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REPORT

Metamorphosis and acquisition of symbiotic algae in planula larvae and primary polyps of *Acropora* spp.

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Abstract Coral planulae settle, then metamorphose and form polyps. This study examined the morphological process of metamorphosis from planulae into primary polyps in the scleractinian corals *Acropora nobilis* and *Acropora microphthalma*, using the cnidarian neuropeptide Hym-248. These two species release eggs that do not contain *Symbiodinium*. The mode of acquisition of freshly isolated *Symbiodinium* (zooxanthellae) (FIZ) by the non-symbiotic polyp was also examined. Non-Hym-248 treated swimming *Acropora* planulae did not develop blastopore, mesenteries or coelenteron until the induction of metamorphosis 16 days after fertilization. The oral pore was formed by invagination of the epidermal layer after formation of the coelenteron in metamorphosing polyps. At 3 days after settlement and metamorphosis, primary polyps exposed to FIZ established symbioses with the *Symbiodinium*. Two–four days after exposure to FIZ, the distribution of *Symbiodinium* was limited to the gastrodermis of the pharynx and basal part of the polyps. Eight–ten days after exposure to FIZ, *Symbiodinium* were present in gastrodermal cells throughout the polyps.

Keywords Metamorphosis · Planulae · *Acropora* · *Symbiodinium* · Hym-248

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Introduction

Scleractinian corals are characterized by a calcium carbonate skeleton produced by the epidermis, and by symbiotic dinoflagellates (zooxanthellae, *Symbiodinium* spp.) harbored in the gastrodermal cells. Corals begin life as soft-bodied planktonic planula larvae and undergo metamorphosis to primary polyps (Harrison and Wallace 1990). Coral planulae develop some tissues but not tentacles or skeletons. In particular, the formation of the mouth and coelenteron is necessary to feed and acquire the *Symbiodinium* for non-symbiotic planula.

Two distinct modes of sexual reproduction occur among scleractinian corals: gamete release followed by external fertilization and development (spawning); and brooding of planulae within the polyp (brooding). Brooding planulae are usually released at an advanced stage of development and possess a mouth, coelenteron and some mesenteries, and tend to already contain *Symbiodinium* when they are released from the parent colony (Harrison and Wallace 1990; Isomura and Nishihira 2001). On the other hand, the planulae of spawning corals usually require up to a week after fertilization to attach to the substrate and then metamorphose into primary polyps (Babcock and Heyward 1986; Harrison and Wallace 1990). Most gametes of spawning coral species do not contain symbionts (Harrison and Wallace 1990). The structure of planulae is different between different species of spawning corals (e.g., Szmant-Froelich et al. 1980; Babcock and Heyward 1986; Hayashibara et al. 1997; Hirose et al. 2000; Hirose and Hidaka 2006).

Acropora is the dominant and most speciose genus of scleractinian corals in the Indo-Pacific (Veron and Wallace 1984), and all *Acropora* species studied thus far have been spawners, releasing eggs that do not contain *Symbiodinium*. New generations of *Acropora* must, therefore, acquire *Symbiodinium*

from the environment. Hayashibara et al. (1997) described the early development of four *Acropora* species (*Acropora hyacinthus*, *Acropora nasuta*, *Acropora florida*, and *Acropora secale*). The planulae of these species had an oral pore and mesogleal layer, but no *Symbiodinium* were present. Since Hayashibara et al. (1997) did not examine the process of metamorphosis, they were unable to show the order of development of a functional mouth, coelenteron, mesenteries, tentacles, and skeleton, and it is still unclear exactly when *Symbiodinium* are acquired in *Acropora* species.

Most coral planulae select a site of permanent attachment using external chemical cues that induce the metamorphosis from planulae into polyps (Morse et al. 1988, 1994, 1996; Heyward and Negri 1999; Iwao et al. 2002; Webster et al. 2004). Iwao et al. (2002) reported that Hym-248 (EPLPIGLWa), a cnidarian neuropeptide of the GLWamide family, induces the metamorphosis of planulae of the genus *Acropora*. Hym-248 appears to act as an internal mediator, after release from neurons following an external stimulus, triggering metamorphosis. Hym-248 induces synchronous metamorphosis and settlement of *Acropora* planulae, and is, therefore, a useful tool for the study of *Acropora* larval metamorphosis.

The aims of this study were (1) to examine the process of metamorphosis from planula larva into the primary polyp in the scleractinian corals *Acropora nobilis* and *Acropora microphthalma* using the cnidarian neuropeptide Hym-248, and (2) to describe the process of acquisition of the freshly isolated *Symbiodinium* (zooxanthellae) (FIZ) by the non-symbiotic polyps.

