Marine Biology (2006) 148: 551–558
DOI 10.1007/s00227-005-0119-x

RESEARCH ARTICLE

E. Hirose · K. Iwai · T. Maruyama

Establishment of the photosymbiosis in the early ontogeny of three giant clams

Received: 16 February 2005 / Accepted: 7 September 2005 / Published online: 25 October 2005

Abstract Distribution and morphology of zooxanthellae were investigated histologically and ultrastructurally in veligers and juveniles of three giant clam species, *Tridacna crocea, T. derassa*, and *T. squamosa*. No zooxanthellal cells were associated with gametes. In veliger larvae, zooxanthellae were ingested and digested in the stomach. Within several days after metamorphosis from veliger to a juvenile clam, the zooxanthellal tube, in which zooxanthellae were packed, elongated from the stomach toward the mantle. Zooxanthellae in the tube appeared in a line, and we designated the appearance of the lined zooxanthellae in the mantle of juvenile clams as the first sign of the establishment of symbiosis. The zooxanthellal tubular system developed as the clams grew, particularly in the mantle margin, and then hypertrophied siphonal tissue formed. In zooxanthellal tubes, zooxanthellae usually had intact ultrastructures suggesting that they were photosynthetically active, while the stomach always contained degraded zooxanthellae that were probably discharged from the zooxanthellal tube. Giant clams probably digest zooxanthellae directly, and ingest the secreted photosynthates from zooxanthellae. There may be a selection mechanism to discharge unhealthy zooxanthellae from the mantle into the stomach. In addition to zooxanthellae, digested diatoms and other unidentified digested materials in the stomach suggest that filter-feeding also contributes to giant clam nutrition.

Communicated by T. Ikeda, Hakodate

E. Hirose (✉)
Department of Chemistry, Biology and Marine Science,
Faculty of Science, University of the Ryukyus, Nishihara,
903-0213 Okinawa, Japan
E-mail: euichi@sci.u-ryukyu.ac.jp
Tel.: +81-98-8958880
Fax: +81-98-8958576

K. Iwai
Yaeyama Branch Laboratory, Okinawa Fisheries Experiment
Station, 907-0453 Ishigaki, Okinawa, Japan

T. Maruyama
Extremobiosphere Research Center, Japan Agency
for Marine-Earth Science and Technology (JAMSTEC),
237-0061 Yokosuka, Kanagawa, Japan

Introduction

Giant clams are well known to have a symbiotic relationship with dinoflagellate of the genus *Symbiodinium*, also known as zooxanthellae. The histological location of the zooxanthellae in hospite has been theorized to be either inside or outside the animal tissue. Yonge (1936) supposed that zooxanthellae are present in the hemocoel and intracellularly digested in the visceral mass, while Mansour (1946a, b) reported that zooxanthellae were located in the lumen of the epithelial tube connecting with the stomach lumen. Yonge (1953) strictly opposed Mansour's view. Most authors have basically supported Yonge's view (e.g., Kawaguti 1966; Fitt and Trench 1981), until Norton et al. (1992) reevaluated Mansour's view and confirmed that epithelial tubes containing zooxanthellae are directly connected with the stomach. Actually, tubes containing zooxanthellae are thought to have a direct connection with the alimentary system in giant clams and these tubes are called zooxanthellal tubes. Moreover, the zooxanthellal tubes were also found in another photosymbiotic bivalve, *Corculum cardissa* (Farmer et al. 2001).

Oocytes and embryos of giant clams do not possess zooxanthellae, and thus clams acquire zooxanthellae in veliger or juvenile stages (LaBarbera 1975; Jameson 1976). The process of symbiont acquisition has not been well documented. Fitt and Trench (1981) reported that veligers and juveniles ingested zooxanthellae and then the symbionts relocated to the hemocoel through an unknown pathway in the giant clam *Tridacna squamosa*. The relocation of the symbionts started after metamorphosis from veliger to juvenile clam. These observations suggest that veliger/juvenile clam acquire zooxanthellae by feeding and the zooxanthellal tube is formed after the

metamorphosis. Aside from Fitt and Trench (1981), there have been few histological or ultrastructural investigations on the early processes of the establishment of clam-zooxanthellae symbiosis.

Zooxanthellae are estimated to supply more than fifty percent of carbon resources required by host clams (Trench et al. 1981; Klumpp and Lucas 1994). Zooxanthellae were formerly thought to be digested intracellularly by amoebocytes in the hemocoel (Yonge 1936; Fankboner 1971). However, it is unlikely that intracellular digestion occurs in the zooxanthellal tube, since the zooxanthellae are not located in the hemocoel but in the space connected with the alimentary lumen. The epithelial cells of the zooxanthellal tube might have been misidentified as hemocytes engulfing algal cells. While radio-labeling studies indicate that zooxanthellae secrete glucose in hospite (Ishikura et al. 1999), the balance of proliferation and excretion of zooxanthellae suggests that some zooxanthellae in clams are digested by host clams (Maruyama and Heslinga 1997). Giant clam possibly utilizes zooxanthellae as food, but it is unclear whether zooxanthellae are digested in the alimentary system of host clams, particularly in veliger and/or early juvenile stages. In this report, we describe the histological location and fine structure of zooxanthellae in veliger larvae and juveniles of the three giant clam species *T. crocea, T. derassa*, and *T. squamosa*, to obtain a better understanding of the process of the establishment of the clam-zooxanthellae symbiosis.

