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Reproductive Cycle and Spawning Rhythm of the Ascidian, *Halocynthia hilgendorfi ritteri*

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Reproductive cycle and spawning rhythm with lunar cycle of the ascidian, *Halocynthia hilgendorfi ritteri* were investigated by histological examination. The specimens were sampled in the coastal waters of Yongdam, northwest of Jeju Island, Korea, from November 2001 to January 2003. *H. hilgendorfi ritteri* is a synchronous hermaphrodite; the gonads are located in the mantle. The reproductive cycle can be grouped into the following successive stages in the ovary: growth (February to June), vitellogenesis (April to September), mature (July to December), spent (November to February), and recovery (December to April). Likewise, in the testis, the stages observed were: growth (October), mature (October to December), spent (November to February), and resting (January to September). Major spawning probably occurs between November and January, when water temperatures decrease. The histological observations of the gonads suggested that this species is a multiple spawner during the spawning period. Spawning occurred between the new moon and full moon, and again between the full moon and new moon, suggesting that the spawning rhythm is influenced by the lunar cycle.

Halocynthia hilgendorfi ritteri is a solitary ascidian that belongs to the family Pyuridae, order Pleurogona, class Ascidiacea. It is generally found on the surfaces of rocks in subtidal water. *H. hilgendorfi ritteri* is a comparatively large species of ascidian. As it is usually covered with algae or hydra and its shape and color are similar to surrounding rock, it is well camouflaged. Its common name in Korean reflects this association; *Dol meong ge* means "stone sea squirts." Ascidians, or sea squirts, are sessile marine animals that are ubiquitous throughout the world. Most live attached to rocks and shells, although some are found on muddy or sandy substrates. Ascidians can be divided into two groups according to their patterns of reproduction and ecology. Some species live as individuals (solitary or simple ascidians), while others form colonies (colonial or compound ascidians). The solitary ascidians are usually hermaphrodites that propagate by sexual reproduction, whereas colonial ascidians reproduce both sexually and asexually by budding or strobilation (Kessel, 1983; Cloney, 1990).

The reproductive cycle of marine animals is controlled by both endogenous rhythms and exogenous environment (Giese and Pearse, 1974; Himmelman, 1980). The spawning periods of ascidians differ according to the reproduction strategy and geographical position (Millar, 1971, Berrill, 1975), and spawning events show species specificity (Fretter, 1984). *Didemnum* sp. breed throughout the year, while the spawning seasons of *Microcosmus sabatieri*, *Halocynthia papillosa* and *Halocynthia roretzi* are limited to two- to three-month periods (Becerro and Turon, 1992; Park et al., 1991). *Polycarpa cryptocarpa kroboja*, *Ciona intestinalis, Styela clava* and *Styela plicata* have spawning seasons that extend over 5 to 8 months (Chen and Dai, 1998; Yang and Lee, 1978; Lee, 1976, 1977). As described above, the reproduction strategies of ascidians are species specific and vary with their ecological distribution and morphological and biological patterns. Therefore, studies on the reproduction of ascidians are essential for understanding their ecological roles. The present study investigated the reproductive cycle based on gametogenesis, stage of gonadal development and monthly changes in oocyte diameter in order to characterize the reproductive biology of *H. hilgendorfi ritteri* inhabiting the coastal waters of Jeju Island, Korea.

Materials and Methods

H. hilgendorfi ritteri was sampled by scuba diving at a depth of 3-9 meters in the coastal waters of Yongdam,

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northwest of Jeju Island, Korea. Samples were harvested monthly from November 2001 to October 2002, and weekly from 5 November 2002 to 17 January 2003. Pieces of the gonad were fixed in Bouin's solution and then embedded in paraffin. The tissues were sectioned at 5-6 μm and stained with Hansen's hematoxylin and 0.5% eosin, and the specimens were examined under a light microscope. To investigate monthly changes in oocyte diameter, about 1,000 oocytes (sectioned through the nucleus) per month were measured by image analysis (Image scope 2.3, Image Line, Inc.). The developmental stages of the gonad were grouped according to Lee's guidelines (1976) in successive stages. In the ovary the stages were growth, vitellogenesis, mature, spent, and recovery. In the testis the stages were growth, mature, spent, and resting.