Material and methods

Preparation of planula larvae

Colonies of *Acropora* spp. (*A. nobilis* and/or *A. microphthalma*) were maintained in an outdoor tank (4 m × 5 m × 1.5 m) supplied with unfiltered running seawater at Okinawa Churaumi Aquarium. Several colonies of both coral species released egg-sperm bundles on 12 June 2006 between 2100 and 2200 hours. The two species of *Acropora* were kept in the same tank and spawned simultaneously, as a result, the embryos could not be differentiated into individual species. However, from preliminary observations, the embryonic development and course of metamorphosis for these two species is known to be very similar. Released gametes were collected and mixed for 1–2 h to allow fertilization, and then transferred to plastic containers filled with seawater filtered through a 0.45 µm-pore (FSW). Embryos were maintained in the containers at room temperature (27–29°C) in FSW changed daily.

Hym-248 treatment

Hym-248 (EPLPIGLWamide) was purchased from Genenet Co. Ltd, Fukuoka, Japan. Swimming planulae 2–16 days after fertilization were washed several times with FSW, and one or two planulae were then incubated in a 20–30 µl drop of peptide solution (4×10^{-6} M Hym-248 in FSW) on waterproof parafilm sheets (Parafilm, Alcan Inc., Wisconsin). The droplets containing planulae were incubated on the sheet in a moist-chamber at room temperature (27–29°C) for 24 h. The parafilm sheets were then fixed with a clamp made of silicon tube and nylon thread and incubated in small containers containing FSW.

Symbiodinium acquisition by primary polyps

In previous experiments, non-symbiotic primary polyps of *A. nobilis* initially took up freshly isolated *Symbiodinium* (zooxanthellae) (FIZ) from the bivalve *Tridacna crocea* more readily than FIZ from *A. nobilis* (parent coral colonies), the soft coral *Xenia* sp. or the sea anemone *Aiptasia* sp. (Y. Higuchi, personal communication). Consequently, in the present study, in order to examine the course of *Symbiodinium* acquisition, primary polyps of *A. nobilis* and *A. microphthalma* were exposed to FIZ from *T. crocea.*

A single specimen of *T. crocea* was collected from the shallow reef lagoon (<1 m) at Bise (26°42′32″N, 127°52′45″E) for *Symbiodinium* isolation. Restriction fragment length polymorphism (RFLP) patterns of 18S rDNA indicated that the *Symbiodinium* from *T. crocea* belonged to clade A (sensu Rowan and Powers 1991). The animal tissue (mantle) of *T. crocea* was cut out and homogenized. The *Symbiodinium* were cleaned several times with FSW and concentrated by centrifugation. Primary polyps of both *Acropora* spp. (1–2 days after Hym-248 treatment) were exposed to FIZ (10^5 cells ml^{-1}) at 27°C under a 12:12 hours light (100 µmol m^{-2} s^{-1}): dark cycle. Six hours after exposure to FIZ, the seawater in the polyp cultures was replaced with FSW. Polyp development was monitored and seawater was changed daily.

Histological observation and electron microscopy

Live planulae and polyps were fixed, dehydrated, embedded and sectioned (Hirose and Hidaka 2006). The skeleton was decalcified with 1% ascorbic acid—0.15M NaCl for 2 days. For scanning electron microscope (SEM), specimens were immersed in *t*-butanol and freeze-dried. Dried specimens were sputter-coated with gold-palladium, and examined under a scanning electron microscope (SEM; JEM-6060LV, JEOL Ltd, Tokyo).

Results

Embryonic development and swimming planulae

The eggs of both *Acropora* species (diameter ~400 μm) did not contain any *Symbiodinium*. About 2 h after spawning (=fertilization), more than 90% of embryo began to cleave. Forty-eight hours after fertilization, embryos became elongated and started to swim using cilia. Gastric cavities (coelenteron) were not present in non-Hym-248 treated swimming planulae until 16 days after fertilization (Fig. 1a, b). These planulae consisted of two layers of cells; epidermis (ectoderm) and gastrodermis (endoderm), which were separated by an extracellular matrix (ECM) called the mesoglea (Fig. 1a, inset). The planula surface was completely covered with cilia surrounded by microvilli (Fig. 1c). Epidermal cells of planulae were not arranged into a monolayer. Most epidermal cells were columnar and contained round granules (ca. 1-μm diameter) in the apical cytoplasm (Fig. 1d). Some round cells were found in the basal half of the epidermis, attached to the mesoglea (Fig. 1d). In contrast, gastrodermal cells were spherical in shape (Fig. 1e) and contained many lipid granules (Fig. 1a).

