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Ultrastructure of Germ Cells during Spermatogenesis and Structural Changes in the Seminal Vesicle in Male *Neptunea (Barbitonia) arthritica cumingii* (Crosse, 1862)

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The ultrastructure of germ cells during spermatogenesis and the structural changes in the epithelial cells of the seminal vesicle with testicular development in male *Neptunea (Barbitonia) arthritica cumingii* were investigated monthly based on electron microscopic and histologic observations. *N. arthritica cumingii* (Gastropod: Buccinidae) undergoes internal fertilization and possesses a modified type of spermatozoon, which is approximately 20 μm long. The axoneme of the tail flagellum consists of nine peripheral pairs of microtubules and one central pair. Many spermatozoa occur in the acini of the testis in the ripe stage and are transported to the seminal vesicles in the accumulating phase. In males, the monthly gonadosomatic index began to increase in September and reached a maximum in February. Subsequently, it decreased rapidly after April. The testis of this species can be classified into four developmental stages: the active (August to September), ripe (October to July), copulation (April to July), and recovery (July to August) stages. Structural changes in the epithelial cells of the seminal vesicles of this species could be classified into three phases: (I) S-I (resting), (2) S-II (accumulating), and (3) S-III (spent) phases. The morphology and structure of the epithelial cells of the seminal vesicle differed in each phase; the cells were cuboidal, squamous, or columnar in the resting, accumulating, or spent phases, respectively.

Key words: Spermatogenesis, Seminal vesicle, *Neptunea (Barbitonia) arthritica cumingii*, Testicular development

Introduction

Neptunea arthritica cumingii (Gastropoda: Buccinidae) is one of commercially important edible gastropods in East Asian countries, including Korea, Japan, China, and Russia (Yoo, 1976; Kwon et al., 1993). On the west coast of Korea, this species is mainly found in silty sand in the subtidal zone. Owing to the recent sharp reduction in the standing stock as a consequence of reclamation work, marine pollution, and reckless overharvesting of this snail, it has been denoted a target organism and fisheries resource that should be managed using a more reasonable fishing regime. For the propagation and management of a living natural resource, it is important to understand its population characteristics with regard to spermatogenesis and testicular development.

In other countries, studies have examined aspects of the reproduction of *Neptunea* spp., 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), and aspects of ecology, including the distribution (Ito and Tachizawa, 1981; Ito, 1982) and growth (MacIntosh and Paul, 1977; Fujinaga, 1987; Suzuki et al., 1996) of *N. arthritica*, and the feeding (Pearce and Thorson, 1967) of *N. antiqua*. However, little information is available on the ultrastructure of germ cells during spermatogenesis and structural changes in the epithelial cells of the seminal vesicle with testicular development in this species (Chung and Kim, 1996). The results of an ultrastructural study

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of spermatogenesis of this species will provide important information on its reproductive mechanism.

Understanding the process of testicular development and the spawning period of *N. arthritica cumingiiwill* provide necessary information for the collection of natural spat and on the recruitment period and age determination for the propagation and management of this species as a living resource.

For gastropods that undergo internal fertilization, many spermatozoa occur in the spermatogenic follicles of the testis and are transported to the seminal vesicle between the ripe stage and the copulation stage. Concerning the internal fertilization species of marine neogastropods, several researchers (Takahashi et al., 1972; Fujinaga, 1985; Chung and Kim, 1997) have reported morphological, structural, and functional changes of the epithelial cells of the seminal vesicle with testicular developmental stage. The cell shape changes from cuboidal to squamous to columnar as development progresses from the resting to the accumulating to the spent phases, respectively. In addition, the yellow granular bodies that appear in the cytoplasm of the epithelial cells in the inner layer of the seminal vesicle have been shown to play an important role in the absorption and digestion of a small number of undischarged spermatozoa in the lumen of the seminal vesicle in the spent and resting phases (during the copulation, recovery, and active stages of the testis) in *Buccinum undatum*, *Nucella lapillus* (Fretter, 1941), *N. arthritica* (Takahashi et al., 1972; Fujinaga, 1985), and *Rapana venosa* (Chung and Kim, 1997).

To understand the reproductive mechanism and mutual relationship between testicular development and structural changes in the seminal vesicle, we conducted a histological study of the structural changes of the epithelial cells of the seminal vesicle in *N. arthritica cumingii*, examining the ultrastructure of germ cells during spermatogenesis and the structural changes of epithelial cells with the developmental stage of the seminal vesicle associated with testicular development in *N. arthritica cumingii*.

Materials and Methods

Sampling

Specimens of *Neptunea* (*Barbitonia*) *arthritica cumingii* (Crosse, 1862) were collected monthly from the subtidal zone at Maldo, Chollabuk-do, Korea, from January 2002 to December 2003.

