Mar Biol (2009) 156:1203–1212
DOI 10.1007/s00227-009-1162-9

ORIGINAL PAPER

Onset of symbiosis and distribution patterns of symbiotic dinoflagellates in the larvae of scleractinian corals

Saki Harii · Naoko Yasuda · Mauricio Rodriguez-Lanetty · Takahiro Irie · Michio Hidaka

Received: 9 October 2008 / Accepted: 11 February 2009 / Published online: 5 March 2009

Abstract The establishment of symbiosis in early developmental stages is important for reef-building corals because of the need for photosynthetically derived nutrition. Corals spawn eggs and sperm, or brood planula larvae and shed them into the water. Some coral eggs or planulae directly inherit symbiotic dinoflagellates (*Symbiodinium* spp.) from their parents, while others acquire them at each generation. In most species examined to date, the larvae without dinoflagellates (aposymbiotic larvae) can acquire symbionts during the larval stage, but little is known regarding the timing and detailed process of the onset of symbiosis. We examined larval uptake of symbiotic dinoflagellates in nine species of scleractinian corals, the onset of symbiosis through the early larval stages, and the distribution pattern of symbionts within the larval host, while living and with histology, of two acroporid corals under laboratory conditions. The larvae acquired symbiotic dinoflagellates during the planktonic phase in all corals examined which included *Acropora digitifera*, *A. florida*, *A. intermedia*, *A. tenuis*, *Isopora palifera*, *Favia pallida*, *F. lizardensis*, *Pseudosiderastrea tayamai*, and *Ctenactis echinata*. The larvae of *A. digitifera* and *A. tenuis* first acquired symbionts 6 and 5 days after fertilization, respectively. In *A. digitifera* larvae, this coincided with the formation of an oral pore and coelenteron. The number of symbiotic dinoflagellates increased over the experimental periods in both species. To test the hypothesis that nutrients promotes symbiotic uptake, the number of incorporated dinoflagellates was compared in the presence and absence of homogenized *Artemia* sp. A likelihood ratio test assuming a log-linear model indicated that *Artemi*a sp. had a significantly positive effect on symbiont acquisition. These results suggest that the acquisition of symbiotic dinoflagellates during larval stages is in common with many coral species, and that the development of both a mouth and coelenteron play important roles in symbiont acquisition.

Communicated by M. Kühl.

S. Harii (✉) · N. Yasuda
Graduate School of Engineering and Science,
University of the Ryukyus, 1 Senbaru,
Nishihara, Okinawa 903-0213, Japan
e-mail: h081771@sci.u-ryukyu.ac.jp

M. Rodriguez-Lanetty
Biology Department, The University of Louisiana at Lafayette,
Lafayette, LA 70504, USA

T. Irie
Sesoko Station, Tropical Biosphere Research Center,
University of the Ryukyus, 3422 Sesoko,
Motobu, Okinawa 905-0227, Japan

M. Hidaka
Department of Chemistry, Biology and Marine Science,
Faculty of Science, University of the Ryukyus, 1 Senbaru,
Nishihara, Okinawa 903-0213, Japan

Introduction

Mutualistic endosymbioses between two organisms are widespread in coral reefs. Marine invertebrates such as foraminiferans, giant clams, and cnidarians (sea anemones, jellyfish and corals) harbor unicellular symbiotic dinoflagellates called zooxanthellae (*Symbiodinium* spp.). Symbiotic dinoflagellates contribute to host nutrition by translocating as much as 95% of their photosynthate to the host, while the symbionts receive inorganic carbon and other nutrients in return (Muscatine 1990).

Acquisition of symbiotic dinoflagellates occurs either vertically, where symbionts are transmitted directly from parent to offspring, or horizontally, where the offspring acquire symbionts from a new environment in each generation (Douglas 1998). Vertical transmission ensures that the host offspring is provided with a complement of symbionts; these vertically transferred symbionts are believed to be genetically identical in offspring and parent colonies. There are several clades of *Symbiodinium* spp. (Baker 2003; Coffroth and Santos 2005) and these are thought to differ in their environmental tolerances (e.g., to high light, Rowan 2004). Accordingly, vertical transmitters, which have a reduced genetic spectrum of *Symbiodinium* spp., may limit their ability to adapt to environmental changes (Weis et al. 2001). Horizontal transmission provides hosts with the opportunity to acquire symbionts that are more suitable for their local environments. However, the disadvantage of horizontal transmission is that the host may fail to acquire symbionts (Weis et al. 2001; Schwarz et al. 2002). Thus, the initial acquisition of symbionts is an important event for corals that have horizontal acquisition.

