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Author(s): Satoko Sekida , Nozomu Iwasaki and Kazuo Okuda
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Gonadal Morphology and Gametogenesis in Japanese Red Coral *Corallium japonicum* (Octocorallia: Alcyonacea) Collected off Cape Ashizuri, Japan

Satoko Sekida[1], Nozomu Iwasaki[2], and Kazuo Okuda[1]*

[1]*Graduate School of Kuroshio Science, Kochi University, 2-5-1 Akebono-cho, Kochi 780-8520, Japan*
[2]*Faculty of Geo-Environmental Science, Rissho University, 1700 Magechi, Kumagaya, Saitama 360-0194, Japan*

Colonies of the Japanese red coral *Corallium japonicum* Kishinouye, 1903 collected off Cape Ashizuri, Japan were gonochoric and produced gonads in siphonozooids annually, mainly during the spring season. Polyp anatomy, gonadal morphology and gametogenesis in this species were revealed by light and electron microscopy. A siphonozooid had a pharynx with a prominent siphonoglyph and eight mesenteries: two sulcal, two asulcal, and four lateral. A rudimentary retractor was found on one side of each mesoglea of these mesenteries. The retractor arrangement in the siphonozooid was reverse of what was described in the autozooids of octocorals. Gonads initiated as small protrusions on the mesenteries, except in the asulcal ones, and even at an incipient stage they were covered with a sac-shaped thin layer of mesoglea, which was continuous with the mesoglea of mesenteries. Gastrodermis enveloped the complete outer surface of the thin layer of mesoglea throughout gametogenesis in both oocytes and sperm cysts. Oocytes produced many microvilli on their cortical surfaces beneath the thin layer of mesoglea concomitantly with the accumulation of lipid globules in the cells, whereas in sperm cysts spermatocytes and spermatids increased in number without microvilli production, followed by synchronous spermiogenesis involving remarkable changes in the shape and position of organelles. Based on the comparison of patterns in gonadal development between octocorals including *C. japonicum*, hexacorals and scyphozoans, octocoral and stauromedusa species may be characterized by the fact that gametogenesis never occurs in the matrix of mesoglea, but rather exclusively within the thin sac of mesoglea surrounded by gastrodermis.

Key words: gonadal morphology, gametogenesis, precious coral, *Corallium japonicum*, ultrastructure

INTRODUCTION

Precious corals, species of which belong to the family Coralliidae within the order Alcyonacea and the subclass Octacorallia, have been commercially exploited for several centuries all over the world because of the use of their skeletons for jewellery and ornaments (Tsounis et al., 2010). Thirty-eight Coralliidae species have been reported from tropical to temperate oceans (Bayer and Cairns, 2003; Simpson and Wartling, 2011; Nonaka et al., 2012; Tu et al., 2012, 2015a, b), and eight of these species are considered precious corals. Recent molecular phylogenetic studies have indicated that the Coralliidae consists of three distinct clades (Ardila et al., 2012; Tu et al., 2015b), and thus Tu et al. (2015b) have recombined the currently known species in the Coralliidae into the three genera: *Corallium*, *Hemicorallium* and *Pleurocorallium*. The present study follows the taxonomic classification proposed by Tu et al. (2015b). The most valuable species of precious corals are the red coral, *Corallium rubrum*, in the Mediterranean Sea, and the Japanese red coral, *Corallium japonicum*, which is distributed in Japanese and Taiwanese waters at depths of 100–300 m (Iwasaki and Suzuki, 2010). Characteristics include an extremely slow growth rate (Luan et al., 2013), low rates of recruitment (Iwasaki et al., 2012) and high commercial value, which make these precious coral populations fragile resources that are easily over-harvested (Chang et al., 2012). It is essential to understand the biological and ecological properties of precious corals to employ accurate fisheries management leading to sustainable use of these natural resources (Bruckner, 2009). However, there is little information on the life history of precious corals, such as reproduction and development, other than their habitat and biomass. This may be because most such species are deep corals (Grigg, 1993; Tsounis et al., 2010).

Colonies of Coralliidae species are dimorphic, where two types of polyp occur: autozooids and siphonozooids (Bayer, 1996; Simpson and Watling, 2011). Gonads are produced within autozooids in the Mediterranean red coral *C. rubrum* (Santangelo et al., 2003; Tsounis et al., 2006), whereas they form within siphonozooids in the Hawaiian species, *Hemicorallium lauuense* and *Pleurocorallium*

* Corresponding author. Tel. : +81-88-844-8314;
Fax : +81-88-844-8356;
E-mail: okuda@kochi-u.ac.jp
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Table 1. *Corallium japonicum*. Annual and monthly frequencies of mature and immature colonies*.

Year	Month												Total
	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	
2006					4F, 5M, (1)	1M	(1)	(1)		(1)			4F, 6M, (4)
2007										(1)	(1)	(1)	(3)
2008	(1)	1F, 1M		1F, 1M	1F								3F, 2M, (1)
2011			1F, 1M	6F, 4M	2M	2F, 1M, (1)	1F, (3)	(2)	2F, (2)	(1)	2F	(2)	14F, 8M, (11)
2012		1M, (6)	1F, 1M, (6)	3F, 4M, (5)	6F, 7M, (2)	2M	(1)	1F, (10)	1F, (6)	(3)	(5)	(2)	12F, 15M, (46)
2013	2F, (4)	(5)	2F, 2M, (7)	5M, (6)	5F, 5M, (10)	(5)		(1)	(2)	(1)	1F, (1)	1F, (1)	11F, 12M, (43)
2014			1M, (3)	1F, (2)	4F, 2M, (3)	1F, (3)	(5)						6F, 3M, (16)
Total	2F, (5)	1F, 2M, (11)	4F, 5M, (16)	11F, 14M, (13)	20F, 21M, (16)	3F, 4M, (9)	1F, (10)	1F, (14)	3F, (10)	(7)	3F, (7)	1F, (6)	50F, 46M, (124)
Frequency of mature colonies	29%	21%	36%	66%	72%	44%	9%	7%	23%	0%	30%	14%	44%

*Number of female (F), male (M) and sterile colonies in parentheses

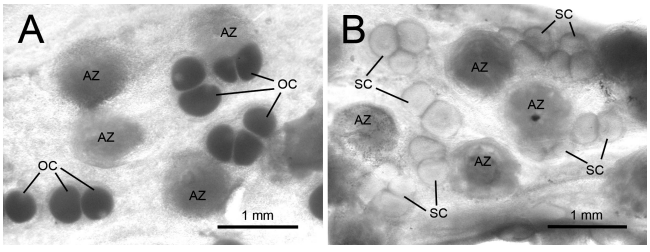


Fig. 1. Female and male gonads observed under a stereomicroscope after decalcification. **(A)** One to three oocytes (OC) in groups produced in single siphonozooids. AZ, autozooids. **(B)** Sperm cysts (SC) in groups produced in single siphonozooids. AZ, autozooids.

secundum (Waller and Baco, 2007). They also form within siphonozooids in the Japanese red coral, *C. japonicum*, the pink coral, *Pleurocorallium elatius*, and the white coral, *Pleurocorallium konojoi* (Kishinouye, 1904; Nonaka et al., 2015). *C. rubrum* is a brooder species that undergoes internal fertilization and broods planula larvae inside autozooids (Santangelo et al., 2003; Tsounis et al., 2006). The planulae are then released from the autozooids between July and August and settle on the seabed in early September (Tsounis et al., 2006). In contrast with *C. rubrum*, no planulae have been observed within siphonozooids in the Hawaiian species (Waller and Baco, 2007) or Japanese species (Kishinouye, 1904; Nonaka et al., 2015), suggesting that they are broadcast spawners (Nonaka et al., 2015). The sexuality of *C. rubrum*, *H. lauuense*, *P. secundum*, *C. japonicum*, *P. elatius* and *P. konojoi* is gonochoric, which is in common in colonies producing either oocytes or spermaries (Santangelo et al., 2003; Tsounis et al., 2006; Waller and Baco, 2007; Kishinouye, 1904; Nonaka et al., 2015).

Histological observations of gonads in the Coralliidae are very limited (Waller and Baco, 2007; Nonaka et al., 2015), although for several other octocorals (species in the Octocorallia), light microscopic features of gonad development are available (Chia and Crawford, 1973; Farrant, 1986; Babcock, 1990; Achituv and Benayahu, 1990; Hellström et al., 2010; Beazley and Kenchington, 2012; Quintanilla et al., 2013). Eckelbarger et al. (1998) investigated oogenesis and spermatogenesis in the sea pen, *Pennatula aculeata*, by electron microscopy. However, no ultrastructural study of gametogenesis in precious corals has been conducted to date. The present study demonstrates gonadal morphology and gametogenetic features in *C. japonicum* observed by light and transmission electron microscopy, related with the seasonal changes of gonad development.

Table 2. *Corallium japonicum*. Proportion of female, male and sterile colonies in seasons.

Season	Total (n = 220)	Female (n = 50; 22.7%)	Male (n = 46; 20.9%)	Sterile (n = 124; 56.4%)	P^1
Spring	120 (54.5%)	35 (70%)*	40 (87%)*	45 (36.3%)	< 0.0001
Summer	42 (19.1%)	5 (10%)	4 (8.7%)	33 (26.6%)*	
Autumn	30 (13.6%)	6 (12%)	0 (0%)	24 (19.4%)*	
Winter	28 (12.7%)	4 (8%)	2 (4.3%)	22 (17.7%)*	

[1]Probability obtained by Chi-square test
*Statistically significant association by adjusted residual analysis ($P < 0.05$)

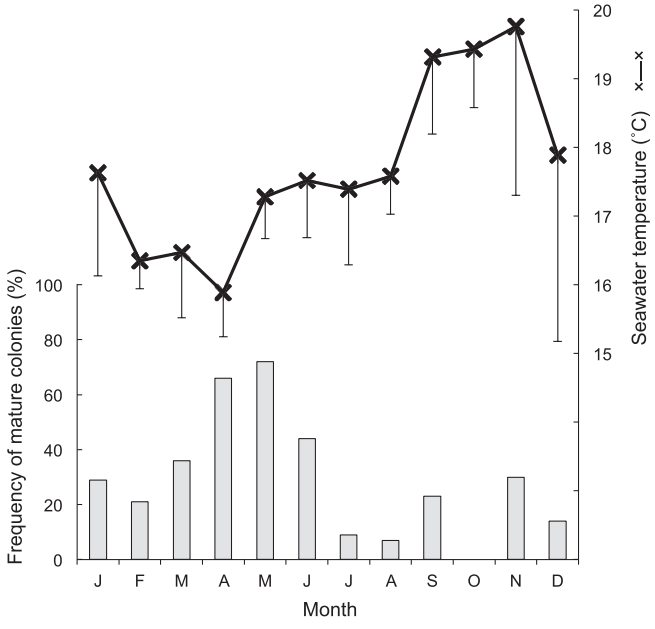


Fig. 2. Monthly changes in frequency of mature colonies and in seawater temperature. Monthly frequency of mature colonies (columns) is based on the data shown in Table 1. Monthly mean seawater temperature was measured 100 m in depth near the collection site of the colonies and indicated by a mean monthly seawater temperature (x) with SD (vertical bars) averaged for six years of 2006, 2008 and 2011–2014.