Gonadosomatic index

A total of 486 individuals were used to calculate the gonadosomatic index (GSI). Monthly changes in the mean GSI were calculated using the following equation (Chung et al., 2002) (Fig. 1):

$$GSI = \frac{\text{The thickness of the gonal} \times 100}{\text{Diameter of posterior appendage including the gonal and Digestive gland}}$$

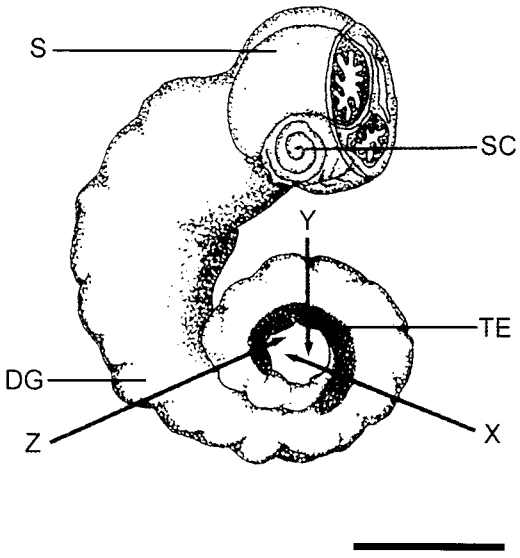

Fig. 1. Anatomy of a male *Neptunea arthritica cumingii* removed from its shell. Posterior appendage showing the testis and digestive gland. X, Y, and Z denote the sections used to measure the gonadosomatic index. The three sections are spaced equally. Abbreviations: DG, digestive gland; S, stomach; SC, stomachal caecum; TE, testis. Scale bar=4 cm.

Ultrastructure of germ cells during spermatogenesis

For electron microscopy, excised pieces of the testes were cut into small pieces and fixed immediately in 2.5% paraformaldehyde-glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h at 4℃. After the initial fixation, the specimens were washed several times with the same buffer and then fixed in 1% osmium tetroxide dissolved in 0.2 M phosphate buffer solution (pH 7.4) for 1 h at 4℃. The 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 the Epon-embedded specimens were cut with glass knives using a Sorvall MT-2 microtome and

a LKB ultramicrotome, at thicknesses of 800-1000 Å. The tissue sections were mounted on collodion-coated copper grids, stained with uranyl acetate followed by lead citrate, and examined using a JEM 100 CX-2 (80 kV) electron microscope.

Testicular development by histological observations

A total of 456 males were used for histological preparations of the testes for light microscopic examination, from January 2002 to December 2003. Testicular tissues were removed from the shells, preserved in Bouin's fixative for 24 h, and then washed with running tap water for 24 h. The tissues were then dehydrated in alcohol and embedded in paraffin molds. The embedded tissues were sectioned at 5-7 μm thicknesses using a rotary microtome. The 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

Position and morphology of the testis

N. arthritica cumingii is a dioecious species, which has well-defined female and male individuals. From visual observations, males can be distinguished easily by the existence of the penis (genital organ), which occurs near the tentacles. The testis is located on the surface of the digestive gland in the spiral posterior region of the shell (Fig. 1). With testicular maturation, the external color of the testis became yellowish brown.

Gonadosomatic index in males and seawater temperatures

The monthly GSI changes in males are shown in Fig. 2. In 2002, the GSI increased slowly after September and reached a maximum (2.77±0.47) in February, when the seawater was very cool. Then, the GSI decreased rapidly beginning in April, when seawater temperatures gradually increased and reached a minimum in June (1.20±0.23), when copulation continued. The monthly GSI changes in 2003 showed similar patterns to those in 2002.

Ultrastructure of germ cells during spermatogenesis

Based on the testicular development and morphological characteristics of germ cells, spermatogenesis can be classified into five phases: (1) spermatogonial, (2) primary spermatocyte, (3) secondary spermatocyte, (4) spermatid, and (5) spermatozoon phases.

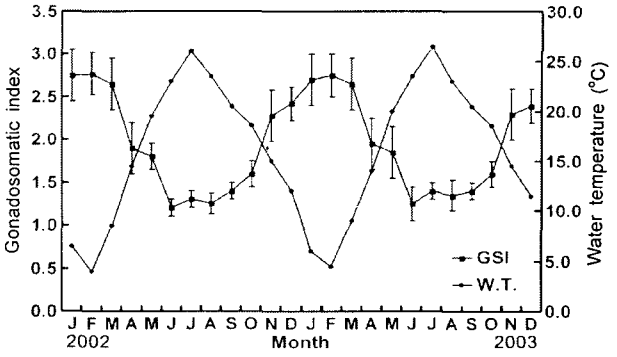

Fig. 2. Monthly changes in the mean gonadosomatic index in male *Neptunea arthritica cumingii* and the mean seawater temperature from January 2002 to December 2003.

