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Gametogenic Cycle, the Spawning Season, First Sexual Maturity, and the Biological Minimum Size in Male *Ruditapes philippinarum* (Bivalvia: Veneridae) in Western Korea

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Abstract: The male gametogenic cycle, spawning season, first sexual maturity, and the biological minimum size in male *Ruditapes philippinarum* were investigated by qualitative and quantitative reproductive analyses. In the study of the male gametogenic cycle by qualitative histological analysis, the gametogenic cycle in male individuals can be classified into five successive stages: (1) early active stage, (2) late active stage, (3) ripe stage, (4) partially spawned stage, and (5) spent and inactive stage. Monthly changes in the gonad index in males measured by qualitative analysis showed a similar pattern to the male gametogenic cycle. In the study of the male gametogenic cycle by quantitative statistical analysis, monthly changes in the portions (%) of areas occupied by the testis areas to total tissue areas showed a rapid increase in March, and reached the maximum in May-June. And also monthly changes in the portions (%) of areas occupied by the spermatogenic stages to the testis area showed a maximum in May and gradually decreased from June to October. Therefore, this species showed a unimodal gametogenic cycle during the year, and the number of spawning seasons occurred once per year, from June to October, with a peak spawning between July and August. The percentage at the first sexual maturity of male clams ranging from 15.1-20.0 mm in shell length was 64.7%, and that of all individuals ranging from over 25.1 mm in shell length was 100%. The biological minimum size (shell lengths at 50% of sexual maturity (RM_{50})) of male mature clams that was fitted to an exponential equation was 17.16 mm (considered to be 1 year old). Because harvesting clams less than 17.16 mm in shell length could potentially cause a drastic reduction in recruitment, a measure indicating a prohibitory fishing size should be enacted for adequate fisheries management.

Key words: Male *Ruditapes philippinarum*, gametogenic cycle, spawning season, first sexual maturity, biological minimum size.

1. Introduction

The Manila clam, *Ruditapes philippinarum* is a commercially important bivalve in East Asian countries, including Korea, Japan, and China [1, 2], and the northwestern coast of the United States [3, 4]. More specifically, in Korea, this species is mainly found in silty sand in the intertidal and subtidal zones of the south and west coasts of Korea [3]. Due to past over-harvesting and parasitic infections, it has been identified as a species requiring a more sustainable fishing regimen [5, 6]. Previously there have been many studies on aspects of reproduction, including the reproductive cycle [5], ovarian maturation [6], spawning season [7-11], and ovarian gametogenic cycle by quantitative analysis [12]. Recently, regarding female *R. philippinarum*, Choi et al. [6] reported on ovarian maturation, including the ultrastructure of the ovary, germ cell differentiation, and vitellogenesis during oogenesis, and the reproductive cycle by qualitative analysis (histological observation). Chung et al. [12] described the ovarian

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gametogenic cycle and the number of spawning seasons by quantitative analysis. Although some studies have been conducted on ovarian maturation in female *R. philippinarum*, including the ovarian gametogenic cycle and the number of spawning seasons, to date, the unimodal (or bimodal) gametogenic cycle, the number of spawning seasons, and the biological minimum size in male *R. philippinarum*have not been previously reported for reproductive ecology and natural resource management.

In this study, understanding information on the male gametogenic cycle and the main spawning season by the quantitative statistical analysis of male individuals will provide the information needed for age determination and recruitment period [12, 13]. However, in case of different populations in the same *R. philippinarum*, the number of spawning seasons of this species have two kinds of spawning seasons: number of the spawning seasons in the northern districts of Tokyo Bay, Japan were once a year [7, 10, 11, 14], while those in the southern districts of Tokyo Bay, Japan occurred twice a year [9]. For that reason, it is difficult to perform age determination or assess population dynamics of this species because the main spawning season and the number of spawning seasons per year has not yet been determined by quantitative analysis. Therefore, in this study, we have to confirm whether the male gametogenic cycle and the number of the spawning seasons of this species show a unimodal or bimodal cycle each year by quantitative analysis using statistics.