Materials and methods

Gametes and fertilization

Adult giant clams of *T. crocea, T. derassa*, and *T. squamosa* were collected from the vicinity of the Yaeyama Islands (Okinawa, Japan) and reared in 4 m^3 concrete aquaria supplied with running seawater. Spawning was induced by the following procedure: the shells were cleaned with a hand brush at 10:00 a.m., the clams were kept out of seawater for 1 or 2 h, and then the clams were put in 500 l polycarbonate aquaria filled with filtered seawater (FSW) with gentle aeration. If no clams spawned within 2 h, we added macerated gonads and, after 15–60 min, exchanged the seawater. Released clam eggs were transferred to another 500 l aquarium filled with FSW, and insemination was carried out by adding sperm-laden seawater (100–200 ml).

Maintenance of larvae and juveniles

Fertilized eggs were incubated in FSW with strong aeration (ca. 6 eggs/ml), until hatching of larvae. Larvae were incubated in aerated FSW (5 or 10 m^3 fiberglass reinforced plastic aquarium; 0.3–0.4 larvae/ml). Larvae were fed with zooxanthellae isolated from parent clams (30–120 cell/ml) starting from the third day after insemination and until the establishment of symbiosis. Half of FSW in the aquaria was exchanged every 5 days, until larvae completed metamorphosis to juvenile clams and zooxanthellae appeared in the mantle of juveniles (10–20 days after the insemination). Juveniles were reared in still FSW for about 2 months and FSW was exchanged regularly.

Microscopy

Larvae and early juveniles were observed daily under a light microscope to check for the presence or absence of zooxanthellae in the alimentary system and mantle. Larvae on day 7–9 and juvenile clams were fixed in 2.5% glutaraldehyde-seawater and stored at 4°C. The specimens were fixed as follows; *T. crocea* (7, 14, 36, 71, and 162 days after the insemination), *T. derasa* (9 and 65 days), and *T. squamosa* (7, 14, and 64 days).

Fixed specimens were briefly rinsed with 0.1 M cacodylate−0.45 M sucrose (pH 7.6) and post-fixed in 1% osmium tetroxide−0.1 M cacodylate (pH 7.6) for 2 h. After a brief rinse with 50% ethanol, the specimens were decalcified with 1% ascorbic acid−0.15 M NaCl for 12 h or more. Specimens were then dehydrated through an ethanol series, cleared with *n*-butyl glycidyl ether, and embedded in low viscosity epoxy resin. Thick sections were stained with toluidine blue for light microscopy. Thin sections were stained with uranyl acetate and lead citrate and observed with a transmission electron microscope (JEOL JEM-1010).

Results

Veliger larvae

Embryos hatched the day after insemination and metamorphosed into veliger larvae after 7–10 days for all three clam species. The shell length reached approximately 180 μm before metamorphosis. Zooxanthellae ingested by veligers were seen in their alimentary systems but were never found in the mantle. In histological sections of pre-metamorphosed larvae (7–9 days), ingested zooxanthellae were found in the stomach lumen and some of them were partly degraded (Fig. 1). In electron microscopic observation, the cell walls of such zooxanthellae tended to be swollen (Fig. 2a). The subcellular structures were disintegrative, while some thylakoids remained (Fig. 2b).

Appearance of zooxanthellal tubes

Zooxanthellae were exclusively found in the stomach of the juveniles just after the metamorphosis from veligers. Differentiation of the zooxanthellal tube was recognized when zooxanthellae in the juvenile clam appeared in a line. We thought that this was the sign of the estab-