Metamorphosis of planula larvae from planula to primary polyp

When swimming planulae were exposed to the cnidarian neuropeptide Hym-248 at 4×10^{-6} M, they first moved actively for a few minutes, then, the elongated planulae

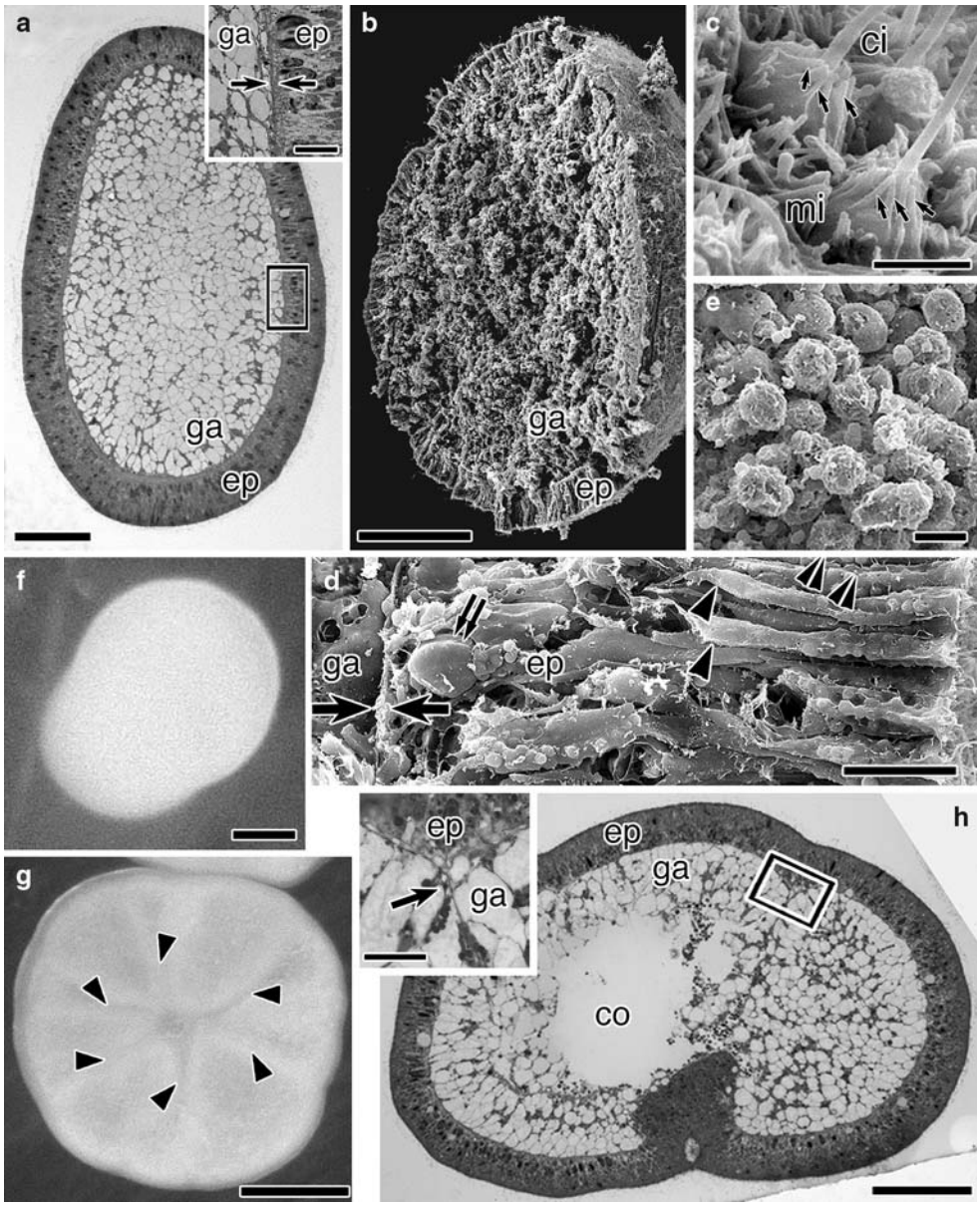

Fig. 1 Swimming planula and initial stages of metamorphosis of *Acropora* spp. **a** Histological section of swimming planula, *square* indicates the area enlarged in inset; inset: *arrows* indicate mesogleal layer (*bar* = 10 μm), **b** SEM micrograph of fractured swimming planula, **c** highly magnified SEM micrograph of epidermal surface of planula. Cilia surrounded by microvilli (*arrows*). **d** SEM micrograph of a fractured epidermal layer of planula. *Arrows* indicate mesogleal layer, *double arrows* indicate round cells in the epidermis, *arrowheads* indicate columnar cells in the epidermis, and *double arrowheads* indicate granules in columnar cell, **e** SEM micrograph of a fractured gastrodermis of planula showing spherical gastrodermal cells, **f** elongated planula attached to parafilm (just after exposure to Hym-248), **g** polyp with six primary mesenteries (6 h after exposure to Hym-248). *Arrowheads* indicate mesenteries, **h** histological section of metamorphosing polyp (6 h after exposure to Hym-248), *square* indicates the area enlarged in inset; inset: *arrow* indicates the just-extended mesogleal layer (*bar* = 10 μm) (*ci* cilium, *co* coelenteron, *ep* epidermis, *ga* gastrodermis, *mi* microvilli). *Bars* = 100 μm in **a**, **b**, **f**, **g** and **h**, 10 μm in **d**, **e**, 1 μm in **c**

attached themselves perpendicularly to the waterproof parafilm sheets and stood upright (Fig. 1f). Six to nine hours after exposure to Hym-248, metamorphosing planulae spread out on the sheets and formed six primary mesenteries, which we designated the "pumpkin-stage" (Fig. 1g). These planulae metamorphosis experiments were performed a total of 12 times for 2–16 days after fertilization, and more than 1,000 planulae were treated with Hym-248. In all cases, more than 90% of the planulae metamorphosed into the "pumpkin-stage" within 9 h. In the "pumpkin-stage" a cavity (coelenteron) could be seen in the larvae (Fig. 1h). This coelenteron appeared to form as a result of secondary delamination during metamorphosis.

