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Source: Zoological Science, 37(6) : 519-528

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zs200080

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ZOOLOGICAL SCIENCE **37**: 519–528 (2020) © 2020 Zoological Society of Japan

Germ Cell Development in Male *Perinereis nuntia* and Gamete Spawning Mechanisms in Males and Females

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Perinereis nuntia **is a fully segmented worm with complete intersegmental septa. A previous study of females revealed that germ cells of this animal originate in the tail end segment, called the pygidium. Germ cells were duplicated in the pygidium, transferred to a newly generated segment, and then settled in the parapodia. Within each segment, the settled germ cells proliferated in the parapodia and then migrated into a body cavity area to begin meiotic development. Currently, there is not much information about differences between male and female germ cell development. Therefore, we conducted monthly in situ hybridization analyses using the germ cell marker Pn-piwi and histological examinations. Germ cells detected by Pn-piwi initially settled in the distal areas of the parapodia on both sides of each segment, then formed a large germ cell cluster in each parapodium, and finally, small germ cell clusters were formed by the separation of the large clusters. The small clusters migrated to the deeper body cavity area during growth by segment addition. Until the female germ cells began vitellogenesis, the sex of germ cells could not be identified by morphological observation. Thus, male and female** ***P. nuntia*** **may have the same mechanism of germ cell provision to all segments. At the time of spawning, sperm were released from nephridiopores at the 2nd through 15th segments from the pygidium, while eggs were released through ruptures in the skin of 2–3 segments between the 10th and 30th segments from the tail.**

Key words: Piwi, annelids, parapodia, germ cell migration, gamete release

INTRODUCTION

Annelida is a diverse and widely distributed animal phylum. Polychaeta, the largest class of Annelida, comprises two subclasses: Errantia (active scavengers or free-moving worms) and Sedentaria (permanent burrowers). Most polychaete species are marine, although a few are freshwater worms (Hartman, 1969; Fauchald and Rouse, 1997; Bakken and Wilson, 2005; Kriska, 2013). Polychaetes play an important role in the marine ecosystem as a food resource for crustaceans, fishes, and shore birds (Pamungkas and Glasby, 2015). Polychaetes are also the essential food in shrimp aquaculture for induction of shrimp gonadal maturation (Olive, 1999; Binh et al., 2008; Techaprempreecha et al., 2011). Most polychaete species live as scavengers, decomposing and recycling nutrients in the marine ecosystem (Fauchald and Rouse, 1997; Nugteren et al., 2009; Ito et al., 2011).

Due to divergence in ecological features such as their habitats, morphologies, and behaviors, polychaetes have evolved various reproductive strategies including hermaphroditism, viviparity, and external fertilization (Pocklington and Hutcheson, 1983; Bartels-Hardege and Zeeck, 1990; Fong, 1993; Simon et al., 2014). Nereididae, one of several families of polychaetes, has long been studied for its unique reproductive strategy, called epitoky. As the breeding season approaches, a sexually immature worm (atoke) transforms into a sexually mature worm (epitoke) with a characteristic pelagic morph. At the time of spawning, the entire body of the epitoke is filled with mature gametes. Epitokes release eggs or sperm with a typical swimming behavior and die shortly after the spawning (Bartels-Hardege and Zeeck, 1990; Fong, 1993; Hébert-Chatelain et al., 2008; Fischer, 1999).

Despite the intensive studies of the reproductive biology of polychaetes, knowledge about gametogenesis and gamete release is still limited, especially in fully segmented species. We previously described the oogenesis of *Perinereis nuntia* (Errantia. Nereididae), which has body segments that are completely compartmentalized by intersegmental septa (Maceren-Pates et al., 2015). During growth, each new segment is added at the anterior border of the pygidium, the tail end segment. Thus, anterior segments are older than posterior segments (Fischer, 1999; Fischer and Dorresteijn, 2004; Bakken and Wilson, 2005). We found that the pygidium was the only source of germ cells in *P. nuntia*, providing germ cells to each new segment at the time of segment generation (Maceren-Pates et al., 2015). This may be a common

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doi:10.2108/zs200080

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mechanism of germ cell distribution to each body segment in fully segmented species of polychaetes. Within each segment, the distributed germ cells migrate and settle in distal areas of parapodia at both sides of the segment, and then proliferate to form one large germ cell cluster in each parapodium. The cluster separates into smaller clusters, which migrate to a body cavity area to begin meiotic development (Maceren-Pates et al., 2015). However, compared to female

