreviations: DG, digestive gland; OV, ovary; ST, stomach; SC, stomachal caecum.

Specimens were then dehydrated in a series of increasing concentrations of ethanol, cleared in propylene oxide and embedded in Epon-Araldite mixture. Ultrathin sections of Epon-embedded specimens were cut with glass knives with a Sorvall MT-2 microtome and an LKB ultramicrotome at a thickness of about 800-1000 Å. Tissue sections were mounted on collodion-coated copper grids, stained with uranyl acetate followed by lead citrate, and examined with a JEM 100 CX-2 (80 kv) electron microscope.

Gonadal Development by Histological Observations

For light microscopic examination of histological preparations, a total of 456 individuals were used for histological analysis of the gonads from January 2004 to December 2005. Gonad tissues were removed from shells and preserved in Bouin's fixative for 24 h and then washed with running tap water for 24 h. Tissues were then dehydrated in alcohol and embedded in paraffin molds. Embedded tissues were sectioned at 5~7 μm thickness using a rotary microtome. Sections were mounted on glass slides, stained with Hansen's hematoxylin-0.5 % eosin, Mallory's triple stain and PAS stain, and examined using a light microscope.

RESULTS

1. Position and Morphology of the Gonads

Neptunea arthritica cumingii is a dioecious species which is composed of well-defined female and male individuals. The ovary is located on the surface of the digestive gland in the spiral posterior region of the shell (Fig. 2). The ovary is composed of numerous oogenic follicles. As the ovary was maturing, it extended to the outer part of the digestive gland. As maturation progresses, the sex of the snail can be distinguishable easily by color: the ovary being pale yellow and testis yellowish-brown. At this time, when it was slightly scratched with a razor, ripe eggs readily discharged from the ovary. But after spawning, the ovary degenerated, and it became difficult to distinguish their sexes by external color or dissection.

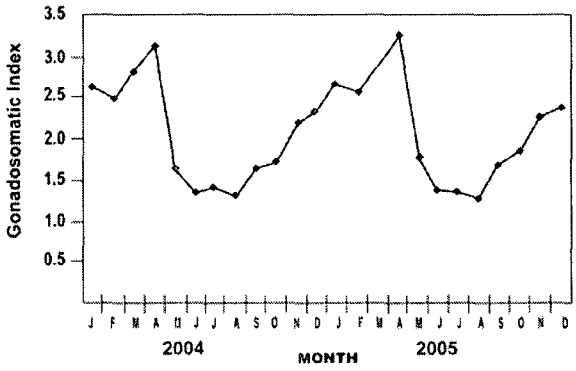

Fig. 3. Monthly changes in the gonadosomatic index of the female *Neptunea arthritica cumingii*, for two years from January 2004 to December 2005.

2. Monthly Changes in the Gonadosomatic Index (GSI)

Monthly GSI changes in females were showed in Fig. 3. In 2004, the GSI slowly increased from September and reached the maximum (mean 3.11) in April when seawater temperature rapidly increased. And then, the GSI rapidly decreased from May, thereafter, the values reached the minimum in August when spawning was completely finished. Monthly changes in the GSI in 2005 showed similar patterns with those in 2004.

3. Germ Cell Differentiation in the ovary by Electron Microscopic Observations

Ultrastructural observations allow the germ cell developmental phases during oogenesis can be divided into 4 phases: (1) oogonial phase, (2) previtellogenic phase, (3) vitellogenic phase, and (4) mature phase. Characteristic features in each stage were as follows;

Oogonial Phase: Oogonia in the oogonial phase, which propagated on the germinal epithelium (follicular wall), were oval and 15 μm in diameter. They commonly were single or formed a cluster on the germinal epithelium. Each oogonium had a large nucleus with chromatin, several mitochondria, and the endoplasmic reticulum, vacuoles in the cytoplasm (Fig. 4A).

Previtellogenic Phase: Previtellogenic oocytes were 25-90 μm in diameter. With cytoplasmic growth, several small mitochondria, a well-developed endoplasmic reticulum and several vacuoles were concentrated around the nucleus in the cytoplasm of the previtellogenic oocyte. The number of Golgi

complexes, scattered from the perinuclear region to the cortical region of the oocyte, increased. At this time, many vacuoles formed by the Golgi complex appeared around the endoplasmic reticulum, several mitochondria, and large vesicles were present in the cytoplasm of the previtellogenic oocyte (Fig. 4B).