Results

Morphological features of H. hilgendorfi ritteri and gonadal structure

H. hilgendorfi ritteri is a comparatively large, barrel-shaped ascidian that attaches to rocks or other substrates. The oral and atrial siphon are located on the anterior end of the body, with the atrial siphon located somewhat lower than the oral siphon. The entire body is invested with a thick covering, and since they are usually covered with algae or hydra, they are hardly distinguishable (Fig. 1A). Once the covering material is removed, *H. hilgendorfi ritteri* has soft yellowish brown muscle tissue (Fig. 1B). It is hermaphroditic, having a pair of gonads inside the right and left body walls. Each gonad consists of a small ovary and peripheral testicular lobes. In the mature gonad, the ovary is red and the testis is white (Fig. 2A). As seen in cross sections of the gonad and the body wall, the outside body wall is composed of fibrous connective tissue including muscular fibers, and the chamber-shaped ovary and peripheral testicular lobes are connected to the outside body wall (Fig. 2B).

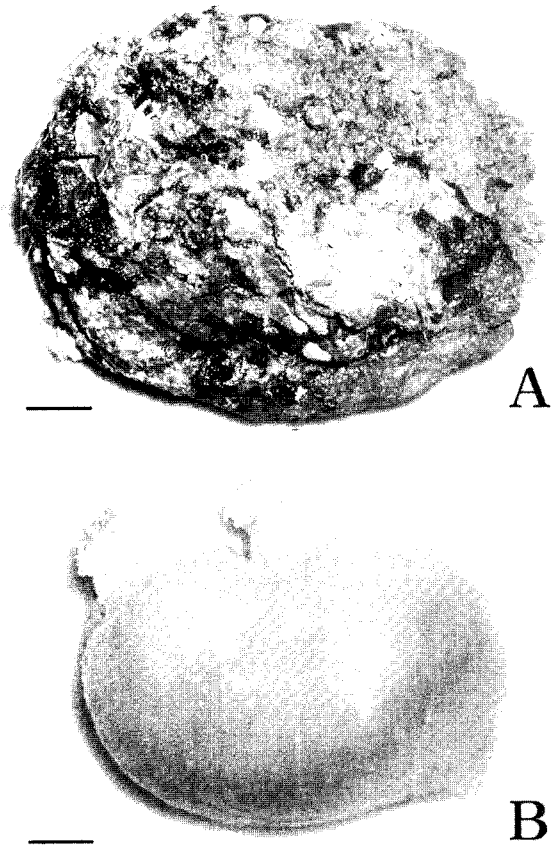

Fig. 1. Morphological feature of *Halocynthia hilgendorfi ritteri*. A, External shape. The entire body is invested with a thick covering. B, Internal shape. *H. hilgendorfi ritteri* has soft yellowish brown muscle tissue. Scale bars=10 mm.

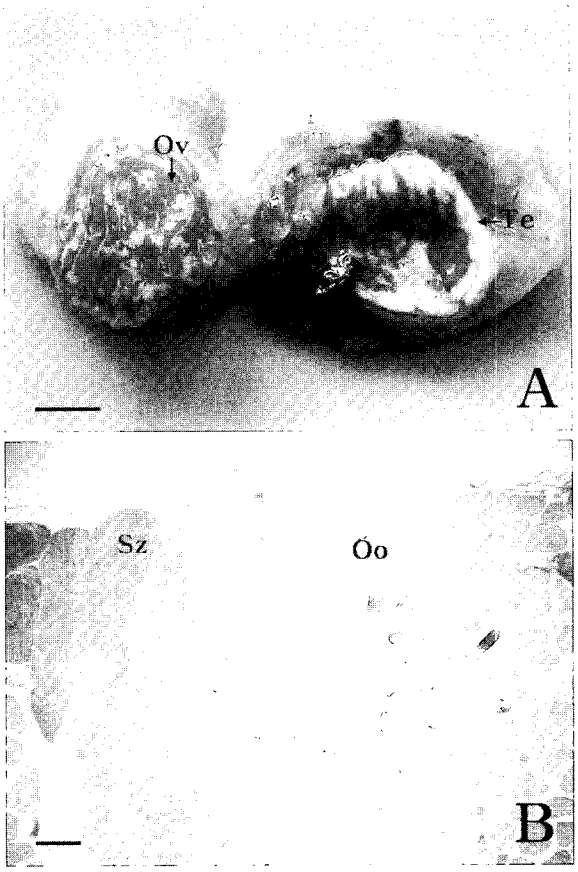

Fig. 2. Appearance of gonad (A) and cross section of gonad (B) of *Halocynthia hilgendorfi ritteri*. A, Each gonad consists of a small ovary and peripheral testicular lobes. B, The chamber-shaped ovary and peripheral testicular lobes are connected to the outside body wall. Oo, oocyte; Ov, ovary; Sz, spermatozoa; Te, testis. Scale bars=10 mm (A) and 200 mm (B).