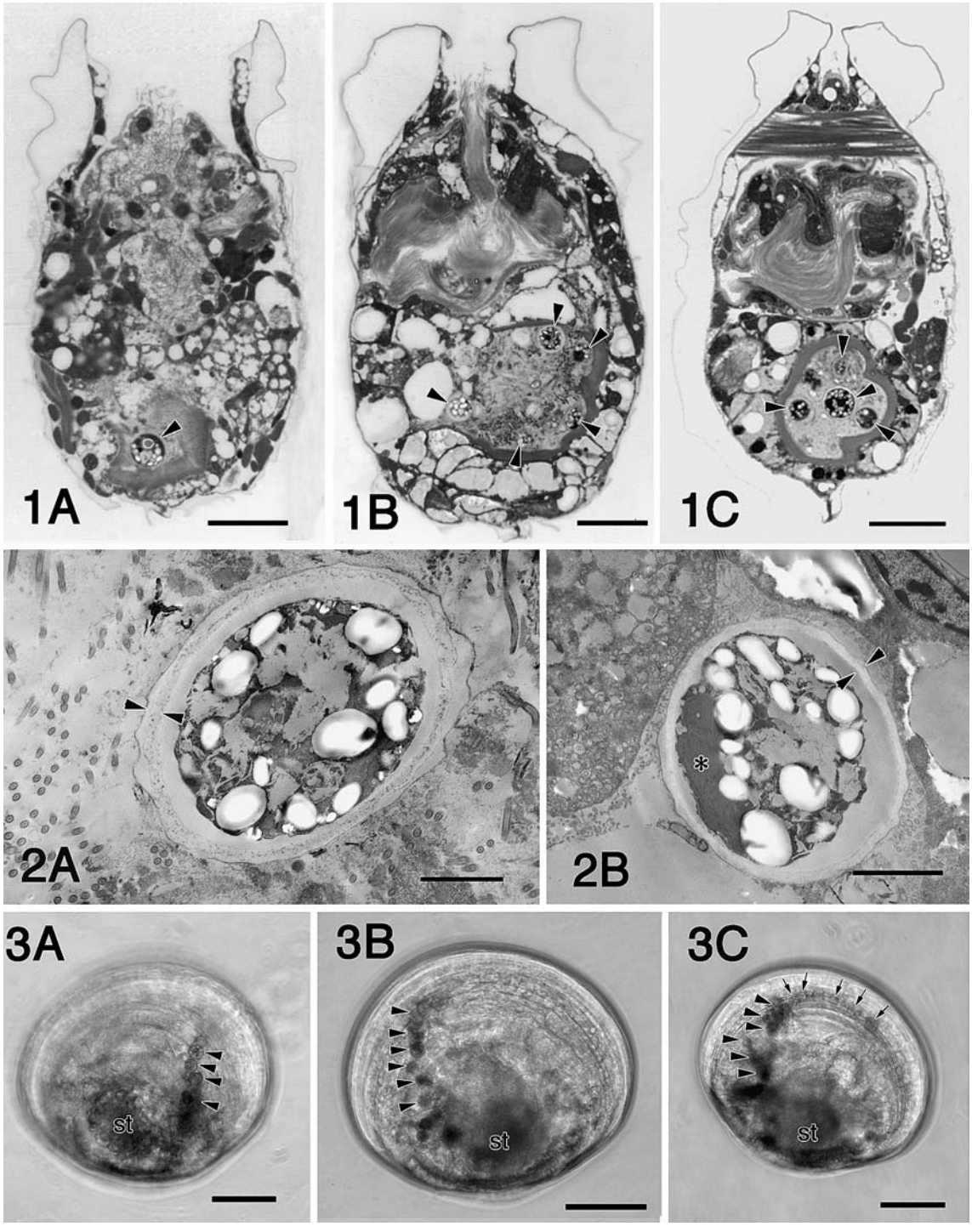

Fig. 1 Cross section of the pre-metamorphosed veliger larvae. **a** *Tridacna crocea* (7 days); **b** *Tridacna derasa* (9 days); **c** *Tridacna squamosa* (7 days). *Arrowheads* indicate ingested zooxanthellae in the stomach lumen. *Scale bar* 20 μm. **Fig. 2** Zooxanthellae in the stomach lumen of veligers. **a** *Tridacna crocea* (7 days); **b** *Tridacna derasa* (9 days). Some thylakoids remained in the cytoplasm (*asterisk*). *Facing arrowheads* indicate cell walls. *Scale bar* 2 μm. **Fig. 3** Juvenile clams (14 days) in which zooxanthellal tubes (*arrowheads*) appeared. **a** *Tridacna crocea*; **b**, **c** *Tridacna squamosa*. *Arrows* indicate zooxanthellae lined along the mantle edge. *Scale bar* 50 μm

lishment of symbiosis. The zooxanthellal tube, in which zooxanthellae were packed, mostly appeared in the juveniles of about 2 weeks after fertilization . At this stage, shell length of juvenile clams was about 200 μm. The zooxanthellal tube extended from the stomach toward the edge of the mantle (Fig. 3), and then the tube further extended along the mantle edge (arrows in Fig. 3c). In the earliest case of our observation, the