Spermatogonial phase: The primary spermatogonia are located near the auxiliary cells. They are 9-10 μm in diameter and more or less oval shaped. The primary spermatogonia divide mitotically to produce secondary spermatogonia (Fig. 3A), which have smaller cells and nuclei than the primary spermatogonia.

Primary spermatocyte phase: Secondary spermatogonia differentiate into primary spermatocytes. The nucleus of the primary spermatocyte contains slightly denser chromatin. The synaptonemal complexes in the nucleus appear in prophase during the first maturation division. Several mitochondria appear in the cytoplasm (Fig. 3B).

Secondary spermatocyte phase: The primary spermatocyte develops into a secondary spermatocyte through the first maturation division (Fig. 3C).

Spermatid phase: After the secondary meiotic division, the secondary spermatocyte is transformed into a spermatid with electron dense chromatin in the nucleus. Spermiogenesis can be divided into four phases based on the characteristics of cell organelle differentiation: Golgi, cap, acrosome, and maturation phases (Figs. 3D-4D). The acrosomal vesicle changes into an acrosome during the acrosome phase, and the acrosome of the spermatozoon shows cone type (Figs. 4A, B).

Spermatozoon stage: At this stage, the centrioles gave rise to the axial filament of the flagellum of the spermatozoon, and sperm morphology showed the modified type, as found in internal fertilization species (Figs. 4C, D). After acrosome formation is

