A process of sexual maturation of *Simplisetia erythraeensis* (Annelida, Nereididae)

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Abstract: We investigated the histological and morphological processes of sexual maturation in the common estuarine polychaete *Simplisetia erythraeensis* (Annelida: Nereididae). Seasonal changes in their sexual maturity were also examined at Omoi River estuary in Kagoshima Bay, Japan. Histological sections of sampled individuals revealed that *S. erythraeensis* is gonochoristic (because specimens had only either oocytes or cell clusters of spermatogenesis) and undergoes extraovarian oogenesis at sexual maturity (because oogenesis was observed in the coelom of mature specimens). Oocytes in mature females possessed a nucleus, a yolk, and a rough-surfaced endoplasmic reticulum-like structure. Female sexual maturation occurred in two stages: F1 and F2. The female F1 stage occurred from March to August, and the F2 stage occurred from July to August when males were filled with cell clusters of spermatogenesis. In addition, the inside portion of the muscular layers of females disappeared with sexual maturity (epitokous metamorphosis) and was replaced by amoebocyte associations around the oocytes (presumably to store nutrition for the oocytes), which enabled the species to spend the majority of its energy for monotelical reproduction. No significant changes were observed in parapodia of F2 in response to epitokous metamorphosis, namely, *S. erythraeensis* does not seem to engage in reproductive swarming.

Key words: Polychaeta; Tidal flats; Oocyte; Histology

Tidal flats are located in calm and semi-closed areas of the coastal zone and provide feeding and nursery grounds for many species of fishes, including fishery species (Kikuchi 1993). In this ecosystem, nereidid polychaetes are common, frequently dominant (Mettam 1981; Sato 2017), and important prey of fishes. Tidal flats also have an ecosystem function that helps prevent eutrophication of coastal areas by removing organic materials and nutrients deposited from rivers (Kikuchi 1993). Because Nereididae in this ecosystem have an important role of decomposing organic material by feeding and defecating (Kurihara 1983; Kumagai and Kurihara 1989), and turning bottom sediments (Sayama and Kurihara 1983; Kikuchi 1987), the

population stability of nereidid polychaetes is crucial for both the fisheries industry and environmental conservation. Therefore, an intensive study of the ecology of these polychaetes, including their life history and reproductive characteristics, is needed (Grassle and Grassle 1974; Connell and Slatyer 1977; Pearson and Rosenberg 1978).

Most species of Nereididae have been reported to be monotelic (Eckelbarger 1983; Olive 1983). Some species change their body morphology at sexual maturity, known as epitokous metamorphosis for reproductive swarming (Schroeder and Hermans 1975; Durchon 1984). For example, parapodia appendages become enlarged, paddle-like lamellae emerge from

the neuropodia, and the dorsal cirrus develops crenulations in *Platynereis dumerilii* (Durchon 1984). And mature males and females swim up synchronously and swarm to spawn (Sato 1999) after developing lobes and acicular parapodia that enable reproductive swarming (Durchon 1984; Sato 1999). The reproductive swarming of *Hediste diadroma* during night high tides in spring apparently assists their mating because synchronized swimming helps them to find mates (Sato 1999). And nereidid species inhabiting low-salinity areas, it appears that much of the guts and longitudinal muscles lysis during metamorphosis (histolysis) when approaching maturity (Fischer and Dorresteijn 2004).

Simplisetia erythraeensis (Fauvel, 1918) (Annelida: Nereididae) has been sampled from estuarine of Kominato Asadokoro in Aomori [prefecture located at the northern tip of Honshu, the main island (40°56′N, 140°58′E)] to Iriomote Island [located among the southwest islands (24°18′N, 123°54′E)] (Iijima 2007). And Imajima (1972) reported that this species is distributed over a wide range along the Japanese coast and occurs as far north as Usu Bay, Hokkaido (approximately 42°13′N). *Simplisetia erythraeensis* has the highest frequency among estuarine polychaete species, occurring in 69 of 157 surveyed tidal flats (Iijima 2007). It seems likely that this species has an important ecological role in the tidal flats ecosystems in Japanese estuaries. However, the population dynamics, including reproductive characteristics, has not been well-studied in *S. erythraeensis* (Ueno et al. 2019), although mating behavior and early development are thought to influence species distribution and population stability (Levin 1984).