Establishment of the cnidarian symbiosis varies interspecifically among host developmental stages. Symbiosis may begin in the embryonic developmental stages (Scyphozoa: *Linuche unguiculata*; Montgomery and Kremer 1995), planula stages (Anthozoa: *Anthopleura elegantissima*; Schwarz et al. 2002; Weis et al. 2002), or polyp stages (Scyphozoa: *Cassiopea xamachana*; Colley and Trench 1983; Sachs and Wilcox 2006). Scleractinian corals spawn eggs and sperm or brood planula larvae (Harrison and Wallace 1990). Planula larvae float for several to tens of days before settling (Babcock and Heyward 1986; Harrison and Wallace 1990). Once these larvae settle, they metamorphose into polyps and grow into coral colonies. Most scleractinian corals spawn gametes that lack symbiotic dinoflagellates and fertilized eggs develop into aposymbiotic planula larvae (Fadlallah 1983; Babcock and Heyward 1986; Harrison and Wallace 1990). Acquisition of symbionts usually occurs at larval or juvenile stages. The endodermal cells of the primary polyp phagocytose symbionts (Hirose et al. 2008a).

Aposymbiotic larvae of corals can acquire symbiotic dinoflagellates from their environment under experimental conditions (Schwarz et al. 1999; van Oppen 2001; Weis et al. 2001; Rodriguez-Lanetty et al. 2004; Baird et al. 2006; Rodriguez-Lanetty et al. 2006). The acquisition of symbionts by the larvae of the coral *Fungia scutaria* occurs 4–5 days after fertilization when the oral pore forms (Schwarz et al. 1999). Although most corals are horizontal transmitters, the acquisition of symbionts during larval stages has been observed only in *Acropora millepora* (van Oppen 2001), *A. muricata* (Baird et al. 2006), *F. scutaria* (Schwarz et al. 1999; Weis et al. 2001; Rodriguez-Lanetty et al. 2004, 2006), *Favia chinensis* and *Goniastrea aspera* (Nozawa and Harrison 2005). Acroporid larvae begin taking up symbionts 6 days after fertilization (Baird et al. 2006); however, Hirose et al. (2008a) suggested that the initial acquisition of symbionts occurs only after metamorphosis, that is, in the initial polyp stage in *A. nobilis* (= *A. intermedia*, Wallace 1999) *and A. microphthalma*. Thus, we know the larvae can acquire symbionts, but we do not know exactly when or how this process occurs. In addition, little is known regarding the relationship between establishment of symbiosis and larval developmental stages.

In the present study, we first confirmed that symbiotic dinoflagellates are acquired during the planktonic stage in nine scleractinian coral species in five genera: *Acropora digitifera*, *A. florida*, *A. intermedia*, *A. tenuis*, *Isopora palifera*, *Favia pallida*, *F. lizardensis*, *Pseudosiderastrea tayamai*, and *Ctenactis echinata*. We experimentally examined the timing of initial symbiosis in acroporid larvae to determine when and how larvae acquire symbionts. In addition, to examine whether symbiont acquisition is enhanced by nutrients, we examined the effect of *Artemia* homogenate on the number of symbionts incorporated by laboratory-reared acroporid larvae.