The mesenteries were primary gastrodermal structures composed of the gastrodermis and mesoglea, and began to form from the anti-substrate side of the polyp (Fig. 1h). In the mesentery forming area, the epidermal layer was concave and gastrodermal cells were rearranged to form mesenteries (Fig. 1h, inset). About 12 h after Hym-248 treatment, secondary mesenteries appeared between each primary mesentery (Fig. 2a). As the gastrodermis was organized and the coelenteron extended, mesenteries grew inward from the body wall (Fig. 2b, c). The columnar epidermal cells tightly adhered laterally and formed a simple epidermal layer, while the granular inclusions were inconspicuous in the apical cytoplasm (Fig. 2c). The gastrodermal surface of the bottom of coelenteron was covered with cilia and there were small spheres present which were ~2–4 μm in diameter (Fig. 2d).

Twelve to twenty-four hours after Hym-248 treatment, the polyps had six tentacular grooves around their mouth and skeletons developed (Fig. 2e, f). The oral pores of two species *Acropora* were formed as simple openings through invagination of the epidermal layer after the formation of the coelenteron in the polyps (Fig. 2g). Some small particles (2–4 μm in diameter) were discharged from the coelenteron through the oral pore (Fig. 2g, h).

Three days after Hym-248 treatment, the gastrodermal layer became thinner and the mesenteries developed (Fig. 2i). Many small particles (2–5-μm diameter) appeared in marginal parts of the coelenteron (Fig. 2j). Although the epidermal surfaces of polyps were covered with cilia and microvilli, the epidermis of the side towards the substrate had a smooth surface without any cilia and microvilli (Fig. 2k).

Symbiodinium acquisition by primary polyp

When non-symbiotic primary polyps (2–3 days after metamorphosis) were exposed to freshly isolated *Symbiodinium* (zooxanthellae) (FIZ) from *T. crocea* (10^5 cells ml^{-1}), FIZ were taken into the coelenteron through the mouth by water currents produced by the cilia on the tentacles (Fig. 3a). Six hours after exposure to FIZ, infected polyps were transferred to FSW. One–two days after exposure to FIZ, many brown cell masses were present in the coelenteron (Fig. 3b). Histological examination showed these brown cell masses to comprise intact *Symbiodinium* and cell debris from both *T. crocea* and *Symbiodinium* (Fig. 3c, d). Some *Symbiodinium* were trapped by cytoplasmic processes from the gastrodermal cells (Fig. 3e). The cilia of the gastrodermal cells were elongated, and sometimes caught some of the *Symbiodinium* in the coelenteron (Fig. 3f).