Ueno et al. (2019) reported that *S. erythraeensis* in southern Kyushu is an annual species that is expected to reproduce in summer. Ovigerous female appeared from March to August and their ratio to individuals with a body width larger than 700 μm (the smallest ovigerous size) was high from May to July in Kagoshima Bay. Since males with cell clusters of spermatogenesis and females with oocytes larger than 80 μm appeared from June to August, it

was expected that their spawning occurred in this period. However, the relationship between oocyte size and maturity stage was not determined in Ueno et al. (2019), then accurate spawning season was not certain. And, it is unknown whether epitokous metamorphosis and reproductive swarming occur in the Japanese population of *S. erythraeensis*, or not, though Dorsey (1981) suggested that this species deposited eggs in an egg tube and did not display external morphological changes associated with reproduction in the Australian population.

Numerous studies in polychaetes have revealed characteristics of the maturation process, such as oogenesis, by examining histological sections (Olive 1978; Jollivet et al. 2000; Prevedelli et al. 2007). The aim of the present study was to describe the sexual maturation process in females of *S. erythraeensis* via histological examinations and observe the morphological changes during maturation in adult males and females.

Materials and Methods

Study sites

We collected specimens of *S. erythraeensis* at two sites: site A (31°42′26.3″N, 130°37′18.8″E) and site B (31°42′11.9″N, 130°37′08.1″E) located at northern and southern side of the mouth of Omoi River, respectively, in Shigetomi Tidal flats in inner Kagoshima Bay of southern Kyushu (Fig. 1). We selected this tidal flats as study sites because the density of *S. erythraeensis* appeared to be high and stable based on previous investigations of benthic fauna by Yamamoto et al. (2009) and Hayashi and Yamamoto (2011).

Collection of specimens

Monthly sampling of specimens of *S. erythraeensis* was carried out at site A during the lowest spring tide from September 2015 to October 2016 to investigate their reproductive seasonality. A long-term sample plot (5 m × 20 m) was established, and we set five quadrats randomly and simultaneously every month inside the plot. Specimens were
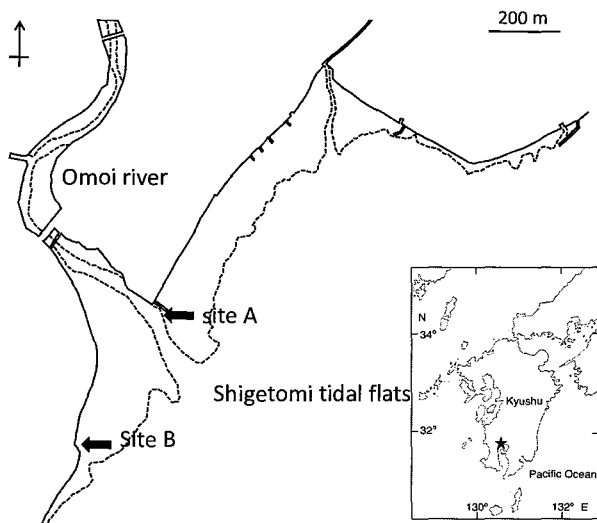
Fig. 1. Two sampling sites (A site and B site) at Shigetomi Tidal flats located at the inner portion of Kagoshima Bay. Black arrows indicate the sampling points.

Table 1. Two-way ANOVA of differences in the mean oocyte diameter during the reproduction season between each chaetiger [anterior (30th to 40th chaetiger), middle (70th to 80th chaetiger), and posterior (120th to 130th chaetiger)], individuals, and the interaction effect. Abbreviations: degrees of freedom (df), F-ratio (F), and P-values (P)

	df	mean square	F	P
chaetiger	2	1588.087	2.746	0.078
specimen	17	53813.122	93.039	<0.001
chaetiger * specimen	34	578.392	4.619	<0.001

obtained from each quadrate (15 cm × 15 cm) by excavating sediment to a 10-cm depth with a shovel. The excavated sediment was sieved by a 0.5-mm mesh sieve from which polychaetes were collected and immediately fixed in 10% formalin seawater.

