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Ultrastructural Study of Oogenesis and Reproductive Cycle of the Female Manila Clam, *Ruditapes philippinarum* in Komso Bay, Korea

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Introduction

The Manila clam, *Ruditapes philippinarum* (Pelecypoda: Veneridae) is present along the coasts of Korea, China and Japan. In particular, it is abundant in the intertidal area of the south and west coasts of Korea where tidal flats are well developed. In Korea, the Manila clam is one of the most important marine resources for human consumption. Recently, due to reclamation of tidal areas along the west coast, marine pollution, and reckless overharvesting of this clam, its standing stock has reduced for a decade. Therefore, it is necessary to manage the population of the clam with a proper fishing regime that will maintain an optimal population size in aqua farm. So far, regarding reproductive ecology of the Manila clam in Korea and Japan, there have been many studies on growth (Chung et al., 1994), population dynamics and secondary production (Yoon, 1992), and the spawning season (Ponurovsky and Yakovlev, 1992). However, little information is available on vitellogenesis during oogenesis and on the reproductive cycle by qualitative and quantitative image analyses of the Manila clam. The purpose of the present study is to understand vitellogenesis during the oogenesis and the reproductive cycle by qualitative and quantitative analysis.

Materials and Methods

Specimens of the Manila clam, *Ruditapes philippinarum*, were collected monthly from the intertidal zone of Komso Bay, west coast of Korea from January to December 1999 (Fig. 1). A total of 485 clams from 8.4 to 54.6 mm in shell length were used for the present study.

1. Ultrastructural study of oogenesis

For electron microscopic observations, excised pieces of gonads were cut into small pieces and fixed immediately in 2.5% paraformaldehyde-glutaraldehyde in 0.1M phosphate buffer solution (pH 7.4) for 2 hours at 4℃. After prefixation, the specimens were washed several times in the buffer solution and then postfixed in 1 % osmium tetroxide solution in 0.2M phosphate buffer solution (pH 7.4) for 1 hour at 4℃.

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

2. Qualitative Reproductive Analysis

Histological preparations were made for analysis of the gonadal phases by light microscopy. Tissues were removed from shells and preserved in Bouins fixative for 24 hours and then the tissues were followed standard histological procedures. Sections were stained with Hansens hematoxylin-0.5% eosin, Mallorys triple stain or PAS stain, and were analyzed using a light microscope.

3. Quantitative Reproductive Analysis

Tissue slides were observed for quantitative analysis by an image analyzer system. Slides were viewed on a stereo-zoom microscope (Nikon, SMZ-U) from where the images were captured by a TOSHIBA Model IK-642K CCD camera and were then viewed on a SAMSUNG color video monitor. The image analyzer (BMI plus, WINATech Co.) is capable of automatic measurement of area and diameter of polygons encircled by the operator, counting objects that are contrasted by background color (in black and white mode), and statistical analysis of numerous characteristics of objects in the

captured images. Measurements were carried out for areas of total tissue, the ovary, the follicles, and the oocytes, the number of the oocytes per unit area, and the diameter of each oocyte. Measurements on total tissue and the ovary area were conducted at magnification of 7.5, at which the field area of captured image was 60 mm2, while the other measurements were done at magnification of 75 (field area: 0.524 mm2). Twenty individuals per month and two fields per slides were analyzed. Areas of total tissue, the ovary, and the follicles were measured by manually tracking the margins of objects with a pointer on the captured image. Counting and measuring the diameter of the oocytes were carried out by conversion of captured color image to a black-and-white image with appropriate threshold, and then automatic measurement procedure provided by the software. From the measured values of image analyses, (1) percent of field occupied by the ovary to total tissues, (2) percent of field occupied by the follicles to total tissue, (3) percent of field occupied by the follicles to the ovarian tissue, (4) percent of field occupied by the oocytes to the ovarian tissue, (5) the number of the oocytes per mm2, and (6) mean diameter of the oocyte in captured image were calculated for each slide. One-way ANOVA (multiple comparison by Duncan's procedure) was applied to compare the means of monthly data. One-way t-tests were used to determine significant differences in data

Results and Discussion

1. Oogenesis observed by electron microscopic observation

Oogenesis occurs in the oogenic follicles of the ovary and can be divided into five successive stages: (1) oogonium, (2) previtellogenic oocyte, (3) vitellogenic oocyte, and (4) mature oocyte stages.

Oogonium Stage: The stem cells, which constituted the boundaries of the follicles, gave rise to oogonia, characterized by a high nuclear-cytoplasmic ratio. Oogonia are small, oval in shape, and 10-11 μm in diameter and contained a large nucleus with chromatin nucleolus, and have several small mitochondria and enodoplasmic reticula in the cytoplasm.

Previtellogenic oocyte stage: The oogonium differentiated into the previtellogenic oocyte with a remarkable nucleolus in the nucleus. At the previtellogenic oocyte, the nucleus and cytoplasm increased in volume, and several small mitochondria and vacuoles were present in the perinuclear region of the cytoplasm, while the microvilli were not present on the vitelline envelope of the oocyte.