Two–three days after exposure to FIZ, the cell masses in the coelenteron disappeared, and *Symbiodinium* appeared in the mesenteries and at the bottom of the gastrodermal layer (Fig. 3g–j). The gastrodermis of the aboral region of polyps was thickened and one gastrodermal cell contained *Symbiodinium* (Fig. 3j).

Four–five days after exposure to FIZ, many *Symbiodinium* were visible in the tentacles (Fig. 3k). Four days after exposure to FIZ, the percentage of *Symbiodinium* undergoing cell division (MI) in the both *Acropora* species polyps were 1.4 ± 0.3% (mean ± SD, $n = 6$). Seven–ten days after exposure to FIZ, *Symbiodinium* were distributed in gastrodermal cells throughout the polyps (Fig. 3l–n). Eight days after exposure to FIZ, the percentage of MI in the both *Acropora* species polyps was 1.3 ± 0.3% ($n = 9$). The number of symbionts were counted at a longitudinal section (including mouth and mesenteries) of the polyps. Four days after exposure to FIZ, 36.5 ± 11.9 algal cells were seen in polyp sections ($n = 4$); 21.8, 68.3, and 9.9% of the symbionts were distributed in the gastrodermis of the aboral region of polyps, the mesenteries, and the gastrodermis of the oral region (=body wall) of the polyps ($n = 4$), respectively. Eight days after exposure to FIZ, 82.3 ± 18.8 algal cells were seen in polyp sections ($n = 3$); 16.2, 47.6, 0.3, and 35.8% of symbionts were distributed in the gastrodermis of the aboral region of polyps, the mesenteries, the gastrodermis of tentacles, and the gastrodermis of the oral region (=body wall) of the polyp ($n = 3$), respectively.

Discussion

Metamorphosis of *Acropora* spp. from planula to primary polyp

Observing larval settlement in nature is inherently difficult, as a result, direct information is very limited. In the laboratory, Babcock and Heyward (1986) observed *Acropora millepora* in culture jars starting to metamorphose 4.5 days after fertilization. Hayashibara et al. (1997) reported that first settlement on plastic walls began 6 days after fertilization in *A. nasuta*, 7 days in *A. hyacinthus*, and 21 days in *A. florida*. In the present study, non-Hym-248 treated planulae