Additional information on the biological minimum size (the size at 50% of sexual maturity) of this species would be very useful for propagation, aquaculture, and resource management. In particular, information on the size at which individuals reach 50% of sexual maturity could be useful in determining a prohibitory measure for adequate natural resource management. Therefore, the aim of this study is to clarify the male gametogenic cycle and the number of spawning seasons by quantitative analysis, and the biological minimum size (the size at 50% of sexual maturity) for reproduction and natural resource management in male *R. philippinarum*.

2. Materials and Methods

2.1 Male Gametogenic Cycle by Qualitative Histological Analysis

2.1.1 Sampling

50-60 specimens of male and female *R. philippinarum* were collected monthly at the intertidal and subtidal zones of Simpo, Jollabuk-do, Korea (Fig. 1) from January to December 2006. The Manila clams ranging from 8.6 mm to 54.6 mm in shell length were used for the present study. After the clams were transported alive to the laboratory, shell length and total weight were immediately measured.

2.1.2 Production of Male Histological Tissue Section Slides for Qualitative Reproductive Analysis

For light microscopic examination of histological preparations, a total of 425 male individuals were used for histological analysis of the gonads. Testicular tissues were removed from shells and preserved in Bouin's fixative for 24 h. They were 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%

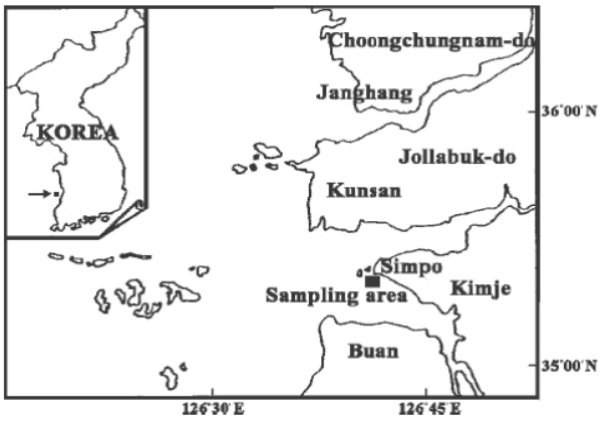

Fig. 1 Map showing the sampling area.

eosin, and examined using a light microscope (Zeiss Axiovert 10 microscope).

2.1.3 Calculation of the Male Gonad Index (GI) According to Scores by the Gametogenic Stages

To explore the spawning season by qualitative analysis (histological observations), the mean GI (gonad index) in males was calculated using a modification of Mann's method [15]. Each histological section of gonadal tissues was also examined in details to assess the gonad developmental stages. Staging criteria of 1 to 5 were employed for spent/inactive (S1 = 1), partially spawned (S2 = 2), early active (S3 = 3), late active (S4 = 4) and ripe (S5 = 5). These categories are only approximations of gonadal development because it is a continuous process and distinctions between stages are not always clear. The monthly GI (gonadal index) in male individuals was determined by multiplying the number of specimens ascribed to each category score, summing all those values and dividing this figure by the total number of clams analyzed.

$$GI = [(N \times RVS1) + (N \times RVS2) + (N \times RVS3) + (N \times RVS4) + (N \times RVS5)] / \text{Total N observed by month}$$

where, N: number of individuals; RVS: ranking value by stage.

2.2 Male Gametogenic Cycle by Quantitative Statistical Analysis

2.2.1 Quantitative Analysis by an Image Analyzer System

Tissue slides were observed for quantitative analysis by an image analyzer system. Slides were viewed on a stereo-zoom microscope (Nikon, SMZ-U) from which the images were captured by a TOSHIBA Model IK-642K CCD camera and 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 performing statistical analysis on numerous characteristics of objects in the captured images. As for males, the areas of total tissue, the testis, and the spermatogenic stages were measured. Measurements on total tissue and the testes areas were conducted at a magnification of 7.5×, at which the field area of the captured images was 60 mm^2, while the other measurements were done at a magnification of 75× (field area: 0.524 mm^2). Twenty individuals per month and two fields per slides were analyzed. Areas of total tissue, the testes, and the spermatogenic stages were measured by manually tracking the margins of objects with a pointer on the captured images. For each male slide, (1) the percent of field occupied by the testis to total tissue, and (2) the percent of field occupied by the spermatogenic stages to the testis area were calculated.