Oogenesis

The ovary has a single germinal epithelium surrounded by basement membrane; the germinal epithelium and basement membrane are connected by fibrous connective tissue. The oogonia multiply in the ovarian cavity and the germinal epithelium of the ovarian tubule. The oogonia are round, approximately 10-20 μm in diameter, and have a small cytoplasmic volume, since the 7-15 μm nucleus occupies the majority of the intracellular space. The early oocytes accumulate uniform granular material in the cytoplasm and reach a

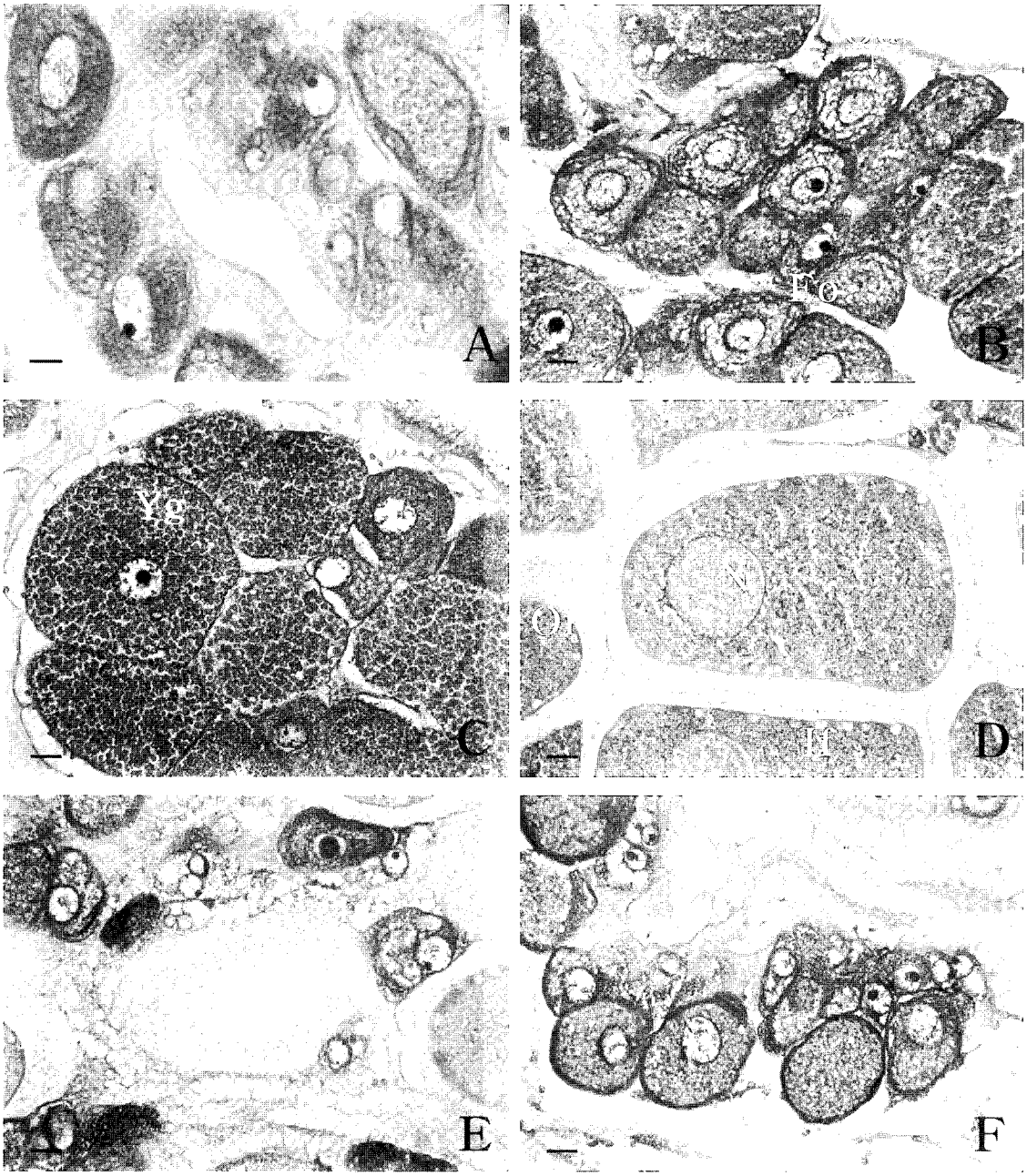

Fig. 3. Photomicrographs of ovarian development of *Halocynthia hilgendorfi ritteri*. A, Ovary in the early growing stage. Oogonia multiplied in the germinal epithelium of the ovarian lobule. B, Ovary in the growing stage. Early oocytes grow into the ovarian lumen. C, Ovary in the vitellogenesis stage. Cytoplasm begins to accumulate yolk granules. D, Ovary in the mature stage. Mature oocyte is surrounded by outer follicle cells, inner follicle cells and test cells. E, Ovary in the spent stage. Empty follicle layer remaining in the lumen has degenerated. F, Ovary in the recovery stage. Oogonia and early oocytes have begun to grow along the epithelium of the lumen again. Ef, empty follicle; Eo, early oocyte; If, inner follicle layer; N, nucleus; Od, oil droplet; Of, outer follicle layer; Oo, oogonia; Tc, test cell. Yg, yolk globule. Scale bars=25 μm

diameter of about 40 μm, containing a voluminous nucleus with a nucleolus (Fig. 3A). The early growing oocytes are about 80-100 μm in diameter with diffuse oil droplets throughout the cytoplasm (Fig. 3B). When the growing oocytes attain a diameter of about 130-200 μm, the cytoplasm begins to accumulate yolk granules and test cells appear (Fig. 3C). The mature oocytes are