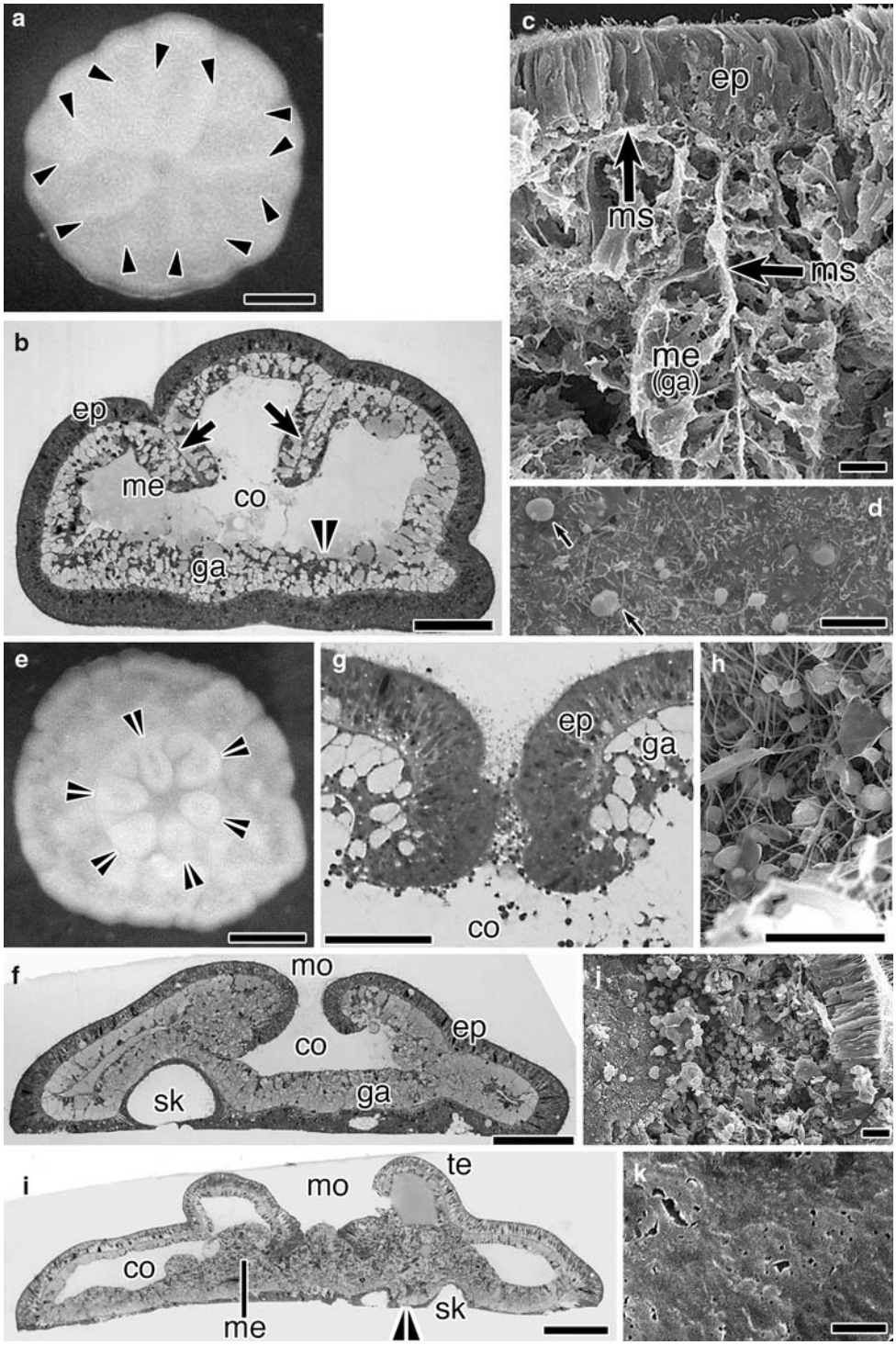

Fig. 2 Further stages of metamorphosis of *Acropora* spp. **a** Polyp initiating secondary mesentery formation (9 h after exposure to Hym-248). *Arrowheads* indicate mesenteries. **b** Histological section of metamorphosing polyp (9 h after exposure to Hym-248). *Arrows* indicate mesogleal layer and *double arrowheads* indicate the site approximately corresponding to **d**. **c** SEM micrograph of a fractured polyp with mesentery, **d** SEM micrograph of the gastrodermal surface of the bottom of coelenteron. *Arrows* indicate spherical objects, **e** polyp with six tentacles and mouth (20 h after exposure to Hym-248). *Double arrowheads* indicate tentacular grooves. **f** Histological section of metamorphosing polyp (24 h after exposure to Hym-248), **g** highly magnified histological section of blastopore of early primary polyp, **h** SEM micrograph of oral region of early primary polyp, **i** histological section of mature primary polyp (3 days after exposure to Hym-248). *Double arrowheads* indicate the site approximately corresponding to **k**. **j** SEM micrograph of marginal region of polyp, **k** SEM micrograph of epidermis of the substratum side (*co* coelenteron, *ep* epidermis, *ga* gastrodermis, *me* mesentery (=gastrodermal tissue), *mo* mouth, *ms* mesentery, *sk* skeleton). *Bars* = 100 μm in **a**, **b**, **e**, **f** and **i**, 10 μm in **c**, **d**, **g**, **h** and **j**, 1 μm in **k**

from *A. nobilis* and *A. microphthalma* in the culture container began to metamorphose on plastic walls or the water surface 5 days after fertilization. However, metamorphosis in both species of the planulae could be induced by Hym-248 treatment after 48 h, continuing until at least 16 days after fertilization. This observation indicates that planulae of *Acropora* spp. acquire the ability to settle and metamorphose just after beginning to swim.

The coelenteron is formed during the planula stage in *Fungia scutaria* (Krupp 1983; Schwarz et al. 1999), *Montipora verrucosa* (Máte et al. 1998), *Pocillopora verrucosa*, *Pocillopora eydouxi* (Hirose et al. 2000) and *Porites cylindrica* (Hirose and Hidaka 2006). In contrast, Hayashibara et al. (1997) reported that the coelenteron or gut did not develop in the planulae of four species of *Acropora* (*A. hyacinthus*, *A. nasuta*, *A. florida*, and *A. secale*) until