Materials and methods

Collection and maintenance of coral larvae

The colonies of the nine coral species, *Acropora digitifera*, *A. florida*, *A. intermedia*, *A. tenuis*, *Isopora palifera*, *Favia pallida*, *F. lizardensis*, *Pseudosiderastrea tayamai* and *Ctenactis echinata*, were collected in Okinawa, Japan, during the spawning season in 2007 and 2008. *Isopora palifera* is a hermaphroditic brooder (Kojis 1986) and *C. echinata* is a gonochoric broadcast spawner (Loya and Sakai 2008), while the others are hermaphroditic broadcast spawners (Richmond and Hunter 1990; Hayashibara et al. 1993; Kitamura et al. 2007). All species examined are common in the Indo-Pacific region and acquire their symbionts horizontally. The spawning corals were collected and maintained in a running seawater aquarium prior to spawning. *Favia pallida* was collected in May 2007 at the Akajima Marine Science Laboratory. *Acropora digitifera* and *A. florida* were collected in May 2007, *C. echinata* in July 2007, and *F. lizardensis* and *P. tayamai* in June 2008 at the Sesoko Station of the Tropical Biosphere Research Center at the University of the Ryukyus. *Acropora tenuis* was collected in May 2008 at the Ishigaki Tropical Station, Seikai National Fisheries Research

Institute of the Fisheries Research Agency. The egg–sperm bundles of *A. intermedia* were provided by the Okinawa Churaumi Aquarium in June 2007. After spawning, egg–sperm bundles were mixed for fertilization. The fertilized eggs of each species were washed twice in filtered (0.22 μm) seawater (FSW) and kept in 2-L plastic containers. All planula larvae were transferred to the Sesoko Station for the experiments. The colonies of *I. palifera* were collected and maintained at the Sesoko Station until they released larvae in June 2007. All planula larvae were transferred to new containers filled with fresh FSW daily.

Preparation of symbiont isolates

Symbiodinium spp. were isolated from coral colonies using a WaterPik (Doltz EW1250, Panasonic, Japan) to remove the coral tissue. Tissue suspensions were homogenized and filtered through nylon mesh (180 μm) and then concentrated using a centrifuge at 2,400 rpm for 4 min. The algal pellets were re-suspended in FSW, filtered (40 μm), and then rinsed three times using a centrifuge at 1,000–1,500 rpm for 4 min. The number of algal cells from 4 subsamples was counted using a hemacytometer in order to know the density of algae. The average number of cells was used. Symbionts were immediately isolated prior to their use in each experiment. Cultured *Symbiodinium* spp. isolated from *Tridacna* sp. were provided by Okinawa Prefectural Fisheries and Ocean Research Center and used to infect the *F. lizardensis* larvae (see below).

Acquisition of symbionts by the larvae of different coral species

The larvae of each species were infected with symbiotic dinoflagellates by one of the two ways. The first infection method was for the preliminary experiment. One to four larvae of *A. intermedia*, *A. florida*, *I. palifera*, or *F. pallida* were incubated in a 40 μl drop of seawater containing isolated symbiotic dinoflagellates (approximately 2.0×10^6 cells ml^{-1}) on a tissue culture dish for 12 h. Approximately 10 drops were made for each species. All larvae in the drops were mixed and washed twice using FSW and moved to a new dish filled with 2 ml FSW. In the second infection method, 10 larvae of *F. lizardensis*, *P. tayamai*, and *C. echinata* were transferred to tissue culture dishes containing 2 ml of FSW with the symbiotic dinoflagellates (same concentration as before) and a few drops of homogenized *Artemia* sp. Four replicates were established. The larvae in each dish were washed twice using FSW after 6 h and moved to a new dish containing 2 ml of fresh FSW. For both methods, the larvae in the final containers were maintained for 12 h. The number of larvae that acquired symbionts and the number of incorporated algal cells were counted carefully under either a light or fluorescence microscope. Either homologous (algae isolated from the same species) or heterologous (algae isolated from different species) algae were used for both methods (Table 1).