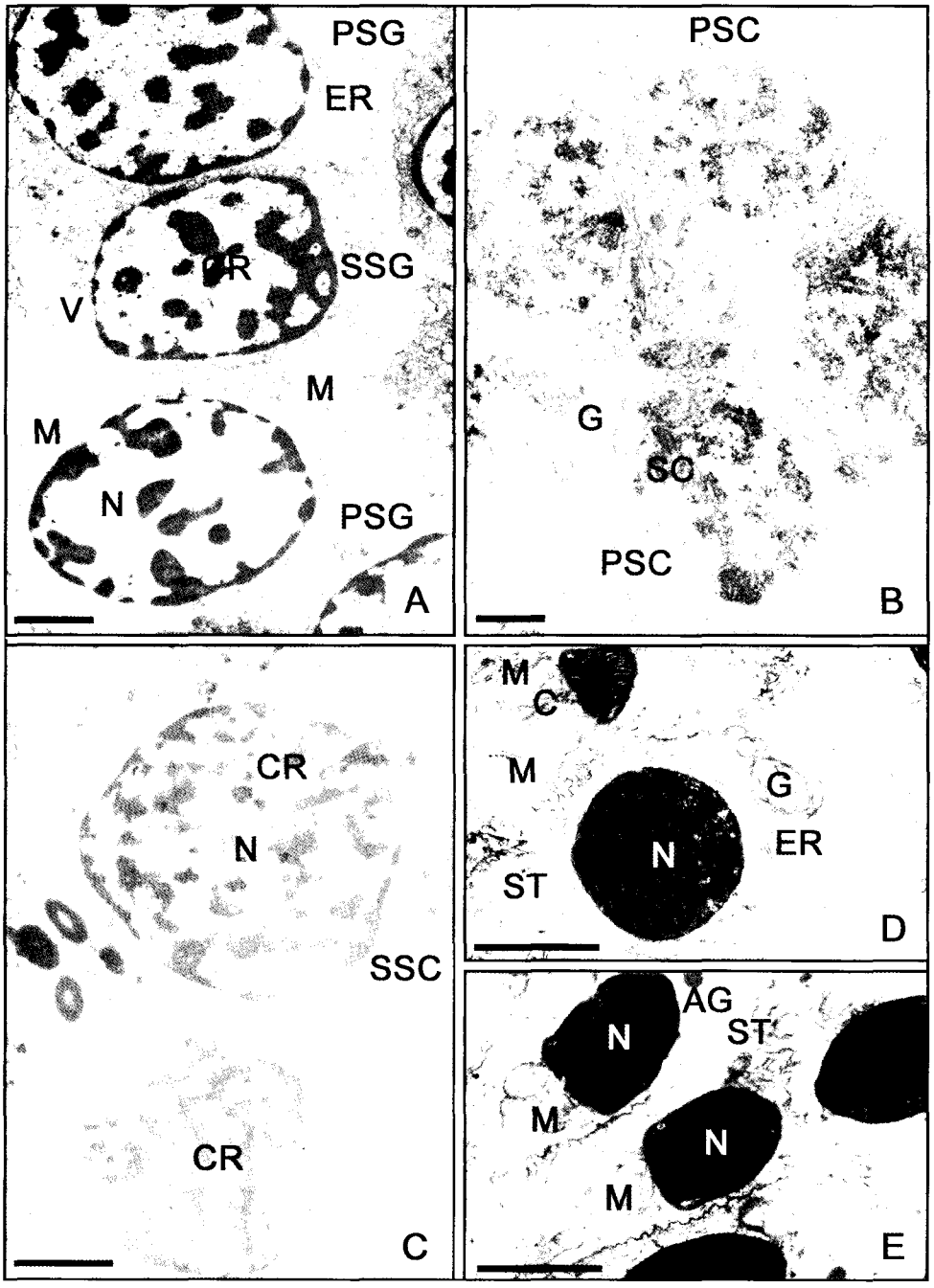

Fig. 3. Electron micrographs of spermatogenesis in *Neptunea arthritica cumingii* (A-E). A, Section of spermatogonia. Note the nucleus containing chromatin, and several mitochondria and the Golgi complex in the cytoplasm. B, Section of primary spermatocytes. Note the synaptonemal complexes in the nucleus during the prophase of meiosis. C, Section of secondary spermatocytes. Note the nucleus with denser chromatin. D, Section of spermatids. Note the aggregated chromatin in the nucleus, and the endoplasmic reticulum and Golgi complex in the cytoplasm. E, Early phase of spermatid differentiation. Note the Golgi complex and acrosomal granule near the nucleus, and the mitochondria and centrosome just behind the nucleus. Abbreviations: AG, acrosomal granule; C, centrosome; CR, chromatin; ER, endoplasmic reticulum; G, Golgi complex; M, mitochondrion N, nucleus PSC, primary spermatocyte; PSG, primary spermatogonium; SC, synaptonemal complex; SSC, secondary spermatocyte; SSG, secondary spermatogonium; ST, spermatid; V, vacuole. Scale bars=2 μm.