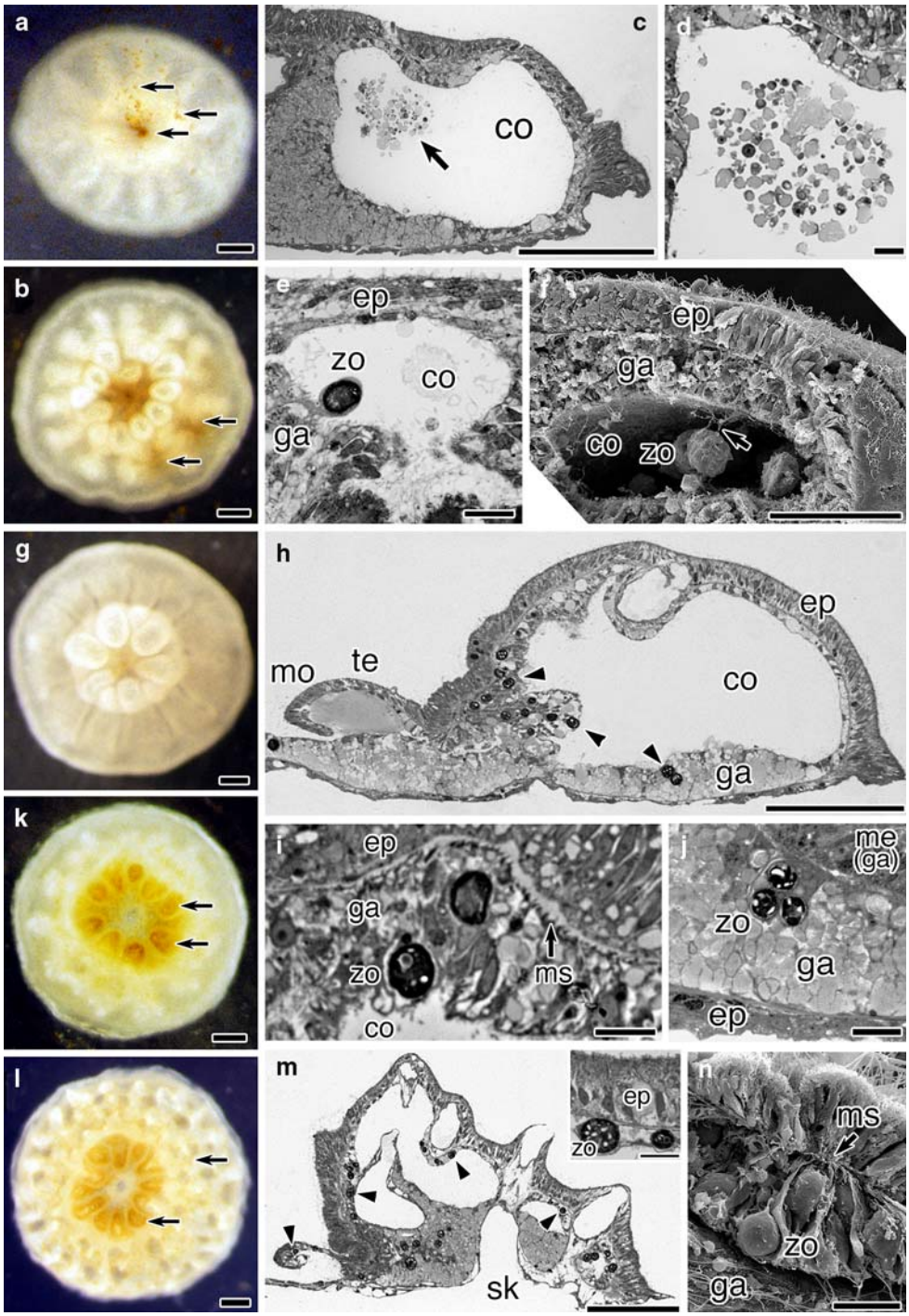

Fig. 3 *Symbiodinium* aqcuistion of primary polyp. **a** Polyp taking up freshly isolated zooxanthellae (FIZ) (10 min after exposure to FIZ). *Arrows* indicate *Symbiodinium*. **b** Polyp showing a clumped cell mass (*arrows*) in the coelenteron (1 day after exposure to FIZ), **c** histological section of primary polyp containing cell mass (*arrow*) in its coelenteron, **d** enlargement of **c**, the cell mass was composed mainly of degraded *Symbiodinium* and other materials, **e** high magnification of histological section of coelenteron, **f** SEM micrograph of fractured polyp. *Arrow* indicates elongated cilia of gastrodermal cells. **g** Polyp with several *Symbiodinium* in basal tissue of coelenteron (3 days after exposure to FIZ), **h** Histological section of primary polyp with *Symbiodinium* in gastrodermal cells. *Arrowheads* indicate *Symbiodinium*, **i** high magnification of histological section of oral regional body wall, **j** high magnification of histological section of aboral region, **k** polyp with many *Symbiodinium* in tentacles (5 days after exposure to FIZ), **l** polyp with *Symbiodinium* in body wall and initiating skeletal formation (9 days after exposure to FIZ). **m** Histological section of polyp. *Arrowheads* indicate *Symbiodinium*. **n** SEM micrograph of fractured mesentery with *Symbiodinium* (*co* coelenteron, *ep* epidermis, *ga* gastrodermis, *me* mesentery (=gastrodermal tissue), *mo* mouth, *ms* mesogleal layer, *sk* skeleton, *te* tentacle, *zo* zooxanthella(e) (=*Symbiodinium*). *Bars* = 100 μm in **a**, **b**, **c**, **g**, **h**, **k**, **l** and **m**, 50 μm in **f**, 10 μm in **d**, **e**, **i**, **j** and **n**