about 220-260 μm in diameter, with numerous yolk granules homogeneously distributed in the cytoplasm. The test cells are arranged near the vitelline coat. The double vitelline coat separates the vitelline coat from the outer follicle layer. The inner follicle layer is formed in the space between the vitelline coat and the outer follicle layer. The mature oocyte is surrounded by outer follicle cells, inner follicle cells and test cells (Fig. 3D). After spawning, the inner follicle layer is discharged together with the mature oocyte; the outer follicle layer is left in the ovarian cavity and undischarged mature oocytes remain and degenerate in the lumen (Fig. 3E). As the undischarged oocytes degenerate, the ovary contracts rapidly. Thereafter, oogonia and early oocytes reappear in the germinal epithelium (Fig. 3F).

Spermatogenesis

The testis forms as testicular lobules peripherally to the ovary. The testicular lobule form a basement membrane in the loose connective tissue, and spermatogonia are distributed in the germinal epithelium. Thereafter, the testicular lobule enlarges and numerous spermatocytes and spermatids develop from germinal epithelium in the median cavity (Fig. 4A). As the testis develops, the testicular lobules enlarge further and numerous spermatozoa occupy the majority of the lumen (Fig. 4B). After spawning, undischarged spermatozoa remain in the lumen of the testicular lobule (Fig. 4C). Thereafter, the undischarged spermatozoa degenerate and spermatogonia reappear along the germinal epithelium (Fig. 4D).

Monthly and weekly changes in oocyte diameter

The range of oocyte diameters was determined monthly from November 2001 to October 2002 (Fig. 5). In November 2001, a mixture of early oocytes (10-80 μm) and mature oocytes (200-260 μm) were present in the