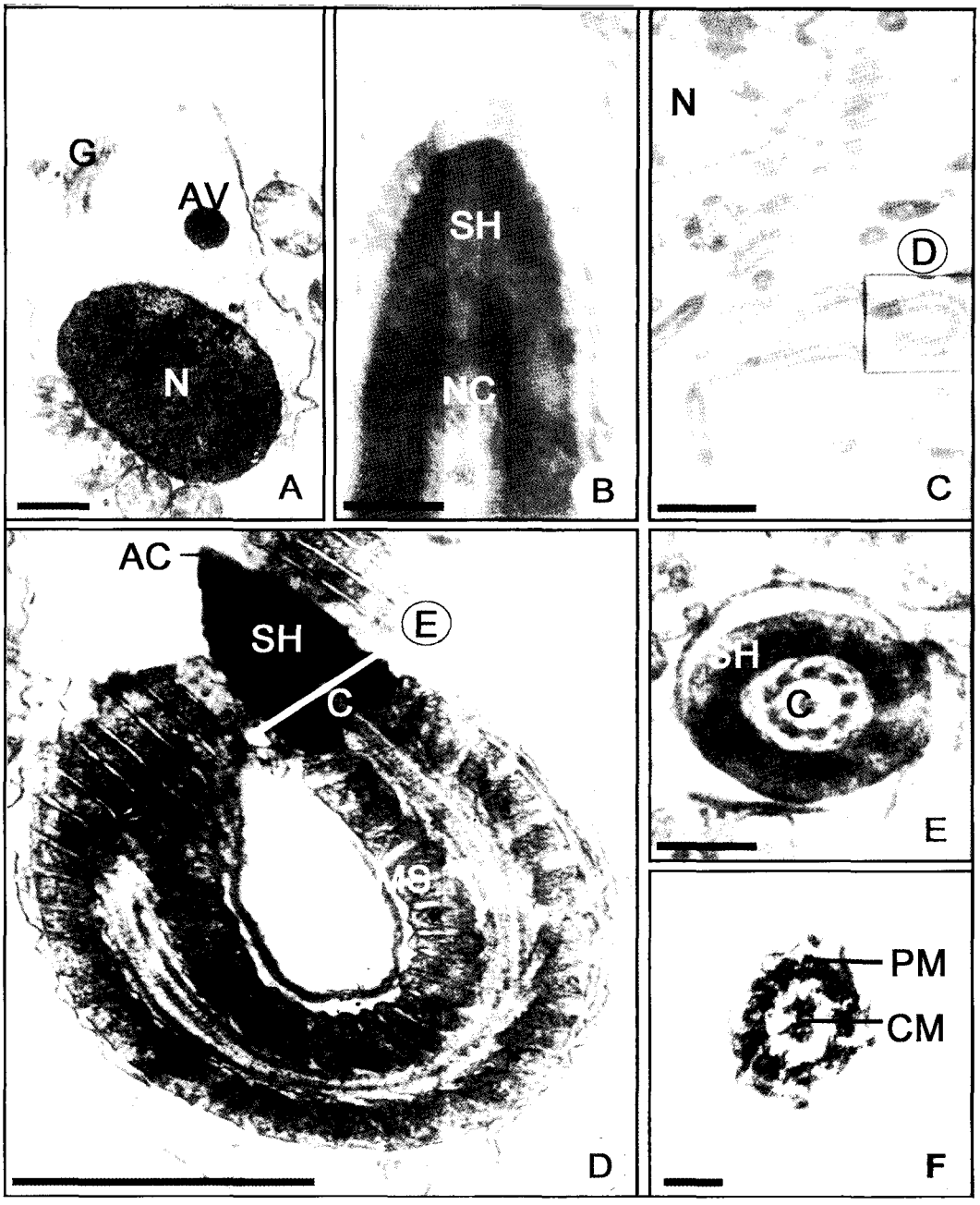

Fig. 4. Electron micrographs of spermiogenesis of spermatids of *Neptunea arthritica cumingii* (A-F). A, Early phase of spermatid differentiation. Note the Golgi complex and acrosomal vesicle near the nucleus, and the mitochondria just behind the nucleus. Scale bar=1 μm. B, C, Acrosome formation and elongated nucleus. Note the elongated acrosome that changed from an acrosomal vesicle, and the elongated nucleus. Scale bars= 1 μm. D in Fig. 4C, schematized diagram of a spermatozoon. Scale bar=1 μm. D, Acomplete spermatozoon. Note the acrosome, sperm head, and axial filament surrounded by a mitochondrial sheath. Scale bar= 1 μm; E, Cross-section of the nuclear canal of the sperm head. Note the centrosome in the central part of the sperm head. Scale bar=0.5 μm. F, Cross-section of the sperm tail. Note the axoneme of the tail flagellum composed of a pair of central microtubules and nine pairs of peripheral microtubules. Scale bar=0.5 μm. Abbreviations: AC, Acrosome; AV, acrosomal vesicle, AX, axial filament; C, centriole; CM, central microtubules; G, the Golgi complex; M, mitochondrion; MS, mitochondrial sheath; N, nucleus; PM, peripheral microtubules; SH, sperm head.

completed, the prominent nuclear canal in the nucleus is present in cross-sections of the sperm head (Fig. 4E). The spermatozoon head is ca. 5 μm long, including the 0.2-μm long acrosome, and its tail is ca. 20 μm long. The axoneme of the tail flagellum of the spermatozoon consists of nine pairs of peripheral microtubules, and one pair of central microtubules (Fig. 4F).

The process of testicular development

Based on germ cell development and the morphological characteristics seen with light microscopy, the development of the testis can be classified into four stages: (1) active, (2) ripe, (3) copulation, and (4) recovery stages. The criteria for defining each stage are as follows.

Active stage: The spermatogonia and spermatocytes appear along the germinal epithelium (acinus wall) (Fig. 5A). As testicular development advances, spermatocytes, spermatids, and a small number of spermatozoa occupy approximately one-third to one-half of the lumina in the acini (Fig. 5B). Individuals in the active stage appeared between August and September, when the seawater was relatively warm.

Ripe stage: The ripe testis is characterized by the formation of a number of spermatozoa in the center of the lumen (Fig. 5C). Individuals in the ripe stage were found from October through July.

Copulation stage: Large numbers of spermatozoa in the acini move to the seminal vesicle. Spermatozoa in the seminal vesicle are discharged into the ovary during copulation. The lumina of the acini and the seminal vesicle are quite empty (Fig. 5D). The copulation stage occurred from April to July, when the seawater gradually warmed.

Recovery stage: A small number of undischarged spermatozoa and residual spermatids are degenerated (Fig. 5E). Thereafter, a few newly formed spermatogonia on the germinal epithelium and connective tissues are rearranged between the acini at this stage (Fig. 5F). Individuals in this stage appeared between July and August.

Structural changes in the epithelial cells of the seminal vesicle with the developmental stage of the testis

Based on the histological and morphological characteristics of the epithelial cells of the seminal vesicle wall, *viz.*, the convoluted part of the vas deferens, and the relative number of spermatozoa accumulated in the lumina of the vesicles, morphological changes in the seminal vesicles could be classified into three phases using the classification of Chung and Kim (1997): (1) S-I (resting), (2) S-II (accumulating), and (3) S-III (spent) phases. These successive phases showed a periodicity with the developmental stage of the testis. The criteria used to define the stages are as follows.

S-I phase (Resting): The fibrous connective tissues that comprise the vas deferens consist of several thick layers, but they are somewhat contracted. The epithelial cells in the inner layer of the seminal vesicle are composed of a single layer of ciliated cuboidal cells and have oval or conical nuclei that are stained darkly with hematoxylin. At this phase, a few yellow granular bodies appear in the cytoplasm of the epithelial cells in the inner layer of the seminal vesicle (Fig. 6A). The resting phase of the seminal vesicle is seen when the testes are in the recovery and active stages.