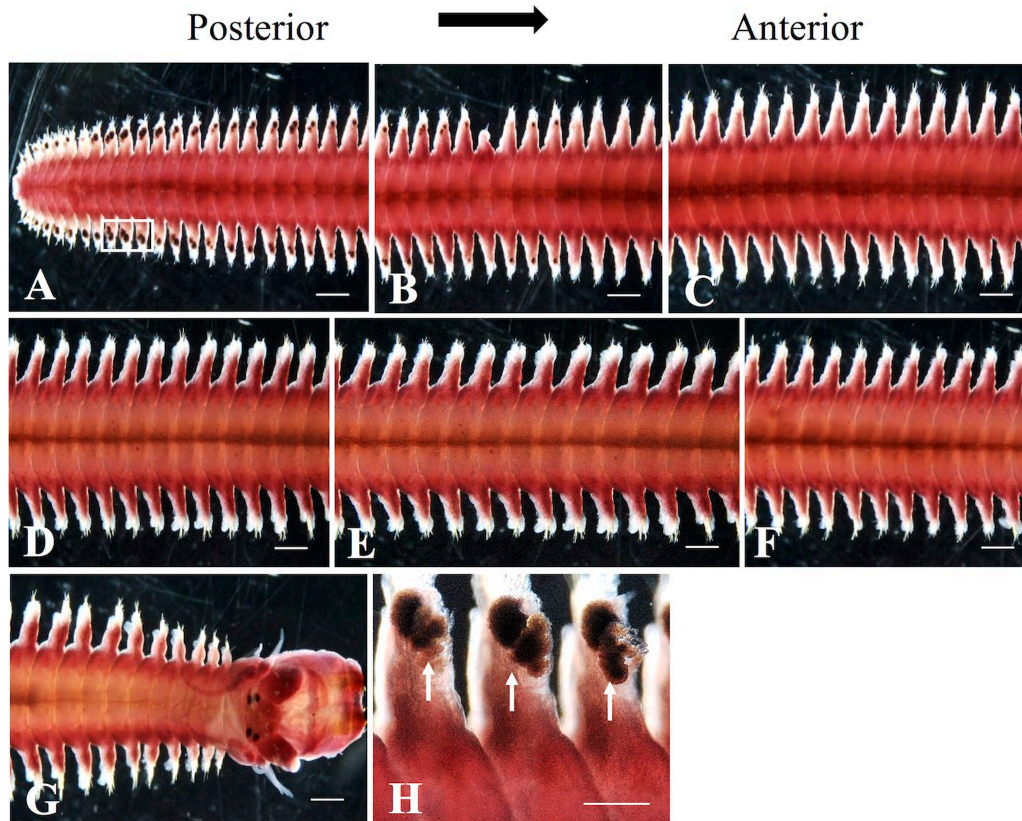

Fig. 1. Whole-mount *in situ* hybridization with the Pn-piwi probe in an adult male worm in late August. Strong signals were detected at the distal ends of the parapodia in the posterior region and signal strength decreased toward the anterior region **(A–G)**. White arrows indicate the Pn-piwi signals at the parapodia **(H)**. Body length = 10 cm. The total number of body segments in this individual was 98. Scale bars for **(A–G)** and **(H)** are 200 µm and 100 µm, respectively.

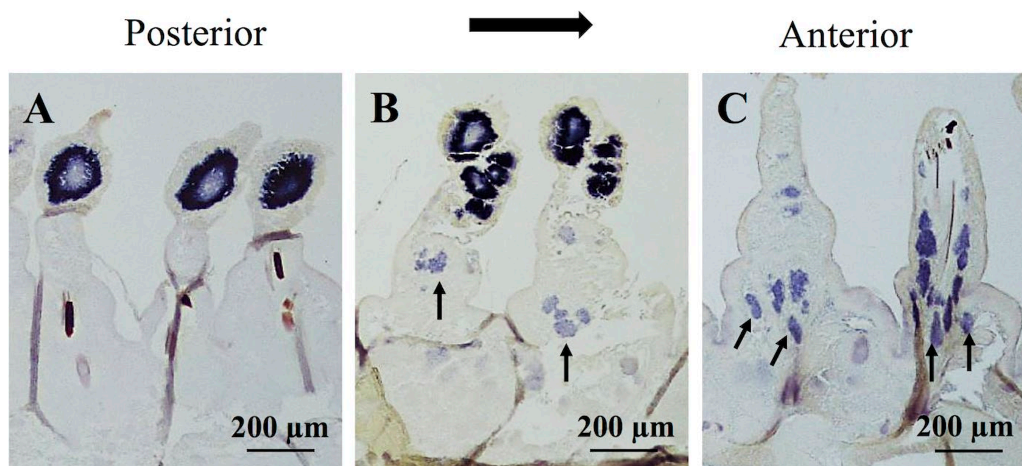

Fig. 2. Horizontal sections of the hybridized worm showing the localization of Pn-piwi expression in an adult male in late August. **(A)** Posterior region; strong Pn-piwi signals were observed at large cell clusters in distal areas of the parapodia. No clusters were detected in trunk areas. **(B)** Mid region; the Pn-piwi signals in the distal areas were decreased, and several smaller clusters were detected in trunk areas. **(C)** Anterior region; the Pn-piwi signal was undetectable in the distal areas. Smaller clusters were observed in the trunk and basal regions of the parapodia. Arrows indicate the smaller clusters of germ cells. Scale bars, 200 µm.

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germ cell development, male germ cell development has never been well described. Furthermore, the mechanisms of gamete release after epitoky are still unclear. To address these matters, we examined 1) germ cell development in males in comparison to oogenesis using histological analyses and gene expression observations and 2) the characteristics of gamete release in males and females.

MATERIALS AND METHODS

Animals

Perinereis nuntia (Polychaeta. Errantia. Phyllodocida. Nereididae) was purchased from a local hatchery in Oita Prefecture and reared in the Fisheries Research Laboratory (FRL) of Kyushu University. Animals were maintained in filtered running seawater with daily feeding. The natural breeding season in Oita Prefecture is in early April, and animals die shortly after spawning in the next April with a one-year life cycle. The embryos and young juveniles were reared at the FRL. Animals grew by segment addition at the anterior border of the tail-end body segment, the pygidium. This extension growth began just after the spawning in April and declined at the end of July. The segment addition was estimated to occur once per day and reached up to 101 ± 7.5 (standard deviation; n = 30) segments in an adult. About 15 animals were sacrificed at least once per month for the analysis of germ cell development. Prior to fixation, animals were anesthetized with 0.3% ethylene glycol monohexyl ether in seawater and then fixed in 4% paraformaldehyde in phosphate-buffered saline. The photographs used in this manuscript show representative figures of similar animals.

In situ hybridization

Vasa, nanos and piwi genes have long been used as germline markers in various annelid species (Cox et al., 1998; Kuramochi-Miyagawa et al., 2004; Rebscher et al., 2007; Giani et al., 2011; Beyret and Lin, 2011; Rebscher, 2014; Özpolat and Bely, 2015; Ponz-Segrelles et al., 2018). In our previous experiments, we used the vasa gene to trace germ cells in female *P. nuntia* (Maceren-Pates et al., 2015). In this study, we cloned the piwi gene and used it to trace germ cells for improved reliability of germ cell detection. For Pn-piwi cloning in this study, primers were designed from the mRNA sequence of a closely related species, *Platynereis dumerilii* (Rebscher et al., 2007) (forward: 5′-ACCACGACTCCAAGCAGAAG-3′; reverse: 5′-ATCTTGTGGGCGTATTGGCA-3′). Total RNA was extracted from unfertilized eggs of mature *P. nuntia* using TRIzol Reagent (Invitrogen) and purified using a RNeasy Kit (Qiagen). cDNA was synthesized using a Prime Script RT-PCR Kit (Takara). Using a pGEM-T Easy Vector System I kit, the 613-bp PCR product was ligated into a plasmid and transformed into *Escherichia coli* DH5. Probes were labelled with digoxigenin and used for whole-mount in situ hybridization, as described previously (Maceren-Pates et al., 2015). The cDNA sequence of Pn-piwi is registered in DDBJ (Accession No. LC564863).