Table 1 Acquisition of symbiotic dinoflagellates in the larvae of nine coral species; brooding species (*)

Species	Days after spawning	Type of symbionts	Infection time (hours)	Infection (±SE)[a] (%)	Average number of algal cells per infected larva (±SE)[a]
Acropora digitifera[b]	6	Homologous	6	60.0 ± 5.8 (4)	24.9 ± 6.2 (4)
A. florida[c]	15	Coral (*A. tenuis*)	12	89.7 ± 4.7 (4)	22.8 ± 5.3 (4)
A. intermedia[c]	4	Homologous	12	0 (30)	0 (30)
	5	Homologous	12	60.0 (40)	24.8 ± 6.5 (40)
	11	Homologous	12	93.3 (30)	111.2 ± 19.9 (30)
A. tenuis[b]	5	Homologous	6	20.0 ± 5.8 (4)	3.4 ± 0.8 (4)
Isopora palifera[c,*]	2	Coral (*A. intermedia*)	12	63.2 (19)	100.9 ± 30.1 (19)
Favia pallida[c]	6	Coral (*A. intermedia*)	12	73.3 (30)	62.1 ± 12.4 (30)
F. lizardensis[b]	6	*Tridacna* sp.	6	100 (4)	58.4 ± 8.8 (4)
Psudosiderastrea tayamai[b]	5	Coral (*A. tenuis*)	6	26.1 ± 6.0 (4)	3.1 ± 1.2 (4)
	12	Coral (*A. tenuis*)	6	63.8 ± 3.2 (4)	47.2 ± 18.8 (4)
Ctenactis echinata[b]	9	Coral (*A. digitifera*)	6	50.0 ± 11.8 (4)	55.8 ± 6.9 (4)

[a] The number in the brackets represents either the number of larvae examined (method 1) or replication (method 2). See also Figs. 2 and 3 for *Acropora digitifera* and *A. tenuis*

[b,c] Refer to the two different infection methods (see Methods "acquisition of symbionts by the larvae of different coral species")

Timing of the onset of symbiosis in *Acropora digitifera* and *A. tenuis*

Thirty larvae each of *A. digitifera* and *A. tenuis* were prepared using the second method described above except 10 larvae of *A. digitifera* were used on 20 days after fertilization because of limited number of the larvae. *Acropora digitifera* was examined on 3–8, 10, and 20 days after fertilization, and *A. tenuis* was examined on 3–8 and 10 days after fertilization. Four replicates were established for each acquisition time. The number of acquired algal cells was counted in ten individuals from each dish under either a light or fluorescence microscope with the exception of day 20 for *A. digitifera* when only five larvae were observed in each dish. The remaining *A. digitifera* larvae in the dishes were fixed for histological observations (see below).

Histological observations

Eggs and larvae of *A. digitifera* were fixed using either 10% formaldehyde in FSW for 10 h for paraffin embedding or 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.4) containing 3% NaCl for 2 h for resin embedding. Specimens for the paraffin sections were moved to 70% ethanol and kept for more than 12 h. They were then dehydrated in a graded series of ethanol and benzene and were embedded with paraffin. The blocks were serially sectioned at 5 μm and mounted on slides, which were stained with Delafield's hematoxylin and eosin and examined under a light microscope. Specimens for the resin embedding were rinsed with 0.2 M phosphate buffer (pH 7.4) overnight and then post-fixed using 1% osmium tetroxide in the same buffer for 2 h. The specimens were then dehydrated in a graded series of ethyl alcohol and embedded in a Quetol 651 resin mixture. For light microscopic observation, 0.5 μm specimen sections were stained with 1% azur II in 1% borax. Larval morphology on histological sections was interpreted referring to Hayashibara et al. (1997), Ball et al. (2002), and Okubo and Motokawa (2007).

Effect of homogenized *Artemia* sp. on acquisition of symbionts

To examine the effects of homogenized *Artemia* sp. on symbiont acquisition in coral larvae, five 14-day-old *A. digitifera* larvae were exposed to an algal suspension using the second type of infection described above, but with or without homogenized *Artemia* sp. (50 μl) for 6 and 12 h. *Artemia* sp. are known to stimulate a feeding response in the coral larvae *Fungia scutaria* (Schwarz et al. 1999). Four replicates (i.e., four tissue culture dishes) were established. After 6 and 12 h, algal counts were performed as described above.

Statistical analysis of the ratio of symbiotic larvae

Since the larvae in a dish scramble for the finite number of symbiotic dinoflagellates, the number of algal cells acquired by each larva may not be statistically independent. To avoid pseudoreplication, we analyzed the number of the larvae that acquired symbiont(s), considering the wells rather than the larvae as the experimental unit.