Vitellogenic oocyte stage: In the early vitellogenic oocyte, the well-developed Golgi apparatus and mitochondria were present near the nucleus, and numerous small vesicles and large vacuoles were scattered from the perinuclear region to the vitelline envelope of the oocyte. Numerous lipid droplets were present in the vacuoles and vesicles, which were formed by the Golgi apparatus near the nuclear envelope and were dispersed toward the cortical layer near the vitelline envelope. At this stage, round or oval microvilli on the vitelline envelope begin to appear. With the initiation of the formation of lipid granules and proteid yolk formation (vitellogenesis), small vesicles and lipid granules and mitochondria were located around the cortical layer. They were mixed each other and became larger ones around the nuclear envelope, and dispersed toward the cortical layer. In the late vitellogenic oocyte, an accumulation of cortical granules occurred in the cortical layer autosynthetically, and proteid substances were present near the annulus lamellae and several well-developed rough endoplasmic reticula. Proteid yolk granules, which formed by the cortical granules and lipid granules in the cytoplasm, were dispersed from the cortical layer to perinuclear cytoplasm. At this time, an amphinucleolus in the nucleus appeared, especially, exogenous lipid granular substances and lots of glycogen particles in the germinal epithelium passed into the ooplasm of the oocyte through the microvilli of the vitelline envelope.

Mature oocyte stage: In the mature oocyte, mature proteid yolk globules appeared in the cytoplasm at the same time many small yolk globules were fused to each other, and became larger mature yolk globules in size. The mature yolk globule in a mature oocyte was composed of three parts: 1) main body, 2) superficial layer, and 3) limiting membrane. At this stage, the type of the microvilli, some of which bifurcate, protruded

and extended just beyond the outer border of the vitelline envelope. The thick vitelline envelope of the mature oocyte was covered with relatively thick jelly coat.

2. Reproductive Cycle by qualitative analysis (histological study)

Gonadal phases of this species showed a periodicity are as follows: 1) *Early Active Stage:* This stage was characterized by the expansion of the follicle and the appearance of oogonia and early developing oocytes along the follicular wall. Individuals in the early active stage appeared between February and March. 2) *Late Active Stage:* Developing oocytes and a few free oocytes were present in the lumen. More than half of the oocytes attached to the follicular wall. Individuals in the late active stage appeared between April and May. 3) *Ripe Stage:* The ripe ovary exhibits distended follicles with mature and fully ripe oocytes. The mean ripe oocyte diameter was 55~60 μm. in diameter. Sexually matured females and males appeared from April to August. 4) *Partially Spawned Stage:* The number of free oocytes in the follicle decreased, and empty and ruptured follicles appeared. Spawning occurred from early June (60%) to October (15%), and one spawning peak of both sexes occurred between July and August. 5) *Spent/Inactive Stage:* After spawning, at the spent stage, the gametes in most follicles of both sexes were degenerated. There was no sign of gonadal activity. Follicles became contracted and degenerated undischarged oocytes in the lumen underwent cytolysis. Newly formed oogonia appear among the connective tissues and phagocytes. Individuals in the spent/inactive stage appeared from August to March.

3. Quantitative results by image analysis

Ruditapes philippinarum showed a unimodal gametogenic cycle. The percent of field occupied by the ovary to total tissue began to increase in March. The ovary area greatly increased from March to May (37.2%~80.4%, p=0.002), reached the maximum in May, and then gradually decreased from June to November (73.7%~40.2%, p=0.015). During winter period (January, February, and December), the ovarian tissue was rarely found so that the proportion of the ovary to total tissue was less than 1%. Variations of the ovary area among individuals were so high that there were no significant differences during March~April, May~June, June~July, August~October, and October~November (one-way ANOVA, p=0.111, 0.126, 0.330, 0.059, and 0.111, respectively). The percent of field occupied by the follicle to total tissue and the percent of field occupied by the follicle to the ovarian tissue showed similar patterns to the ovary area. But higher values (more than 50%) of the follicle area in the ovary lasted longer than that in total tissue. The follicle area in the ovary increased rapidly from near 0% in February to 67.1% in March (p=0.005). It increased more to over 80% during April~June and then decreased slowly until November. Still high proportion of the follicle area to the ovary was observed in November (53.0%). During winter period (January, February, and December), percent of the follicle area were lower than 1%. There was no significant difference in the follicle area to the ovary during April~August (one-way ANOVA, p=0.385). The percent of ovarian tissue occupied by the oocyte, the number of the oocyte per unit ovary area, and the mean diameter of the oocyte also showed similar patterns to the ovary area. Proportion of oocyte area ranged from 8.7% to 33.4% with a peak in May. During September~November, the oocyte area greatly decreased to less than half of that during April~August, while the follicle area during same period remained high. The number of the oocyte also decreased greatly during September~November. Mean oocyte diameter reached maximum in Ma μm]. Large sized eggs were found even in November [24.7 2.23 (S.E.) μm]. There was good consistence in the time of peaks in May among the ovary area, the follicle area, the oocyte area, and the oocyte diameter. Only the number of the oocyte reached its maximum in June.

Summary

R. philippinarum is dioecious and oviparous. In the early vitellogenic oocyte, the Golgi apparatus and mitochondria present in the perinuclear region are involved in the formation of lipid droplets and in lipid granule formation. In the late vitellogenic oocyte, the endoplasmic reticulum, mitochondria in the cytoplasm are involved in the formation of proteid yolk granules. At this time, exogenous lipid granular substance and glycogen particles in the germinal epithelium are passed into the

ooplasm of oocyte through the microvilli of the vitelline envelope. Ripe oocytes are about 55-60 μ m in diameter.

The spawning period was once a year between early June and early October, and the main spawning occurred between July and August when seawater temperature was approximately 20 C. The reproductive cycle of this species can be categorized into five successive stages: early active stage (February to March), late active stage (April to May), ripe stage (April to August), partially spawned stage (June to October), and spent/inactive stage (August to March). Gonad developmental phases by histological qualitative analysis showed similar results with those of quantitative image analysis.

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