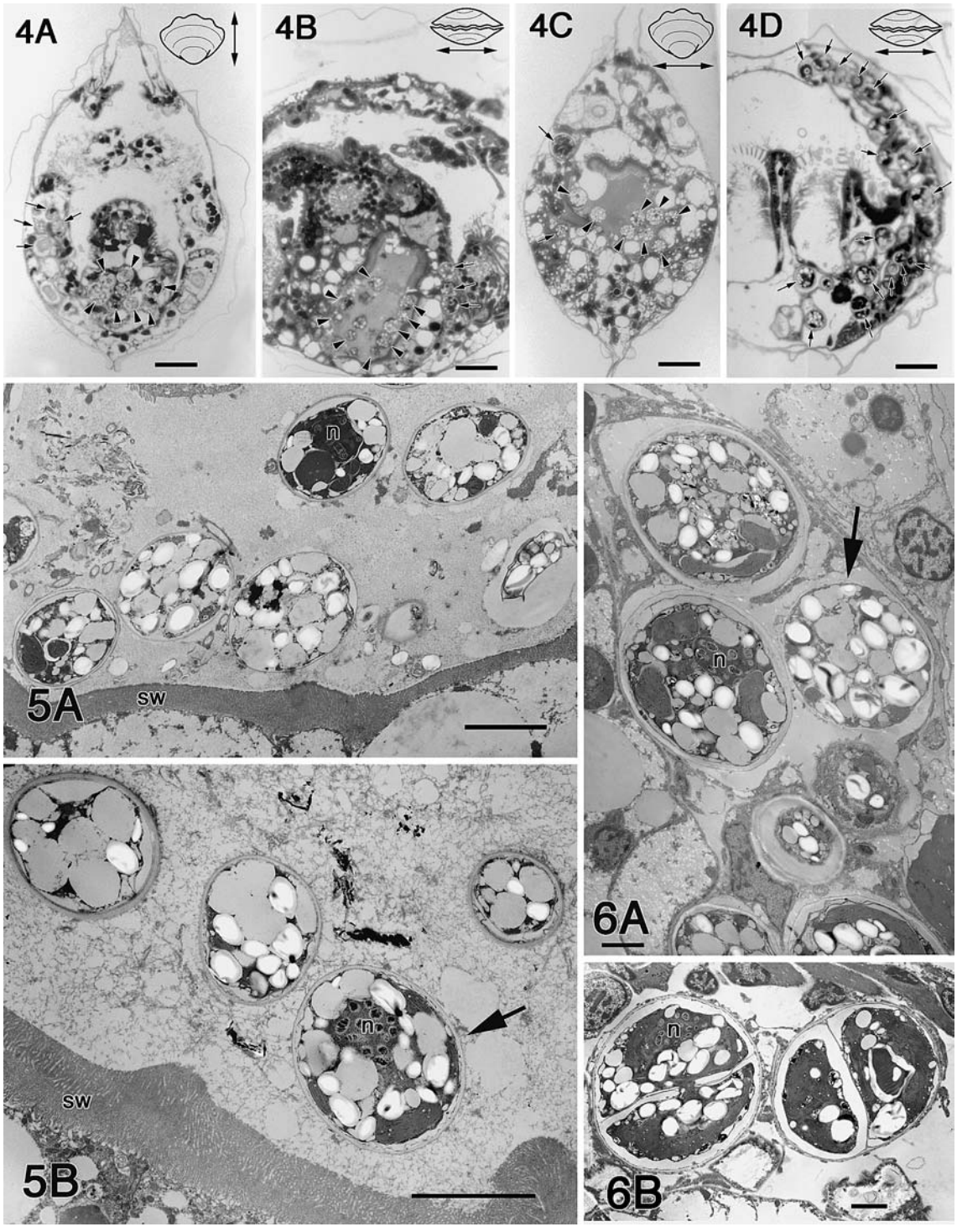

Fig. 4 Histological section of juveniles (14 days). **a, b** *Tridacna crocea*; **c, d** *Tridacna squamosa*. Icons at the upper *right corners* indicate the direction of the sectioning. *Arrows and arrowheads* respectively indicate the zooxanthellae in the zooxanthellal tube and the stomach. *Scale bar* 20 μm. **Fig. 5** Zooxanthellae in the stomach of juvenile (14 days). **a** *Tridacna crocea*; **b** *Tridacna squamosa*. *Arrow* indicates relatively intact zooxanthellae. *n* nucleus of zooxanthellae; *sw* stomach wall. *Scale bar* 5 μm for **a**, 2 μm for**b**. **Fig. 6** Zooxanthellae in the zooxanthellal tube of juvenile (14 days). **a** *Tridacna crocea*; **b** *Tridacna squamosa*. *Arrow* indicates the zooxanthella with disintegrative subcellular structures. *n* nucleus of zooxanthellae. *Scale bar* 2 μm

zooxanthellal tube appeared in most of the juvenile clams on day 10 and all the juveniles of all three species had established the symbiosis by day 20.

In histological sections, zooxanthellae were found in the mantle as well as the stomach (Fig. 4). These zooxanthellae were surrounded by an epithelial sheet that is