MATERIALS AND METHODS

Colonies of *C. japonicum* were collected off Cape Ashizuri in Kochi, Japan (32°30′ N, 132°50′ E) at least once per month from May to October in 2006, from October 2007 to May 2008 and from March 2011 to July 2014. They were harvested by drawing a tangle net which is composed of a few pieces of net that hang from a chain sinker on the seabed, at depths of between 100–150 m. Branch tips of an axial skeleton of colonies were immediately transferred into a sample tube filled with 50 mL of 5% formalin-seawater on boat. Thirty-seven per cent formalin was neutralized by adding 25 mg/mL sodium tetraborate and filtered before use. A total of 220 colonies was thus collected and fixed. Seawater temperatures throughout this study were measured by the Kochi Prefectural Fisheries Experimental Station, at a fixed point (32°37.4′ N, 133°1.0′ E) approximately 100 m deep.

Fixed colonies were decalcified in 5% ethylenediamine-*N,N,N',N'*-tetraacetic acid (pH 7.2) containing 0.2 M sucrose (Kurahashi, 1965) for 3–4 d. Decalcified colony samples were observed under an Olympus SZX16 stereomicroscope (Olympus Co. Ltd., Japan) to examine the presence or absence of gonads and determine the colony's sexuality, if gonads were present. All gonads produced in each colony were counted and photographed by a digital camera set on the stereomicroscope to measure the diameters of gonads using the image analysis software, Image J (Wayne Rasband, National Institutes of Health, USA). Sample data including sample code, collection date, depth, sexuality and size of gonads are shown in the list of sample colonies (Supplementary Table S1 online). The number of gonads produced in individual siphonozooids was counted under the stereomicroscope in selected colonies. These colonies had gonads of different mean diameters and consisted of five females (the sample codes were A12-9/22-06, A11-6/29-02, A12-3/29-05, A11-4/11-06 and A06-5/21-09) and five males (the sample codes were A14-5/17-13, A06-5/21-08, A11-4/11-16, A13-5/25-17 and A13-5/21-13).

Histological analysis was performed in five female colonies (the sample codes were A11-3/31-01, A11-4/1-02, A11-4/3-03, A11-4/11-06 and A11-11/6-01) and seven male colonies (the sample codes were A08-2/19-15, A11-4/11-17, A11-4/14-08, A11-5/15-16, A12-2/12-22, A12-5/28-29 and A12-5/30-34). Tissue fragments with siphonozooids containing gonads were excised

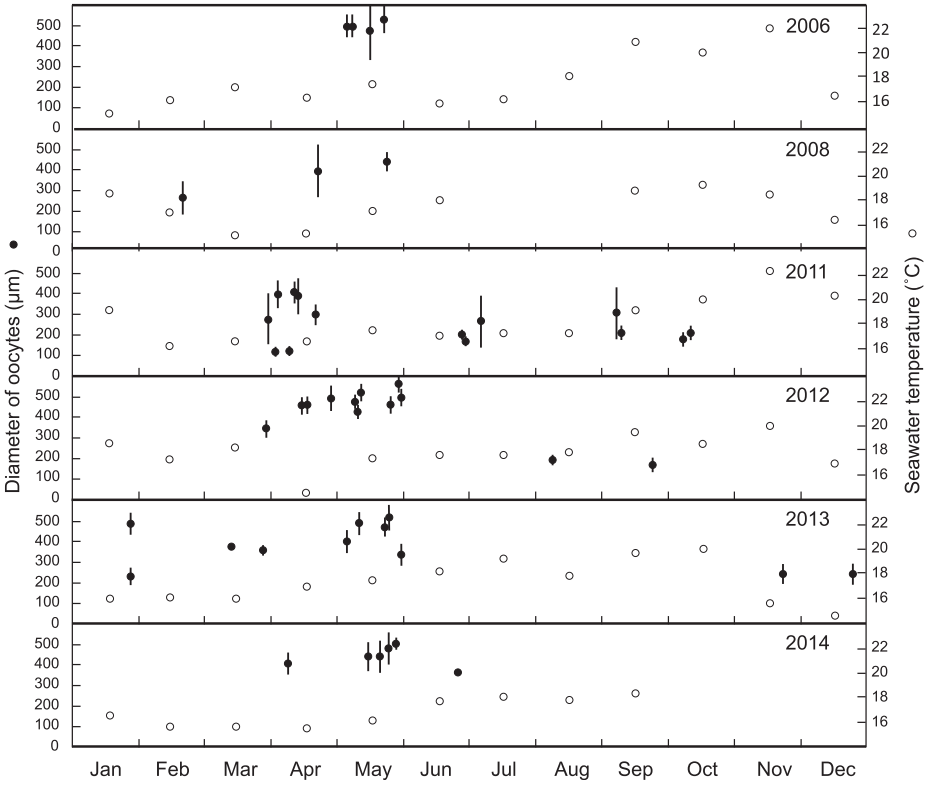


Fig. 3. *Corallium japonicum*. Annual and monthly changes in mean diameter (solid circles) ± SD (vertical bars) of oocytes in female colonies collected in 2006, 2008 and 2011–2014 and in monthly mean seawater temperature (open circles).

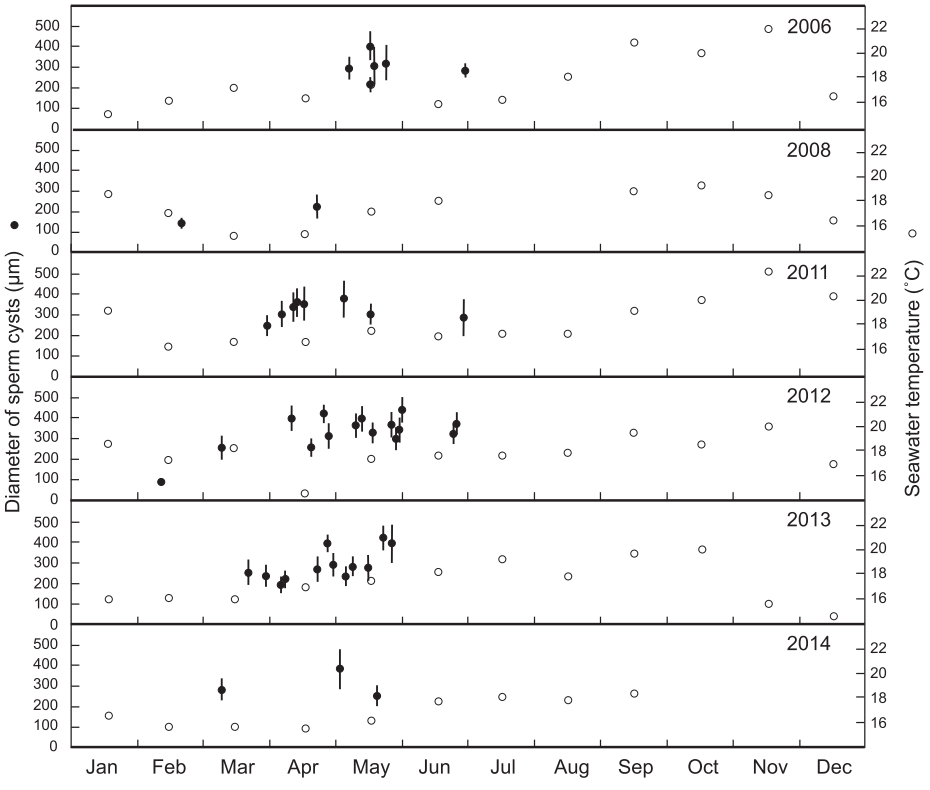


Fig. 4. *Corallium japonicum*. Annual and monthly changes in mean diameter (solid circles) ± SD (vertical bars) of sperm cysts in male colonies collected in 2006, 2008 and 2011–2014 and in monthly mean seawater temperature (open circles).

from decalcified colonies and post-fixed in 0.1 M phosphate buffer (pH 7.2) containing 1% osmium tetroxide for 16 h at 5°C. Post-fixed specimens were dehydrated in acetone series for 2.5 h and embedded into Spurr's resin. Semi-thin sections (1–2 μm thick) were cut by a glass knife using a Leica Ultracut UCT ultra-microtome (Leica Microsystems, Germany), stained with 1% toluidine blue O and observed through an Olympus BX-52 light microscope (Olympus Co. Ltd., Japan). Thin sections were made by a diamond knife on the ultra-microtome, stained with 1% uranyl acetate followed by lead citrate, and examined with a Jeol JEM 1010T electron microscope (JEOL Co. Ltd., Japan).

RESULTS

Seasonal changes in gonad development

Gonads, if formed, were readily observed under the stereomicroscope in decalcified tissues that were almost transparent (Fig. 1A and B). Oocytes of female gonads appeared yellowish in colour (Fig. 1A), whereas sperm cysts of male gonads had a grey or translucent appearance (Fig. 1B). Gonad-bearing colonies had either oocytes or sperm cysts in siphonozooids, indicating that *C. japonicum* exhibited gonochorism. No colony with planulae in polyps was observed. Of 220 colonies examined, 50 were female colonies, 46 were male, and 124 were sterile and sexuality was undetermined (Table 1). No male colony was found in January or between July to December throughout this study. In October, no female colonies were found.