2.2.2 Statistical Analysis

A one-way ANOVA (multiple comparisons by Duncan's procedure) was applied to compare the means of monthly data. One-way *t*-tests were used to determine significant differences in the data of two adjacent months. All statistical analyses were done using the SPSS package.

2.3 Calculations of First Sexual Maturities by the Size Classes According to the Qualitative Analysis

For determination of the size at 50% of first sexual maturity, a total of 135 males histological preparations (8.4-54.6 mm in shell length) were examined the size at 50% of first sexual maturity (= biological minimum size) by histological observations from January to December, 2006. The percentage (%) of first sexual maturity = No. of mature individuals × 100 / No. of total individuals investigated.

2.4 Calculation of the Biological Minimum Size (RM_{50}) by the Quantitative Analysis

To calculate the biological minimum size (the size at the rate (50%) of group sexual maturity) after fitting the rate of sexual maturity to an exponential equation,

the size equivalent to the size at 50% of sexual maturity was estimated to be the sexually mature length of the population [16]. The exponential equation of the rate of sexual maturity is as follows: RM = 100/1 + exp (a-b*x*), where, RM: rate of sexual maturity; a, b: constants, *x*: shell length.

3. Results and Discussion

3.1 Male Gametogenic Cycle with Spermatogenic Stages by Qualitative Histological Analysis

Spermatogenesis occurred in the acini in the testis. Based on morphological features and sizes of the germ cells and accompanying cells, the gametogenic cycle with spermatogenic stages in male individuals of this species can be classified into five successive stages: early active, late active, ripe, partially spawned, and spent/inactive stages (Fig. 2). The spermatogenic stages in the testes and the criteria used in defining them are as follows:

Early active stage: In males, at this stage, the acinus walls of the acini in the testis were relatively thick, the spermatogonia and spermatocytes wereapproximately 7-8 μm and 5-6 μm in diameter, respectively. They appeared along the acinus wall of the acini in the testis (Fig. 3A). In particular, gamete differentiation of *R. philippinarum* began between February and March when water temperatures are relatively low (under 9 °C). Male individuals in the early active stage appeared from February to March when sea water temperatures are relatively low (Fig. 2).

Late active stage: In the testis, a few spermatogonia and a number of spermatocytes (about 5-6 μm in diameter) and spermatids (about 3 μm in diameter) appeared in the acini. At this time a small number of spermatozoa began to transform into differentiated spermatozoa in the center of the lumen of the acinus (Fig. 3B). Male individuals in the late active stage were found from April to May when seawater temperatures are gradually increased (Fig. 2).

Ripe stage: At this stage, number of spermatids begin to transform into differentiated spermatozoa in the centre of the lumen, and numerous spermatozoa appeared in the center of the lumen of the acinus (Fig. 3C). During the ripe stage, the testis of *R. philippinarum* reached a maximum under the conditions of the optimum high water temperature and abundant food supply. Male individuals in the ripe stage appeared from April to August when sea water temperatures are higher than 17 °C (Fig. 2).

Partially spawned stage: The lunina of the aciniwere empty because over 50% of the spermatozoa have been discharged but undischarged spermatozoa as well as spermatids remain in the lumen of the acinus (Fig. 3D). Male individuals in the partially spawned stage were found from June through early October, with the main spawning event occurring from July to August when seawater temperatures are relatively high (Fig. 2).

Spent/inactive stage: A small number of remaining spermatozoa and spermatids degenerated and the products of gamete atresia were resorbed, thereafter, the rearrangement of a few newly formed spermatogonia and connective tissue occurred in the