Vitellogenic Phase: In the early vitellogenic oocyte, especially, well-developed endoplasmic reticulum and vacuoles in the cytoplasm were concentrated around the nucleus having nucleoli. At this time, the follicle cell, which lied adjacent to the early vitellogenic oocyte, had an elongated nucleus. In particular, electron-dense granules and several lipid droplets were accumulated in the cytoplasm of the follicle cell (Fig. 4C). With the initiation of yolk formation, lipid droplets were accumulated in the vacuoles formed by the Golgi complex in the perinuclear region. Lipid droplets diffused toward the cortical layer, and then glycogen particles appeared around the mitochondria at the cortical region of early vitellogenic oocytes (Fig. 4D). At this time, after electron-dense materials were accumulated in the Golgi complex (Golgi sac, Golgi vacuoles and Golgi vesicles), lipid droplets were formed by secretion of electron-dense materials in the large vacuoles and small vesicles which were formed by the Golgi vacuoles and Golgi vesicles (Fig. 4E). On the other hand, relatively large lipid droplet was surrounded by the endoplasmic reticulum, the mitochondria and glycogen particles in the cytoplasm of the early vitellogenic oocyte (Fig. 4F). In the late vitellogenic oocyte, lots of yolk granules appeared between the rough endoplasmic reticulum and the mitochondria at the cortical layer in the cytoplasm (Fig. 5A). At this time, the multivesicular bodies, which were formed by the modified cristae of the mitochondria, appeared near the nuclear envelope of the nucleus in the late vitellogenic oocyte. Yolk precursors such as glycogen particles, lipid droplets, yolk granules and multivesicular bodies were accumulated in the cytoplasm (Fig. 5B). Eventually, proteid yolk granules were formed by yolk granules and multivesicular bodies (Fig. 5C).

Mature Phase: Mature oocytes were about 180-250 x 300-450 μm in diameter. In the mature oocyte, various sizes of proteid yolk granules were intermingled with small lipid yolk granules, and it became a small mature yolk granule (Fig. 5C). Relatively small mature yolk granules were continuously mixed with each other and became large mature yolk granules in the cytoplasm. A fully mature yolk granule is composed of three components: (1) main body, (2) superficial layer, and (3) a limiting membrane (Fig. 5D).

4. Reproductive Cycle with the Gonad Developmental Stage

Based on the morphological features and sizes of germ cells and the tissue cells around them, the reproductive cycle with gonadal phases can be classified into five stages in females. Especially, the reproductive cycle and monthly changes in water temperatures showed similar patterns in 2004 and 2005 (Fig. 6). The criteria in defining of each stage are as follows;

Early Active Stage: The gonadal volume was small, and the follicles occupied approximately 25 % of the gonad. The follicular walls were relatively thick. Oogonia and the previtellogenic oocytes propagated along the oogenic follicular walls and mesenchymal tissues of the ovary. The oogonia and previtellogenic oocytes are about 15-25 μm in size, respectively. At this time, early vitellogenic oocytes of 25-50 μm in diameter formed an egg-stalk attached to the walls (Fig. 7A). The individuals in the early active stage were found from September to October when seawater temperatures were gradually decreasing.

Late Active Stage: This stage is characterized by the presence of developing early vitellogenic oocytes. Follicular walls (germinal epithelium) were thin. A number of early vitellogenic oocytes of 100-140 μm in diameter were attached to the follicular walls through each egg-stalk. With the initiation of yolk formation, there were numerous yolk granules in the cytoplasm of late vitellogenic oocytes of 150-200 x 250-300 μm in diameter. Some fully mature oocytes were free in the lumen of the follicle (Fig. 7B, C). The individuals in the late active stage appeared from November to February.