Each year was divided into four seasons: spring consisting of March, April and May; summer of June, July and August; autumn of September, October and November; and winter of December, January and February. Table 2 shows the numbers of all female, male and sterile colonies collected in these four seasons throughout 2006–2008 and 2011–2014. There was a significant difference in the proportion of female, male and sterile colonies between the four seasons (Chi-square test, $X^2 = 42.278$, $df = 6$, $P < 0.0001$). More females (70%) and males (87%) appeared in the spring than any other seasons based on adjusted residual analysis. The frequency of sterile colonies was more prevalent in the summer, autumn and winter than in the spring. Seawater temperature near the collection site ranged from 15.89 ± 0.60°C in April to 19.76 ± 2.25°C in November (Fig. 2). Mature colonies including females and males tended to increase during the time when sea temperature began to rise from the lowest level. However, there was no significant correlation between the frequency of mature colonies and seawater temperature (Spearman rank correlation, $rs = -0.441$, $N = 12$, $P = 0.152$).

Figures 3 and 4 show the annual and monthly changes in gonad size in fertile colonies. The mean diameter of oocytes ranged from 119 μm to 565.9 μm (Fig. 3). Among all female colonies collected through 2006, 2008 and between 2011 and 2014, oocytes in March, April and May were larger than those in the other months (Mann–Whitney U test, $U = 58$, $N1 = 15$, $N2 = 35$, $P < 0.0001$). No significant difference in oocyte size was found between March and April, but oocytes in May were larger than those in March (Mann–Whitney U test, $U = 4$, $N1 = 4$, $N2 = 20$, $P = 0.0023$) and those in April (Mann–Whitney U test, $U = 38$, $N1 = 11$, $N2 = 20$, $P = 0.0022$). The mean diameter of sperm cysts ranged from 90.3 μm to 441.6 μm (Fig. 4). No significant difference in size distribution of sperm cysts appeared between male colonies collected in 2011, 2012 and 2013 (Kruskal–Wallis test, $H35 = 4.04$, $P = 0.132$). There was also no significant difference in size of sperm cysts between male colonies collected in April and May during 2011–2013 (Mann–Whitney U test, $U = 67.5$, $N1 = 13$, $N2 = 13$, $P = 0.397$). Oocytes were larger than sperm cysts among all mature colonies collected throughout this study (paired t test, $t80 = 3.12$, $P = 0.0025$).

More than one gonad produced within a single siphono-

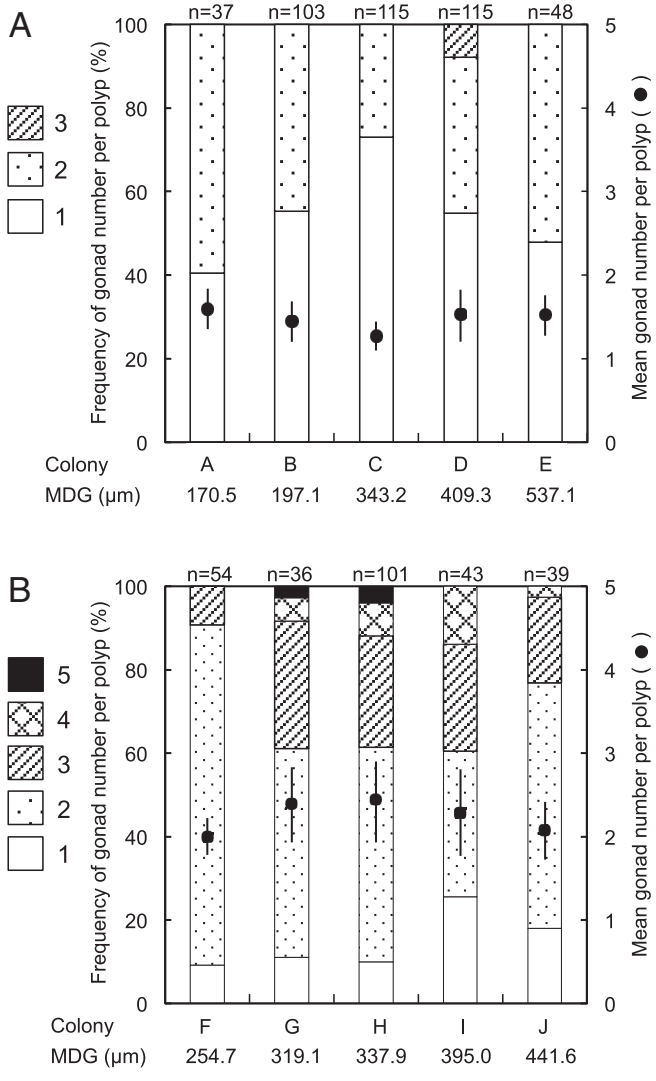
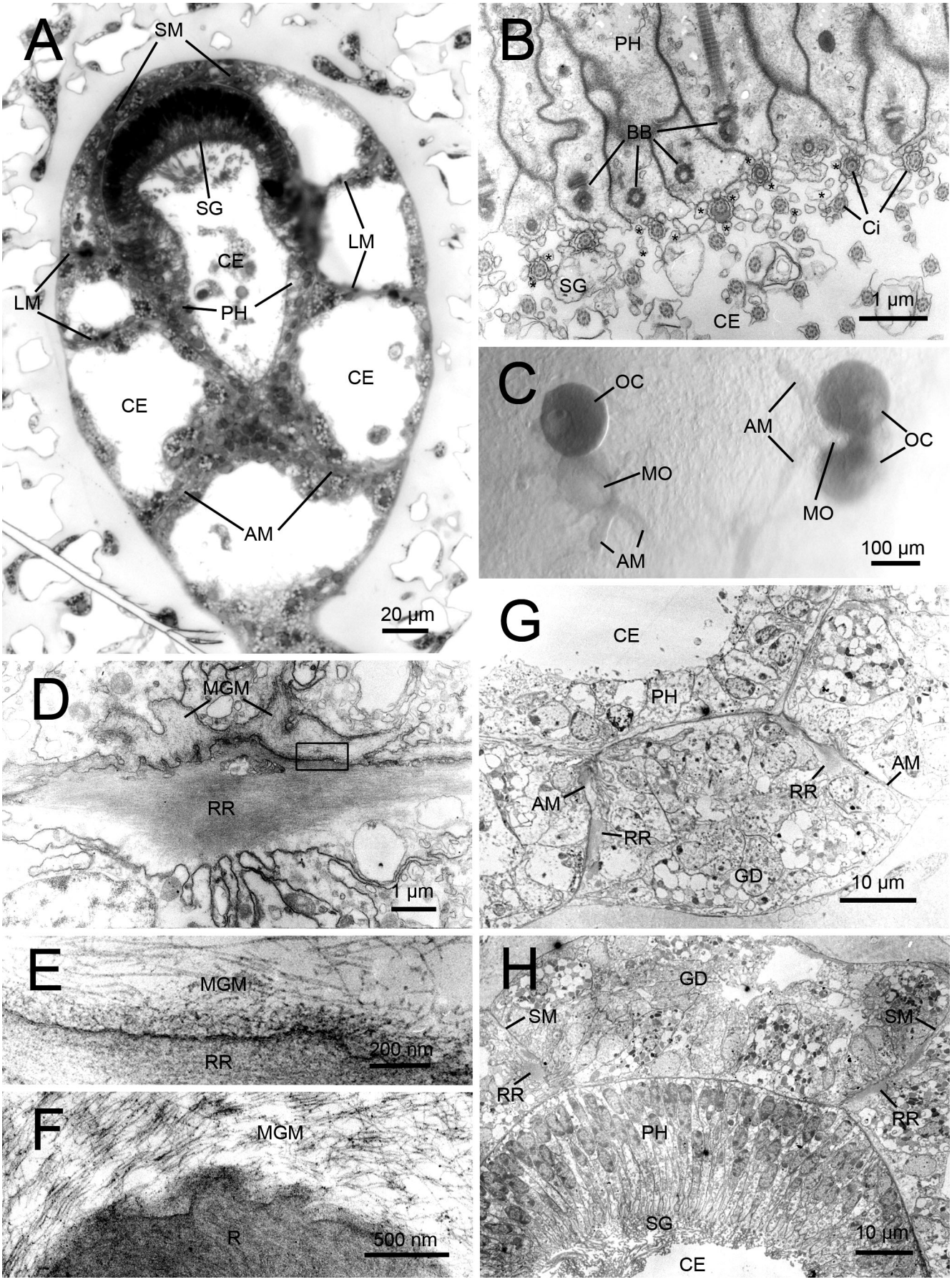


Fig. 5. *Corallium japonicum*. Frequency of gonad number per polyp (vertical columns) and mean gonad number per polyp (solid circles) ± SD (vertical bars). **(A)** Female colonies **(A–E)** with different mean diameter of gonads (MDG) are data from the sample codes A12-9/22-06, A11-6/29-02, A12-3/29-05, A11-4/11-06 and A06-5/21-09, respectively. The frequency of polyps yielding one (white), two (dots) and three (oblique lines) oocytes shown in each column. n = number of siphonozooids observed. **(B)** Male colonies **(F–J)** with different mean diameter of gonads (MDG) are data from the sample codes A14-5/17-13, A06-5/21-08, A11-4/11-16, A13-5/25-17 and A13-5/21-13, respectively. The frequency of polyps yielding one (white), two (dots), three (oblique lines), four (meshes) and five (black) sperm cysts shown in each column. *n* = number of siphonozooids observed.


A
SM
SG
LM
CE
LM
PH
CE
CE
AM
20 μm
B
PH
BB
Ci
SG
CE
1 μm
C
OC
AM
OC
MO
MO
AM
100 μm
D
MGM
RR
1 μm
E
MGM
RR
200 nm
F
MGM
R
500 nm
G
CE
PH
AM
RR
AM
RR
GD
10 μm
H
GD
SM
SM
RR
RR
PH
SG
CE
10 μm

zooid. Frequency of gonad numbers per polyp varied by polyps and colonies. The number of oocytes in single polyps ranged between one and three (Fig. 5A), whereas one to five sperm cysts formed in single polyps (Fig. 5B). Mean numbers of oocytes per polyp in the female colonies A–E were significantly lower than those of sperm cysts in the male colonies F–J (Mann–Whitney U test, $U = 25$, $N1 = 5$, $N2 = 5$, $P = 0.0079$). Mean numbers of gonads per polyp were not correlated to mean diameters of gonads both in the female colonies A–E (Spearman rank correlation, $rs = -0.2$, $N = 5$, $P > 0.05$) or in the male colonies F–J (Spearman rank correlation, $rs = 0.1$, $N = 5$, $P > 0.05$).