Histological analysis

After whole-mount in situ hybridization, animals were sectioned for histological observation to detect germ cells inside the body segments, as described previously (Maceren-Pates et al., 2015). The same batch of animals was also prepared for staining with hematoxylin and eosin (H&E). Sections were cut at 7 μm.

Spawning observation

Swimming epitokes (2–4 males per female) were placed in a transparent container filled with filtered seawater and observed until spawning occurred naturally. This observation was repeated several times to locate the spawning sites on the body. Several epitokes were quickly fixed at the beginning of spawning to analyze the spawning sites. After fertilized eggs were collected, embryos were reared for use in further experiments.

RESULTS

Germ cell localization in male *P. nuntia*

To examine the localization of germ cells in the body segments of males for comparison with the previous observations of female *P. nuntia*, we used in situ hybridization with Pn-piwi and Pn-vasa anti-sense probes on adult males at first. Although both probes showed similar expression patterns in whole-mount in situ hybridization, the Pn-piwi probe

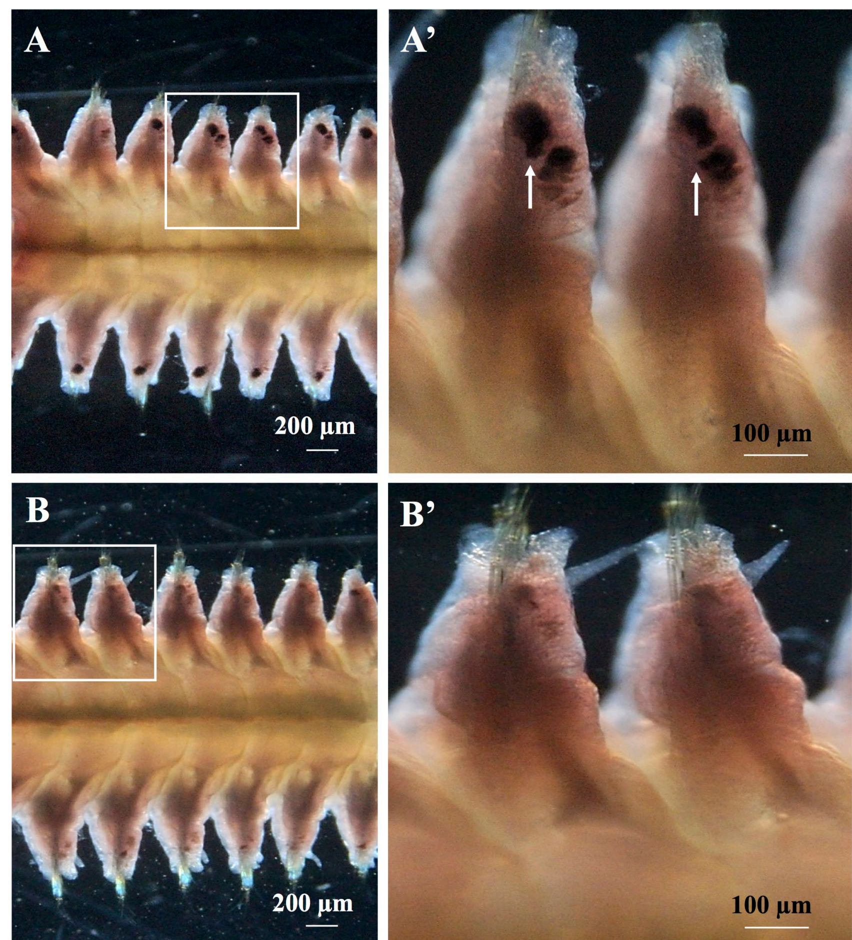

Fig. 3. Specificity of the Piwi probe in in situ hybridization. **(A)** Germ cell clusters were detected by in situ hybridization with use of the anti-sense probe of Pn-piwi. **(B)** No signal was detected with use of the sense probe. **(A’, B’)** Higher magnification views of boxed area in **(A, B)**, respectively. Arrows indicate germ cell clusters. Scale bars, 200 μm in **(A, B)**; 100 μm in **(A’, B’)**.

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tended to give a stronger signal compared with the vasa-probe. Therefore, we decided to use the Pn-piwi probe in this study. The Pn-piwi probe was strongly hybridized at the distal areas of parapodia on both sides of the segments (Fig. 1). The signal strength gradually decreased from the posterior to the mid-body, and then disappeared in the anterior region of the body (Fig. 1). Horizontal sections of worms stained by in situ hybridization showed cell clusters in the parapodia in the posterior region, but no signal was detected in the trunk area (Fig. 2A). In the medial body segments, we observed large cell clusters with the Pn-piwi signal at the distal areas of the parapodia, and several smaller clusters were separated from the large cluster and several Pn-piwi signals in the trunk and basal areas of parapodia (Fig. 2B). In the anterior segments, the large cell clusters were not detected in the parapodia, but several signals were observed in the trunk areas of the parapodia and in the deeper body cavity area (Fig. 2C). The Pn-piwi signals were decreased in the trunk and deeper body cavity areas compared to the clusters at the distal areas of the parapodia. Figure 3 shows that no signal was detected with use of a sense probe.

Sexual development of gametes in *P. nuntia*

Figure 2 shows the continual changes of germ cell localization with the structural changes of germ cell clusters. These results were similar to those previously observed in females (Maceren-Pates et al., 2015). This shows that germ cells migrate from parapodia to the deeper area of the body cavity. The migration was observed in the early specimens of whole mount in situ hybridization in May, which indicated that the migration had already begun in young juveniles by 40 dpf (Fig. 4). The migration was initiated from anterior segments, as those were formed earlier than posterior segments. During the migration period, it was not possible to distinguish the sex of each animal because there was no morphological difference in germ cells (Fig. 5A, A').