Our interest was in the time course of increase in the proportion of symbiotic larvae, defined as the number of larvae with at least one algal cell divided by the total number of larvae examined in a dish. We assumed that the proportion follows a logit-normal distribution and analyzed the ratio by fitting a generalized linear model. We did not fit the model to transformed data because recent studies have criticized this method as inexact (e.g., Kasuya 2004; Wilson 2007).

For each *A. digitifera* and *A. tenuis*, the ratio of symbiotic larvae, y, was related to the number of days since fertilization, x, by the equation

$$\mathrm{logit}(y) = \alpha + \beta x, \tag{1}$$

where α and β are constants that are estimated. Setting $y = 0.5$ and solving Eq. (1) for x provides the timing at which half of the larvae acquire symbiotic dinoflagellates as

$$x_{0.5} = -\alpha/\beta. \tag{2}$$

Based on the observed data for x and y, the maximum estimates of α and β were calculated by maximizing the log-likelihood of the logit-normal distribution. Sample sizes were 24 (four dishes over 6 days) for both species; data from day 20 were removed from the analysis in *A. digitifera* because of their negative effect on the goodness-of-fit of the model, and thus on the estimation accuracy.

Statistical analysis of the average number of algal cells

The average number of algal cells was calculated for all larvae examined in a dish, and was analyzed as the experimental unit. We used generalized linear models with lognormal distribution because this score is non-negative and the variance appears to co-vary with the mean.

In the first analysis, we examined whether the average number of symbiotic algae increased as time advanced after fertilization. For each *A. digitifera* and *A. tenuis*, we fit the relationship between the average algal number per larva (y) and the number of days after fertilization, x, to the equation

$$\ln(y) = \alpha + \beta x, \quad (3)$$

where α and β are constants to be estimated. The maximum log-likelihood was calculated based on the observed data and the null hypothesis that the dependent variable is independent of time (i.e., $\beta = 0$) was tested using likelihood ratio tests (see Pawitan 2001). Sample sizes were 24 (4 dishes × 6 days) for both species.

A similar generalized linear model was applied to the data with regard to the effect of *Artemia* homogenate on symbiont acquisition by *A. digitifera*. In this analysis, independent variables were assumed to be nominal (i.e., presence/absence of *Artemia* sp. and dichotomous timing of observation; see Fig. 4) instead of the covariate x in the above analysis. Accordingly, the natural logarithm of average algal number was related to a two-way crossed ANOVA model with *Artemia* supply, timing of observation and their interaction as explanatory variables. Significant testing was conducted based on the likelihood ratio of the full model to null models constructed by removing each of the main effects and their interaction from the full model.

Results

Eggs and larvae of all corals examined did not initially contain *Symbiodinium* spp. when they were released. All of the larvae acquired symbiotic dinoflagellates under laboratory conditions (Table 1; Fig. 1). The larvae that acquired symbionts were active swimmer generating particle movement from the aboral to the oral side using their cilia. The larvae of *Acropora intermedia* began to acquire symbionts 5 days after fertilization, however, the larvae of the brooder *Isoopora palifera* acquired symbionts 2 days after release (Table 1).

Symbionts were first observed in the larvae of *A. digitifera* 6 days after fertilization, and thereafter more than 60% of larvae had symbionts (Fig. 2a). The logit normal model estimated the day that half of the larvae contained symbionts as $x_{0.5} = 5.7$. A likelihood ratio test indicated that the average number of symbiotic algae per larva significantly increased with time (Fig. 2b; $n = 24$, $\chi^2 = 19.1877$, $df = 1$, $P < 0.0001$). Similar patterns were observed in *A. tenuis*, but the day that the larvae first acquired symbionts was 1 day earlier than that of *A. digitifera* (Fig. 2c), and the maximum likelihood estimate of $x_{0.5}$ was 6.1. The average number of symbiotic algae per larvae also significantly increased with time after fertilization in *A. tenuis* (Fig. 2d; $n = 24$, $\chi^2 = 20.1136$, $df = 1$, $P < 0.0001$).