Anatomy of siphonozooids

Siphonozooids had eight mesenteries extending centripetally from their bodies and reaching a tube, where the stomodaeum formed a pharynx, leading from the mouth into the coelenteron (Fig. 6A). A siphonoglyph was located in a thick part of the pharynx between two sulcal mesenteries (Fig. 6A), and many cilia and microvilli extended from the siphonoglyph toward the interior of the coelenteron when they were observed through the electron microscope (Fig. 6B). Two asulcal mesenteries were located at the opposite side of the siphonoglyph and were associated with deeper portions of siphonozooids than the other mesenteries (Fig. 6C). Figures 6D–H are electron micrographs. A small swelling appeared on one side of every mesentery (Fig. 6D) and contained fibrous materials, which were distinct in electron density and thickness of fibrils from mesoglea materials of the mesentery (Fig. 6E). The morphological distinction between the swellings and the mesenteries in siphonozooids was also observed between the retractor muscles and the mesenteries in autozooids (Fig. 6F). The fibrous materials of the siphonozooid swellings actually corresponded to those of the retractor muscles of autozooids. The swellings present on the mesenteries of siphonozooids were regarded as rudimental retractors, as they were much smaller than the retractor muscles of autozooids. The rudimental retractors on the two asulcal mesenteries faced each other (Fig. 6G), whereas those on the two sulcal and four lateral mesenteries lay on the sides directed away from the siphonoglyph (Fig. 6H) in the siphonozooids of *C. japonicum*. Gonads developed on the mesenteries except in the asulcal ones.

Morphogenesis of early gonads

Early oocytes in the female colony A11-4/1-02 (mean gonad diameter = 119.0 μm) (Fig. 7A and B) and early sperm cysts in the male colony A08-2/19-15 (mean gonad diameter = 142.2 μm) (Fig. 8 A and B) were attached to mesenteries. Figures 7B, 8B and C are electron micrographs. These early oocytes and sperm cysts were completely covered with a sac-shaped thin layer of mesoglea, which extended from the mesoglea of a mesentery. Gastrodermis enveloped the complete outer surface of the thin layer of mesoglea. Gastrodermal cells accumulated around the mesentery, located near the proximal part of the early gonads (Figs. 7A and 8A). A single, large oocyte and a small amount of cytoplasmic content below the base of the oocyte were packed together in a thin sac of mesoglea (Fig. 7B). A much smaller oocyte covered with a thin sac of mesoglea also protruded from the mesentery toward the gastrodermis (Fig. 7B). A part of the cytoplasmic contents of the gastrodermis appeared to be engulfed by arm-like extensions of the mesenteric mesoglea (Fig. 7B). In early sperm cysts, many spermatocytes were already contained within a thin sac of mesoglea (Fig. 8B). The small sac of the mesoglea was closely packed with several cells and found in the gastrodermis (Fig. 8C). The mesenteric mesoglea produced irregular sinuses both in the female and male siphonozooids (Figs. 7B, 8B and C). We did not observe any germ cells entering the mesoglea of mesenteries from the gastrodermis, in either female or male siphonozooids.

General morphology of developing oocytes and sperm cysts

Serial sections of developing oocytes in the female colony A11-4/3-03 (mean gonad diameter = 398.8 μm) were cut parallel to the direction of the skeletal axis, as shown in the animation (Supplementary File S1 online). In Fig. 9A, one of the serial oocyte sections contained a large nucleus with a nucleolus and many metachromatic granules that were distributed uniformly in the cytoplasm. Smaller, juvenile oocytes also occurred in the same siphonozooids but contained fewer granules in the cytoplasm. A cross section of developing sperm cysts from the male colony A11-4/11-17 (mean gonad diameter = 353.1 μm) shown in Fig. 9B, is represented in another animation (Supplementary File S2 online). Sperm cysts contained basophilic materials and were hollow in the centre. Since sperm cysts developed in the ventral side of the pharynx where sulcal and lateral mesenteries were situated, the position of the pharynx was biased from the centre axis of the siphonozooid to the dorsal side where asulcal mesenteries lay (see the animation shown in Supplementary File S2 online). Neighboring siphonozooids communicated with each other by solenia near the bottom of the siphonozooids.

Fig. 6. Structure of siphonozooids. **(A)** Light microscopic section of a siphonozooid cut parallel to skeletal axis of colony. Two sulcal mesenteries (SM) on the pharynx (PH) at the side of siphonoglyph (SG), two asulcal mesenteries (AM) at the opposite side of siphonoglyph (SG) and the other four lateral mesenteries (LM). CE, coelenteron. **(B)** Electron micrograph of siphonoglyph (SG). Cilia (Ci) and microvilli (*) extending from basal bodies (BB) of epidermal cells in pharynx (PH). CE, coelenteron. **(C)** Light microscopic micrograph showing mature siphonozooids with oocytes (OC), mouths (MO) and long asulcal mesenteries (AM). **(D–H)**, electron micrographs. **(D)** Rudimentary retractor (RR) on one side of the mesoglea of mesentery (MGM) in siphonozooid. **(E)** Magnification of a square area in **(D)** showing morphological distinction between rudimentary retractor (RR) and the mesoglea of mesentery (MGM). **(F)** Retractor muscle (R) and the mesoglea of mesentery (MGM) in autozooid. **(G)** Asulcal mesenteries (AM) have rudimentary retractors (RR) facing each other. CE, coelenteron; GD, gastrodermis; PH, pharynx. **(H)** Rudimentary retractors (RR) of sulcal mesenteries (SM) at the side of siphonoglyph (SG) directed away from each other. CE, coelenteron; GD, gastrodermis; PH, pharynx.

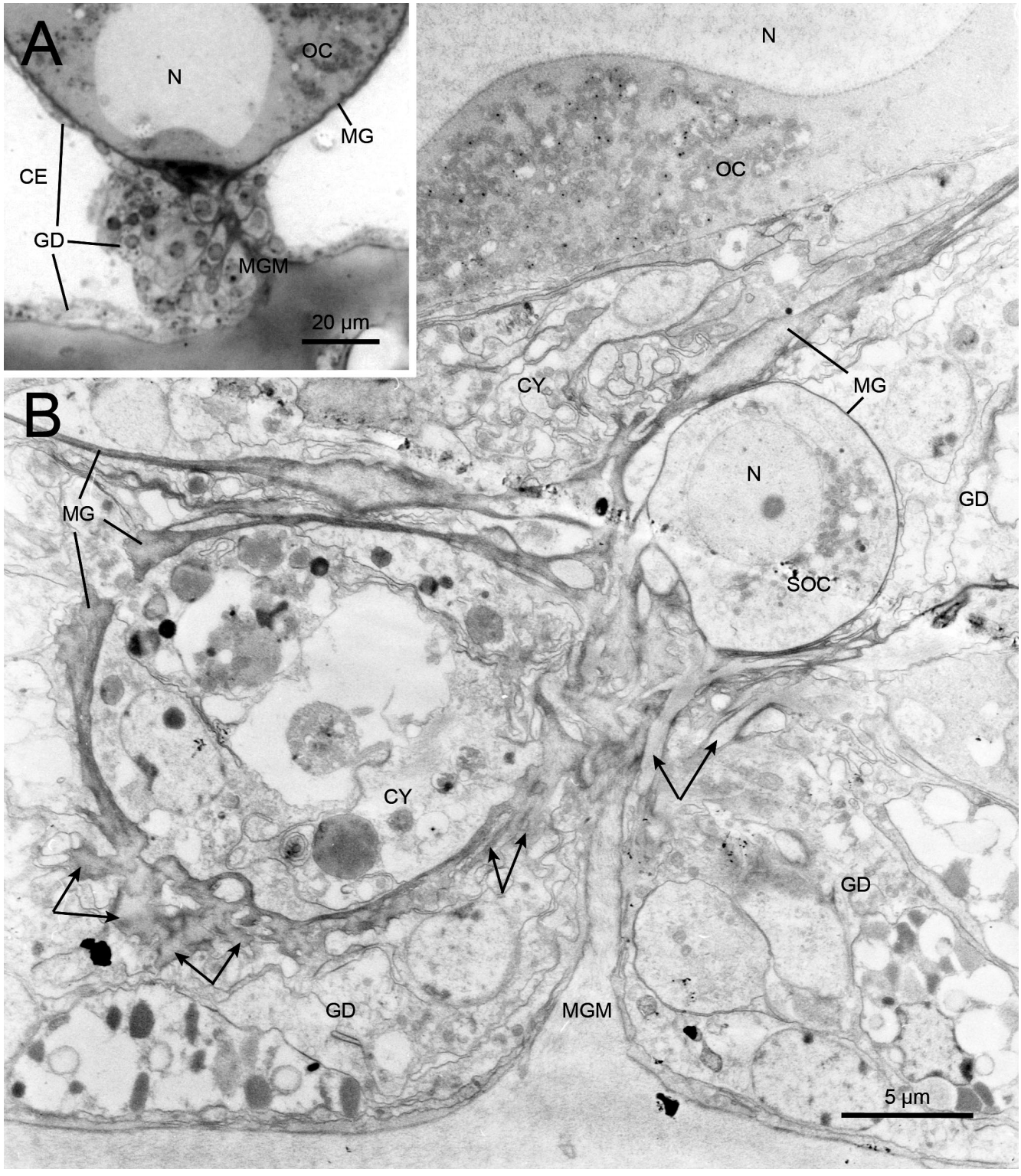


Fig. 7. *Corallium japonicum*. **(A)** Light microscopic section of an early oocyte. Early oocyte (OC) covered with a thin sac of mesoglea (MG) and attached to the mesoglea of mesentery (MGM) surrounded by gastrodermis (GD). N, nucleus. **(B)** Electron micrograph at the basal portion of an early oocyte. The mesoglea of mesentery (MGM) is continuous to the mesoglea (MG) of a thin sac that confines oocyte (OC) and cytoplasmic contents (CY). Small oocyte (SOC) and cytoplasmic contents (CY) enveloped by the thin layer of mesoglea (MG) that extends from the mesoglea of mesentery (MGM). Parts of mesoglea thickened and ramified irregularly (arrows). GD, gastrodermis; N, nucleus.