In September, the female germ cell became discriminable from the male germ cell by its enlargement with an accumulation of vitellogenin (Fig. 5B, B'). After September, 30 more animals were analyzed and were equally divided into males and females; thus, the sex ratio of *P. nuntia* was assumed to be 1:1. The vitellogenesis began simultaneously in all body segments, but the size of vitellogenic germ cells varied, even within the same segment during this season (Fig. 5B, B'). However, nearly all eggs were observed to attain equal size just before the spawning season (Fig. 6A). By contrast, male germ cells were observed to have a longer proliferation period (Fig. 5C), which lasted until March, and they entered meiosis at the same time, in late March, in all body segments (Fig. 6B, B').

The developing male germ cells were categorized according to morphological changes in the clusters at each stage (Fig. 7). In the early stage of proliferation, nuclei and nucleoli were clearly visible, and the cytoplasm was filled with granular particles (Fig. 7A). These cells were observed in September and categorized as spermatogonia I. In this stage, the average cell size varied among clusters, even within the same segment. In November, each cluster was filled with proliferating germ cells (Fig. 7B), and by January, the clusters enlarged in size due to the highly proliferating

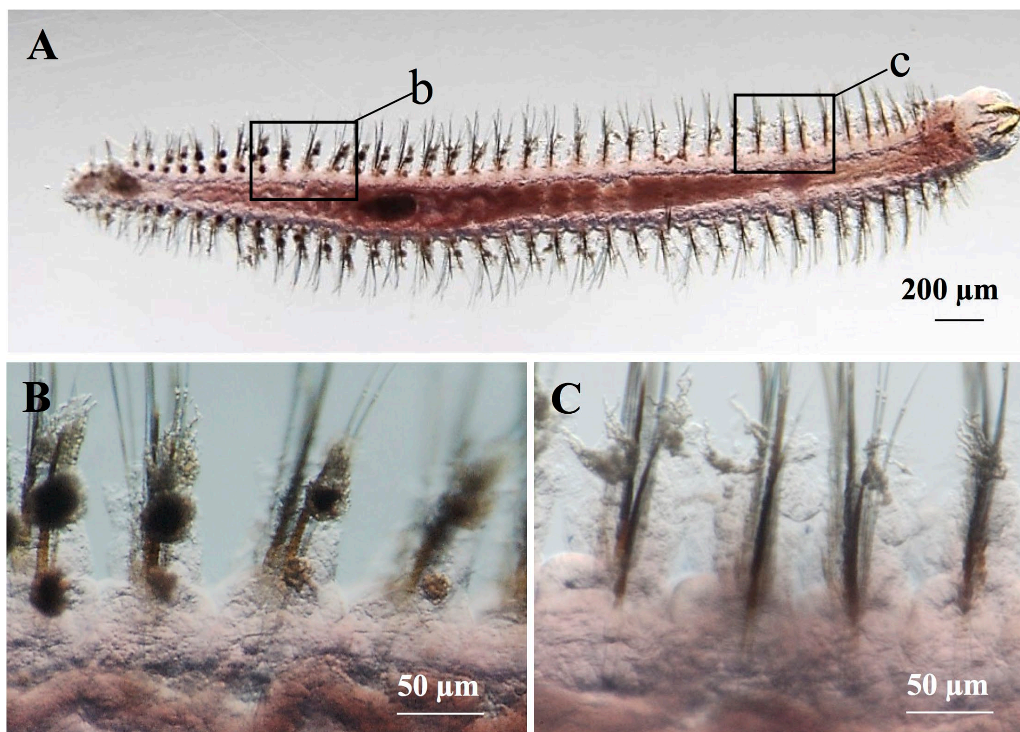

Fig. 4. Germ cell clusters in a juvenile worm. **(A)** Germ cell clusters shown by in situ hybridization at parapodia of a juvenile worm at 40 dpf. **(B)** Enlarged view of parapodia of the posterior region indicated by a black box (b) in **(A)**. Germ cell clusters are shown at distal areas of each parapodia. **(C)** Enlarged view of parapodia of the anterior region indicated by a black box (c) in **(A)**. Germ cell clusters are not observed at these parapodia. Scale bars, 200 µm in **(A)**, 50 µm in **(B, C)**.