The egg and early stage of larva of *A. digitifera* did not contain symbionts (Fig. 3a–c). In a 4-day-old larva, the epidermal layer (ectoderm) on the oral side was invaginated in *A. digitifera* (Fig. 3b). The oral pore was observed but the coelenteron (gastric cavity) was not developed in a 5-day-old larva. Symbiotic dinoflagellates were not detected in the larvae at this time (Fig. 3c). On the other hand, a few symbionts were found in the endodermal tissue of the larvae 6 days after fertilization (Fig. 3d). At this stage, the coelenteron had developed and connected to the oral pore. In 8- and 10-day-old larvae, the coelenteron was well developed with wide space (Fig. 3e, f). Several symbionts occurred in the endodermal tissue around the oral pore and the coelenteron of the larvae (Fig. 3e, f).

The proportion of larvae with symbionts was 95.0 ± 5.0 (±SE) % for both 6 and 12 h after infection in the presence of homogenized *Artemia* sp., on the other hand, without *Artemia* supply, it was 70.0 ± 10.0 and 25.0 ± 5.5% for 6 and 12 h after infection, respectively. Two-way crossed ANOVA assuming lognormal distribution indicated that the positive effect of *Artemia* supply on the average number of symbiotic dinoflagellates was statistically significant ($\chi^2 = 6.1785$, $df = 1$, $P = 0.0129$), and both observation timing and interaction effects were non-significant ($P > 0.05$; Fig. 4).

Discussion and conclusions

The present study examined acquisition of *Symbiodinium* spp. by larvae of several reef-building corals and is the first to report the detailed relationship between symbiont acquisition and developmental stages of planula larvae. The larvae (nine species in five genera) horizontally acquired symbiotic dinoflagellates in infection experiments. The larvae of some corals are not able to take up dinoflagellates (e.g., Hirose et al. 2008a), whereas the larvae of other coral species do have acquisition capability (e.g., Schwarz et al. 1999; Nozawa and Harrison 2005; Baird et al. 2006). The current study corroborates the results of previous studies showing that larval stages of many coral species have the ability to obtain symbionts from their environments. Interestingly, we proved in this study that larvae of the coral *A. intermedia*, which Hirose and collaborators were unable to infect with symbionts (Hirose et al. 2008a), can indeed acquire dinoflagellates at the larval stage. The difference between studies might be due to differences in the developmental stages of larvae used (see below). The developmental speed of coral larvae may vary depending on culture conditions.

In this study, all coral larvae exposed to heterologous symbionts acquired them regardless of whether the symbiotic dinoflagellate strains were freshly isolated or cultured (Table 1). The larvae of *Fungia scutaria* are able to obtain heterologous symbionts, although the homologous symbiont has a significantly better association with

