

Timing of Spawning and Early Development of *Palythoa tuberculosa* (Anthozoa, Zoantharia, Sphenopidae) in Okinawa, Japan

MAMIKO HIROSE^{1,2,*}, MASAMI OBUCHI¹, EUICHI HIROSE², AND JAMES D. REIMER^{1,3}

¹Molecular Invertebrate Systematics and Ecology Laboratory, Rising Star Program, Transdisciplinary Research Organization for Subtropical Island Studies (TRO-SIS), University of the Ryukyus, Senbaru 1, Nishihara, Okinawa, 903-0213, Japan; ²Faculty of Science, University of the Ryukyus, Senbaru 1, Nishihara, Okinawa, 903-0213, Japan; and ³Marine Biodiversity Research Program, Institute of Biogeoscience, Japan Agency for Marine-Earth Science and Technology (JAMSTEC), 2-15 Natsushima, Yokosuka, Kanagawa 237-0061, Japan

Abstract. The spawning behavior and early embryogenesis of *Palythoa tuberculosa* (Anthozoa, Zoantharia) were observed in August 2009 off Okinawa Island, Japan. *P. tuberculosa* released zygotes just after high tide around new moon nights. The mean diameter of zygotes was $365.6 \pm \text{s.d.} 14.8 \mu\text{m}$, and zygotes did not contain any symbiotic algae (zooxanthellae). About 2 h after spawning, the first cleavage furrow appeared on one side of the zygotes, although it was uncertain when eggs were fertilized. After second cleavage, the arrangement of blastomeres was pseudospherical. At 9 h after spawning, the embryo became a concave-convex dish shape, then gastrulation occurred and the blastopore was formed. Seven-day old larvae were ellipsoid and about $700 \mu\text{m}$ long, with an open mouth at one end. Two weeks after spawning, the larvae developed a longitudinal band of long cilia (= ventral ciliate band) that is characteristic of zoanthella larvae. In *P. tuberculosa*, larvae show a non-radial body plan and then metamorphose to almost-radial (in outward appearance) polyps after settlement. These results may support a hypothesis that a common ancestor of Cnidaria had a bilateral body plan that has been secondarily lost in some extant cnidarians.

Introduction

Zoantharia (Cnidaria, Anthozoa, Hexacorallia) is an order of benthic cnidarians, and zoanthid species are widespread

globally from the intertidal zone to the deep sea. They are characterized by (1) a distinctive arrangement of radially disposed septa (= mesenteries) and two rows of tentacles; (2) the lack of skeleton (except for the genus *Savalia*), with most species incorporating sand grains into their tissue; (3) a distinctive, modified planula larva, either “zoanthella” or “zoanthina.” Zoanthids, with exception of the solitary *Sphenopus*, generally form clonal aggregations or colonies. They are often an abundant component of marine communities, particularly in tropical ecosystems (see Fadlallah *et al.*, 1984). Despite its ecological and phylogenetic importance, this taxon has been the subject of few studies on its reproductive biology (reviewed by Ryland, 1997), probably because of its confused taxonomy (Burnett *et al.*, 1997; Reimer *et al.*, 2004) and difficulty in internal observation due to sand encrustation (Reimer *et al.*, 2010).

The order Zoantharia (=Zoanthidea) is divided into two suborders by arrangement of their mesenteries. Colonies in the suborder Macrocnemina are, with few exceptions, gonochoric. On the other hand, colonies are commonly hermaphroditic in the suborder Brachycnemina, which includes the genera *Palythoa* and *Protopalythoa*, although small samples may yield only unisexual polyps (Ryland, 1997). Shallow-water zoanthids ranging from higher latitudes (54–61°N) to tropical areas often have annual cycles of gametogenesis (see Ryland, 1997, 2000), while *Epizoanthus* species in deep water breed continuously (Muirhead *et al.*, 1986). To date, there have been a few reports on the spawning behavior of zoanthids in the laboratory (Kimura *et al.*, 1972) and in the field (Ryland and Babcock *et al.*, 1991), and internal

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* To whom correspondence should be addressed. E-mail: mhirose@zenno.jp

brooding has been confirmed only in *Isozoanthus giganteus* (mentioned in Ryland, 1997).

Two types of larvae have been reported for zoanthids. The zoanthella larva (named by van Beneden, 1897) was first described by Semper (1867); this larva is an almost cylindrical planula that has a long, mid-central ciliary band extending from the blastopore to the anterior pole. Semper's "second" larva, designated the zoanthina larva (named by van Beneden, 1897), has a spherical or somewhat elongated body with an equatorial ciliary band. Menon (1902, 1914, 1926) revealed that the genera *Sphenopus* and *Protopalythoa* (family Sphenopidae) produce zoanthella, and the genera *Isaurus* and *Zoanthus* (family Zoanthidae) produce zoanthina. Kimura *et al.* (1972) later reported that *Palythoa* (Sphenopidae) also produces zoanthella. Most descriptions of zoanthid larvae have been based on specimens collected with plankton tows (Menon, 1902; Scheltema, 1971; Nielsen, 1984), and species identification of the larvae has often been impossible. Babcock and Ryland (1990) raised the fertilized eggs of *Protopalythoa* sp. to zoanthella larvae, and to date this is the only description of zoanthid development from spawning to settlement.