S-II phase (Accumulating): In the early accumulating phase of the seminal vesicle, the epithelial cells change into cuboidal cells with round nuclei (Fig. 6B), and the yellow granular bodies disappear. Subsequently, as the sperm masses produced in the acini enter the seminal vesicle, the vesicles become milky white. In the late accumulating phase, a number of sperm masses are present in the lumina of the vesicle, and the cuboidal cells in the epithelium of the seminal vesicle change into squamous cells. Each squamous cell has an elongated nucleus, and the connective tissues in the outer layer of the vesicle become thin (Fig. 6C). The accumulating phase of the seminal vesicle continued during the ripe stage of the testis.

S-III phase (Spent): During this phase, a number of discharged sperm masses are still present in the seminal vesicles. The ciliated epithelial cells of the inner layer change into columnar cells, which become taller. Furthermore, the fibrous connective tissues of the outer layer increase in thickness remarkably (Fig. 6D). This phase was found during the period of copulation.

Discussion

Spermatogenesis in *Neptunea arthritica cumingii* is very similar to that in other neogastropods that undergo internal fertilization (Chung and Kim, 1997). However, fine structural differences in molluscan sperm structures, which are associated with the evolution of the species, are sometimes used as criteria

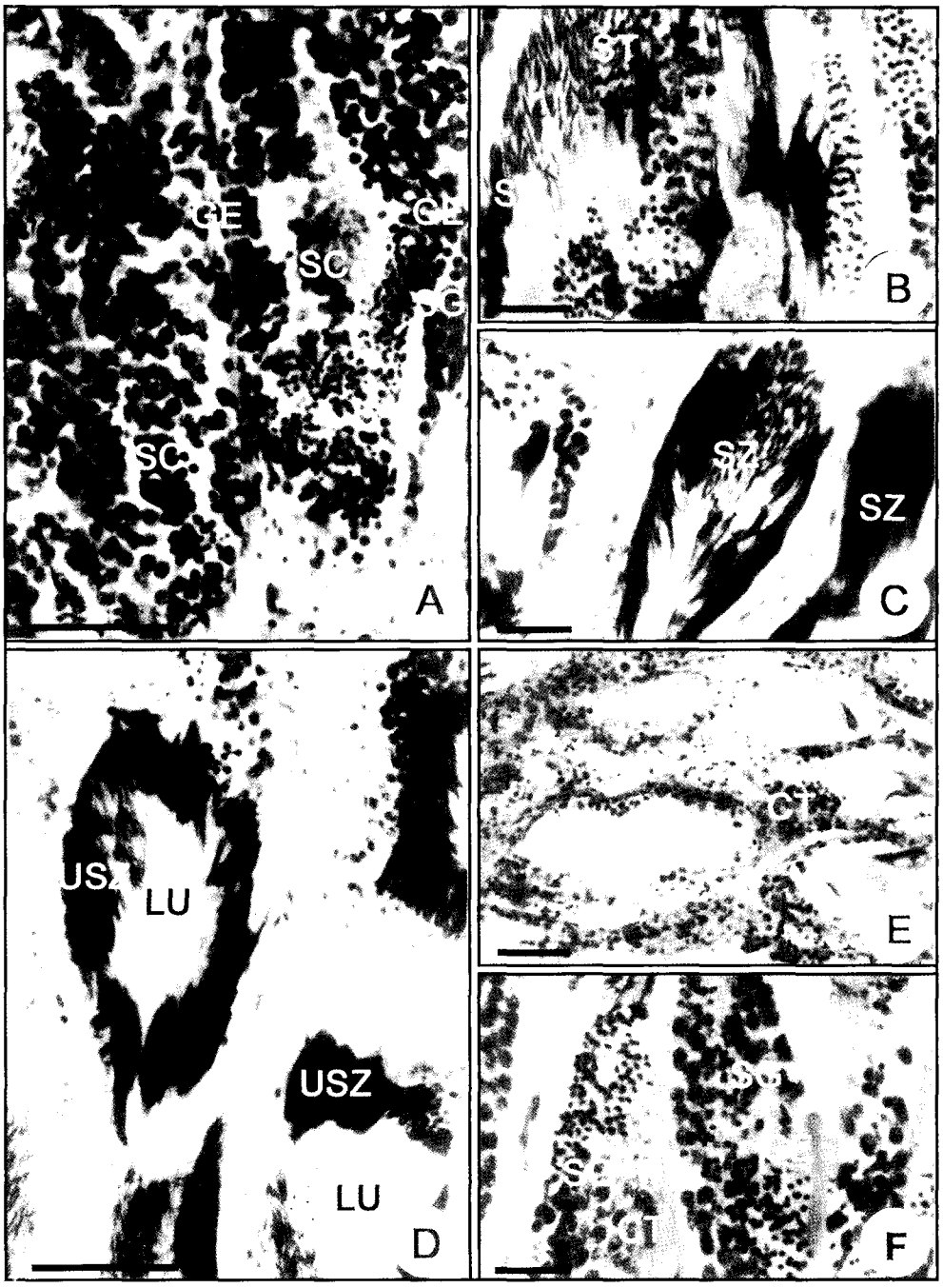

Fig. 5. Photomicrographs of the gonadal phases of male *Neptunea arthritica cumingii* as seen with light microscopy. A, B, Sections of the acini in the active stage. C, Sections of the acini in the ripe stage. D, Section of the acini in the copulation stage. E, F, Sections of the acini in the recovery stage. Abbreviations: CT, connective tissue; GE, germinal epithelium; LU, lumen; RS, residual body; SC, spermatocyte; SG, spermatogonium; ST, spermatid; SZ, spermatozoon; USZ, undischarged spermatozoon. Scale bars=50 μm.

for classification (Popham, 1979). Franzen (1956) divided molluscan sperm morphology into two types: 1) the primitive type found in external fertilization species and 2) the modified type found in internal fertilization species. Verdonk et al. (1983) divided sperm morphology into four types: 1) primitive, 2) modified, 3) biflagellate, and 4) aflagellate types. In addition to the primitive type and partially modified