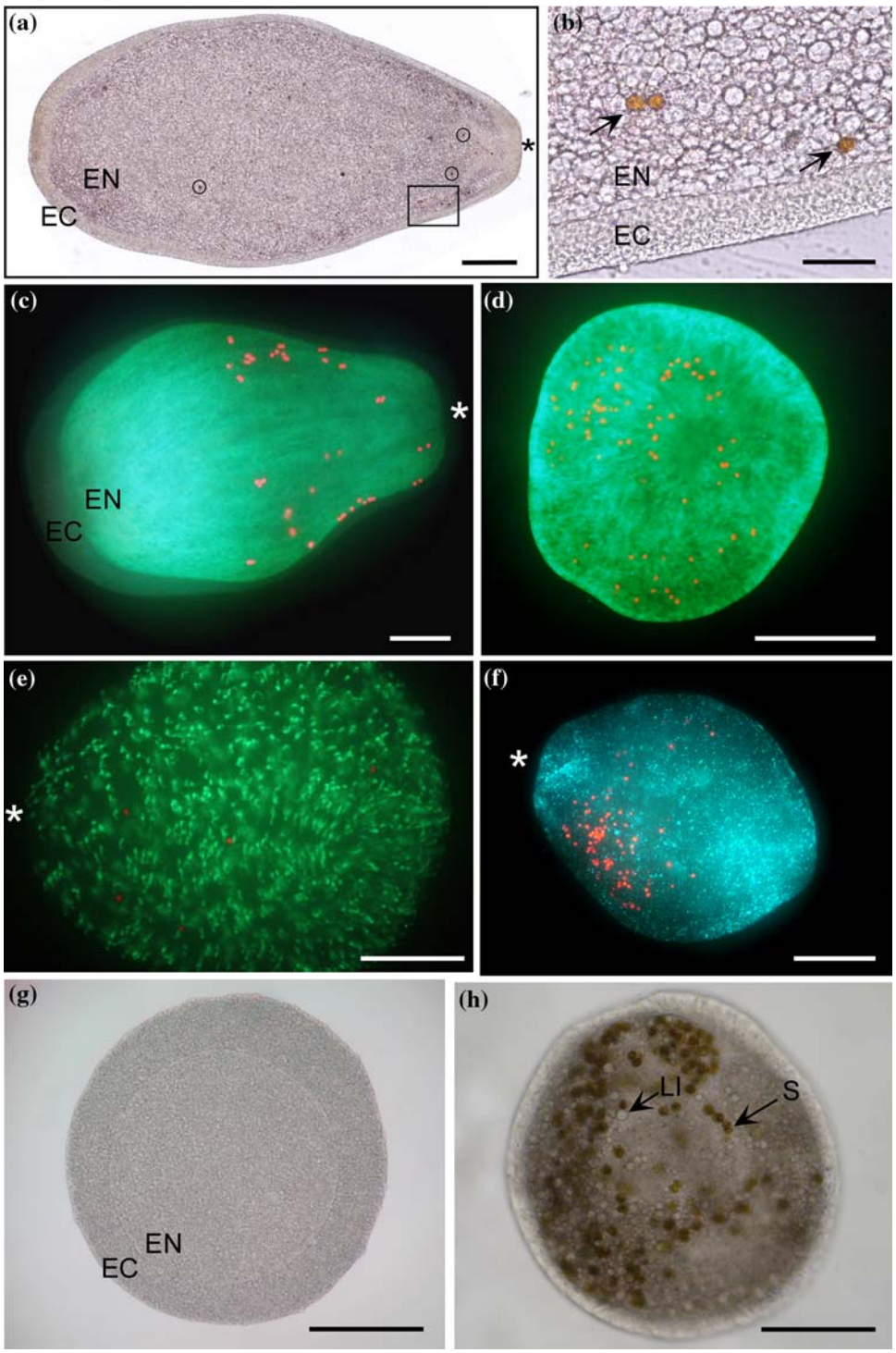

Fig. 1 **a–h** *Acropora tenuis, A. digitifera, Favia pallida, F. lizardensis, Pseudosiderastrea tayamai* and *Ctenactis echinata.* Microphotographs of the larvae with or without *Symbiodinium* spp. **a** Ten-day-old larvae of *A. tenuis* with acquired symbionts; **b** higher magnification of the box area in **a**; **c–f** larvae of *A. digitifera* (**c**, 7-day-old), *F. pallida* (**d**, 6-day-old), *F. lizardensis* (**e**, 6-day-old), and *P. tayamai* (**f**, 12-day-old) under a fluorescence microscope. *Symbiodinium* spp. in the larvae are shown as red particles. **g** Three-day-old larvae of *C. echinata* without symbionts, and **h** 9-day-old larvae of *C. echinata* with acquired symbionts (*circles*); ectoderm (EC); endoderm (EN); *Symbiodinium* spp. (S); lipids (LI); oral pore (*). *Scale bars* = 200 μm (**a**, **c–g**), 100 μm (**h**) and 50 μm (**b**)

the host larvae than do heterologous symbionts (Weis et al. 2001; Rodriguez-Lanetty et al. 2004, 2006). The early polyp stage of soft corals and scleractinian corals tend to acquire heterologous symbionts, in contrast to the adults (Coffroth et al. 2001; Little et al. 2004; Gómez-Cabrera et al. 2008). It is likely that larvae or initial polyps of corals do not have strict symbiont specificity and may lack mechanisms for symbionts identity recognition.