To collect maturing adults for histological examinations, 52 live specimens were collected at site B at low tide during spring tides from May to July of 2018 (on 4 and 30 May, 14, 28 and 29 June, and 26 July). These specimens were collected manually by using 12-cm long trowels to excavate the sediment. We decided on this sampling regime that was based on a previous study conducted by Ueno et al. (2019).

Reproductive traits

For all specimens, the maximum body width was measured between the base of the right and left parapodia at the anterior end of the body (10th–20th chaetiger). All measurements were made within an accuracy of 25 μm by using a stereomicroscope (SMZ-U, Nikon Corp, Tokyo, Japan).

The midbody approximately 50th chaetiger was dissected on a glass to evaluate the sexual maturity of each specimen. When oocytes or cell clusters of spermatogenesis were identified in the coeloms of bodies, the specimen was defined as either female or male, respectively.

For each ovigerous female, we randomly chose 20 to 30 oocytes and acquired digital images of them under optical microscope (E600, Nikon Corp, Tokyo, Japan). We measured the diameter of oocytes on ImageJ software using images taken under the microscope (DS-L3, Nikon Corp, Tokyo, Japan).

In a pilot study, we also used ImageJ software to measure oocytes in the anterior (30th–40th chaetiger), middle (70th–80th chaetiger), and posterior (120th–130th chaetiger) portion of specimens sampled on June 2018. We applied a two-way analysis of variance to examine the differences in oocyte diameters among chaetigers and individuals. Because the mean oocyte diameter was not significantly different among these measured chaetigers (Table 1), we thereafter used the mean diameter of oocytes from the 50th chaetiger as an indicator of oocyte size for each specimen.

After transporting specimens collected for histological sections to the laboratory, they were raised overnight to excrete sand from their guts. Histological sections were then prepared from the specimens, fixed in Bouin fluid, and thereafter preserved in 80% ethanol. After dehydrating with 90% ethanol, 100% ethanol and xylene serially, the specimens were permeated with and embedded in paraffin wax. Wax sections were cut into 5-μm-thick sections and stained with hematoxylin–eosin. The stained sections were photographed with a digital camera (DP73, Olympus Corp, Tokyo, Japan) attached on an optical microscope (BX43, Olympus Corp, Tokyo, Japan).

We picked up females with green body color that spawned oocytes easily by impulsion

during sampling on reproductive season (13 June, 2018) (Figs. 3A, B). Images of raw 24 oocytes from this female were taken (in seawater) to measure oocyte diameter, and the female was processed into histological sections. Measurement of oocytes diameter and preparation of histological sections were processed as described above. Another female with green body color and oocytes (29 June, 2018) and specimen collected period of non-reproductive season (11 January, 2016) were compared for their morphology of the parapodia on chaetigers of the anterior and middle portions.

Results

A total of 73 females of 590 specimens collected at site A from March to August 2016 had oocytes, while 997 specimens collected from September to February had no oocyte. Figure 2 shows the relationship between the body width and the mean oocyte diameter of each female. The body width of ovigerous females ranged from 700 μm to 1200 μm. The mean oocyte diameter varied from approximately 110 μm (in a specimen with a body width of 900 μm) to 25 μm (in a specimen with a body width of 725 μm) (Fig. 2). No correlation was detected between body width and oocyte diameter ($r^2 <$ 0.001; $P > 0.800$; $n = 73$), though specimens with large oocytes (≥ 80 μm) ranged in body width
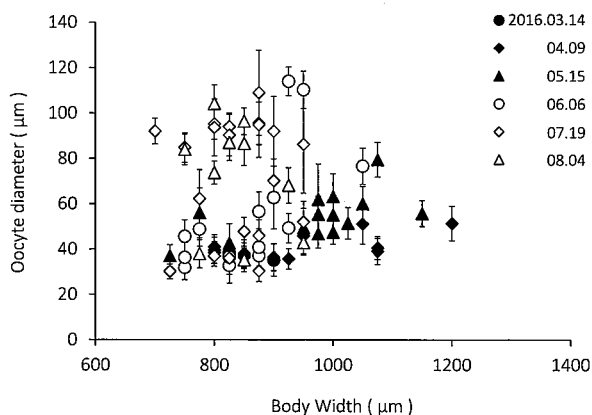