Species of the genus *Palythoa* are colonial, generally zooxanthellate, and commonly found in shallow coral reefs worldwide. Recent studies using sequences of the internal transcribed spacer of ribosomal DNA (ITS-rDNA) from *Palythoa* specimens in Japan have suggested that the very closely related *Palythoa* species "liberae" *Palythoa mutuki*, "immersae" *P. tuberculosa*, and "intermediate morphology" *Palythoa* sp. have experienced reticulate evolution (Reimer *et al.*, 2007a). However, there is little information about sexual reproduction of *Palythoa* spp. in Japan: (1) *P. tuberculosa* produces gametes in summer (Yamazato *et al.*, 1973; Shiroma and Reimer, 2010); and (2) eggs were observed to be released from laboratory-cultured colonies in August and developed into zoanthellae (Kimura *et al.*, 1972).

In August 2009, we observed natural spawning of *Palythoa tuberculosa* Klunzinger, 1877 in the field and the release of zygotes that developed into zoanthellae. Here, we report on the embryology and fine structure of zoanthella larvae of *P. tuberculosa*.

Materials and Methods

Collection and observation of early development

In Okinawa, *Palythoa tuberculosa* often forms large aggregations, consisting of many colonies, in the intertidal and shallow subtidal zones. Released zygotes of *P. tuberculosa* from a large aggregation (up to 4 m²) were collected by scuba at Mizugama, on the west coast of Okinawa Island, Japan (26°21'38 N, 127°44'20 E) on 19 and 20 August 2009. Released zygotes were caught *in situ* and brought to the laboratory in 300-ml plastic bottles filled with seawater.

A few colonies were collected from the same aggregation at the same time and fixed with 10% formalin-seawater.

Embryos and larvae were reared in 200-ml plastic jars filled with 0.45- μ m-pore filtered seawater (FSW) at room temperature (27–29 °C), and FSW was changed daily. The embryos and larvae were observed under a light microscope, and photomicrographs were taken.

Microscopy

Embryo and larvae were fixed in 2.5% glutaraldehyde in FSW and stored at 4 °C. Specimens were then rinsed with 0.1 mol l⁻¹ cacodylate–0.45 mol l⁻¹ sucrose and post-fixed in 1% osmium tetroxide–0.1 mol l⁻¹ cacodylate for 2 h. After dehydration through a graded ethanol series, some specimens were immersed in *t*-butanol, freeze-dried, sputter-coated with gold-palladium, and examined under a scanning electron microscope (SEM: JEOL JSM-6060LV) at low vacuum mode (backscattered electron images at about 30 Pa). Other specimens were immersed in *n*-butyl glycidyl ether and embedded in epoxy resin (Quetol 812, Nissin EM). Sections of 0.5–1 μ m thick were stained with toluidine blue for light microscopy. Thin sections were stained with lead citrate and uranyl acetate and examined with a transmission electron microscope at 80 kV (TEM: JEOL JEM-1011).

Results

Observation of spawning

Some polyps of *Palythoa tuberculosa* had no gonads, and some developed either testes or ovaries (Fig. 1A); no polyp was hermaphroditic. Zygote spawning of *P. tuberculosa* was observed between 2030 and 2200 h on both 19 and 20 August 2009 at a shallow reef. *P. tuberculosa* spawned just after high tide on new moon nights (Fig. 1D). We confirmed that several colonies within the same aggregation released their zygotes on both days. However, we do not know whether colonies are clones or genetically different colonies because individual-level genetic markers do not yet exist for zoanthids. The zygotes (fertilized eggs) were released through the mouth as tightly packed clusters. Packed zygotes (fertilized egg bundles) were highly buoyant (Fig. 1B, C). The diameter of packed zygotes was 2–3 mm. Zygote spawning was not strictly synchronized within and among colonies. The zygotes were orange and did not contain any symbiotic microalgae (zooxanthellae). The diameter of zygotes was $365.6 \pm 14.8 \mu\text{m}$ (average \pm SD) (Fig. 2A). We did not see the release of sperm during the observation period. However, the collected egg bundle, containing potentially fertilized eggs (=zygotes), began to develop (see Discussion).