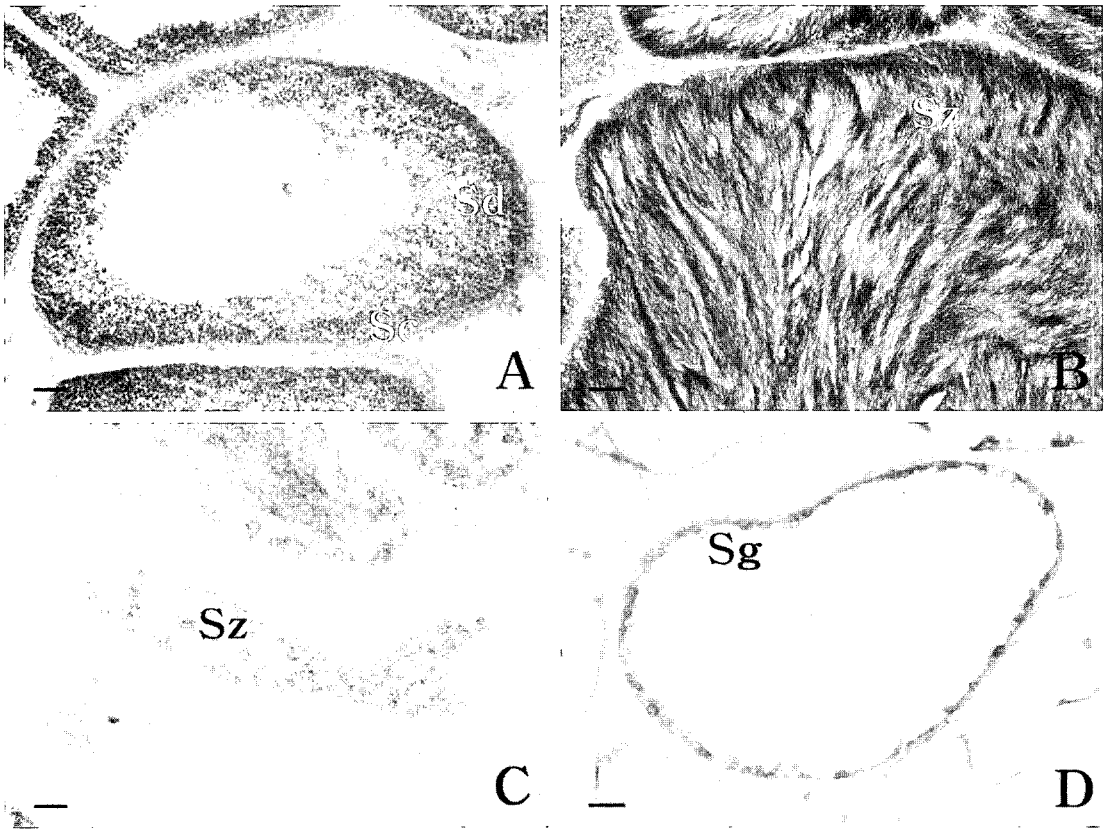

Fig. 4. Photomicrographs of testicular development of *Halocynthia hilgendorfi ritteri*. A, Testis in the growing stage. Numerous spermatocytes and spermatids develop from germinal epithelium in the median cavity. B, Testis in the mature stage. Numerous spermatozoa occupy the majority of the lumen. C, Testis in the spent stage. Undischarged spermatozoa remain in the lumen of the testicular lobule. D, Testis in the resting stage. Spermatogonia reappear along the germinal epithelium. Sc, spermatocytes; Sd, spermatid; Sg, spermatogonia; Sz, spermatozoa. Scale bars=25 μm.