Fig. 2. Relationship between body width and oocyte diameter by sampling date. One plot shows an average of 20 to 30 oocytes for one ovigerous female (mean ± SD). The legend shows the sampling dates from 14 March to 4 August. ($r^2 < 0.001$; $P = 0.981$; $n = 73$).

from 800 μm to 950 μm. Female *S. erythraeensis* with small oocytes (< 80 μm) appeared from March to May 2016, whereas those with large oocytes (≥ 80 μm) appeared June to August (Fig. 2). Specimens with oocyte diameters ranging from 60 μm to 80 μm were rare.

A total of 52 specimens of *S. erythraeensis* including 26 females and six males were collected in May and June 2018 from site B, and histological sections made from all collected females and males. During sampling period, no reproductive swarming was observed. The female (collected in 13 June, 2018) released oocytes by sampling impulsion, and her body color was green (Figs. 3A, B). Specimen of Figures 3A and 3B are the same female, and Figures 3F and 3H show the histological section of this female. The mean oocytes dimeter of this female was approximately 167 μm.

In histological sections, *S. erythraeensis* have centrally located gut, a nerve cord, dorsal vessels, and ventral vessels. Most of the coelom is occupied by layers of muscular tissue in individuals that could not be determined as male or female (Fig. 3C). In specimens with coelomic oocytes and cell clusters of spermatogenesis, their muscular tissues degenerated (Figs. 3D, F, and G). In females of this stage, muscular tissues on the dorsal side dissolved, and the muscular layers became thinner as their oocytes grow (Figs. 3D, F). The diameters of oocytes at early stages of maturity (Fig. 3D) were smaller than 60 to 80 μm, whereas oocytes at later stages were approximately 100 μm in diameter and filled with yolk (Fig. 3F). In the later stage, amoebocyte associations began to appear around large oocytes (almost 100 μm in diameter) in the coelom and parapodium, and as reported by Eckelbarger (1992) and Eckelbarger and Rice (1988), oocytes have a nucleus, a yolk, and a rough-surfaced endoplasmic reticulum (RER)-like structure (Figs. 3F, H). The images of Figures 3B, 3F, and 3H were provided by the specimen of Figure 3A. In males, only a few cell clusters of spermatogenesis were released into the coelom in the early stages of maturity (Fig. 3E). At later maturity stage, the interior coelom and parapodium
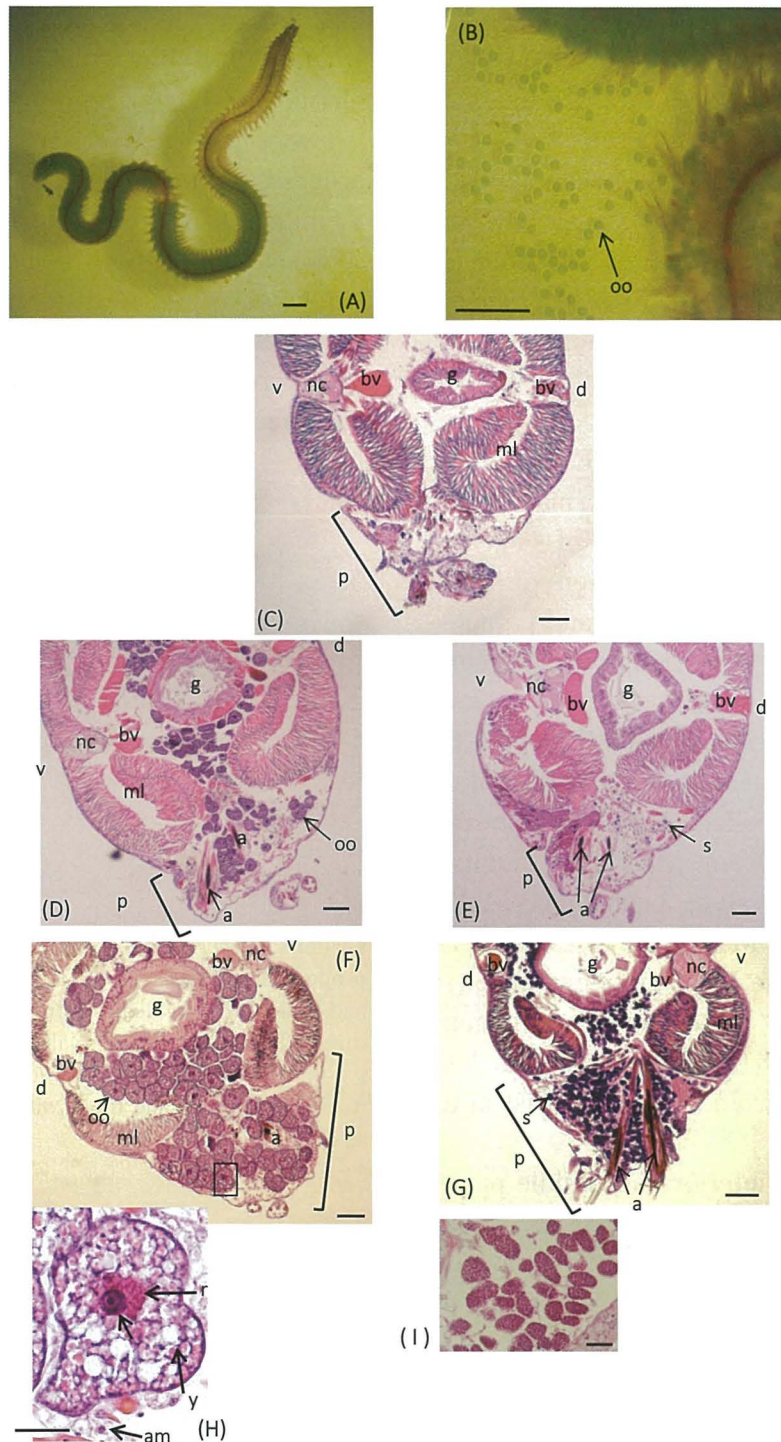
Fig. 3. Morphological characteristics of *Simplisetia erythraeensis*. Panels: (A) sexually mature female of a living *S. erythraeensis* (with damaged pygidium) and (B) oocytes of a living *S. erythraeensis*. Scale bars = 1 mm. Panels: histological sections of (C) a sexually immature individual (in May), (D) a moderately sexually mature female (in June), (F) a sexually mature female (in June), (E) a moderately sexually mature male (in June), and (G) a sexually mature male (in June), with panels showing the aciculae (a), blood vessels (bv), dorsum (d), oocytes (oo), gut (g), muscular layers (ml), nerve cord (nc), parapodium (p), spermatocytes (s), and venter (v). Scale bars = 100 μm. Panel (H): oocytes of panel (F) showing a central nucleus (arrow), amoebocyte (am), yolk (y), and a perinuclear cytoplasm (r) that demonstrates a rough-surfaced endoplasmic reticulum (RER). (I): spermatocytes of panel (G). Scale bar is 20 μm.