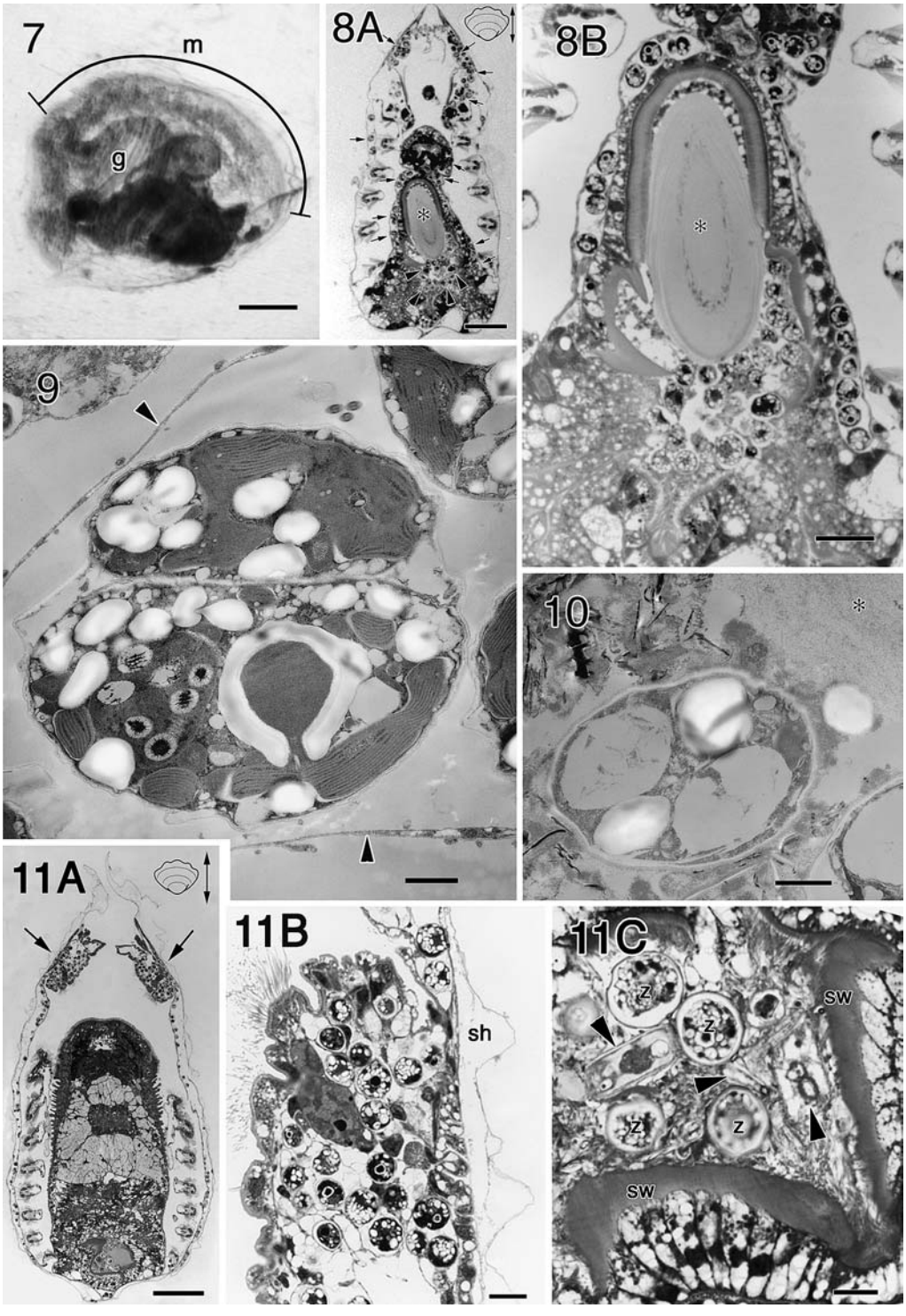

Fig. 7 A juvenile of *T. crocea* on day 36. The shells were decalcified. *g* gill; *m* mantle margin. *Scale bar* 0.1 mm. **Fig. 8** **a** A cross section of the juvenile of *T. crocea* (36 days). **b** Enlargement of the stomach in (**a**). *Arrows and arrowheads* respectively indicate zooxanthellae in the zooxanthellal tube and the stomach. *Asterisk* crystalline style. *Scale bar* 50 μm in (**a**), 20 μm in (**b**). **Fig. 9** A dividing zooxanthella in the zooxanthellal tube of *T. crocea* (36 days). *Arrowheads* indicates thin epithelial walls of zooxanthellal tube. *Scale bar*: 1 μm. **Fig. 10** A degraded zooxanthella in the stomach of the *T. crocea* juvenile (36 days). *Asterisk*, crystalline style. *Scale bar*: 1 μm. **Fig. 11** *Tridacna squamosa* (64 days). **a** A cross section of the whole clam. **b** Enlargement of the mantle edge. **c** Stomach. *Arrows* indicate swelling of the mantle margin. *Arrowheads* indicate diatoms in the stomach. *sh* shell; *sw* stomach wall; *z* zooxanthellae. *Scale bar* 100 μm for (**a**), 10 μm for (**b**) and (**c**)