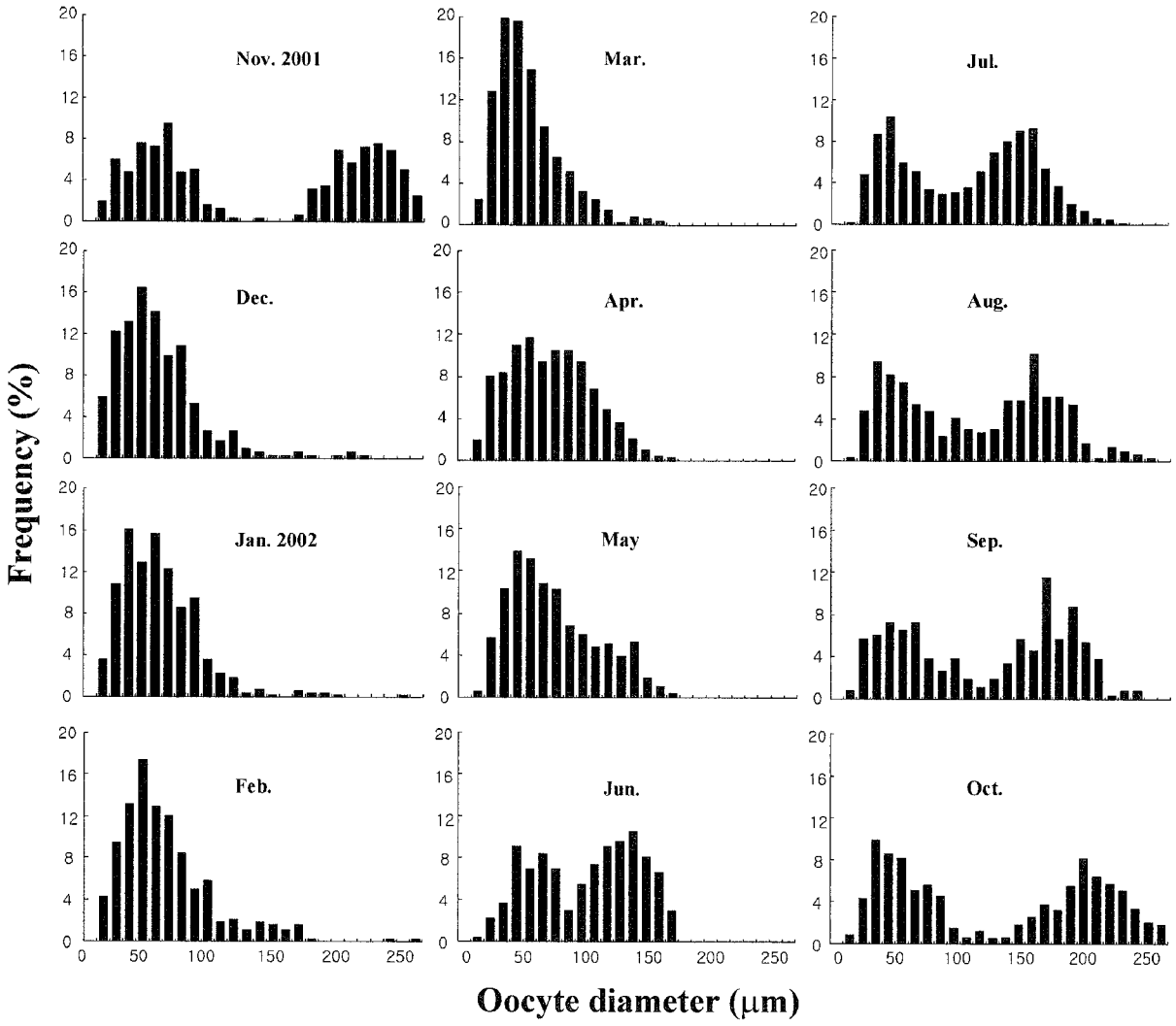

Fig. 5. Monthly changes of oocytes diameter in the ovary of *Halocynthia hilgendorfi ritteri* from November 2001 to October 2002.

ovary. Some undischarged mature oocytes were observed in December, but early oocytes occupied the majority of the ovary. From March 2002, oocytes over 100 μm in diameter began to appear, and from May to July, the oocytes ranged from 130 to 200 μm in size. In August, oocytes over 200 μm in diameter began to appear, and from September to October, mature oocytes (220-260 μm) occupied the majority of the ovary. To investigate the spawning rhythm based on the lunar cycle (new moon, first-quarter moon, full moon and last-quarter moon), weekly changes in oocyte diameter were measured from 5 November 2002 to 17 January 2003 (Fig. 6). During this period, a mixture of early oocytes (under 100 μm) and mature oocytes (over 200 μm) were present in the ovary. However, in November and December, the presumed major spawning period, the proportion of mature oocytes decreased on 13 November (first quarter moon), 28 November (last quarter moon), and 14 December (first quarter moon).

Reproductive cycle

Based on gametogenesis, gonadal development, monthly changes in oocyte diameter, and reproductive cycle of *H. hilgendorfi ritteri* could be grouped into successive stages. In the ovary, the stages were growth, vitellogenesis, mature, spent, and recovery; in the testis, the stages were growth, mature, spent, and resting (Fig. 7).

Growth stage

In the ovary, oogonia multiplied in the germinal epithelium of the ovarian lobule and early oocytes grew into the ovarian lumen. They appeared from February to June. In