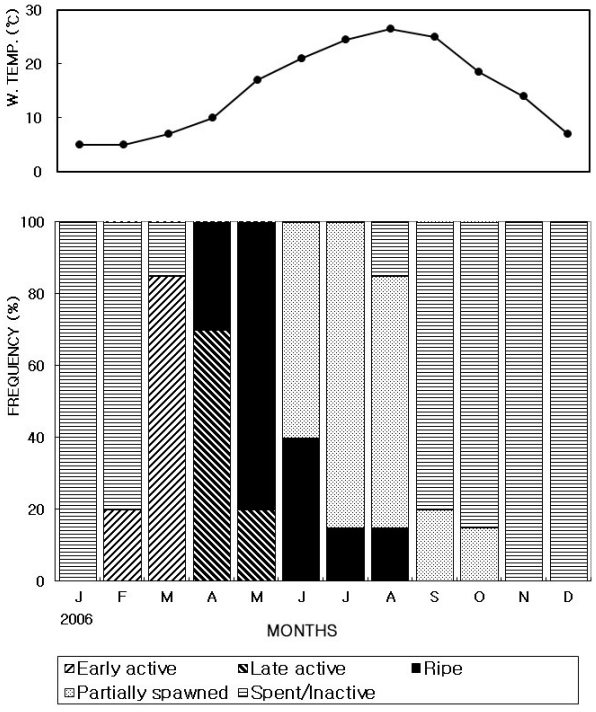

Fig. 2 Frequency of gonadal phases in male *Ruditapes philippinarum* and the mean sea water temperature from December, 2006.