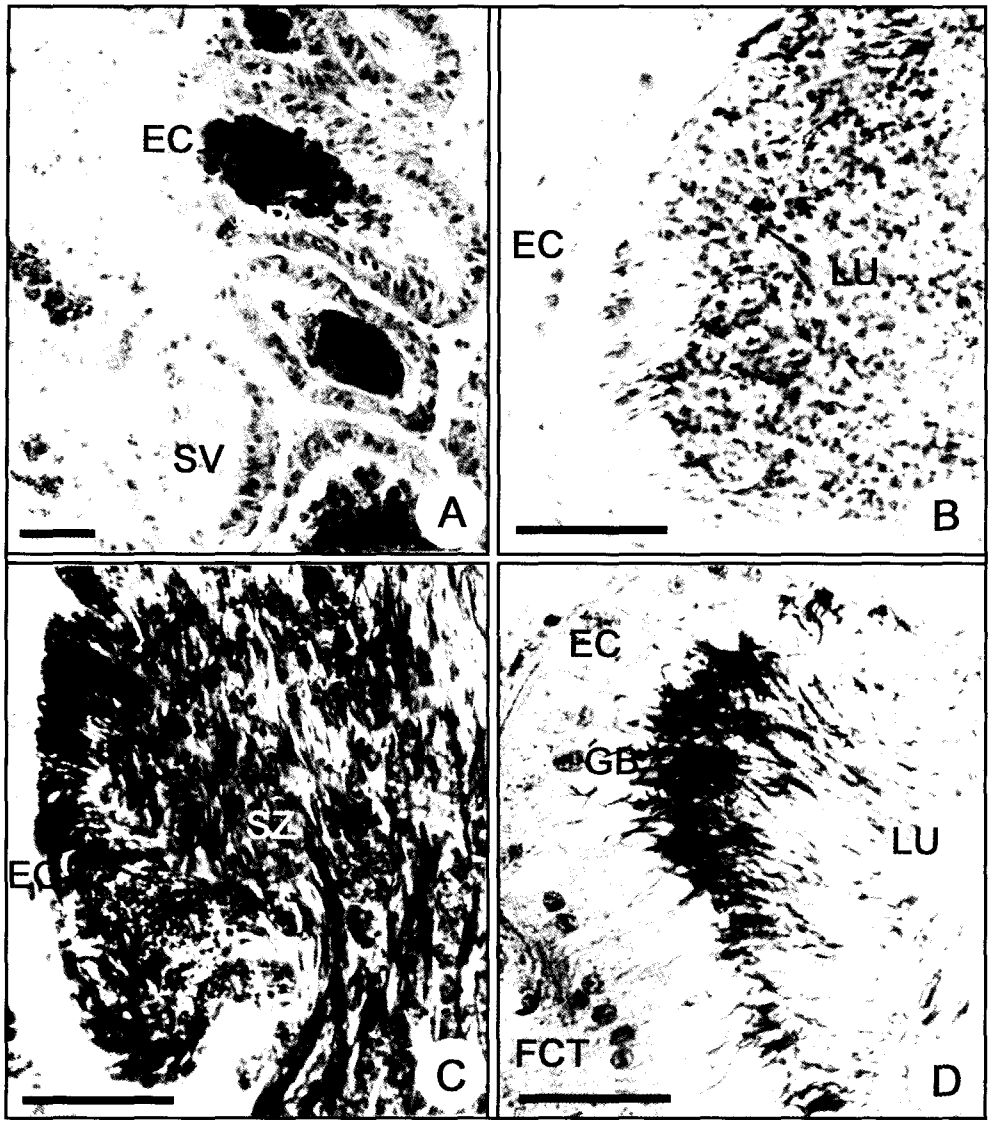

Fig. 6. Morphological and structural characteristics of the epithelial cells of the seminal vesicle in male *Neptunea arthritica cumingii*. A, Section of epithelial cells of the inner layer of the seminal vesiclein the resting phase. Note the ciliated cuboidal cells with oval or conical nuclei in the epithelial cells of the inner layer, and the relatively thick fibrous connective tissue in the outer layer. B, Section of the epithelial tissue of the seminal vesicle in the early accumulating phase. Note the small number of spermatozoa in the lumen of the seminal vesicle with cuboidal cells. C, Section of epithelial cells of the seminal vesicle in the late accumulating phase. Note the spermatozoa in the lumen of the seminal vesicle with ciliated squamous cells. D, Section of the epithelial cells of the seminal vesicle in the spent phase. Note the undischarged spermatozoa in the lumen of the seminal vesicle with ciliated columnar cells in the inner layer, and the remarkably thick fibrous connective tissue in the outer layer. Abbreviations: CT, connective tissue; EC, epithelial cell; FCT, fibrous connective tissue; GB, granular body; LU, lumen; SV, seminal vesicle; SZ, spermatozoon. Scale bars= 30 μ m.

type of molluscan sperm, a biflagellate type is seen in the triploid *Corbicula fluminea* and *C. leana* in natural populations (Komaru and Konishi, 1996; Komaru et al., 1997; Choi, 2004). An aflagellate type is also found in a few crustacean (Kim, 2001). *Neptunea arthritica cumingii* undergoes internal fertilization and possesses the modified type of spermatozoon, unlike the primitive type found in most

bivalves.

The acrosome morphology of the sperm head differs markedly between species (Popham, 1979). The acrosome shape can be classified into four types: cone, cap, elongate-modified cone, and modified cap types. Moreover, sperm nucleus types vary with molluscan species. The sperm nuclei are cylindrical in *Septifer virgatus* and some *Mactra* and *Pernidia venulosa*; global in *Spisula sachalinensis* and *Tersus keenae*; ovoid in the Ostreidae, P*inctata fucata martensii*, and *Atrina pinnata japonica*; vase-shaped in the Pectinidae; dome-shaped in *Mytilus*; elongate or modified cylindrical in the Veneridae; jar-shaped in *Solen grandis* and arrow-shaped in *Corbicula japonica* (Kim, 2001).