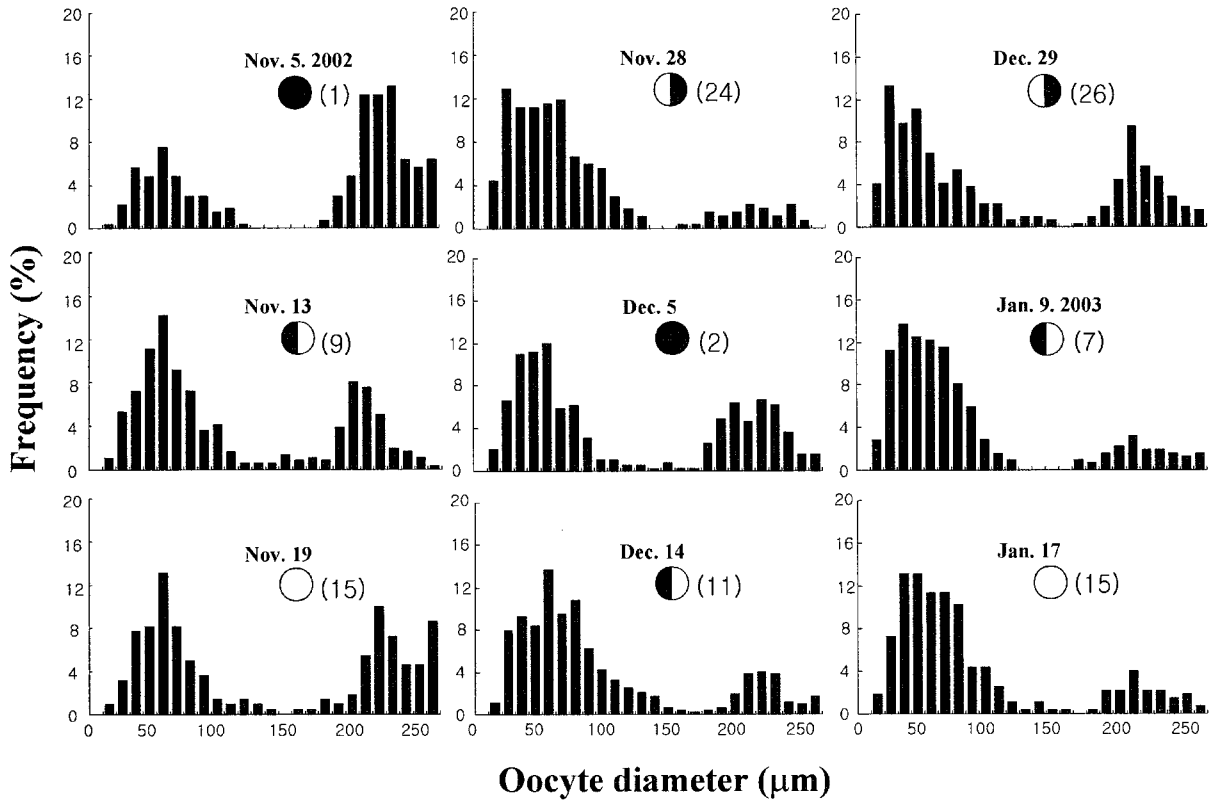

Fig. 6. Weekly changes of oocytes diameter in the ovary of *Halocynthia hilgendorfi ritteri* from November 2002 to January 2003.

the testis, a few spermatocytes and numerous spermatogonia were observed along the germinal epithelium, and a few spermatids and spermatozoa were also observed. This stage appeared in October.

Vitellogenesis stage

When early oocytes reached approximately 80-100 μm in diameter, the cytoplasm rapidly increased and was surrounded by primary follicle cells. Thereafter, oocytes grew to approximately 130-200 μm in diameter, and test cells appeared in the cytoplasm. From this time, yolk granules accumulated and grew rapidly in the cytoplasm. They appeared from April to September.

Mature stage

The mature ovary had a few early growing oocytes, but mature oocytes occupied the majority of the lumen. The mature oocytes were about 200-260 μm in diameter, with numerous yolk granules homogeneously distributed in the cytoplasm. The mature oocytes were surrounded by outer follicle cells, inner follicle cells and test cells. They appeared from July to December. In the testis, the number of spermatocytes decreased, and numerous spermatids and spermatozoa occupied the majority of the lumen. The haematoxylin-stained heads of the spermatozoa were oriented toward the wall of the testicular lobule, with the eosin-stained tails in the lumen. This stage appeared from October to December.

Spawning stage

The mature oocytes were released into the ovarian lumen from the outer wall. During spawning, the inner follicle layer was discharged together with the mature oocyte; the outer follicle layer was left in the ovarian cavity. Thereafter, oogonia and early oocytes appeared in the germinal epithelium, and the undischarged mature oocytes and outer follicle layer remaining in the lumen degenerated. This stage appeared from November to February. When the testis was spent, a few of the spermatozoa remained in the lumen; spermatocytes and spermatids in the germinal epithelium of basement membrane degenerated. They appeared from November to February.

Recovery and resting stages

In the ovary during the recovery stage, oogonia (10-20 μm) and early oocytes (40-60 μm) began to grow along the epithelium of the lumen again. The fibrous connective tissue developed into the lumen from the outer wall, and the undischarged mature oocytes in the lumen mostly degenerated. They appeared from December to April. In the resting stage testis, undischarged spermatozoa