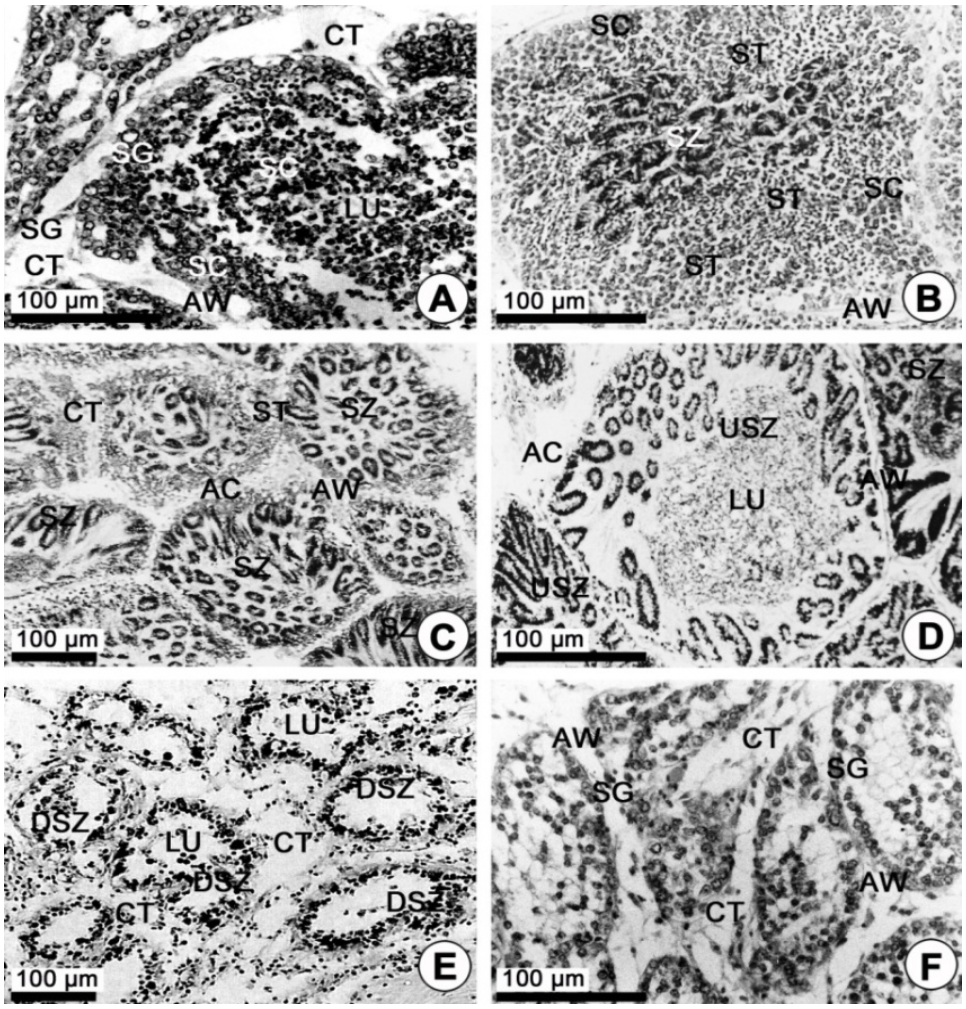

Fig. 3 Photomicrographs of gonadal phases in male *Ruditapes philippinarum* as seen by light microscopy.
(A): Transverse section of the acini in the early active stage; (B): Section of the acini in the late active stage; (C): Section of the acini in the ripe stage; (D): Section of the acini in the partially spawned stage; (E) & (F): Sections of the acini in the spent/inactive stage. AC: acinus; AW: acinus wall; CT: connective tissue; DSZ: degenerating spermato zoon; LU: lumen; SC: spermatocyte; SG: spermatogonium; ST: spermatid; SZ: spermatozoon; USZ: undischarged spermatozoon.

acini (Figs. 3E, 3F). Male individuals in the spent/inactive stage appeared from August to March when sea water temperatures are gradually decreased and relatively low (Fig. 2).

3.2 Monthly Changes in the Gonad Index (GI) Calculated by the Scores of the Gametogenic Stages

Monthly changes in the GI (gonad indice) in male individuals by qualitative analysis of this species are shown in Fig. 4. The GI began to gradually increase in March, and reached a maximum (GI, 4.8) in May. And then the GI values gradually decreased from June

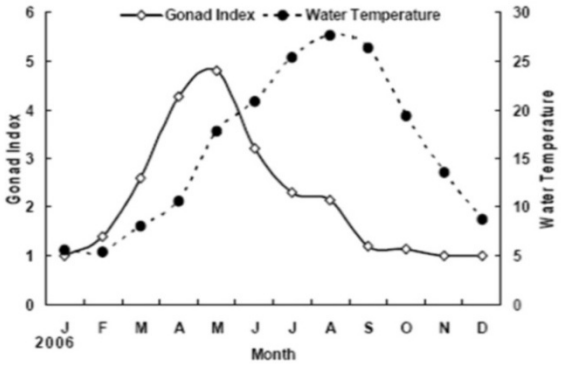

Fig. 4 Monthly changes in the GI (gonad indice) in male *Ruditapes philippinarum* by qualitative analysis and seawater temperatures (°C) from January to December, 2006.

to October when spawning occurred and relatively high water temperatures were maintained. Thereafter, the GI values temporarily reached a minimum from November (GI, 1.0) to January (GI, 1.0). Monthly changes in the GI in males in 2006 showed a peak in May during the year, and the spawning period of *R. philippinarum* in Korea showed once per year (a unimodal gametogenic cycle).

3.3 Male Gametogenic Cycle with the Spermatogenic Stages by Quantitative Statistical Analysis

*R. philippinarum*in males showed a unimodal gametogenic cycle (Figs. 5A, 5B). The percent (%) of field areas occupied by the testis to total tissue area began to increase in February. The testis area greatly increased from February to June (9.8%-80.3%, $P < 0.001$) and reached a maximum in June, and continued at a relatively high level until September, and then decreased from September to December (73.3%-18.1%, $P < 0.001$). During the winter period, the percent of the testis area was lower than 20%. There was no significant difference in the testis area to total tissue area during March-April, May-September, or October-November (one-way ANOVA, $P = 0.095$, 0.207, 0.095, respectively) (Fig. 5A).

The percent of field areas occupied by the spermatogenic stages to the testis areas began to increase in February. It increased rapidly from February to May (1.4%-84.4%, $P < 0.001$) and reached a maximum in May, and then decreased gradually until December (84.4%-1.5%, $P < 0.001$). During the winter period, the percent of the spermatogenic stages to the testis area was lower than 2%. There was no significant difference in spermatogenic stages to the testis area during July-August or October-November (one-way ANOVA: $P = 0.130$, 0.055, respectively) (Fig. 2B). Especially, in quantitative reproductive analysis (statistical analysis) using an image analyzer system, the patterns of monthly changes in the percent (%) of the areas occupied by spermatogenic stages to the testis area in