Ultrastructure of gametogenesis

The large nucleus of an oocyte had a conspicuous nucleolus and was filled almost entirely with euchromatin (Fig. 10A). The cytoplasm of an early oocyte in the female colony A11-4/1-02 was dotted with small groups of lipid granules (Fig. 10A), whereas a developing oocyte in the

female colony A11-4/3-03 was full of a large number of lipid granules and globules, which were distinctive in size and electron density (Fig. 10C). These lipid globules were present not only in the oocyte but also in the gastrodermal cells that lay on the outer surface of the mesoglea layer (Fig. 10B). The layer of mesoglea covering the early oocyte was thin (Fig. 10B) and became thicker when the oocyte developed (Fig. 10D). This was concomitant with an increase in the number and length of the microvilli that extended from the surface of the oocytes toward the inner surface of the layer of mesoglea (Fig. 10B and D).

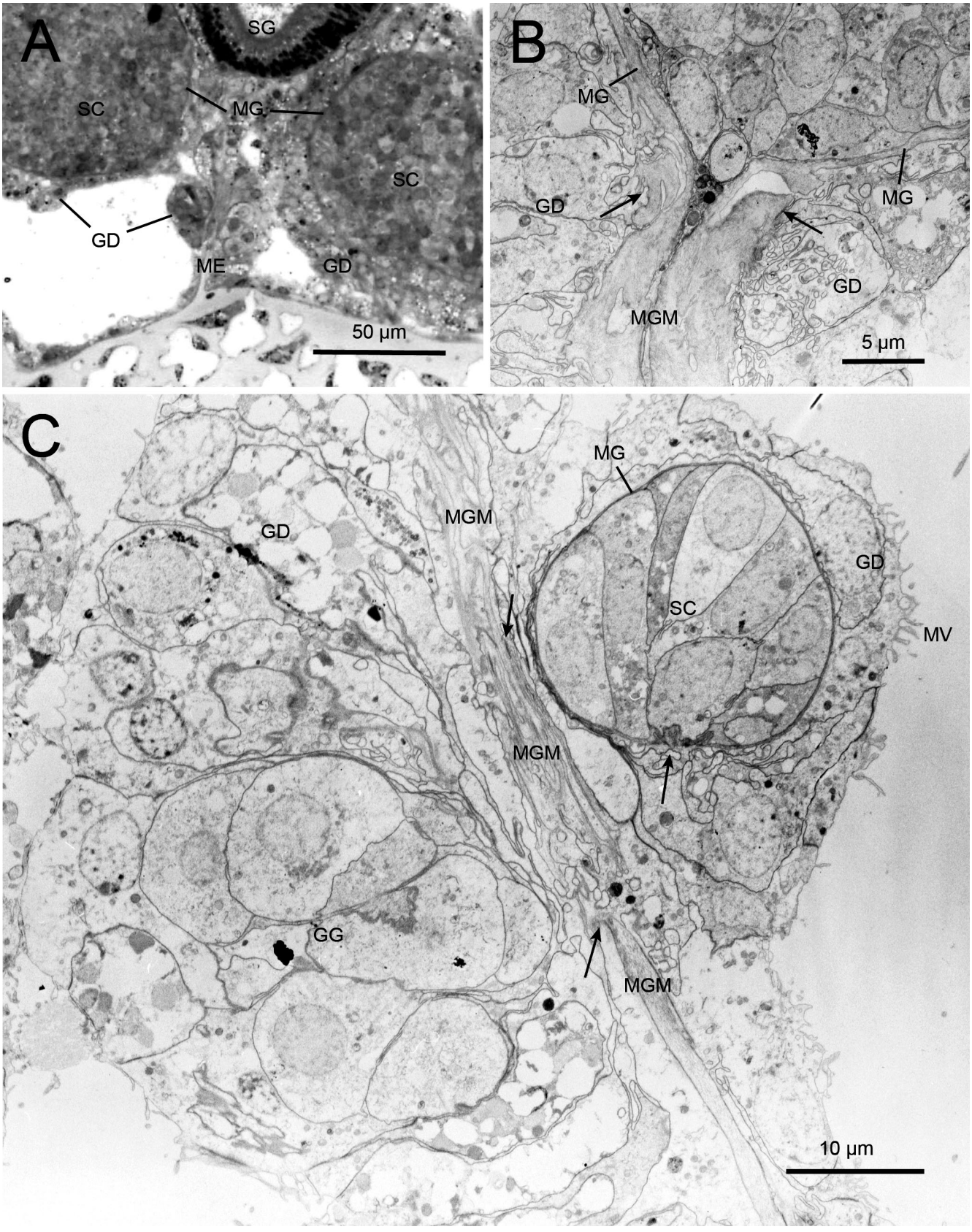
Spermatogenesis after the initial occurrence of sperm cysts progressed by at least three successive steps, each of which almost synchronized within the same sperm cysts. In the first step, spermatocytes increased in number to produce spermatids, accompanied by an increase in the size of the sperm cysts in the male colony A08-2/19-15 (mean gonad diameter = 142.2 μm). Spermatocytes closely packed in the sperm cyst were polygonal in shape and had a nucleus containing a nucleolus, with heterochromatin distributed throughout (Fig. 11A). The second step involved the elongation of flagella from spermatids (Fig. 11B), which was found in the male colony A11-4/11-17 (mean gonad diameter = 353.1 μm). A flagellum extended from the centriole that was located just under the plasma membrane of the spermatid, distant from the nucleus (Fig. 11C). Flagella were arranged in bundles centripetally to the centre of the sperm cysts, and spermatids lay between the bundles of flagella. Nuclei were spherical to ovoid in shape. Heterochromatin of nuclei was not so condensed that it was still distinguishable from nucleoli (Fig. 11D). The final step was spermiogenesis and was observed in the male colony A12-5/30-34 (mean gonad diameter = 441.6 μm). Spermatozoa, which were not motile, were lined up along the bundles of flagella (Fig. 12A). Undifferentiated cells failing to complete spermiogenesis were also observed in the sperm cyst (Fig. 12B). The nuclei were condensed, conical in shape and had a depression at their posterior part where the two distal and proximal centrioles lay (Fig. 12C). A cup-shaped, basal plate was localized between the nuclear depression and the centrioles. The distal centriole extended a flagellum, whereas the proximal centriole was shorter and connected to the lateral side of the distal centriole. Since the plasma membrane invaginated around the flagellum, the cytoplasm below the nucleus formed a bell-shaped lobe. Transverse sections of the cells showed regular arrangement of organelles. Flattened vesicles or cisternae containing electron-dense materials in a single layer surrounded the nucleus (Fig. 12D). At the level of the posterior part of the nucleus, 13 lipid globules were arranged circumferentially (Fig. 12E), and several mitochondria were located in the lobe encircling the flagellum (Fig. 12F). Layers of mesoglea covering sperm cysts remained thin when spermatids developed (Fig. 11C), whereas they swelled up and became thicker when spermiogenesis occurred (Fig. 12B). Gastrodermis surrounding the sperm cysts (Fig. 11B) was not as thick as that observed in oocytes (Fig. 10C). No microvilli extended from any of the cell membranes of spermatocytes and spermatids throughout spermatogenesis (Figs. 11C and 12B), compared with what was observed for oocytes (Fig. 10D).

DISCUSSION

Sexual reproduction of *C. japonicum*

Since there is no externally morphological difference between female and male colonies in octocorals including species in the Coralliidae, the presence of oocytes or sperm cysts is the only characteristic that allows us to determine the sexuality of these colonies (Kahng et al., 2011). Dimorphic octocorals are composed of two types of polyp, autozooids and siphonozooids. Autozooids possess eight pinnate tentacles surrounding the mouth and function in capturing and digesting food. Siphonozooids are smaller than autozooids, characterized by the absence or highly reduced nature of tentacles and specialized by powerful siphonoglyphs to circulate water through fleshy colonies (Williams et al., 2012). Gonads occur in siphonozooids (Kishinouye, 1904; Cordes et al., 2001) and in both siphonozooids and autozooids (Utinomi and Imahara, 1976) but most of the species produce gonads in autozooids (Simpson, 2009; Kahng et al., 2011). According to Kahng et al. (2011), of 159 octocoral species where sexuality was reported, 89% of the species are gonochoric and 9% are simultaneously hermaphroditic, with the rest having mixed reproductive patterns. The present study showed that all 96 mature colonies of *C. japonicum* collected off Cape Ashizuri were gonochoric and produced gonads in siphonozooids. These results fundamentally corresponded to those described for this species by Kishinouye (1904) and Nonaka et al. (2015). The present study also demonstrated that the proportion of female, male and sterile colonies collected in the spring season from March to May was different from that in the other seasons, and more mature colonies appeared in the spring. Oocytes became the largest in female colonies collected in May, disappearing almost immediately after May. No male colony was found in January or from July to December. However, female colonies with relatively small oocytes did occur in January and between July and December. These facts and the lack of observation of embryos and planulae in the siphonozooids suggest that *C. japonicum*, which is found off Cape Ashizuri, becomes fertile and develops oocytes and sperm cysts mainly during the spring season. Broadcast spawning of gametes occurs subsequently until the end of June.

Nonaka et al. (2015) reported that a few colonies of *C. japonicum* collected in the Ryukyu Archipelago, which is located 300–1250 km distant from Cape Ashizuri, exhibited hermaphroditism and produced gonads not only in siphonozooids but also in autozooids. According to Nonaka et al. (2015), colonies with larger and mature gonads (Stage IV) appeared during the summer season from May to August or September in *C. japonicum* living in the Ryukyu Archipelago, and the mean diameters of the mature oocytes and sperm sacs (Stage IV) were 362.1 μm and 222.2 μm, respectively. These sizes of gonads are smaller than those in *C. japonicum* from Cape Ashizuri, because the oocytes and sperm cysts of mature colonies collected off Cape Ashizuri in May had mean diameters of 471.4 μm and 343.3 μm, respectively. Nonaka et al. (2015) have suggested that the cohort of Stage IV oocytes may be released one year in *C. japonicum* in the Ryukyu Archipelago, and the next cohort of immature oocytes (Stage II or III) may be released the following year,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MG
SC
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GD
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GD
SC
MV
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GG
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10 μm

since there were two peaks in oocyte size in the female colonies collected from May to September. The differences in sexuality, the season of gonad development and the size of mature gonads between two populations of *C. japonicum*, from the Ryukyu Archipelago and Cape Ashizuri, may be attributable to distinct environmental conditions in their deep-sea habitats or to the geographical, genetic diversity in this species (Nakajima et al., 2010).