Ripe Stage: In females, the majority of oocytes grew

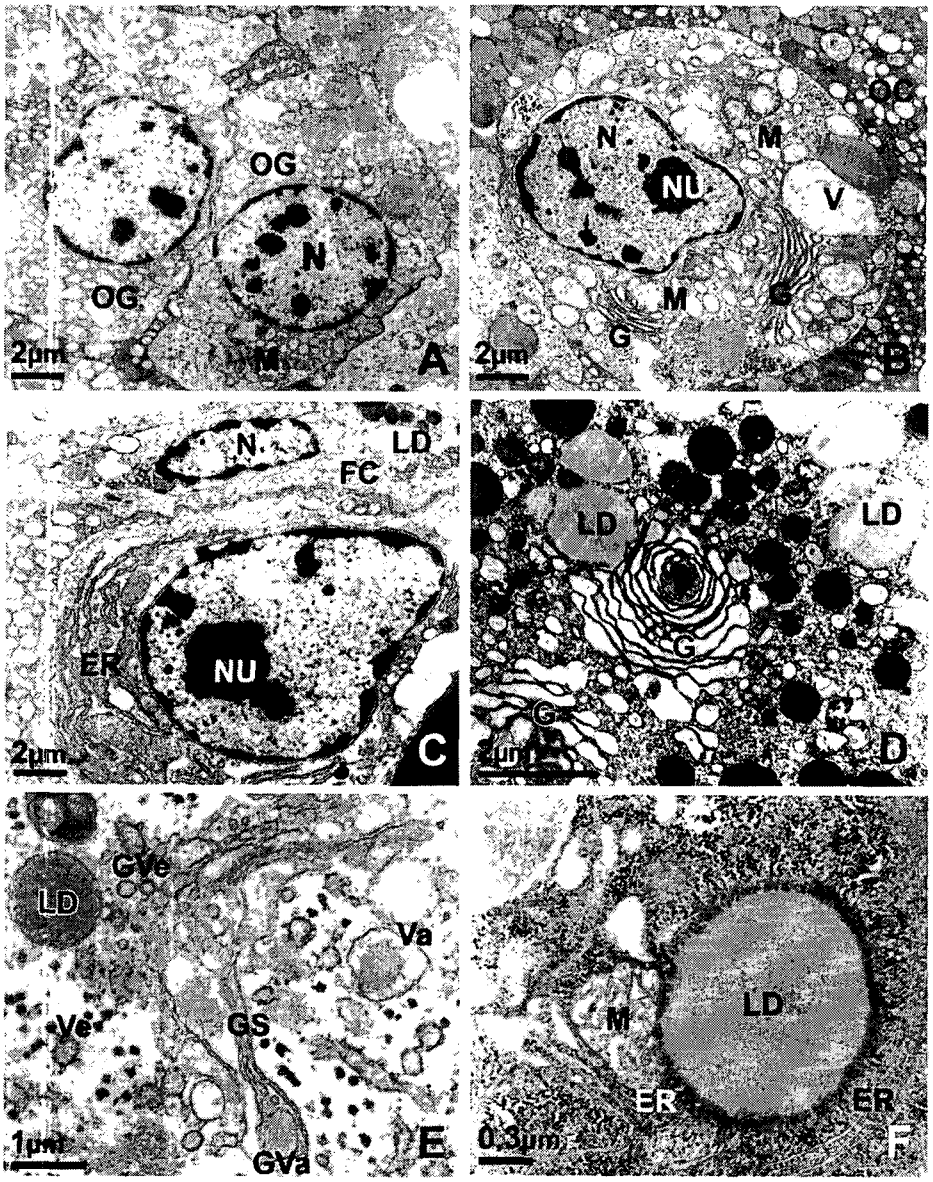

Fig. 4. Electron micrographs of the previtellogenic and early vitellogenic phases during oogenesis of *Neptunea arthritica cumingii* (A-F). **A**, oogonia (OG) in the oogonial phase, with a large nucleus (N) and several mitochondria (M) in the cytoplasm. **B**, a previtellogenic oocyte, with a large nucleus (NU) with a few nucleolus (N) and several mitochondria (M), the Golgi complex (G) and vacuoles (V) in the cytoplasm. **C**, an early vitellogenic oocyte attached to a follicle cell (FC), with a large nucleus (NU) containing chromatin and a number of vacuoles and well-developed endoplasmic reticulum (ER) in the cytoplasm. **D**, the early vitellogenic oocytes, with well-developed Golgi complex (G), glycogen particles and lipid droplets (LD). **E**, an early vitellogenic oocytes, with lipid droplets (LD) formed by secretions in vacuoles (Va) and vesicles (Ve). **F**, an early vitellogenic oocyte, with a lipid droplet (LD) surrounded by the endoplasmic reticulum (ER) and the mitochondria (M).
Abbreviations: GS, Golgi sac; GVa, Golgi vacuole; GVe. Golgi vesicle.