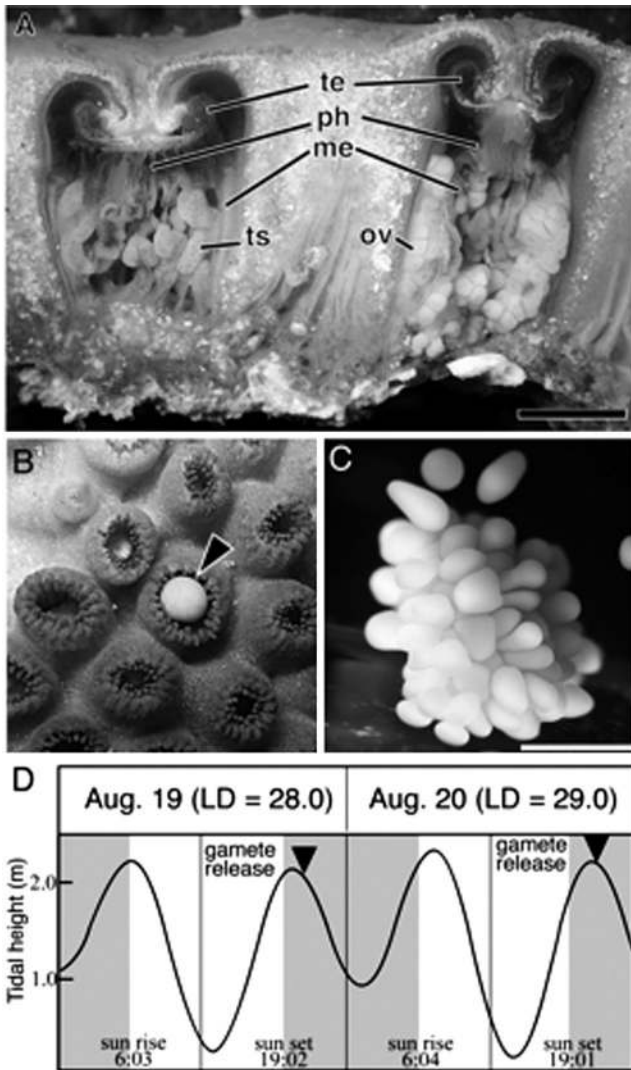


Figure 1. (A) Cross section of a hermaphroditic colony of *Palythoa tuberculosa* (20 August 2009). Male polyp (left) has numerous white testes; female polyp (right) has pink ovaries. (B) A polyp of *P. tuberculosa* releasing a bundle of fertilized eggs (arrowhead). (C) Fertilized eggs disaggregating from the released bundle. (D) Timing of spawning in relation to day length and tidal cycle. Arrowheads indicate the start of spawning: times of sunrise and sunset are referenced from National Astronomical Observatory of Japan (<http://www.nao.ac.jp/koyomi/koyomix/koyomix.html>), and tidal height from Japan Meteorological Agency (<http://www.data.kishou.go.jp/kaiyou/db/tide/suisan/index.php> for Naha (26°13'N, 127°40'E)). me, mesentery; ov, ovary; ph, pharynx; te, tentacle; ts, testis. Scale bars: 2 mm in A; 1 mm in C.

Embryogenesis of *P. tuberculosa*

About 2 h after spawning, the first cleavage furrow appeared on one side of the zygotes, producing a heart-shaped cleavage stage (Fig. 2B). The first cleavage was equal and holoblastic (Fig. 2C). After the second cleavage, the arrangement of blastomeres was pseudospherical (Fig. 2D), but subsequent cleavages appeared to be less regular, re-

sulting in cells of different sizes (Fig. 2E–I). Cleavage occurred at intervals of 30–45 min. At 9 h after spawning, embryos consisted of a bilayer of cells with no definite blastocoel. The embryos then became concave-convex dish-shaped (Fig. 3A, B, C). At 12 h after spawning, embryos were roughly spherical, with a blastopore (Fig. 3D, E, F). At 24 h after spawning, the surface of embryos became smooth, and the spherical larvae crept along the bottom of the plastic jar (Fig. 3G, H). At this stage, the blastopore was closed and there was no coelenteron (= gastrovascular cavity) observed in histological examination (Fig. 3I). Seven-day-old larvae were ellipsoid and about 700 μ m long and 350 μ m wide, with an anterior, open mouth (Fig. 3J). The surfaces of larvae were slightly concave, and a shallow groove formed a line along the long axis (Fig. 3K, arrowhead). At this stage, the body of the larvae consisted of two germ layers (ectoderm and endoderm) that were separated by the mesoglea. Ectodermal cells were columnar and stained weakly with toluidine blue, while endodermal cells were irregularly shaped and highly vacuolated due to lipid storage (Fig. 3L, M). At the mouth opening, the ectoderm turned inward to form a ciliated lining of the mouth. The mouth lumen was continuous with the coelenteron that was lined with endoderm (Fig. 3L). A cross section of the concave groove in a 7-day larva showed that ciliary bundles emerged from the bottom of the shallow groove (Fig. 3N). Larvae rotated on the oral-aboral axis and swam in an aboral direction, regardless of the movement of the cilia.