Timing of sexual reproduction in octocorals

In many species of octocorals, sexual reproduction takes place during a definite period, once per year. In some species, every gonad of mature colonies widely releases female and male gametes and then disappears after the spawning period (Benayahu and Loya, 1983; Babcock, 1990; Santangelo et al., 2003; Gutiérrez-Rodríguez and Lasker, 2004; Putron and Ryland, 2009; Mercier and Hamel, 2011; Quintanilla et al., 2013; Coelho and Lasker, 2014). In this situation, both oocytes and sperm cysts develop to release gametes within a year, and *C. japonicum* off Cape Ashizuri correlates with this pattern. Although annual spawning occurs, not all gonads are known to develop synchronously. This includes species where oocytes and sperm cysts that are only reaching maturity, can engage in reproduction in the annual spawning period, whereas the other immature oocytes remain in the colonies (Yamazato et al., 1981; Coma et al., 1995; Excoffon et al., 2004, 2011; Hwang and Song, 2007; Ribes et al., 2007; Edwards and Moore, 2008; Hellström et al., 2010; Mercier and Hamel, 2011; Beazley and Kenchington, 2012). In these species, oogenesis takes longer than spermatogenesis, requiring more than one year until oocytes ripen. This is exemplified by small, immature oocytes being maintained as a reservoir in each colony throughout the year, and an oocyte group developing from the reservoir to mature and become ready for the next annual spawning (Ribes et al., 2007; Edwards and Moore, 2008). According to Yamazato et al. (1981), three groups of oocytes in *Lobophytum crassum* grow together in the same colony and need two years for maturation, consequently triggering spawning at a yearly interval. In other species, both oocytes and sperm cysts, both of which are small and immature, are present throughout the year (Farrant, 1986; Kruger et al., 1998; Tsounis et al., 2006), suggesting that they also serve as a reservoir. Farrant (1986) reported that asynchronous development of gonads occurs within each colony, and between colonies and allows prolonged spawning of *Capnella gaboensis*. In contrast with the phenomena of the apparent annual spawning mentioned above, there are species where asynchronous gametogenesis continues, and breeding takes place several times over a year (Dahan and Benayahu, 1997; Kahng et al., 2008; Mercier and Hamel, 2011; Barbosa et al., 2014; Waller et al., 2014).

Environmental factors influencing sexual reproduction

Reproduction and breeding in octocorals may be influenced by external factors such as seawater temperature (Grigg, 1977; Ben-David-Zaslow et al., 1999), food availability (Ribes et al., 2007; Barbosa et al., 2014) and the lunar cycle (Benayahu and Loya, 1983; Coma et al., 1995; Kruger et al., 1998; Gutiérrez-Rodríguez and Lasker, 2004; Putron and Ryland, 2009). There are at least three patterns in which the timing of gonad development and gamete release is related to changes in seawater temperature. In the first pattern, gonads appear at the end of a high temperature phase but retain their small size until the temperature declines, then develop rapidly from a low temperature phase to the beginning of the increase in temperature and finally spawn prior at the highest temperature, as reported in the Italian red coral (Santangelo et al., 2003) or just after temperature has reached its maximum, as evidenced in the Australian blue coral (Babcock, 1990). In the second pattern, new gonads initiate when temperature reached their lowest level, followed by development from a low temperature phase to the time when the temperature begins to rise. This includes *Alcyonium coralloides* off the Medes Islands in Spain, in which male gametes are released on a 2°C increase from a low temperature phase (Quintanilla et al., 2013). In the same climate, *Paramuricea clavata* also exhib-

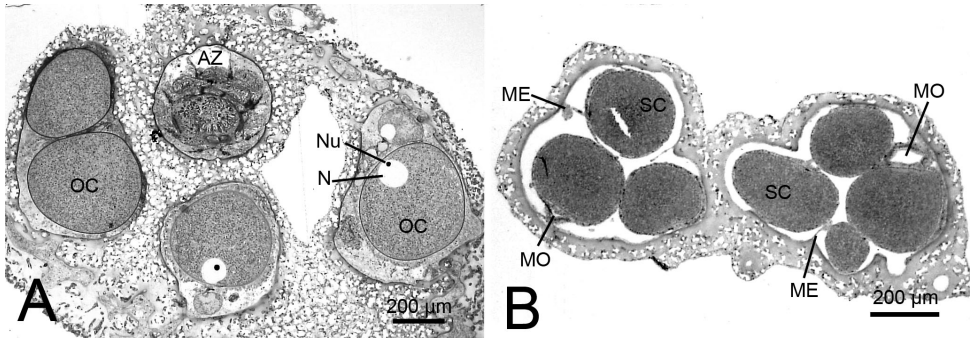


Fig. 9. *Corallium japonicum*. **(A)** Light microscopic section of female colony showing developing oocytes (OC) in siphonozooids. N, nucleus; Nu, nucleolus; AZ, autozooid. **(B)** Light microscopic section of male colony showing developing sperm cysts (SC) in siphonozooids. ME, mesentery; Mo, mouth.

Fig. 8. *Corallium japonicum*. **(A)** Light microscopic section of early sperm cysts adjacent to a mesentery. Early sperm cysts (SC) covered with a thin sac of mesoglea (MG) near mesentery (ME) and pharynx at the side of siphonoglyph (SG). GD, gastrodermis. **(B)** Electron micrograph at the basal portion of an early sperm cyst. Early sperm cysts (SC) attached to mesentery (ME) and enveloped by a thin layer of mesoglea (MG) continuous to the mesoglea of mesentery (MGM). GD, gastrodermis. **(C)** Electron micrograph of cells associated with a mesentery in male siphonozooids. Several cells (SC) closely packed within a thin sac of mesoglea (MG) intervene between gastrodermis (GD) and the mesoglea of mesentery (MGM) at the upper right side of the photo. Gastrodermal cells in a group (GG) adjacent to the mesoglea of mesentery (MGM) at the lower left side of the photo. MV, microvilli; arrows, ramifications of the mesoglea of mesentery.

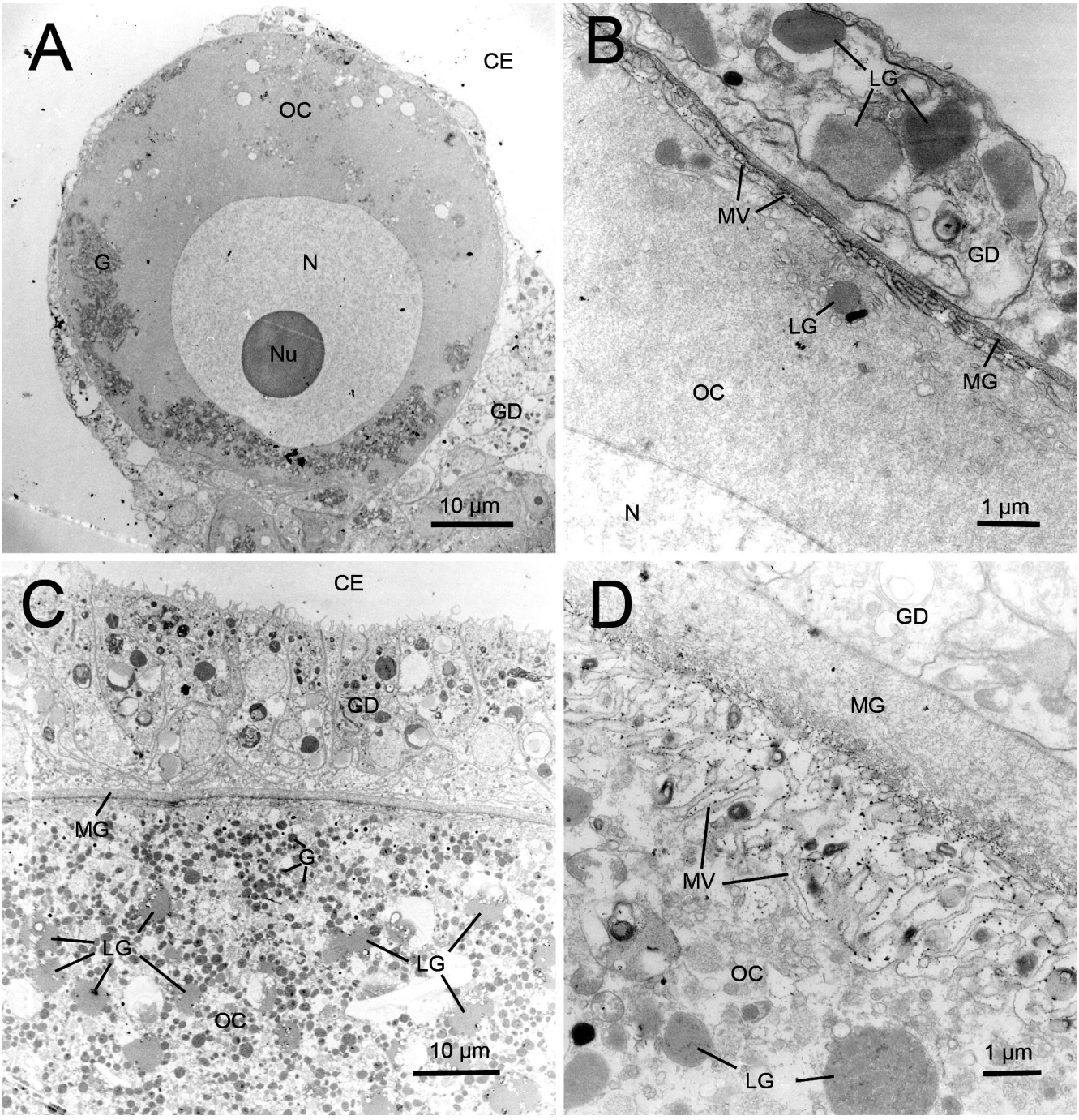


Fig. 10. Electron micrographs showing oogenesis. **(A)** Oocyte (OC) at early stage. N, nucleus; Nu, nucleolus; G, lipid granules; GD, gastrodermis; CE, coelenteron. **(B)** Short microvilli (MV) at the boundary between oocyte (OC) at early stage and a thin layer of mesoglea (MG). GD, gastrodermis; LG, lipid globules; N, nucleus. **(C)** Developing oocyte (OC) containing many lipid globules (LG) and granules (G). MG, mesoglea; GD, gastrodermis; CE, coelenteron. **(D)** Irregular, long microvilli (MV) at the boundary between oocyte (OC) at latter stage and a thick layer of mesoglea (MG). LG, lipid globules; GD, gastrodermis.