to 160-180 μm in diameter, occupied over 70% of the gonad, and follicular walls became very thin. Mature oocytes growing up to 180-250 x 300-450 μm in diameter became tetragonal or polygonal in shape, and contained a number of mature yolk granules (Fig. 7D). Mature or ripe ovaries were found in February through June when seawater temperatures gradually increased.

Partially Spawned Stage: Since about 50-70% of the oocytes in the follicles were discharged, the lumen of the follicles became considerably empty. Spawned ovaries were characterized by the presence of a few undischarged vitellogenic oocytes as well as previtellogenic oocytes in the follicles (Fig. 7E). The individuals in this stage appeared from May to August, and the main spawning occurred between June and July when the seawater temperature rose to approximately 16-23℃.

Recovery Stage: After spawning, the undischarged vitellogenic oocytes in the lumen of the follicle undergo cytolysis, and each follicle was contracted, and then degeneration or resorption of undischarged vitellogenic or mature oocytes occurred. Thereafter, the rearrangement of newly formed connective tissues,

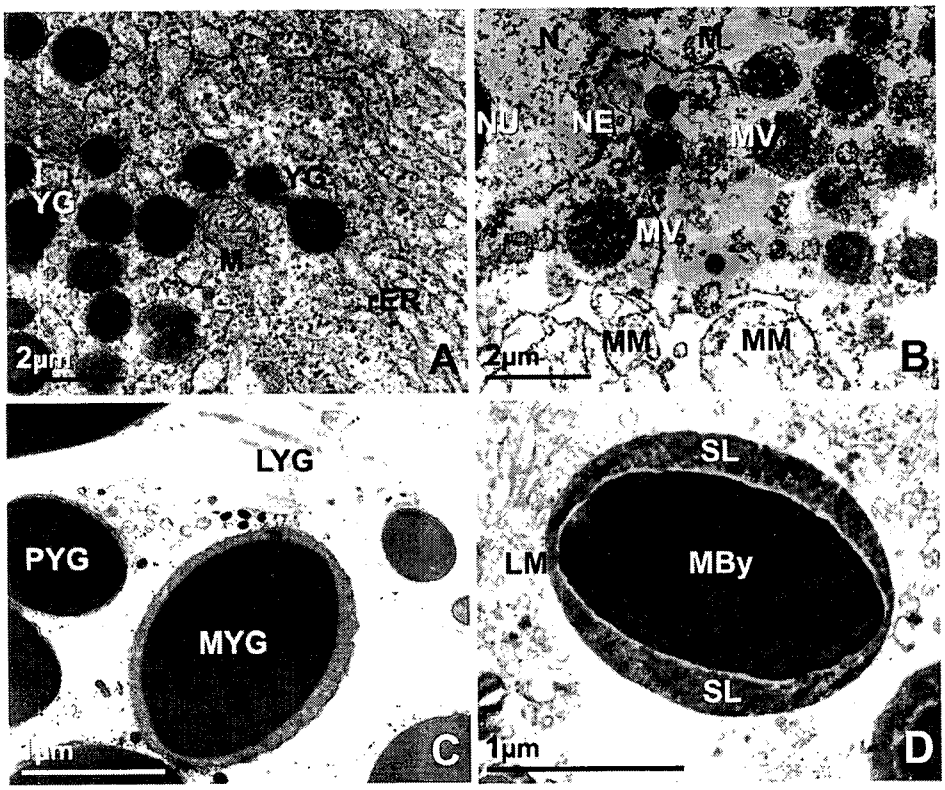

Fig. 5. Electron micrographs of late vitellogenic and mature phases during oogenesis of *Neptunea arthritica cumingii* (A-D). **A**, a late vitellogenic oocyte, with yolk granules (YG) between the rough endoplasmic reticulum (rER) and the mitochondria (M). **B**, a late vitellogenic oocyte, with a number of multivesicular bodies (MV) formed by modified mitochondria (MM). **C**, a late vitellogenic oocyte, with proteid yolk granules (PYG) and mature yolk granules (MYG). **D**, mature oocytes, with a mature yolk granule being composed of the main body (MBy) (central core), superficial layer (SL) and a limiting membrane.
Abbreviations: LM, limiting membrane; LYG, lipid yolk granule.

a few oogonia and previtellogenic oocytes appeared on the newly formed follicular walls (Fig. 7F). The individuals in the recovery stage appeared from June to August.