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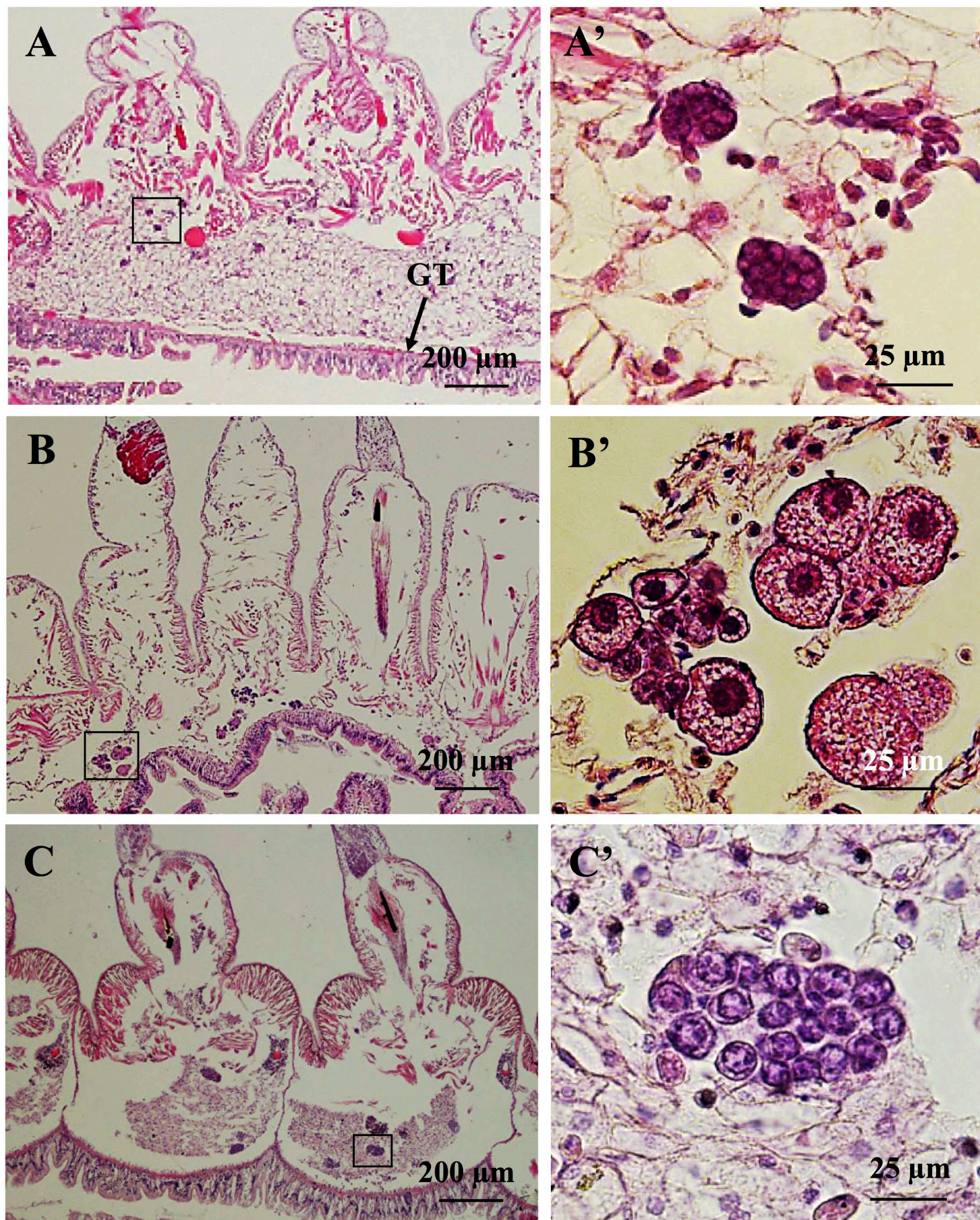

Fig. 5. Germ cells before and after the start of vitellogenesis. **(A)** Sex is difficult to distinguish morphologically in germ cells of 4-month-old juveniles in early August. **(B)** Growing female germ cells with vitellogenesis in September. **(C)** Proliferating male germ cells in clusters in September. **(A', B', and C')** are enlarged images of the boxed area in **(A, B, and C)**, respectively. **(B')** shows oocytes of various sizes in the same segment. **GT**: Gut tract. Stained with H&E. Scale bars, 200 µm in **(A, B, C)**, 25 µm in **(A', B', C')**.

germ cells; the cells decreased in size and the nuclei and nucleoli could not be distinguished due to chromosome condensation. The cells in these clusters were categorized as spermatogonia II (Fig. 7C). The enlarged clusters loosened throughout February, divided into smaller clusters (Fig. 7D), and then transitioned into spermatocytes in March (Fig. 7E). In April, a few spermatids were observed as tetrads, although the majority were single sperm cells (Fig. 7F).

Gamete spawning sites in *P. nuntia*

Perinereis nuntia body segments are completely separated from each other by intersegmental septa. Each segment is filled with proliferating germ cells during the breeding season, and these germ cells are all released during characteristic swimming behavior. However, the means by which *P. nuntia* releases gametes has never been clearly described. To examine the spawning mechanism, we observed the animals' behavior during spawning to identify the gamete release site, and also examined the fine structure of this release site.

The intersegmental septa were broken during the swimming while spawning and gametes were mixed between segments in males and females (Fig. 8). In males, sperm were continuously released as streams from several

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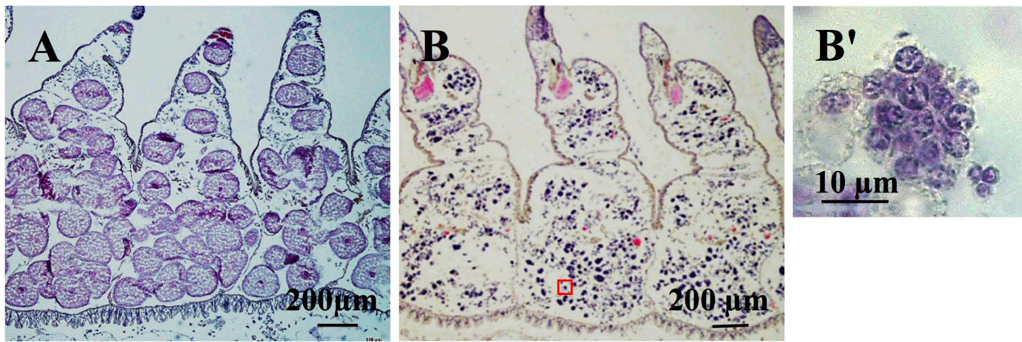

Fig. 6. Germ cells filling body cavities of each segment in March (mid-body region). **(A)** Female body segments are filled with fully grown oocytes. **(B)** Male segments are filled with many germ cell clusters. **(B')** An enlarged image of the boxed area in **(B)**. Stained with H&E. Scale bars, 200 μm in **(A, B)**, 10 μm in **(B')**.