its this pattern of reproduction (Coma et al., 1995). In the third pattern, gonad appearance and development accompany an increase in temperature (Farrant, 1986; Putron and Ryland, 2009). Average seawater temperature at 100 m depth, off the Cape Ashizuri, varied between 15.9°C and 19.8°C and consisted of four phases, a low temperature phase from February to April, a temperature-rising phase from May to August, a high temperature phase from September to December and a temperature-declining phase from December to February (Fig. 2). In *C. japonicum* off Cape Ashizuri, mature colonies began to increase during the low temperature phase. The gonads developed during an early period of the temperature-rising phase and then released gametes during the later period (Fig. 3). However,

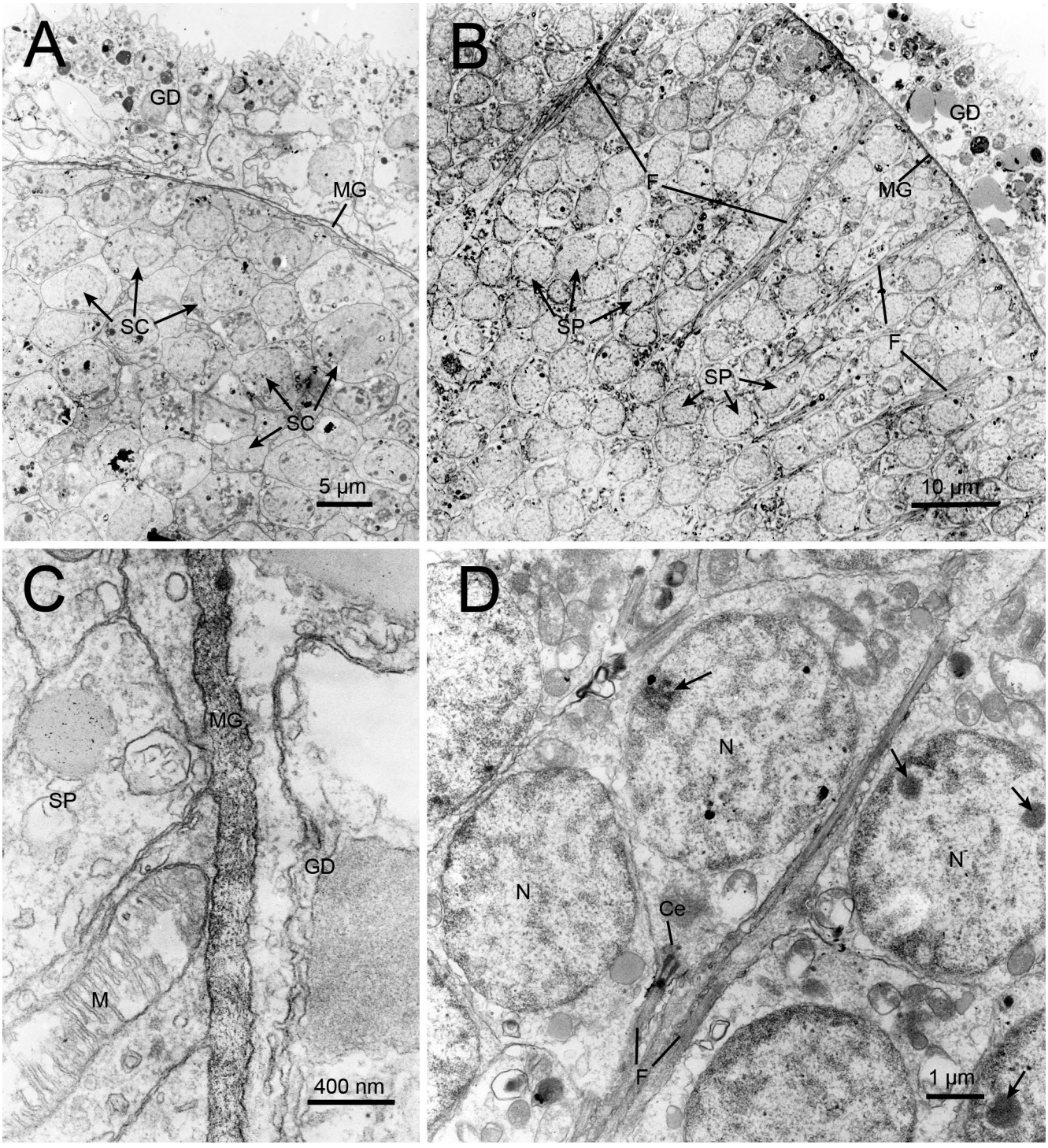
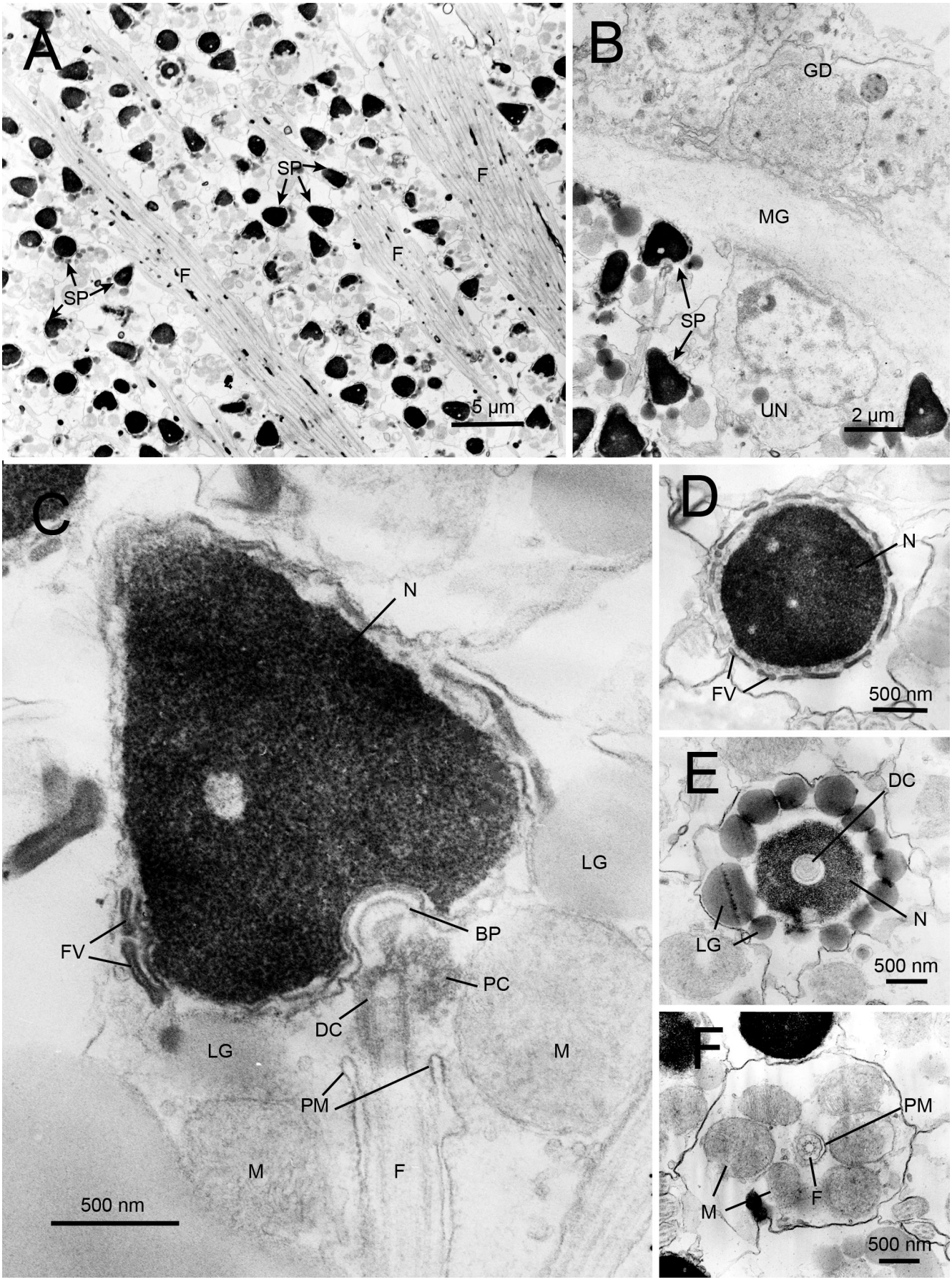


Fig. 11. Electron micrographs showing spermatogenesis. **(A)** Sperm cyst at early stage containing spermatocytes (SC) polygonal in shape. MG, mesoglea; GD, gastrodermis. **(B)** Bundles of flagella (F) arranged centripetally in sperm cyst at mid stage. MG, mesoglea; GD, gastrodermis, SP, spermatids. **(C)** Spermatids (SP) directly contacting to a thin layer of mesoglea (MG). GD, gastrodermis; M, mitochondrion. **(D)** Spermatids extending flagella (F) from one of centrioles (Ce). N, nuclei; arrows, nucleoli.

only female gonads were produced even during the high temperature phase and the temperature-declining phase. This indicates that the gonad development and spawning of *C. japonicum* off Cape Ashizuri fundamentally fall into the category of the second pattern.

Another factor that may affect the seasonality of gonad development is the supply of food, the source of which is derived from blooms of phytoplankton (Ribes et al., 2007; Edwards and Moore, 2008; Mercier and Hamel, 2011). Hirota and Ichikawa (2012) have investigated the seasonality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of phytoplankton in Tosa Bay, approximately 120 km distant from the collection site in the present study, and reported that phytoplankton significantly increases in the year when there is an upwelling current of deep sea water, which continues for more than one month. This can cause blooms at depths of 40–80 m in February to March, 40–50 m in May to June, and 20–40 m in August and November. Although the extent of the bloom between February to March may reach the habitat of *C. japonicum*, the fact that the blooms occur intermittently through the year suggests that the food supply from phytoplankton does not necessarily promote gonad development of *C. japonicum* especially in the spring season. Torrents and Garrabou (2011) have reported that fecundity i.e. the number of gonads per polyp in the Mediterranean red coral *C. rubrum,* varies significantly depending on different cave zones and by geographic area. Fecundity may also be affected by local water flow, food availability, and temperature (Torrents and Garrabou, 2011).