30 days after insemination, and they found a coelenteron only in metamorphosing planulae. In the present study, the non-Hym-248 treated swimming planulae did not have a coelenteron until 16 days after fertilization. However, following the induction of metamorphosis by Hym-248, the polyps formed coelenterons and mesenteries. This suggests that settlement may be a cue for the formation of coelenteron and mesenteries in *Acropora* spp.

In planulae from *Favia fragum* (Szmant-Froelich et al. 1985), *Porites porites* (Tomascik and Sander 1987), and *Montipora digitata* (Hirose and Hidaka 2006), the formation of the oral pore occurs by invagination of part of the epidermal layer before the coelenteron (gastric cavity) starts to appear. In the present study, the oral pore of two species *Acropora* was formed by invagination after the formation of the coelenteron in polyps. This suggests that the oral pore or mouth does not need to be psesent in order for the coelenteron to form in *Acropora* spp.

In polyps, the cilia were found in the gastrodermis (Fig. 2d). This is similar to the findings of Benayahu et al. (1988) in planulae and polyps of the octocorallia, *Xenia unbellata*. These ciliated gastrodermal cells probably produce water currents to move material into the coelenteron. The mesenteries consisted of the elongated endodermal layer together with the mesoglea. Although little is known about the syntheses of mesoglea components in cnidarians, the result of this study indicates that the mesoglea in *Acropora* may be synthesized by gastrodermal cells forming mesenteries during metamorphosis.

Acquisition of *Symbiodinium* by *Acropora* spp.

Non-Hym-248 treated swimming planulae of the two species of *Acropora* did not establish symbioses with *Symbiodinium* until at least 16 days after fertilization even if the swimming planulae were exposed to freshly isolated *Symbiodinium* (zooxanthellae) (FIZ). Hayashibara et al. (1997) also reported in four species of *Acropora* that no *Symbiodinium* were acquired during the planula period until 30 days after formation. However, van Oppen (2001) found that cultured *A. millepora* planulae established stable symbioses and that these symbiotic larvae survived for more than 4 months, although she did not show any histological data or describe the culture conditions. Whether there is in fact variation between acroporids in the timing of initial infection, or whether reported differences in timing of infection reflect differences in experimentation or culturing, remains to be determined.

Intact *Symbiodinium* and cell debris from both *T. crocea* and *Symbiodinium* were found in the coelenteron during this study. However, most *Symbiodinium* in the gastrodermal cells of the polyps were intact. Lesser (1997) reported that damaged *Symbiodinium* were discharged selectively by adult corals. Young polyps may also have the ability to differentiate between intact and damaged *Symbiodinium*. The percentage of *Symbiodinium* MI in both *Acropora* species polyps was 1.3–1.4%, which is consistent with previous reports of MI indexes of 0.5–7.0% in *Acropora* spp. (e.g., Wilkerson et al. 1988; Jones and Yellowlees 1997; Cervino et al. 2003), and 0.8–11.5% in *Tridacna* spp. (Ambariyanto and Hoegh-Guldberg 1997; Maruyama and Heslinga 1997). Most algal symbioses are characterized by relatively constant densities of symbionts (e.g., Muscatine and Pool 1979; Jones and Yellowlees 1997; Smith and Muscatine 1999; Fitt and Cook 2001; Titlyanov et al. 2004), giving rise to the hypothesis that symbiont numbers are regulated by their hosts. Young polyps may also regulate the density of *Symbiodinium*. Recently, Yuyama et al. (2005) reported host genes involved in the establishment or maintenance of *Symbiodinium* symbiosis in *Acropora tenuis*. These molecular tools might assist in future analyses of ontogenic establishment of symbioses between coral and *Symbiodinium*.

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