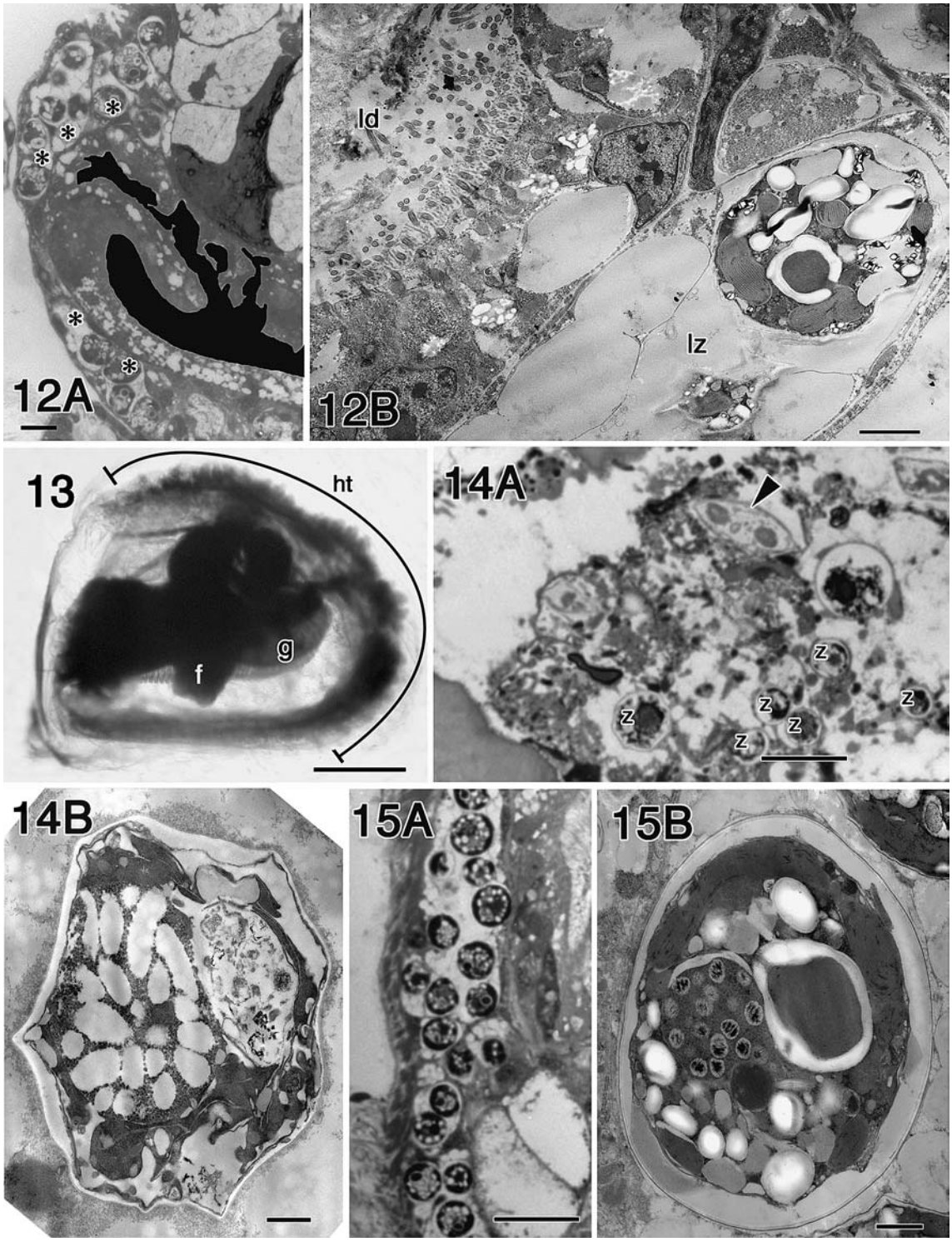

Fig. 12 *Tridacna derasa* (65 days). Some zooxanthellal tubes (*asterisks*) are contiguous with digestive diverticula. Shaded area in (**a**), lumen of the alimentary canal; *ld* lumen of the digestive diverticulum; *lz* lumen of the zooxanthellal tube. *Scale bar* 10 μm for (**a**) and 2 μm for (**b**). **Fig. 13** *Tridacna crocea* on day 162. The shells were decalcified. *f* foot; *g* gill; *ht* hypertrophied siphonal tissue. *Scale bar* 0.5 mm. **Fig. 14** Zooxanthellae (*z*) digested in the stomach (*T. crocea*, 162 days). *Arrowhead* indicates a diatom. *Scale bar* 20 μm for (**a**), 1 μm for (**b**). **Fig. 15** Zooxanthellal tube (**a**) and zooxanthellae in the tube (**b**) (*T. crocea*, 162 days). *Scale bar* 20 μm for (**a**), 1 μm for (**b**)

probably the wall of the zooxanthellal tube. The tube appeared to extend bilaterally from the stomach and then extend toward the mantle edge along the shell (Fig. 4d). However, we could not find the junction of the zooxanthellal tube and the alimentally canal despite our histological observations. In the stomach lumen, most of

the zooxanthellae were degraded, but cell walls remained in some cells (Fig. 5). There were some zooxanthellae with relatively intact ultrastructures (arrow in Fig. 5b). On the other hand, in zooxanthellal tubes, most zooxanthellae had intact structures (Fig. 6). Whereas some zooxanthellae were disintegrative in subcellular structure (arrow in Fig. 6a), others were apparently healthy and some were occasionally in cell division (Fig. 6b).