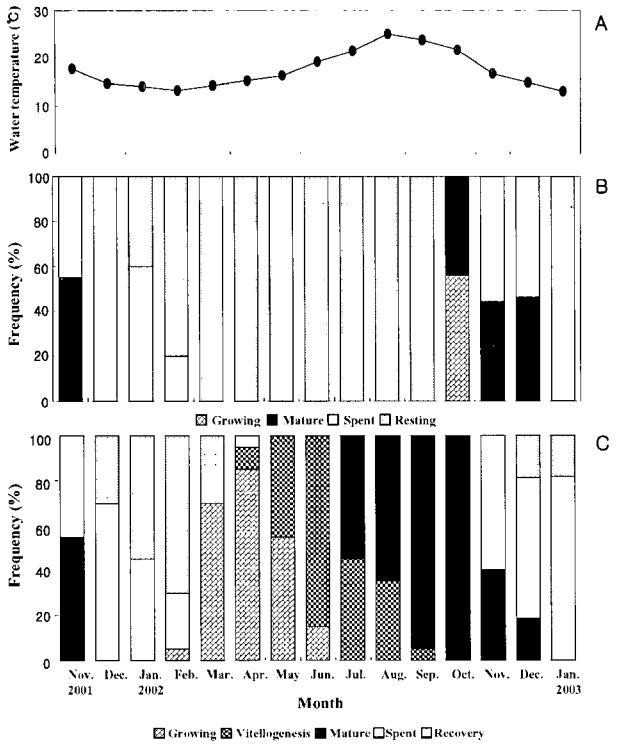

Fig. 7. Monthly changes in water temperature (A), frequency of testes (B) and ovary (C) developmental phase of *Halocynthia hilgendorfi ritten* from November 2001 to January 2003.

in the lumen mostly degenerated; spermatogonia appeared along the germinal epithelium. This stage appeared from January to October.

Discussion

Gametogenesis of ascidians has been well studied with respect to the growth of oocytes and the role of the follicle cells. The envelope surrounding oocytes is composed of test cells, inner follicle cells and outer follicle cells. The follicle cells and test cells arise from undifferentiated cells of the germinal epithelium (Berrill, 1975). According to Kessel and Kemp (1962), the inner follicle cells can synthesize or store yolk precursors, and test cells are considered to be able to transfer their granules to the oocyte. It has also been suggested that test cells are secretory cells (Knaben, 1936), produce pigment granules (Kessel and Beams, 1965), and provide the oocyte with yolk precursors (Mancuso, 1965). In this study, the test cells appeared in the vitellogenesis stage and were arranged near the vitelline coat of mature oocytes (220-260 μm). These results suggest that the test cells correlate with the maturation of oocytes, including the accumulation of yolk precursors.

Gonadal development and reproductive cycles of marine invertebrates are controlled by endogenous rhythms, with an annual or lunar phase, and by the exogenous environment, including temperature, photoperiod, salinity, food abundance, and mechanical stimulation (Giese and Pearse, 1974; Himmelman, 1980). Temperature and photoperiod or lunar cycle are generally recognized as major factors in controlling the maturation of gonads and spawning (Pearse and Eernisse, 1982; Olive and Garwood, 1983). Reproductive season is also related to geographic distribution of the species and its specific reproductive strategy (Millar, 1971; Berrill, 1975). In the Caribbean, *Didemnum* sp. is found breeding throughout the year, while the reproductive season of *Trididemnum solidum* and *Eudistoma* sp. lasts for five months (Millar, 1974). In the Mediterranean Sea, the spawning of *M. sabatieri* and *H. papillosa* occurs after the period of highest temperature, between September and November (Becerro and Turon, 1992). On the northern coast of Taiwan, *P. cryptocarpa kroboja* has a spawning season that extends from April to November (Chen and Dai, 1998). In Korea, the spawning of *S. plicata*, *S. clava* and *C. intestinalis* occurs between March and July, April and October, and April and December, respectively (Lee, 1976, 1977; Yang and Lee, 1978). However, the spawning season of *H. roretzi* is limited to two months (November to December) (Park et al., 1991). In this study, the gonadal development of *H. hilgendorfi ritteri* coincided with rising temperatures, and spawning occurred from November to February, when the temperature was low. These results suggest that the gonadal development and spawning of this species are closely correlated with temperature.

Many marine invertebrates have a lunar reproductive cycle as a strategy for survival and reproduction, in order to increase fertilization, protect against predators and help to retain free-living larvae in the nursery sites (Omori, 1995). The female of *Pilumnus vespertilios* releases larvae 1-3 days before the full moon, coinciding with a high tide (Kyomo, 2002), and *Trochus niloticus* spawns during the new moon phase (Hahn, 1993). The spawning of *H. hilgendorfi ritteri* occurred between the new moon and full moon, or between the full moon and new moon. These results suggest that the spawning rhythm of this species is probably influenced by the lunar cycle.

Our results show that *H. hilgendorfi ritteri* is a type of winter breeder that spawns mostly between November and January, when the temperature falls. Through histological observation of the gonad, we gathered evidence that suggests that this species spawns multiple times during the spawning period. Further study on the effects of environmental factors on gonad maturation must be conducted, because the gonadal development, reproductive cycle and spawning rhythm are closely correlated with changes in water temperature, To examine the relationship between the lunar cycle and the spawning rhythm, it will be necessary to study the spawning rhythm under controlled conditions.

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