Zoanthella larvae of *P. tuberculosa*

About 13 days after spawning, larvae developed a longitudinal band of long cilia (= ventral ciliate band) that is characteristic of zoanthella larvae (Fig. 4A). The longitudinally enlarged band moved rhythmically right and left along the oral-aboral axis in a coordinated wave-like fashion. However, the larva rotated on the oral-aboral axis with the enlarged ciliate band. The enlarged band appeared not to play a major role in the locomotion of larvae, as it occasionally stopped but the larvae continued to move. At this stage, a pharynx with six mesenteries was formed (Fig. 4B, C, D), although only two mesenteries were observed in the posterior half of the larvae (Fig. 4E). At 22 days after spawning, several nematocysts were found in the ectoderm, and the larvae still contained many lipid drops in the endodermal layer (Fig. 4F). Each cell of the ventral ciliate band carried one cilium and one microvillus (Fig. 4G, inset). Each cilium was associated with an accessory centriole and striated ciliary root. Septate junctions were found at the latero-distal part of the ectodermal cells (Fig. 4G).

Discussion

Shallow-water zoanthids have been shown to have annual cycles of gametogenesis: *Zoanthus* spp. and *Palythoa*

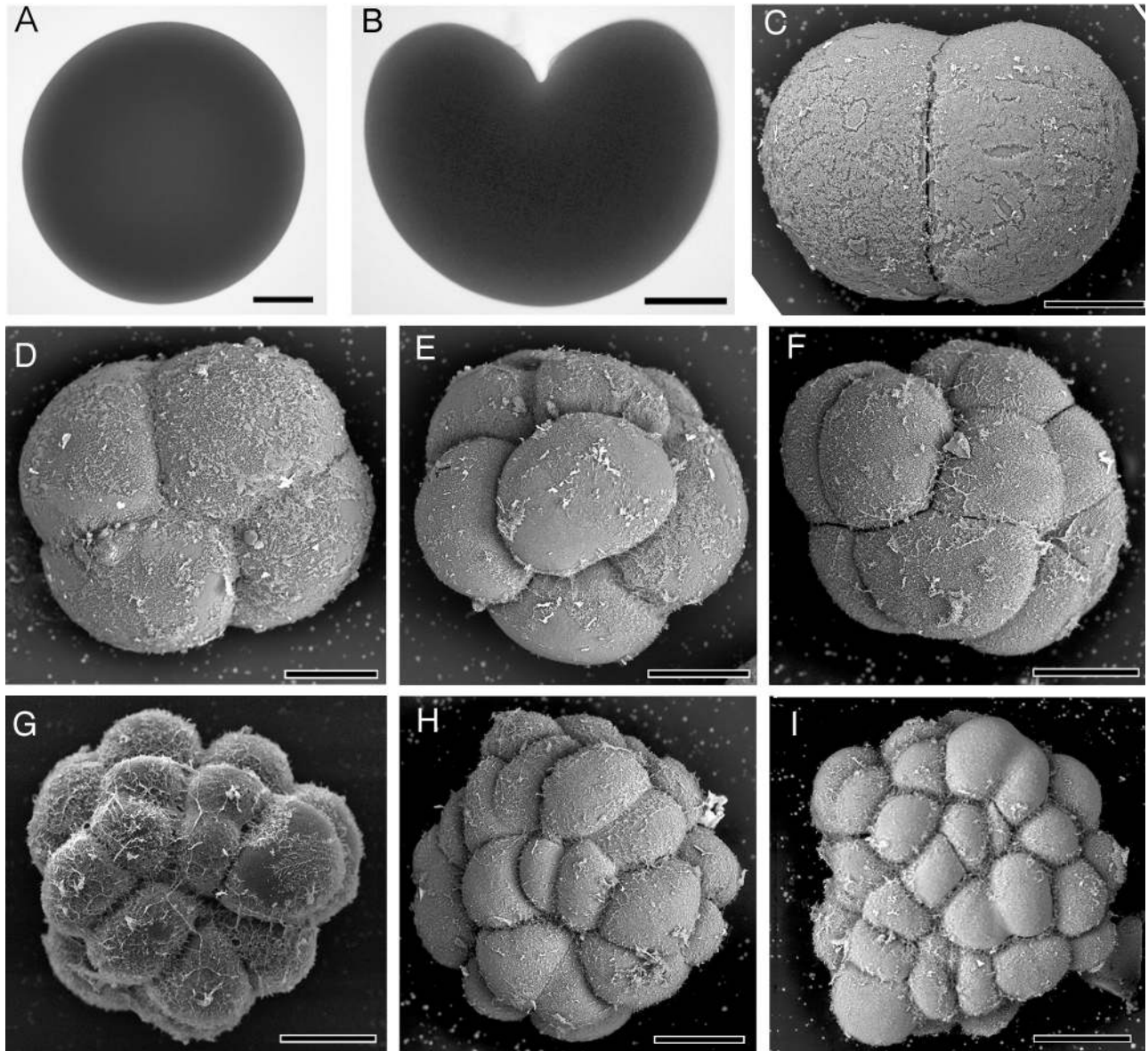


Figure 2. Early development of *Palythoa tuberculosa* (one-cell to morula stage). (A) One-cell stage. (B) First cleavage. The cleavage furrow appeared on one side of the blastomere, resulting in a heart-shaped cleavage stage. (C) Two-cell stage. (D) Four-cell stage. (E) Eight-cell stage. (F) 8–16-cell stage. (G) 16–32-cell stage. (H) 32–64-cell stage. (I) Morula stage. Scale bars: 100 μm .