Zooxanthellae in juvenile clams

One month after fertilization, zooxanthellae were widely spread in the mantle margin (Fig. 7). Zooxanthellal tubes were well developed and many intact-looking zooxanthellae were crowded in the tube, while the stomach contained many degraded zooxanthellae (Fig. 8). The luminal space of zooxanthellal tubes appeared to be expanded compared to that in the juveniles of day 14. Cytoplasm of zooxanthellae in tubes was filled with chloroplast and pyrenoids (Fig. 9). The tubular wall had a very thin epithelium (arrowheads in Fig. 9) and hemocytes were not found in the lumen. In the stomach, zooxanthellae were highly vacuolated and seemed to be digested in a crystalline style (Fig. 10).

The shell length reached about 1 mm by 2 months after fertilization. Zooxanthellae were distributed throughout the mantle, and the formation of the zooxanthellal tube network seemed to be basically completed. The mantle edge formed a swelling in which numerous zooxanthellae were found (Fig. 11a, b). In the stomach, degraded zooxanthellae were always found with diatoms and other unidentified contents (Fig. 11c). Although we could not find a direct connection between the zooxanthellal tubes and the alimentally canal, some zooxanthellal tubes were contiguous to the digestive diverticula (Fig. 12).

In *T. crocea* on day 162 (Fig. 13), hypertrophied siphonal tissue that contained numerous numbers of zooxanthellae had developed. There were many degraded zooxanthellae in the stomach lumen (Fig. 14). They seemed to be digested with diatoms and other unidentified stomach contents. On the contrary, most of the zooxanthellae in the zooxanthellal tube had intact ultrastructures (Fig. 15).

Discussion

Larvae of giant clams acquire their photosymbionts from the environment, because no zooxanthellae are associated with their gametes. We observed veligers ingesting zooxanthellae by feeding. The zooxanthellae were exclusively found in the alimentary canal. The algae were degraded in morphology and appeared to be digested by the host clam as food at this stage. Within several days after the metamorphosis into juvenile clams, zooxanthellae were appeared in a line in juvenile clam about 2 weeks after the insemination, indicating the establishment of symbiosis. The lined zooxanthellae extended from the stomach region toward the mantle edge, and then grew along the mantle margin. These zooxanthellae were located in a tube of thin epithelium (zooxanthellal tube) and usually had intact ultrastructures that suggest photosynthetic activity. In many microscopic images, some zooxanthellae were in the process of cell division, indicating active zooxanthellae proliferation in the tubes. The zooxanthellal tubes were directly connected with the digestive diverticula, as seen in Norton et al. (1992). Although we could not locate the connection between the zooxanthellal tubes and the diverticula, some zooxanthellal tubes were contiguous with the diverticula in some sections (Fig. 12). The timing of the appearance of zooxanthellae in the mantle and the associated morphological features were essentially the same as the observations of Fitt and Trench (1981). However, Fitt and Trench (1981) theorized that the tubular system containing zooxanthellae was a hemal sinus. The zooxanthellal tubular system developed as the clam grew, especially in the mantle margin, and then the hypertrophied siphonal tissue was formed.

Regardless of the presence of symbiosis or not, degraded zooxanthellae were always found in the stomach of veligers and clams. They appeared to be digested with other stomach contents, such as diatoms. Therefore, the giant clams probably utilize the zooxanthellae not only as photosymbionts, but also directly as foods. In *T. derasa* (shell length 5–6 cm), since the sum of daily increased zooxanthellae in the mantle and discharged zooxanthellae in the feces was estimated to be only about 20% of the newly formed zooxanthella population in the clam, it has been proposed that the missing zooxanthellae are digested by the clam (Maruyama and Heslinga 1997). While zooxanthellae are believed to supply the host with photosynthetic product, digestion of zooxanthellae may also be an important nutrient source for giant clams. Filter-feeding also contributes to giant clam nutrition (Klumpp et al. 1992; Klumpp and Lucas 1994), and the diatoms and unidentified stomach contents we observed here were probably ingested by filter-feeding (Figs. 11, 14).

When the symbiosis was established within several days after metamorphosis into juvenile clams, some zooxanthellae entered the newly formed zooxanthellal tube and became founders of the zooxanthellae population in the mantle. Most of zooxanthellae in the zooxanthellal tube were intact, while those in the stomach were degraded. The host clams might have a mechanism to select the zooxanthellae for photosymbionts: the zooxanthellal tube should keep healthy zooxanthellae and excrete poorly functional ones into the stomach lumen. On the other hand, in the early juvenile, almost intact zooxanthellae were occasionally found in the stomach (Fig. 5b), and some degraded zooxanthellae were found in the tube (Fig. 6a). Therefore, the zooxanthellae population in the mantle may result from domination of the progenies of the healthy zooxanthellae in the tube, while the founder zooxan-

thellae probably entered the tube at random from stomach. According to Maruyama and Heslinga (1997), many of the zooxanthellae in the stomach originated from the zooxanthellal tubular system. There may be a selection mechanism to discharge unhealthy zooxanthellae from the mantle into the stomach.