In *N. arthritica cumingii*, the acrosome is the cone type, although it lacks the acrosomal rod seen in *Corbicula japonica* (Lee et al., 2004) and other bivalves. The sperm nucleus is the modified cylindrical type seen in internal fertilization species. As in a few marine neogastropods that undergo internal fertilization, there are no axonemal lateral fins on the sperm tail (Chung and Kim, 1997). Sperm morphology is species-specific and thus is a good tool for identifying molluscan groups (Kim, 2001).

Takahashi et al. (1972) and Fujinaga (1985) described the structural changes and functions of the genital ducts. In the present study, we observed similar morphological and structural changes in the epithelial cells of the seminal vesicle: they changed into cuboidal, squamous, and columnar cells in the resting, accumulating, and spent phase, respectively, as seen in *Neptunea arthritica* (Takahashi et al., 1972; Fujinaga, 1985) and *Rapana venosa* (Chung and Kim, 1997). Therefore, we assume that the morphology and structure of the epithelial cells of the seminal vesicle vary with the developmental stage of the testis.

In the seminal vesicles of *Neptunea arthritica*, the epithelial cells in the inner layer of the vesicle underwent remarkable morphological changes with developmental stage. In the resting phase before sperm accumulation in the seminal vesicle, yellow granular bodies were seen in the epithelial cells in the inner layer, and these bodies disappeared during sperm accumulation. However, they reappeared during the spent phase of the seminal vesicle. Fretter (1941) reported that yellow granular bodies in the cytoplasm of the epithelial cells in the inner layer in *Buccinum undatum* and *N. lapillus* play an important role in the absorption and digestion of residual sperm. Moreover, Takahashi et al. (1972) reported that yellow granular bodies in the cytoplasm of epithelial cells of the seminal vesicle of *N. arthritica* have a lysosome-like structure. Chung and Kim (1997) reported that, in *Rapana venosa*, yellow granular bodies in the cytoplasm of the inner layer of epithelial cells in the seminal vesicle appeared in the spent and resting phases, and disappeared during the accumulating phase. They postulated that the yellow granular bodies in the epithelial cells play an important role in the absorption and digestion of residual sperm.

In this study, we observed granular bodies in the cytoplasm of epithelial cells of *Neptunea arthritica cumingii* during the spent and resting phases, as reported by Fretter (1941), Takahashi et al. (1972), and Chung and Kim (1997). We came to similar conclusions; however, further electron microscopic study is needed to examine the structure, function, and fate of the yellow granular bodies.

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