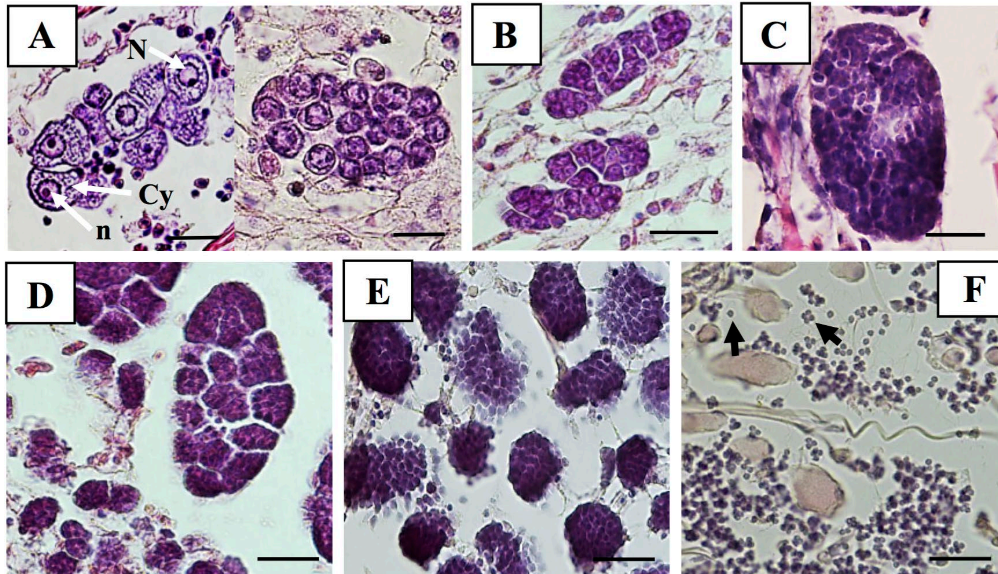

Fig. 7. Time course of development of male germ cells. **(A)** Early spermatogonia in September. **(B)** Proliferating spermatogonia in November. **(C)** Proliferating spermatogonia in January. **(D)** A large cluster in early March. **(E)** Spermatocytes in mid-March. **(F)** Spermatids and sperm in April (breeding season). N: Nucleus, n: nucleolus, Cy: Cytoplasm. Black arrows in **(F)** show a tetrad spermatid and a single sperm. Stained with H&E. Scale bars,

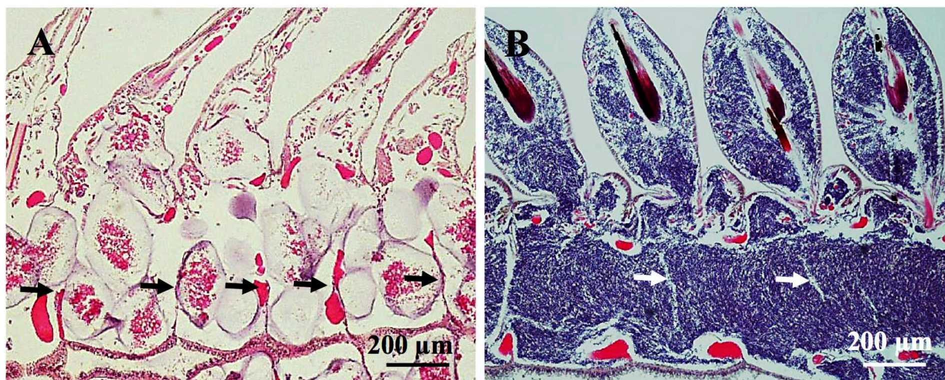

Fig. 8. Horizontal sections of a swimming worm just prior to spawning in April **(A)** Female. **(B)** Male. Arrows indicate broken septa. Stained with H&E. Scale bars, 200 μm.

segments in the tail region (Fig. 9A). The total number of segments with sperm release varied among individuals, ranging from 10 to 14 segments from the 2nd to 15th segments from the tail end (Fig. 9B). The gamete release sites were located near the intersegmental septum at the anterior side of each segment (Fig. 10A, A'). A tube-like structure

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from the body cavity was observed connecting to this opening site, which could possibly be a nephridium (Fig. 10B). The sites were opened during sperm release (Fig. 10C).

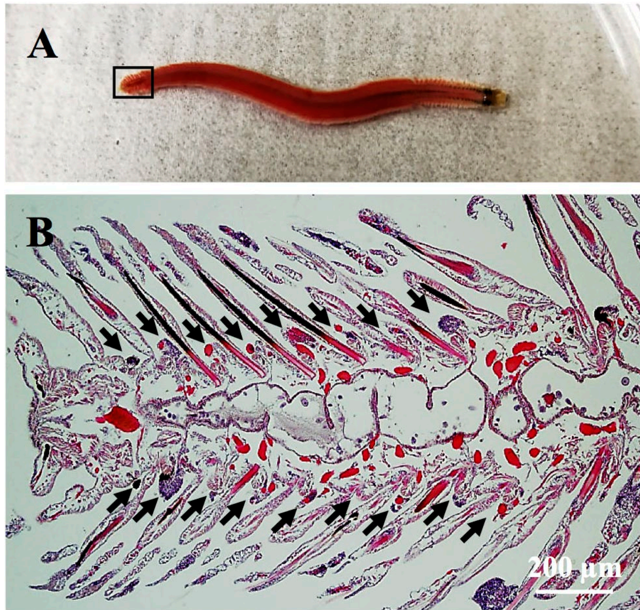

Fig. 9. Sperm spawning area in the tail region of a male. **(A)** The box indicates the area of sperm spawning. **(B)** A horizontal section of the tail indicated in **(A)**. Arrows indicate sperm released from both sides of limited segments. Stained with H&E. Scale bar, 200 μm in **(B)**.

In females, eggs were released from only 2–3 segments in the tail region (Fig. 11A). The exact segments differed between individuals but were in the range of the 10th to 30th segment from the pygidium. As the epithelial structure was severely ruptured by egg spawning, it was difficult to identify the exact point and detailed structure of egg release (Fig. 11B–D). Ten animals were fixed and sectioned to analyze the internal structures for each sex. Figures 8–11 show typical examples.

DISCUSSION

Germ cell localization and gamete production in *P. nuntia*

In this study, we compared germ cell development between males and females of *P. nuntia*. *Perinereis nuntia* grows by adding new body segments at the anterior boarder of the tail-end segment, the pygidium, and all segments are completely compartmentalized by intersegmental septa. In our previous report, we identified an interesting mechanism by which germ cells are allocated to all body segments, that is, a newly generating segment receives copies of germ cells from the pygidium, the only source of germ cells in *P. nuntia* (Maceren-Pates et al., 2015). In each segment, germ cells settled first at the parapodia on each side of the segment and proliferated to form a large cell cluster. Then, the large cluster separated into several smaller clusters, which migrated to the inner body cavity area (Maceren-Pates et al., 2015).