caribaeorum in the North Atlantic and Caribbean (Karlson, 1982; Fadlallah *et al.*, 1984), *Zoanthus pacificus* and *Palythoa vestitus* in Hawaii (Cooke, 1976), *Protopalythoa* sp. in the Great Barrier Reef (GBR) (Ryland and Babcock, 1991), *Palythoa caribaeorum* and *Protopalythoa variabilis* in Brazil (Boscolo and Silveira, 2005), and *Zoanthus sansibaricus* in Kagoshima, Japan (Ono *et al.*, 2005). However, there are few reports about timing and mode of spawning events in zoanthids. In the GBR, *Protopalythoa* sp. was confirmed both by field and laboratory observations to release gametes three to five

nights (from 1930 to 2200 h) after the full moon in early summer (November) (Babcock and Ryland, 1990; Ryland and Babcock, 1991). In Bermuda, a major spawning of *Parazoanthus parasiticus* was inferred through histological study to occur one to two nights after the full moon in summer (September) (Ryland, 1997). Kimura *et al.* (1972) reported that *Palythoa tuberculosa* released its eggs in August in the laboratory in Okinawa; however, they did not describe the exact day or time of the spawning. In the temperate zone of Japan, *Zoanthus sansibaricus* spawning was estimated through histological study to

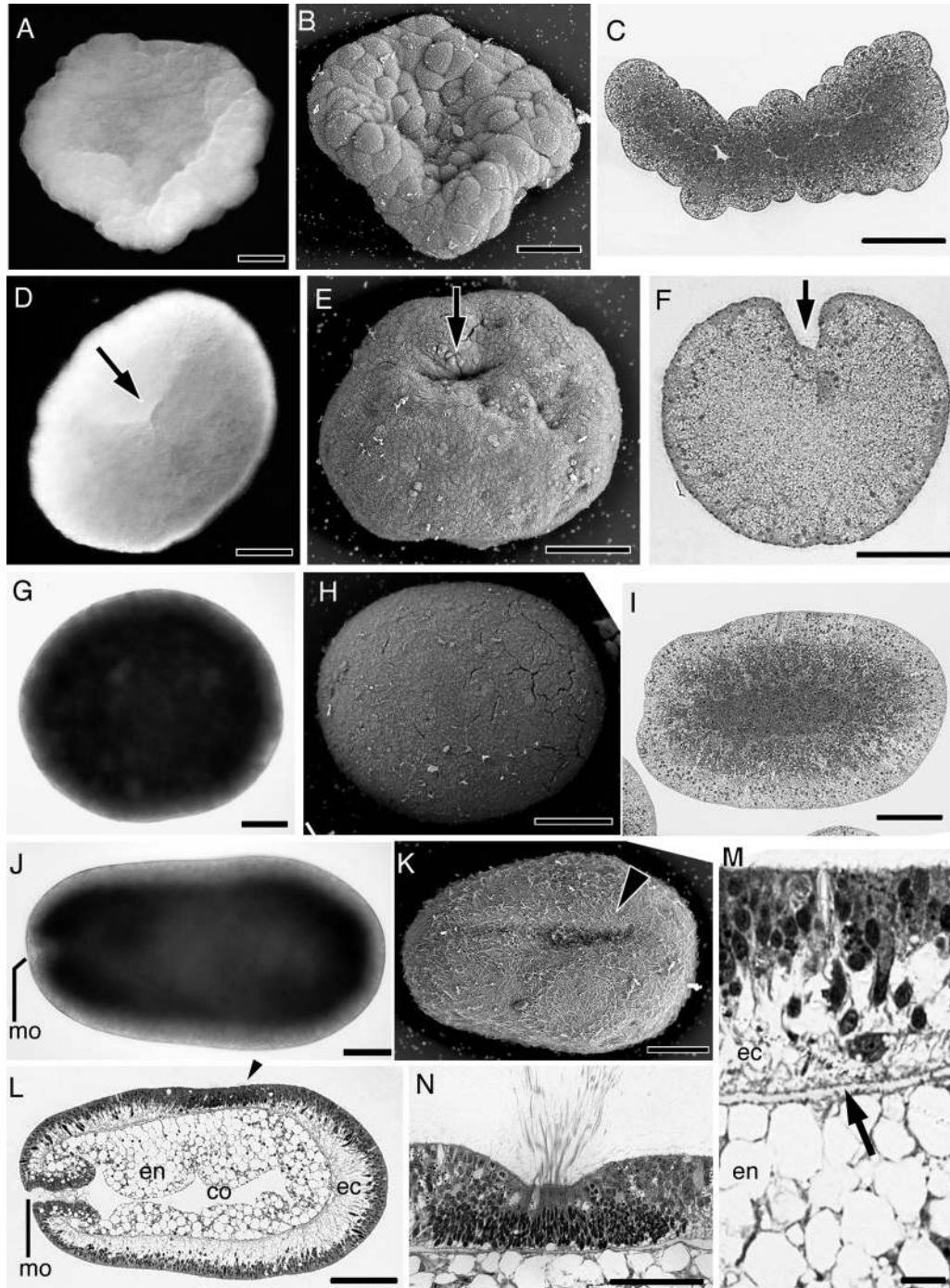


Figure 3. Early development of *Palythoa tuberculosa* (gastrulation to young larva). (A) Early gastrulation (whole mount). (B) Early gastrulation (scanning electron microscopy [SEM]). The blastula begins to flatten and take a concave-convex dish shape. (C) Section of early gastrulation. (D) Late gastrulation (whole mount). (E) Late gastrulation (SEM). (F) Section of late gastrulation. Arrows in D, E, and F indicate blastopore. (G) Immature larva (2-day-old, whole mount). (H) Immature larva (2-day-old, SEM). (I) Section of immature larva (2-day-old). The blastopore was closed and there was no coelenteron (gastrovascular cavity) in this stage. (J) Young larva (7-day-old, whole mount). (K) Young larva (7-day-old, SEM). (L) Section of a 7-day-old larva. Larvae were slightly concave (arrowhead). (M) Section of part of a 7-day-old larva. The ectoderm and endoderm are separated by mesoglea (arrow). (N) Cross section of the concave part of a 7-day-old larva. Bundles of cilia emerged from the bottom of the shallow groove. co, coelenteron; ec, ectoderm; en, endoderm; mo, mouth. Scale bars: 100 μ m in A–L; 50 μ m in N; 10 μ m in M.