Retractor arrangement in octocoral polyps

In octocorals, gonads develop on mesenteries except for the two asulcal mesenteries that are situated at the dorsal side, opposite from a siphonoglyph (Hyman, 1940). This also applied to the case of *C. japonicum*. There have been few studies on the structure of siphonozooids in octocorals. Electron microscopic observations in the present study revealed the presence of a rudimentary retractor muscle on every mesentery in the siphonozooid of *C. japonicum*. This is based on the fact that the rudimentary retractor was morphologically identified with the retractor muscle of the autozooids. The rudimentary retractors of the asulcal mesenteries faced each other, whereas those of the sulcal ones faced the opposite direction, with the lateral mesenteries having the retractors at the sides away from the siphonoglyph. For the autozooids of octocorals, the retractors of sulcal mesenteries face each other, whereas those of asulcal mesenteries face away from each other (Hyman, 1940; Beklemishev, 1969; Bayer, 1974). Thus, the retractor arrangement in the siphonozooids of *C. japonicum* is the reverse of what is described in the autozooids of octocorals (Fig. 13). It might be assumed that the sulcal mesenteries could have been confused with asulcal mesenteries in the present study. However, in the mature siphonozooids of *C. japonicum*, the gonads bulged in the coelenteron below the ventral or lateral side of the pharynx but not on the dorsal side, so that the position of the pharynx was often shifted from the center towards the dorsal side of siphonozooids (see the animation in Supplementary File S2 online). The mesenteries at the dorsal side were thus always sterile, as described by Hyman (1940) and regarded as asulcal mesenteries by definition. Therefore, the present study has found that the asulcal mesenteries have rudimentary retractors facing each other in the siphonozooids of *C. japonicum*.

Gonadal morphology and gametogenesis

Gonads of octocoral species develop as buds, which emerge from the mesenteries, and each of these is attached to the mesentery by a pedicle (Chia and Crawford, 1973; Benayahu and Loya, 1983; Farrant, 1986; Benayahu et al., 1989; Achituv and Benayahu, 1990; Eckelbarger et al., 1998; Kruger et al., 1998; Excoffon et al., 2004; Gutiérrez-Rodríguez and Lasker, 2004; Hwang and Song, 2007; Hellström et al., 2010; Beazley and Kenchington, 2012). In these species, including *C. japonicum*, the gonad is covered with an inner layer of mesoglea and an outer layer of gastrodermis, both of which are continuous with their corresponding layers of the mesenteries. Different terms have been used for the gastrodermis layer surrounding the gonad. Both the layers surrounding oocytes and sperm cysts are termed follicle cells, a follicular cell layer or a follicular layer (Benayahu and Loya, 1983; Eckelbarger et al., 1998; Beazley and Kenchington, 2012); in this study, we referred to these as gastrodermis. Only the layer surrounding oocytes is termed a follicular layer (Kruger et al., 1998; Benayahu et al., 1989; Excoffon et al., 2004; Hellström et al., 2010). In cases where the layer surrounding the oocytes is discriminated from the layer surrounding sperm cysts, the former is termed follicle cells or the follicular layer, whereas

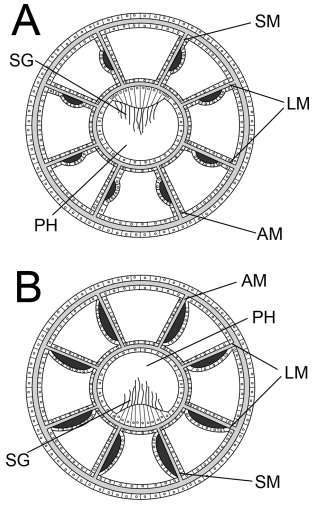

Fig. 13. Comparison in the retractor arrangement of mesenteries between polyps. **(A)** A siphonozooid in *C. japonicum*. **(B)** An autozooid in known octocorals after Hyman (1940). Retractors represented by black convex portions. SM, sulcal mesentery; LM, lateral mesentery; AM, asulcal mesentery; PH, pharynx; SG, siphonoglyph.

Fig. 12. Electron micrographs showing spermatogenesis. **(A)** Spermatids (SP) and bundles of flagella (F) in sperm cyst during spermiogenesis. **(B)** Swelling mesoglea (MG) surrounded by gastrodermis (GD) and undifferentiated cell (UN) failing to complete spermiogenesis in sperm cyst. SP, spermatids. **(C)** Longitudinal section of spermatid showing condensed nucleus (N) conical in shape, proximal centriole (PC) connected to the distal centriole (DC) that extends flagellum (F) and invagination of the plasma membrane (PM) around the base of flagellum (F). **(D)** Transverse section of nucleus (N) showing circular arrangement of flattened vesicles (FV). **(E)** Transverse section cut through the level of posterior part of nucleus (N) showing circular arrangement of 13 lipid globules (LG). DC, distal centriole. **(F)** Transverse section cut through the level of the base of flagellum (F) showing circular arrangement of mitochondria (M). PM, plasma membrane.

the latter is designated a tubule wall (Chia and Crawford, 1973), an envelope cell (Achituv and Benayahu, 1990) or in histological terms, an endoderm (Gutiérrez-Rodríguez and Lasker, 2004) and gastrodermis (Hwang and Song, 2007). The term follicle is generally used for an ovary consisting of an oocyte surrounded by epithelial granulosa cells (Cross and Mercer, 1993), but follicle cells are used to define the patterns of oogenesis found in invertebrates (Barnes et al., 2001). The follicle cells may play an important role in the transport of macromolecules to the cytoplasm of oocytes and cover the surface of oocytes (Barnes et al., 2001). Eckelbarger et al. (1998) have suggested that the follicle cells surrounding the oocytes in the sea pen, *Pennatula aculeata,* may be involved in heterosynthesis, such as nutrient production, transport and/or mediation of yolk precursors to the oocytes. This is based upon ultrastructural analyses. However, the follicle cells surrounding the sperm cysts in this species were thinner than those surrounding the oocytes and did not appear to be endocytotically active, as described by Eckelbarger et al. (1998). This corresponds with the results in the present study and indicates that the follicle cells (gastrodermis) surrounding gonads may transform their function depending on the sexuality of gonads because the development of sperm cysts does not appear to require the supply of yolk precursors compared with that of oocytes. The thickening of the layer of mesoglea covering oocytes relates to the occurrence of numbers of microvilli elongating from the surface of oocytes possibly to promote nutrient absorption in *C. japonicum* (Fig. 10D). In the last stage of maturation of gonads in *C. japonicum*, the oocytes notably accumulated distinct lipid globules, whereas the spermatids changed drastically in ultrastructural features during spermiogenesis. This included condensation of nuclei, extension of flagella from centriolar complexes and regular arrangement of flattened vesicles, lipid globules and mitochondria (Fig. 12C–F). The morphology of spermatids near the final stage of spermatogenesis of *C. japonicum* was similar to that of *P. aculeata,* as reported by Eckelbarger et al. (1998). Eckelbarger and Larson (1988) have found the earliest oocytes at the zygotene/pachyten stage of meiosis, which later differentiate into vitellogenic oocytes, in the scyphozoan *Aurelia aurita*. Oocytes in most animals remain arrested in prophase I of meiosis for prolonged periods while they grow in size and accumulate yolk materials (Sagata, 1996). Although no mitotic nor meiotic division during oogenesis was observed in the present study, the developing oocytes that accumulated lipid globules in *C. japonicum* might be in an arresting stage.

Characteristics of gonad development in *C. japonicum*

The characteristic of gonad development in octocorals, where gonads protrude from mesenteries and develop within a thin sac of mesoglea surrounded by gastrodermis, is clear when compared with that of other cnidarian species, such as hexacorals and scyphozoans. In hexacorals it has been reported that early oocytes and male germ cells arise from the gastrodermis of the mesenteries and migrate towards the interior of the mesenteries, within which they differentiate into gametes (Chornesky and Peters, 1987; Fautin and Mariscal, 1991; Goffredo et al., 2005; Eckelbarger et al., 2008). In scyphozoan species belonging to the orders Semaeostomae, Rhizostomae and Coronatae, gonads are produced in the gastric filaments filled with mesoglea, which protrude from the floor of the gastric pouches (Eckelbarger and Larson, 1988; Morandini and Silveira, 2001). The oocytes of these species originate from germ cells in the gastrodermis, migrate into the mesoglea of the gastric filaments and then develop to mature in the mesoglea (Widersten, 1965; Eckelbarger and Larson, 1988, 1992; Morandini and Silveira, 2001; Lucas and Reed, 2010; Ikeda et al., 2011). Spermatogenesis of these medusas occurs in the 'follicle' that has migrated from the gastrodermis into the mesoglea of the gastric filaments (Widersten, 1965; Lucas and Reed, 2010). Thus, hexacorals and scyphozoan species belonging to these three orders have the common characteristic of germ cells of the gastrodermis, which enter the mesoglea and differentiate into gametes staying in the mesoglea. On the other hand, the gonads of scyphozoan species belonging to the Stauromedusae form as small vesicles that protrude from the lateral side of inter-radial septa (Miranda et al., 2013). Gastrodermis covers the vesicles, and the vesicular content that differentiates into germinal epithelium is separated from the outer gastrodermis by a thin layer of mesoglea that is continuous with the mesoglea of the inter-radial septa (Eckelbarger and Larson, 1993; Miranda et al., 2013). Germ cells do not enter the mesoglea of the septa, and the oocytes and spermatids develop within the vesicles. The gonad development of the above stauromedusan species is analogous to that of octocorals, in which gametogenesis does not occur in the matrix of mesoglea, but rather within a thin sac of mesoglea surrounded by gastrodermis that projects into a coelenteron. It is unknown how germ cells are confined within a thin sac of mesoglea in octocorals and stauromedusa. In the present study we found that even very incipient gonads were covered with a thin sac of mesoglea and appeared in the gastrodermis out of a core of the mesoglea of mesenteries in *C. japonicum* (Figs. 7B and 8B). We also found that extensions from the surface mesoglea of the mesenteries engulfed a part of the gastrodermis in *C. japonicum* (Fig. 7B). Based on these observations, we propose that the mesoglea of the sulcal and lateral mesenteries grow out and engulf germ cells from the gastrodermis to produce gonads in *C. japonicum*.

ACKNOWLEDGMENTS

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