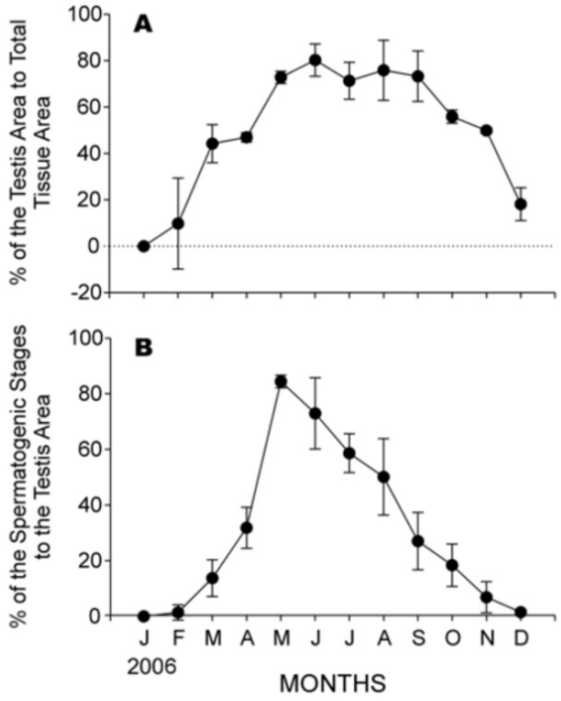

Fig. 5 Monthly changes in quantitative reproductive traits in Male *Ruditapes philippinarum*. (A): Percents of area occupied by testes to total tissue area; (B): Percents of areas occupied by spermatogenic stage areas to the testis area.

males showed a maximum in May, and then rapidly dropped from June to October. Thereafter, they reached the minimum value from December to February, 2006 (Fig. 5B).

3.3.1 A Comparison of the Male Gametogenic Cycle by Qualitative and Quantitative Analyses

To compare some characteristics of the gametogenic cycle by qualitative histological analysis and quantitative statistical analysis in male *R. philippinarum*, the authors investigated some characteristics and patterns between monthly changes in the gonad index and monthly changes in percents (%) of the tesis area to total tissue area (or percents (%) of the spermatogenic areas to the testis area).

In the results of the male gametogenic cycle, monthly changes in the GI (gonad index) in male individuals began to increase in March and reached a maximum in May, and then gradually decreased from June to October (Fig. 4). Therefore, monthly changes in the GI showed a unimodal gametogenic cycles showing a maximum maturity in May and one

spawning season per year from June to October. The gametogenic cycle in male *R. philippinarum* by qualitative histological analysis was classified into five successive stages: the early active stage (January to March), late active stage (February to May), ripe stage (April to August, a maximum in May), partially spawned stage (June to October, with peak spawning between July and August), and spent/inactive stage (August to February).

According to the results of the male gametogenic cycle by quantitative statistical analysis, in this study, the results of monthly changes in percents (%) of the tesis area to total tissue area showed a rapid increase in March, and reached the maximum in May-June. Thereafter, the percent (%) of the testis area to total tissue area gradually decreased from July to October when spawning occurred. And also monthly changes in the percent of spermatogenic stage areas to the testis area showed a rapid increase in March and reached a maximum in May. Thereafter, the percent (%) of the spermatogenic stage areas to the testis area rapidly decreased from June to October when spawning occurred, and the main spawning occurred between July and August. In qualitative histological observations, the peak spermatozoon level occurred in May followed by a significant decrease from June to October which indicated spawning, and the main spawning occurred between July and August. Therefore, the results of the male gametogenic cycle and maturation by qualitative histological analysis coincided with those studied by quantitative analysis.

Giese and Pearse [17] and Sastry [18] observed latitudinal differences in timing of the reproductive cycles of marine molluscs in general. Some authors [19-21] reported that *Mya arenaria* and *Mercenaria mercenaria* in bivalve molluscs exhibited a change from a unimodal to a bimodal cycle with decrease in latitude [22]. However, several authors [23, 24] reported that several other bivalves (i.e, *Geukensia demisa*, *Crassostrea virginica* and *Spisula solidissima* similis) showed unimodal gametogenic cycle in the southeastern U.S. waters [22]. In this study, the gametogenic cycle in male *R. philippinarum* by quantitative statistical analysis showed a unimodal gametogenic cycle.