Individual giant clams often harbor heterogeneous assemblages of zooxanthellae (Carlos et al. 2000). The stomach contents of the clams (Figs. 11, 14) showed the occurrence of active filter-feeding and the clams would occasionally ingest free-living zooxanthellae in seawater. Thus, it is possible that giant clams constantly acquire zooxanthellae from the environment and some of them are recruited to the zooxanthellae population in the zooxanthellal tube. If this is the case, some mechanism(s) should be present to capture and select the zooxanthellae at the entrance of the zooxanthellal tube, although we could not demonstrate such a mechanism(s) morphologically. Alternatively, zooxanthellae in the tube may be all progeny of the zooxanthellae that entered in the zooxanthellal tube within several days after the metamorphosis originally. In this case, zooxanthellae heterogeneity would be invariable throughout the life of the clams, although the population ratio among the clones originated from each founder zooxanthella may change depending on environmental stress. Plasticity and maintenance of the zooxanthellae population in the mantle is an important key in better understanding the physiology of symbiosis between zooxanthellae and giant clams.

Acknowledgments The present study was supported by the twenty-first century COE program of the University of the Ryukyus. We are indebted to Dr. Yasunori Saito (Shimoda Marine Research Center, University of Tsukuba) for providing the facilities for electron microscopy and Dr. Hideyuki Yamashiro (Meio University) for his advice on the decalcification of specimens. Dr. James D. Reimer (JAMSTEC) was acknowledged for his valuable comments and correcting the English. This report includes the contribution form Shimoda Marine Research Center (# 713).

References

Carlos AA, Baillie BK, Maruyama T (2000) Diversity of dinoflagellate symbionts (zooxanthellae) in a host individual Mar Ecol Prog Ser 195:93–100

Fankboner PV (1971) Intracellular digestion of symbiotic zooxanthellae by host amoebocytes in giant clams (Bivalvia: Tridacnidae), with a note on the nutritional role of the hypertrophied siphonal epidermis Biol Bull 141:222–234

Farmer MA, Fitt WK, Trench RK (2001) Morphology of the symbiosis between *Corculum cardissa* (Mollusca: Bivalvia) and *Symbiodinium corculorum* (Dinophyceae) Biol Bull 200:336–343

Fitt WK, Trench RK (1981) Spawning, development, and aquisition of zooxanthellae by *Tridacna squamosa* (Mollusca, Bivalvia) Biol Bull 161:213–235

Ishikura M, Adachi K, Maruyama T (1999) Zooxanthellae release glucose in the tissue of a giant clam, *Tridacna crocea* Mar Biol 133:665–673

Jameson SC (1976) Early life history of the giant clams *Tridacna crocea* Lamark, *Tridacna maxima* (Röding), *Hippopus hippopus* (Linnaeus) Pac Sci 30:219–233

Kawaguti S (1966) Electron microscopy on the mantle of the giant clam with special references to zooxanthellae and iridophores Biol J Okayama Univ 12:81–92

Klumpp DW, Bayne BL, Hawkins AJS (1992) Nutrition of the giant clam *Tridacna gigas* (L.). I. Contribution of filter feeding and photosyhthate to respiration and growth J Exp Mar Biol Ecol 155:105–122

Klumpp DW, Lucas JS (1994) Nutritional ecology of the giant clams *Tridacna teborea* and T. derasa from Tonga: influence of light on filter-feeding and photosynthesis Mar Ecol Prog Ser 107:147–156

LaBarbera M (1975) Larval and post larval developments of the giant clams *Tridacna maxima* and *Tridacna squamosa* (Bivalvia: Tridacnidae) Malacologia 15:69–79

Mansour K (1946a) Communication between the dorsal edge of the mantle and the stomach of *Tridacna* Nature 157:844

Mansour K (1946b) Source and fate of the zooxznthellae of the visceral mass of *Tridacna elongata* Nature 158:130

Maruyama T, Heslinga GA (1997) Fecal discharge of zooxanthellae in the giant clam *Tridacna derasa*, with reference to their in situ growth rate Mar Biol 127:473–477

Norton JH, Shepherd MA, Long HM, Fitt WK (1992) The zooxanthellal tubular system in the giant clam Biol Bull 183:503–506

Trench RK, Wethey DS, Porter JW (1981) Observation on the symbiosis with zooxanthellae among the Tridacnidae (Mollusca, Bivalvia) Biol Bull 161:180–198

Yonge CM (1936) Mode of life, feeding, digestion and symbiosis with zooxanthellae in the Tridacnidae Sci Rep Gr Barrier Reef Exped (1928–29), Brit Mus (Nat Hist) 1:283–321

Yonge CM (1953) Mantle chambers and water circulation in the Tridacnidae (Mollusca) Proc R Soc Lond 123:551–561