DISCUSSION

Germ Cell Development and Vitellogenesis

As vitellogenesis commences the nucleus of the oocytes increased in size. Early vitellogenesis is characterized by proliferation of endoplasmic reticulum and mitochondria, both of which are closely associated with lipid droplets. According to our electron microscope observations of early vitellogenic oocytes of *N. arthritica cumingii,* the Golgi apparatus is thought to be involved in a number of vacuoles and small vesicles in the perinuclear region in the cytoplasm, with carbohydrate (glycogen) particles filling the vacuoles. Lipid droplets and lipid yolk granules are then added to the vacuoles and vesicles formed by the Golgi complex (referred as autosynthetic by Taylor and Anderson, 1969), as in *Ilyanassa obsoleta* (Taylor and Anderson, 1969), *Biomphalaria glabrata* (de Jong-Brink *et al.,* 1976), *Mytilus edulis* (Reverberi, 1971), *Rapana venosa* (Chung *et al.,* 2002), *Siphononaria capensis* (Pal and Hodgson, 2002), *Pattella barbara, P. argenvillei, P. granularis, P. oculus, P. miniata* and *Helcion pectunculus* (Hodgson and Eckelbarger, 2000). Therefore, this study suggests that the Golgi complex and various sizes of vacuoles are involved in the formation of lipid droplets in the early vitellogenic oocytes. From our observations of oogenesis, it is assumed that the mitochondria and the endoplasmic reticulum near lipid droplets are involved in the formation of lipid droplets in the early vitellogenic oocyte. However, we did not find pinocytotic tubules which are thought to be involved in yolk production as seen in the vitellogenic oocytes of *Agriolimax reticulatus* (Hill and Bowen, 1976; Dohmen, 1983). In the late vitellogenic oocyte we also did not observe microvilli on the vitelline envelope which is thought to be involvedin helping in absorption, transportation and secretion of egg envelopes (NØrrevang, 1968) as seen in *Mactra chinensis* (Chung, 1997), *M. veneriformis* (Chung and Ryou, 2000), and *Siphonaria serriata* (Pal and Hodgson, 2002).

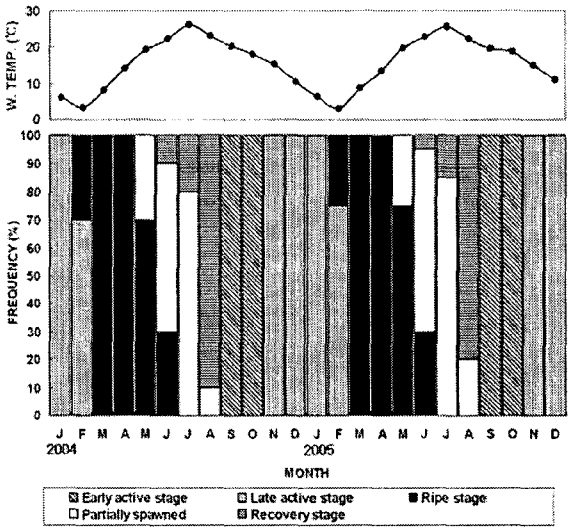

Fig. 6. Frequency of gonadal phases of *Neptunea arthritica cumingii* and the mean water temperatures, for two years, from January 2004 to December 2005.