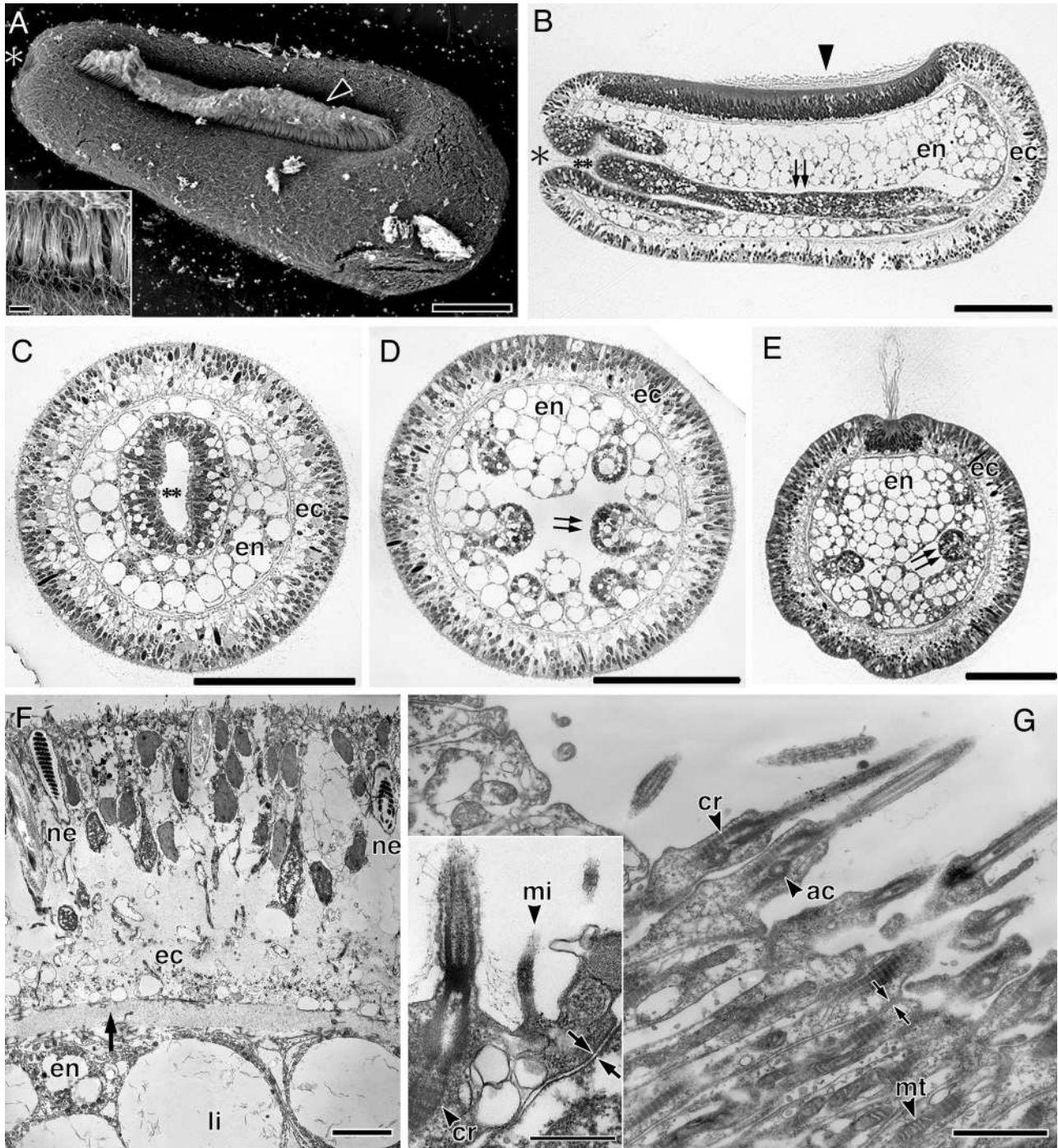


Figure 4. Zoanthella larva of *Palythoa tuberculosa*. (A) A 22-day-old larva (ventral side) with a longitudinal band of long cilia (= ventral ciliate band, arrowhead). (B) Longitudinal section of a 22-day-old larva that has a mouth (asterisk), pharynx (double asterisks), and mesenteries (double arrows). (C) Cross section of pharynx of a 13-day-old larva. (D) Cross section of the middle part of a 13-day-old larva. Six mesenteries (double arrows) are visible. (E) Cross section of a 13-day-old larva including ventral ciliate band. Two mesenteries (double arrows) are visible. (F) Longitudinal section of a 22-day-old larva (transmission electron microscopy [TEM]). Arrow indicates mesoglea. (G) Section of the basal part of the ventral ciliate band (TEM). Ectodermal cells were laterally adhered with septate junction (facing arrows). ac, accessory centriole; ec, ectoderm; en, endoderm; cr, ciliary root; li, lipid drop; mi, microvilli; mt, mitochondrion; ne, nematocyst. Scale bars: 100 μm in A–E; 20 μm in the inset of A; 10 μm in F; 1 μm in G; 0.5 μm in the inset of G.

occur three to five nights after the full moon in summer (July) (Ono *et al.*, 2005).

In this study we observed the spawning behavior of *P. tuberculosa* one day before and on the night of the new moon in mid-summer (August) in the field. In the Okinawa Islands, most scleractinian corals release their gametes around full moon nights during the summer (June to August) (Heyward *et al.*, 1987; Richmond, 1997). *Pocillopora verrucosa* (Hirose *et al.*, 2000) and *Fungia repanda* (Loya *et al.