3.3.2 Number of Spawning Seasons per Year by Quantitative Analysis

Regarding the number of the spawning season of different local populations of *R. philippinarum*, it is well-known that the number of spawning seasons by qualitative histological analysis varied with latitudinal gradients. In case of the northern districts of Tokyo Bay, Japan, Momoyama and Iwamoto [25] reported that the number of spawning seasons of *R. philippinarum* in Hokkaido, Japan was once a year during the summer season. However, in case of the southern districts of Tokyo Bay, Japan, Ko [26] described that the number of spawning seasons in Sasebo Bay, Nagasaki, Japan were twice per year: the first spawning season (April to July) and the second spawning season (September to November). And Tanaka [8] also reported that from the southern part area of Kando district to Kumamoto district, Japan, the number of spawning seasons of this species were twice per year: the first spawning season (spring) and the second spawning season (autumn). Although the number of spawning seasons of this species in southern districts of Tokyo Bay, Japan showed twice per year by qualitative analysis, however, their analysis were not correct because these results were not confirm by quantitative statistical analysis using an Image Analyzer System. Especially, in case of two spawning seasons per year, it needs to confirm to get accurate results by quantitative statistical analysis. Regarding the number of spawning seasons of this species in Korea, Kurashige [27] described that the number of spawning season of *R. philippinarum* in two different districts of Korea was once a year: from May to early October in Taeya, Chungcheongnam-do, Korea, and from mid-May to late October in Dadepo, Busan, Korea. In this study, the number of spawning seasons of this species by qualitative analysis

(histological observations) was once a year from early June to early October in Simpo, Jeollabuk-do, Korea [6]. Therefore, the results on the spawning periods by quantitative analysis coincided with those studied by qualitative analysis. In consequence, the spawning periods by qualitative and quantitative analyses in Simpo, Korea were about one month later than the results reported by Kurashige [27]. Therefore, it is assumed that some local variations and timing of spawning of this clam might be related to the geographical differences in the water temperatures, time of the food production (phytoplanktons), and some other environmental factors.

3.4 Size at First Sexual Maturity (%) by Histological Observations of Male Gonadal Tissue Section

For the investigation of first sexual maturity and the biological minimum size of population, a total of 135 male individuals of *R. philippinarum* were investigated histologically to determine the shell lengths that reach maturation and participate in reproduction from May (maturation before spawning) to late October (completion of spawning). As shown in Table 1, the percentage of smaller individuals ranging from 8.4-10.0 mm in shell length was 0%, and those individuals were in the early active stage, which is characterized by a small number of spermatogonia and the appearance of a number of spermatocytes in the acini of the testis. It is supposed that their sizes at sexual maturity could not have been reached until late October when spawning was completed. The percentage of first sexual maturity of male clams ranging of 10.1-15.0 mm shell length is 16.7%, and those individuals were in the early active, late active and ripe stages during the period between June and August, when spawning was observed among older individuals. However, younger animals had a small number of spermatozoa and spermatids, a number of spermatocytes and spermatogonia in acini of the testis. It is supposed that sexual sizes of most individuals could not be reached until October when spawning of a few mature individuals was completed. In addition, the percentage of first sexual maturity of male clams ranging from 15.1 mm to 20.0 mm in shell length is 64.7%, but those individuals were in the early active, late active, ripe, and partially spawned stages during the breeding season. The percentage of sexual maturity of all individuals of shell length greater than 25.1 mm is 100%, and those individuals were in the late, ripe, partially spawned, and spent/inactive stages. Therefore, it is assumed that most individuals can reach full maturity by late October if they are larger 25.1 mm in shell length at that time. In this study, the percentage of sexual maturity of male Manila clams ranging from 15.1 mm to 20.0 mm was 64.7% (over 50.0%). So, the authors could not understand the accurate size at 50% of sexual maturity of male individuals. Therefore, the authors have to calculate its shell length at 50% of sexual maturity by quantitative analysis, however, this percentage was 100% for male clams over 25.1 mm length.