were filled with cell clusters of spermatogenesis (Fig. 3G). As reported by García-Alonso et al. (2006) that studied *Alitta virens*, blackberry-shaped cell clusters of spermatogenesis were observed in a section of the relatively late stage of maturing male (Fig. 3I).

Based on our observation of histological sections of a total 26 females, the following two

stages of the maturation could be distinguished: F1 defined as females with small oocytes (< 80 μm in oocyte diameter), and F2 defined as females with large oocytes (≥ 80 μm) with a nucleus and a yolk were distinguished. And since the oocytes of these two stages did not occur in any single specimen. Furthermore, we observed degeneration of muscular layers in both stages, and the degeneration seemed to have progressed more in F2 than in F1 (Figs. 3D, F).

The males of stage of Figure 3E had only released a few cell clusters of spermatogenesis into the coelom, and the males of stage of Figure 3G were filled with cell clusters of spermatogenesis in the interior coelom and parapodium. We had difficulty discerning two stages of maturation in males.

Proportions of maturing females (F1 and F2) and males were examined for total of 73 females and males that were collected by our monthly sampling at Site A from March to August 2016 (Fig. 4). The F1 stage began increasing in March, peaked in May (> 30%), and then decreased, whereas the F2 stage began in June (4%), peaked in July (27%), and then began decreasing in August (13%). Maturing males appeared in samples from June to August at a steady rate of 15%.