*, 2009) release their gametes a few days after new moon mornings (spawn at 0600–0700 and 0300–0500, respectively), but there are no reports of corals spawning around new moon nights in Okinawa. This is the first report of new moon night spawning in either scleractinian corals or zoanthids in Okinawa. The seasonal reproduction patterns and the timing of gamete release in hexacorals are influenced by various factors—for example, temperature, salinity, food availability, moon phases, and tides—but it is unclear how these factors may interact in series of complex events (Jokiel *et al.*, 1985; Harrison and Wallace, 1990; Perse *et al.*, 1991; Richmond, 1997). The spawning behavior of *Protopalpythoa* sp. on the GBR was observed at about the same time as scleractinian coral mass spawning there (Babcock and Ryland, 1990; Ryland and Babcock, 1991), while the spawning of *P. tuberculosa* is not synchronized with scleractinian corals in Okinawa. The cue or cues for *P. tuberculosa* spawning may be different from those for both *Protopalpythoa* sp. on the GBR and for most scleractinian corals in Okinawa.

Traditionally, the identification of *Palythoa* species has been based on morphological characters such as polyp shape and size, colony color, as well as locality and habitat (see Ryland and Lancaster, 2003). However, species diversity, identification, and taxonomy within the genus *Palythoa* remain very difficult and confused, as *Palythoa* species are distributed worldwide and show large intraspecific variations in morphology (Muirhead and Ryland, 1985; Ryland and Muirhead, 1993; Burnett *et al.*, 1994, 1997). The present study demonstrated the timing and mode of spawning and embryogenesis of *P. tuberculosa*. The results of this study will help to examine whether reproductive isolation exists among *P. tuberculosa*, *P. mutuki*, and *Palythoa* sp., which are implicated in reticulate evolution by DNA molecular analyses (Reimer *et al.*, 2007a, b), and exist sympatrically on coral reefs in southern Japan.