During this period, germ cells proliferated and developed in each segment. In our previous paper, we found that

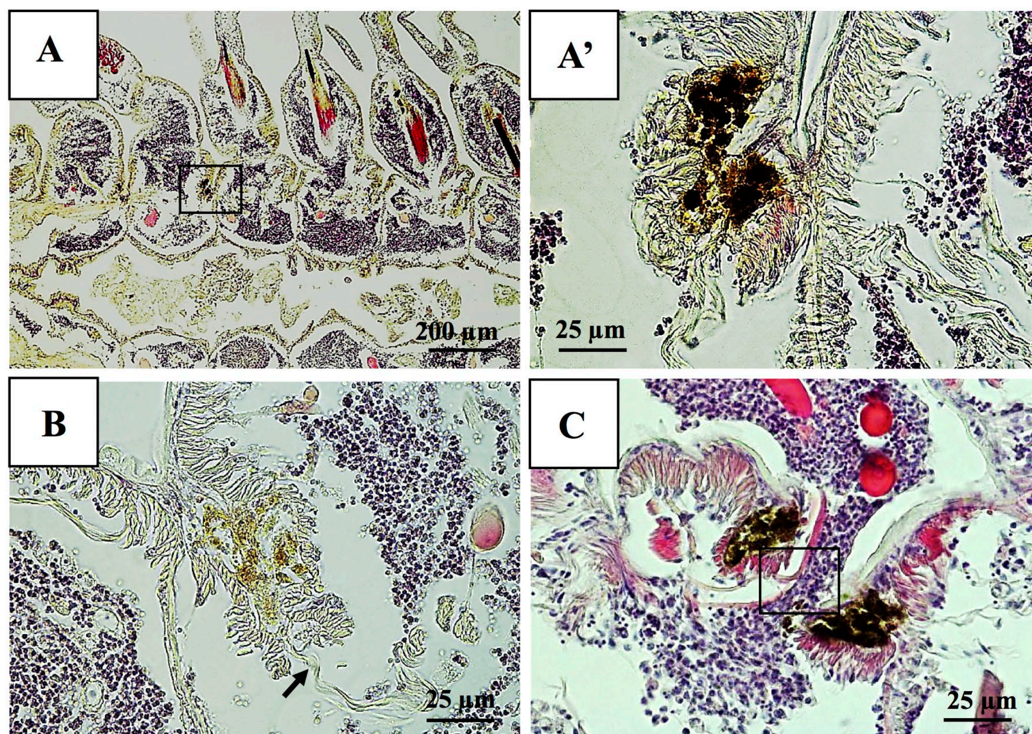

Fig. 10. Histological observation of sperm release sites in *P. nuntia*. **(A)** A horizontal section of sperm-releasing segments. **(A')** An enlarged sperm release site indicated by the boxed area in **(A)**. **(B)** A sperm release site with a tube-like structure indicated by a black arrow. **(C)** Sperm release from the opening of a structure similar to that shown in **(A')**. The box indicates the opening point. Stained with H&E. Scale bars, 200 μm in **(A)**, 25 μm in **(A', B and C)**.

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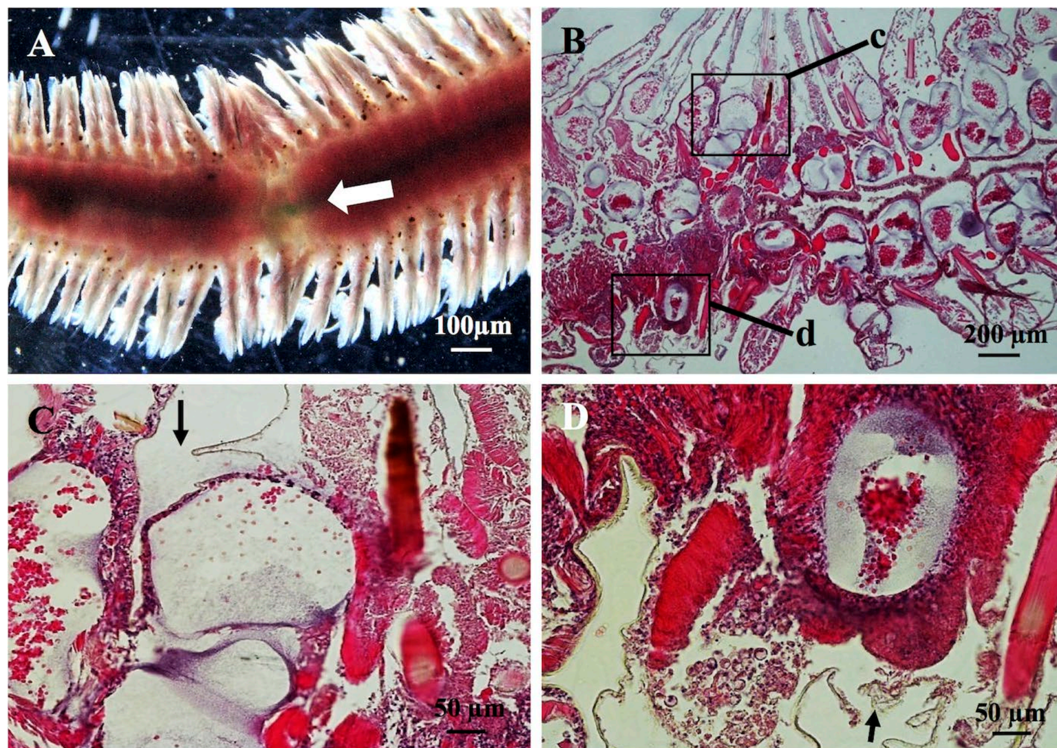

Fig. 11. Histological observation of egg release sites in *P. nuntia*. **(A)** The white arrow indicates the segments where eggs were released. **(B)** The egg release sites are indicated by black boxes c and d. Eggs are released from breaks at both sides of the segment. **(C)** and **(D)** are enlarged images of boxed areas (c) and (d), respectively. Black arrows indicate the break points of the egg-releasing segment. Animals were fixed during spawning. Stained with H&E. Scale bars, 200 μm in **(B)**, 100 μm in **(A)**, 50 μm in **(C, D)**.

the large germ cell clusters were already formed in the anterior segments of young juveniles at 10 dpf (Fig. 9 of Maceren-Pates et al., 2015). However, at 40 dpf, the Pn-piwi signal disappeared from the parapodia of the anterior segments (Fig. 4). The germ cell clusters translocated into the deeper body cavity area might be difficult to be observed because the Pn-piwi signal decreased during migration (Fig. 2). These observations indicate that germ cells proliferate to form a large cluster in the parapodia and then migrate to the inner body cavity area within 30 days after the cluster formation. However, at this stage, it was impossible to distinguish the sex of animals because there were no morphological differences between males and females (Fig. 5A, A').