Morphology of the anterior and middle parapodia and chaetae was compared between asexually immature specimen (sex unknown) collected on 11 January, 2016 at site A and a probable mature female of F2 stage with the largest oocytes (approximately 127 μm in diameter) on 13 June, 2018 at site B (Fig. 5). No morphological differences were not observed between them; the epitokous metamorphosis including the development of some additional parapodial lobes and the replacement of atokous chaetae by paddle-like natatory chaetae did not occur in the female.

Discussion

In this study, we investigated maturity process of common estuarine polychaete *S. erythraeensis* at Kagoshima Bay by using histological examinations, and examined whether they have any morphological characteristic indicating a reproductive swimming.

Two stages were detected in female mature process, and in the later stage (F2), a part of their body was slightly metamorphosed for reproduction. However, no morphological or behavioral characteristic indicating reproductive swimming was not observed.

In previous studies, several species of polychaetes have been reported to be
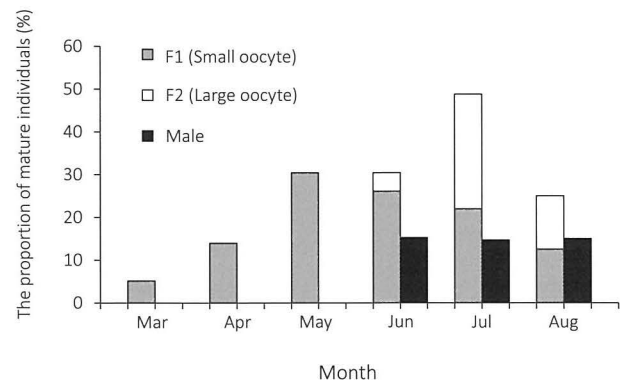

Fig. 4. Seasonal change (March–August 2016) in the proportions of each stage of females and mature males. The proportion is defined as the ratio of each stage of females and males to individuals with a body width > 700 μm (a threshold defined by the smallest sexually mature individual in the sample). Females with small oocytes (< 80 μm) were defied as F1 and those with large oocytes (≥ 80 μm) as F2.
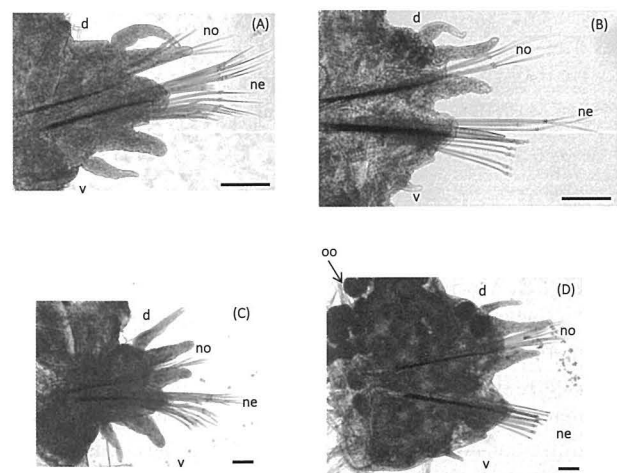

Fig. 5. Anterior view of the chaetiger of *Simplisetia erythraeensis*. (A) Fifth Chaetiger of a sexually immature specimen, (B) 60th chaetiger of a sexually immature specimen, (C) parapodium fifth of a specimen in the F2 stage, and (D) 70th chaetiger of a specimen in the F2 stage, showing the venter (v), dorsum (d), notopodium (no), neuropodium (ne), and oocytes (oo). Scale bars (black line) = 100 μm.

hermaphroditic, although many species are gonochoristic (Schroeder and Hermans 1975). And *S. erythraeensis* is speculate gonochoristic because specimens with only oocytes or cell clusters of spermatogenesis appeared in our samples. Given that the diameter of oocytes of *S. erythraeensis* did not significantly differ among different parts of its body and that the histological sections of one female showed oocytes in only one stage of maturation, we concluded that all oocytes of each female mature synchronously. Because the mean oocyte diameter was not affected by a female's body size, oocyte diameter can be used to indicate a female's stage of sexual maturation. In histological sections, oocytes and cell clusters of spermatogenesis not only filled their coeloms, but they also filled the insides of their parapodia. This characteristic has often been observed in other species of extraovarian Nereididae (Çinar et al. 2002; Jesús-Florse et al. 2015).