The eggs of *Protopalpythoa* sp. on the GBR were 300 μm in diameter and contained symbiotic algae (zooxanthellae) (Babcock and Ryland, 1990). In contrast, *P. tuberculosa* zygotes in Okinawa were about 365 μm in diameter and did not contain zooxanthellae. However, the patterns of early embryogenesis, especially modes of gastrulation, are very similar in both species. This gastrulation pattern is similar to that of *Acropora* spp. (Anthozoa, Scleractinia, Cnidaria) (Miller and Ball, 2000; Okubo and Motokawa, 2007) and is

very unique. In addition, both zoanthellae larvae appeared at 2 weeks after spawning. Recently, Reimer *et al.* (2006, 2007a) demonstrated by DNA phylogenetic analyses that the genus *Palythoa* should include both *Palythoa* species and species formerly assigned to the genus *Protopalpythoa*. The similarity of early embryogenesis and larval types supports the results of these molecular phylogenetic analyses.

Babcock and Ryland (1990) confirmed that three types of gamete release occur simultaneously in one *Protopalpythoa* species on the GBR—egg-sperm bundles, egg bundles without sperm, and freely shed sperm—and they then observed early embryogenesis of this species. However, it is impossible to ascertain when gametes of *Protopalpythoa* sp. were fertilized, because the authors did not describe natural or artificial fertilization of their samples. In this study, we confirmed the presence of mature testes in colonies at spawning time, but we did not observe freely shed sperm or egg-sperm bundles in the field. However, most “eggs” that were captured from the water column began to cleave within 2 h after spawning. It is possible that “eggs” were fertilized in the gastric cavity before release as an “egg bundle,” or that they were fertilized in the water column by a very dilute concentration of sperm. In scleractinian corals, most spawning species release their gametes simultaneously and fertilization takes place in the water column or at the water surface within hours (Harrison and Wallace, 1990). The mode of sexuality and the spawning behavior of zoanthid species appear to be rich in variety and different from those of scleractinian corals.

Acropora is one of the most speciose and widely distributed scleractinian corals (Veron and Wallace, 1984), and the egg diameters of *Acropora* spp. are 300–400 μm (Harrison and Wallace, 1990), comparable to those of *Protopalpythoa* sp. (Babcock and Ryland, 1990; Ryland and Babcock, 1991) and *P. tuberculosa* (this study). Most *Acropora* larvae settle on substrate within 9–10 days after spawning under experimental conditions (Nozawa and Harrison, 2008), and these larvae settle mainly within a local population (e.g., Ayer and Hughes, 2000; Nishikawa and Sakai, 2005; Nakajima *et al.*, 2009, 2010; Hemond and Vollmer, 2010). On the other hand, it takes more than 2 weeks for *Protopalpythoa* sp. (Babcock and Ryland, 1990) and *P. tuberculosa* (this study) to develop mature, motile zoanthella larvae. *Palythoa* spp. (including genus *Protopalpythoa*) are widespread in the subtropical and tropical zones, and molecular phylogenetic analyses have shown the number of species to be much less than previously assumed, also with potentially much lower levels of local endemism than previously believed (Burnett *et al.*, 1997; Reimer *et al.*, 2006, 2007a; Reimer and Hickman, 2009). A long floating, planktonic period may be one reason for the wide distribution ranges and low endemism in *Palythoa* spp.

Cnidarians are generally regarded as having a radially

symmetrical body plan with a single major axis of polarity, the oral-aboral axis. In most anthozoans, a radial polypoid stage starts after larvae settle on substrate and metamorphose into primary polyps. The shape of planula larvae is cylindrical with a radially symmetrical body plan, but some gene expression patterns indicate that planula larvae are asymmetrical along an axis perpendicular to the oral-aboral axis, the directive axis (Hayward *et al.*, 2002; Finnerty *et al.*, 2004; Matus *et al.*, 2006a, b; Rentzsch *et al.*, 2006; Saina *et al.*, 2009). Thus, the common ancestor of Cnidaria may have had a bilateral body, which was secondarily lost in non-bilateral cnidarians (the Medusozoa) (see Manuel, 2009). Recent morphological and molecular phylogenetic evidence indicates that the class Anthozoa is the most basal group in the extant cnidarians (Bridge *et al.*, 1992, 1995; Odorico and Miller, 1997; Kim *et al.*, 1999; Medina *et al.*, 2001). Anthozoa is divided into two subclasses (Hexacorallia and Octocorallia), and recent molecular phylogenetic analyses show that Actinaria or Zoantharia (or both) are the most ancestral group within extant Hexacorallia species (Medina *et al.*, 2006; Brugler and France, 2007; Sinniger *et al.*, 2007; Fukami *et al.*, 2008; Sinniger and Pawlowski, 2009). Both polypoid and larval stages of zoanthid internal structures display biradial symmetry (see Ryland *et al.*, 2000), and zoanthea larvae have a ciliated band along the oral-aboral axis. Expression patterns of axis-related genes, such as *Dpp/BMP* and *chording/sog*, and their functional analyses should be examined in zoanthea larvae to clarify the regulation and determination of the axes and body plans in primitive Radiata.

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