3.5 Biological Minimum Size (Size at the Rate (50%) of Sexual Maturity (RM_{50}))

As shown in Fig. 6, the biological minimum size (shell length at 50% of sexual maturity, (RM_{50})) that was fitted to an exponential equation by von Bertalanffy's equation was 17.16 mm in males.

As shown in Table 1, the percentages of sexual maturity of male individuals of 15.1 mm to 20.0 mm in shell length were 64.7% in males. Those percentages

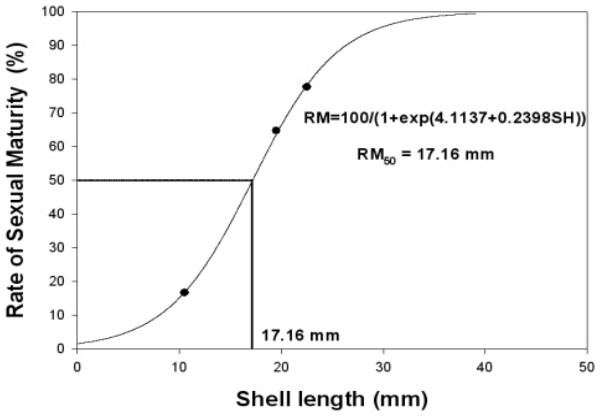

Fig. 6 Relationship between the rate of group sexual maturity (%) and shell length (mm) in male *Ruditapes philippinarum*.

Table 1 Shell lengths of first sexual maturity in male *Ruditapes philippinarum* from May to October, 2006.

Shell length	Male						
	EA	LA	RI	PS	SP/IA	No. of ind.	Maturity (%)
8.4-10.0	15					15	0.0
10.1-15.0	15	1	2			18	16.7
15.1-20.0	6	3	5	2	1	17	64.7
20.1-25.0			5	2	2	9	77.7
25.1-30.0		2	6	8	2	18	100.0
30.1-35.0		2	8	7	3	20	100.0
35.1-40.0			7	6	2	15	100.0
40.1-45.0		2	4	3	3	12	100.0
45.1-50.0			2	3	1	6	100.0
50.1-54.6			2	2	1	5	100.0
Total	135						

Ind. means the number of individuals; EA: early active stage; LA: late active stage; RI: ripe stage; PS: partially spawned stage; SP/IA: spent/inactive stage.

are 100% for male Manila clams greater than 25.1 mm in shell length. According to growth curves for the mean shell length fitted to the von Bertalanffy equation by Chung et al. [5]. Ages (year) and mean shell lengths (mm) are as follows: 1 year (13.80 mm), 2 years (24.59 mm), 3 years (33.38 mm), and 4 years (40.27 mm).

In this study, male individuals ranging from 15.1 mm to 20.0 mm (shell length at 50% of sexual maturity, RM_{50} = 17.16 mm) in shell length (Fig. 3) are considered to be 1 year old. We assumed that this male population achieve maturity and begin reproduction at 1 year of age.

Because harvesting clams less than 17.16 mm in shell length could potentially cause a drastic reduction in recruitment, a measure indicating a prohibitory fishing size should be enacted for adequate fisheries management.

4. Conclusions

The male gametogenic cycle by qualitative histological analysis can be classified into five successive stages: (1) early active stage; (2) late active stage; (3) ripe stage; (4) partially spawned stage; and (5) spent/inactive stage. Monthly changes in the gonad index in male individuals showed a unimodal gametogenic cycle showing maximum maturity and one spawning season per year from June to October. The results of the male gametogenic cycle by quantitative statistical analysis showed similar patterns to those by qualitative histological analysis. The authors found that *R. philippinarum* belongs to the summer breeder class. In the present study, the percentage of first sexual maturity of male clams ranging from 15.1 mm to 20.0 mm in shell length was 64.7%, and 100% for clams over 25.1 mm shell length. It is assumed that this male population begins reproduction about one year of age.

The biological minimum size (shell lengths at 50% of sexual maturity (RM_{50})) of male mature clams that was fitted to an exponential equation was 17.16 mm (considered to be 1 year old). In terms of natural resource management, the present study suggests that harvesting clams less than 17.16 mm in shell length (< 1 year old) can potentially lead to a drastic reduction in recruitment. A prohibitory measure should be taken for adequate natural resources management.

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