Ueno et al. (2019) have expected that the spawning of *S. erythraeensis* occurred from June to August in southern Kyushu, however the accurate spawning season was not certain. According to our observation of histological sections, F2 (more mature female) appeared from June to August, and the proportion was particularly high in July (Fig. 4). Therefore, we can say that *S. erythraeensis* spawn from June to August at the peak on July in Kagoshima Bay.

Muscular layers on the dorsal side of sexually maturing males (Fig. 3G) were thinner than those in sexually immature specimens (Fig. 3C). Similarly, the inside portion of the dorsal muscular layers in females disappeared with sexual maturity (Fig. 3F). It is reported that some species of Nereididae lose the much of the guts and longitudinal muscles lysis during metamorphosis (histolysis) approaching maturity (epitokous metamorphosis) (Fischer and Dorresteijn 2004 on *P. dumerilii*). And amoebocyte associations made from muscular layers to provide a depository for oocyte nutrition (Eckelbarger 1983) appeared around oocytes in our specimens (Fig. 3H). *Simplisetia erythraeensis* exhibited an annual life cycle and reproduced only once at Kagoshima

Bay (Ueno et al. 2019). Therefore, we concluded that the muscles of *S. erythraeensis* exhibit histolysis as they sexually mature and that this species probably expends the majority of its energy reserves supporting monotelic reproduction. However, guts muscles lysis reported by Fischer and Dorresteijn (2004) was not detected.

Significant morphological changes like developing lobes and acicular of parapodia in response to epitokous metamorphosis were not observed in females of F2 stage (Fig. 5). Reproductive swarming is a well-known reproductive behavior in polychaetes, wherein the parapodia change in reproductive individuals as they sexually mature (epitokous metamorphosis) (Durchon 1984). For example, in *Platynereis dumerilii*, parapodia appendages become enlarged, paddle-like lamellae emerge from the neuropodia, and the dorsal cirrus develops crenulations (Durchon 1984), and reproductive swimming is thought to assure fertilization (Sato 1999). Dorsey (1981) reported that population of *S. erythraeensis* in Australia displayed no morphological changes and deposited its eggs in an egg tube, suggesting that females of this species would spawn eggs in tubes and have no reproductive swarming. According to this report and our observation in which females developed no lobes neither acicular of parapodia, we can say that in the population of Japanese coast, *S. erythraeensis* does not engage in reproductive swarming.

In this study, male with sperm in coelom were not collected. The final stage of male spermatogenesis may progress in a short time. In any case, the reproductive behavior, such as spawning and fertilization, and early development of this species are not known. An observation study of the reproductive behavior in the laboratory and sampling of mature females and males in the field sites are needed to clarify reproductive characteristic of this species.

Acknowledgments

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コケゴカイ（環形動物門：ゴカイ科）の性成熟過程

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日本沿岸に普遍に分布する多毛類コケゴカイ *Simplisetia erythraeensis*（環形動物門：ゴカイ科）について，鹿児島湾の思川河口に生息する個体群を対象に，性成熟のプロセスとその季節変化を調査した。組織切片では，卵母細胞と精母細胞が別々の個体で見られ，卵母細胞が体腔で観察されたため，本種は雌雄異体であり，雌は卵巣を持たず体腔内で卵形成を行うことが明らかになった。雌の性的成熟は2つの段階（F1とF2）が確認され，F2は核，卵黄，および粗面小胞体様構造を有する事が確認された。F1は3月から8月に，F2は雄が精母細胞を持つ7月から8月に発生し，特に7月は出現率が高かったことから，繁殖期のピークは7月であることが示唆された。また，性的に成熟した雌と雄は筋肉層の大部分を消失していた（生殖変態）ことから，本種は1回繁殖でエネルギーの大部分を費やすと考えられる。さらに，成熟した雌は生殖変態した疣足を有していなかった為，本種は生殖群泳をしないであろうと示唆される。