Formation of cortical granules is a prominent feature of late vitellogenic oocytes in most bivalves such as *Mactra chinensis* (Chung, 1997) and *M. veneriformis* (Chung *et al.,* 2000). Regarding formation of cortical granules during oogenesis, Hodgson and Eckelbarger (2000) described that Golgi complexes appeared predominately in the cortical region of the ooplasm and secrete electron-dense, cortical granule-like organelles in the vitellogenic oocytes of *Patella barbara.* And they stated that Golgi complexes synthesize cortical granules. In the present study, however, such structures were not observed in the vitellogenic oocytes as in *Iliana obsoleta* (Taylor and Anderson, 1969) and *Rapana venosa* (Chung *et al.,* 2002). Compared with *Patella barbara,* that is prominent characteristics during oogenesis and a remarkable difference of *N. arthritica cumingii.* In the present study, proteid yolk granules, which appeared near the rough endoplasmic reticulum and modified mitochondrial structure (multivesicular bodies) as seen in *Hypselodoris tricolor* and *Godiva banyulensis* (Medina *et al.,* 1986), were observed at the cortical region of the cytoplasm. Accordingly, it is assumed

that the endoplasmic reticulum and multivesicular bodies are involved in the formation of proteid yolk granules (Taylor and Anderson, 1969) as yolk precursor. In the present study, although the follicle cell, which lied adjacent vitellogenic oocyte, contains electron-dense granules and lipid droplet, we could not observe clear evidence of secretion into the vitellogenic oocyte. Therefore, it is assumed that *N. arthritica cumingii* synthesize yolk autosynthetically as in the majority of gastropods except for some gastropod species (*Planorbarius corneus, Lymnaea stagnalis, Hypselodoris tricolor, Godiva banyulensis,*