In September, all germ cells began vitellogenesis in females, which was recognizable by the increase in size (Fig. 5B, B'). By contrast, male germ cells continued proliferation (Fig. 5C, C'). With these differences, animals could be identified as male or female. At this time, the sex ratio of *P. nuntia* was 1:1, and it was the same in the breading season, similar to other species (Reish, 1954, 1957; Barels-Hardege and Zeeck, 1990; Fischer and Dorresteijn, 2004). Before the start of vitellogenesis, although we did not identify the sex of the animals used for the histological analysis, we assumed that the animals included males and females at a ratio of 1:1. Therefore, we may conclude here that there is no clear morphological difference between male and female germ cells in the early developmental phase.

In late September, germ cells had reached the inner body cavity area in all body segments and simultaneously began vitellogenesis. Naturally, the germ cells in the anterior segments reached the body cavity area earlier than those in the posterior segments. However, the anterior germ cells delayed vitellogenesis while waiting for the posterior germ cells to reach the body cavity area. This may suggest that there is some unidentified mechanism to control the initiations of migration and vitellogenesis in female germ cells. As the oocytes reached a maximum size before the spawning season, vitellogenesis seemed to continue until just before spawning. The oocytes varied in size in the early phase of vitellogenesis, and vitellogenesis was not well synchronized among germ cells, even in the same segment (Fig. 5B, B'). This variation in oocyte sizes in the early stages has been observed in several polychaete species (Olive and Garwood, 1981; Fischer, 1974; Golding and Yuwono, 1994). However, as the breeding season approached, all oocytes attained equal size (Fig. 6A).

By contrast, males had a longer germ cell proliferation phase to maximize the number of sperm produced, and they entered meiosis in late March, as shown in Fig. 6B and B′. The developing male germ cells in *P. nuntia* were clearly distinct in morphology between stages (Fig. 7). In many polychaetes, morphological characteristics have been used as the criteria for germ cell development. In *Nereis virens*, spermatogenesis was categorized into five stages based on the morphology of germ cells; spermatogonia I, spermatogonia II, spermatocytes, tetrads, and spermatozoa (Schenk and Hoeger, 2010). In this study, the observed *P. nuntia* spermatogenesis stages closely resembled those of *N. virens* (Schenk and Hoeger, 2010). In *P. nuntia*, male germ cells in September might be categorized as spermatogonia I

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by clear visibility of big nuclei and nucleoli (Fig. 7A). However, as proliferation continued through January, it resulted in the formation of thick and tightly packed clusters of germ cells, which were categorized as late spermatogonia II (Fig. 7B, C). In February, this tightly packed cluster (spermatogonia II) loosened and began to form several smaller clusters; these were clearly separated by March and were categorized as spermatocytes (Fig. 7D, E). In April, the breeding season, some germ cells were observed in groups of four spermatids, whereas the majority were individual sperm cells (Fig. 7F).

Gamete spawning sites in *P. nuntia*

Perinereis nuntia has complete septa to compartmentalize each segment of the body. Therefore, we hypothesized that *P. nuntia* would spawn mature gametes from various areas of the body. However, in this study, both males and females were found to release gametes from limited regions in the posterior body (Figs. 9 and 11). Males released sperm from 10–13 segments, and females released eggs from only a few segments. Most germ cells were released from those segments during typical spawning swimming. The spawning swimming was characterized by a strong and flowing serpentine motion of the body. We presume that this strong motion may help to split the intersegmental septa and move germ cells toward the posterior region. This septum breakage appeared to precede germ cell release in both sexes (Fig. 8). If the serpentine motion effectively pushed germ cells into the posterior region, it would increase the internal pressure to break the skin and release germ cells. In females, the germ cell release occurred from only a few neighboring segments in the posterior third of the body (10th–30th segments from the tail). This may indicate that the egg release site is formed randomly in females. By contrast, in males, sperm were released from ~10 segments in the tail region (Fig. 11). The location of the release sites did not appear to vary among individuals, suggesting the existence of some common structure related to sperm release, unlike in females. Several previous papers have reported the sites of germ cell release in polychaetes. Most of the nereidids are reported to spawn by rupturing the body wall (Fischer and Dorresteijn, 2004) or by dehiscence (Goodrich, 1945). However, in Syllidae, metanephridia of the fertile segments are modified into metanephromixia during sexual maturity to act as gonoducts (Goodrich, 1945; Franke, 1999). In this study, we observed that sperm were released by splitting/opening at the nephridiopore area in *P. nuntia* (Fig. 11). This is quite similar to sperm release in Syllidae. However, we could not identify the detailed structures as metanephromixia. Although these opening sites were generally found in most segments, the sperm release occurred at limited nephridiopores in the posterior 10 segments. Therefore, in males, some unknown mechanism may be involved in opening the limited nephridiopores for sperm release.

ACKNOWLEDGMENTS

We thank Mr. Yasunobu Masuo, the owner of the polychaete hatchery in Oita Prefecture, Japan for the supply of animals during the whole period of this research.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

MJP, MMP, YK and MY designed the experiments; MJP performed the experiments; MJP, MMP, YK, GP Jr and MY analyzed the results and wrote the manuscript. All authors read and approved the final manuscript.

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(Received May 29, 2020 / Accepted August 17, 2020 /
Published online October 29, 2020)

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