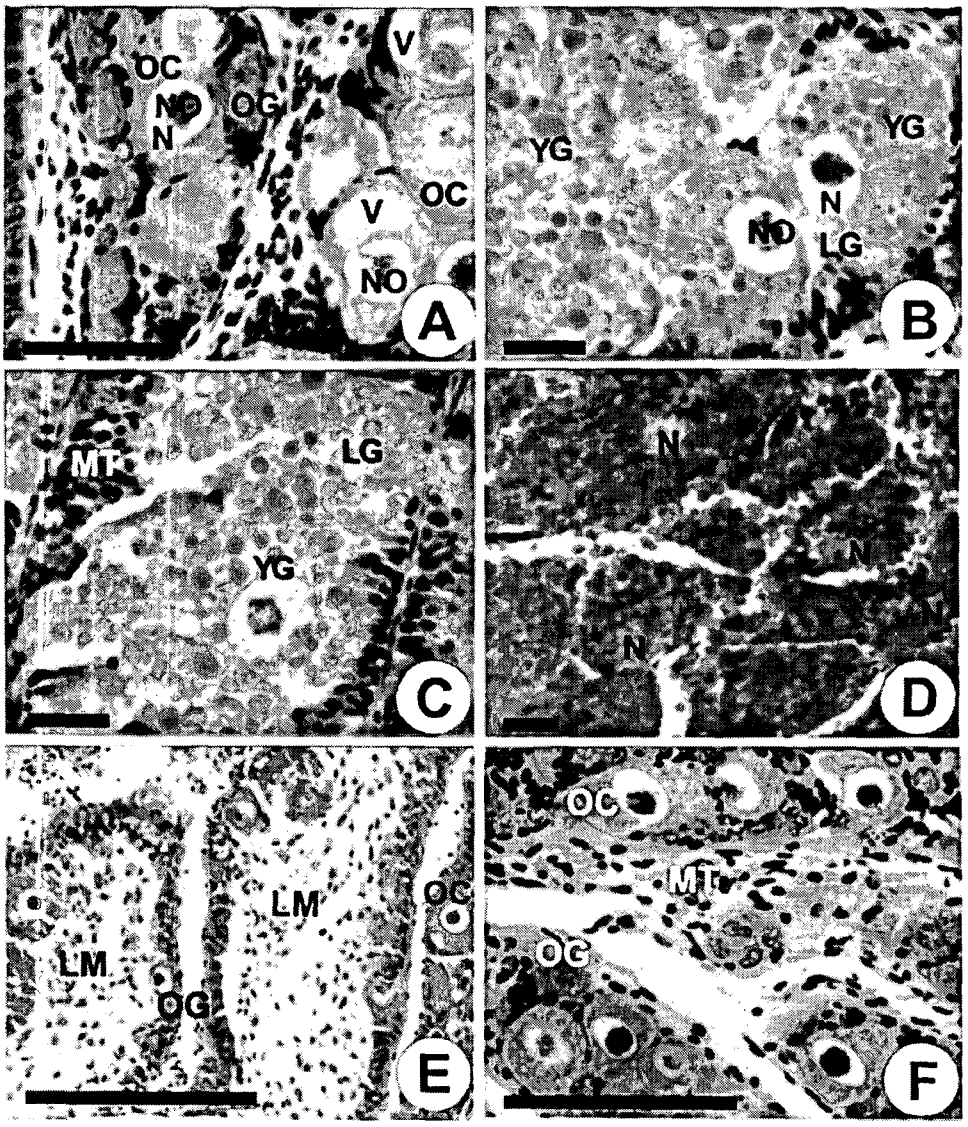

Fig. 7. Photomicrographs of the gonadal phases in female *Neptunea arthritica cumingii*. A, Transverse section of oogenic follicles in the early active stage; B- C, section of follicles in the late active stage; D, section of ripe oocytes in the ripe stage; E, section of follicles in the partially spawned stage; F, section of the follicles in the recovery stage. Scale bar = 50 μm.
Abbreviations: LG, lipid granule; LM, lumen; MT, mesenchymal tissue; N, nucleus; NO, nucleolus; OC, oocyte; OG, oogonium; V, vacuole; YG, yolk granule.

Table 1. A comparison of the spawning seasons of Buccinidae in each locality.

Species	Spawning season	Locality	Author
Neptunea arthritica cumingii	May-August	Biin Bay, Korea	Present study
N. cumingii	July-August	East China Sea, China	Amio, 1963
N. arthritica	May-June	Usu Bay, Hokkaido, Japan	Fujinaga, 1985
N. arthritica	May-August	Saroma, Hokkaido, Japan	Kawai *et al.*, 1994
N. constricta	December	East Sea, Korea	Son, 2003
Siphonaria cassidariaeformis	December	East China Sea, China	Habe, 1960

Siphonaria capensis and *S. serrata*) which synthesize yolk autosynthetically and heterosynthetically (Bottke *et al.*, 1982; Medina *et al.*, 1986 Pal and Hodgson, 2002).

Gonadal Development and Maturation

We observed that gametogenesis of *N. arthritica* cumingii initiates at a temperature of about 3.0℃, with maximum gonadal maturation occurring in April 2004 and 2005 when water temperatures rose (Fig. 7) and phytoplankton was very abundant. Periods of high food abundance and gonad development were nearly coincident. In Korean coastal waters, growth and production of *Meretrix lusoria* and *Ruditapes philippinarum* are very high in the spring - early summer seasons (Kim *et al.*, 1977; Chung *et al.*, 1994; Lee, 1995) due to the abundant phytoplankton that occurs with increasing water temperatures. Especially, *Ruditapes philippinarum, Meretrix lusoria* and other clams are commonly used as food organisms of *N. arthritica cumingii*. At this time, abundant food can be supplied to *N. arthritica cumingii* during the period of gonadal development and maturation. Therefore, it is suggested that gonadal development and maturation of *N. arthritica cumingii* is closely related to water temperature and food availability.

Breeding Pattern

As shown in Table 1, our histological observations show that spawning of *N. arthritica cumingii* on the west coast of Korea occurs from late May to August in 2004 and 2005 when sea water temperatures were high. The spawning season of *N. cumingi* collected by the trawl net in the East China Sea occurs between July and August (Amio, 1963). And *N. arthritica* in Japan has been reported to spawn once a year from May and June in Usu Bay, Japan (Fujinaga, 1985). Therefore, it is assumed that the spawning period of *N. arthritica cumingii* on the west coast of Korea occurred somewhat earlier than that in the East China Sea. On the whole, *N. arthritica cumingii* in Korea is a summer breeder, based on the criteria outlined by Boolootian *et al.* (1962) for marine mollusks. In general, it is assumed that spawning of *N. arthritica cumingii* and *N. arthritica* in Korea and Japan occur between May and August. However, spawning of *N. constricta* and *Siphonalia assidariaeformis* (Buccinidae) occurs during December as a winter breeder (Table 1). Therefore, the slight discrepancy in the spawning period between these studies might be related to geographic differences in water temperature and food availability (Chung *et al.*, 2002).

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