The timing of symbiont acquisition varies by coral species. The onset of symbiosis in the larvae of *A. digitifera*, *A. intermedia*, and *A. tenuis* occurred 5–6 days after fertilization (Fig. 2; Table 1), which is later than in *Fungia scutaria*, whose larvae begin to acquire symbionts from 3 days after fertilization (Schwarz et al. 1999). In the brooding coral *Isopora palifera*, the larvae acquired symbionts 2 days after release (Table 1). The differences

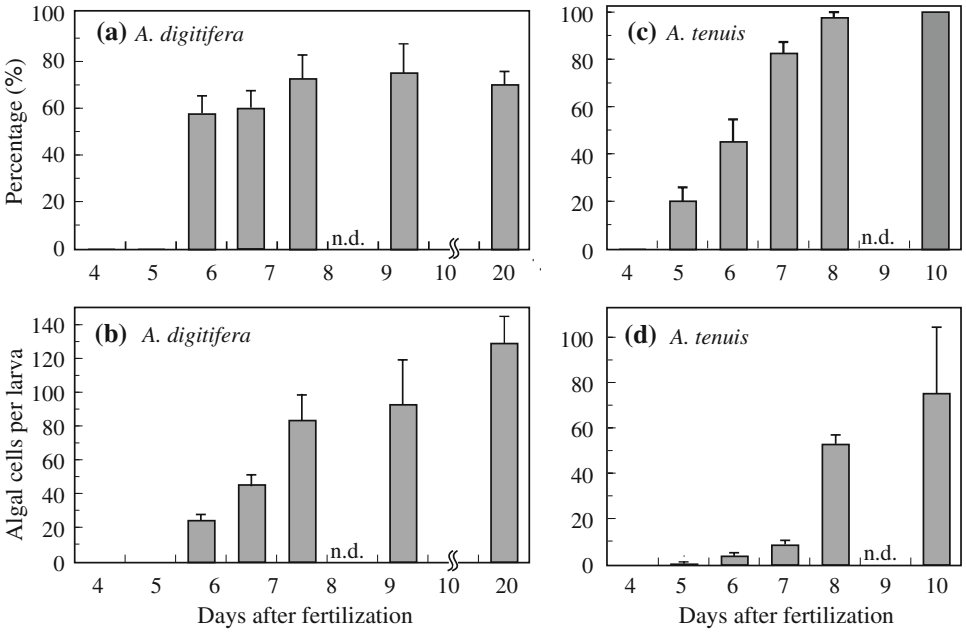

Fig. 2 *Acropora digitifera* and *A. tenuis*. Temporal changes in the percentage of larvae with symbionts (**a** and **c**) and in the number of symbiotic dinoflagellates in the larvae (**b** and **d**). *Error bars* indicate ± SE, $n = 4$

among the coral species in the timing of larval symbiont acquisition may be related to differences in the developmental stages at which they were exposed to symbionts. In *A. digitifera* larvae, the oral pore developed by day 4, and the coelenteron was formed on day 5, when symbiont acquisition occurred. Okubo and Motokawa (2007) reported that larvae of five acroporid corals, including *A. digitifera*, had formed the coelenteron and mesenterial filaments by the ninth day after fertilization. The larvae of *F. scutaria* acquired symbionts following oral pore and coelenteron development (Schwarz et al. 1999). *Isopora palifera* larvae, which have a well-developed coelenteron when released (Kojis 1986), acquired symbionts 2 days after release. In contrast, the coelenteron is not formed 16 days after fertilization in *A. intermedia* and *A. microphthalma*, and larvae of these species do not take up symbionts during the period before the coelenteron appears (Hirose et al. 2008a). Together, these findings suggest that the onset of symbiosis in coral larvae occurs after oral pore formation and subsequent coelenteron development. The pore allows symbionts entry into the coelenteron. However, in some cases, acquisition of symbionts may occur through the epithelial cells of the blastoderm (Marlow and Martindale 2007). The differences in larval developmental stages among coral species may explain the differences in the timing of symbiont acquisition by larvae.

Our study demonstrated experimentally that the number of algal cells acquired per day by coral larvae increased over time (Fig. 2b, d). This may be related to the continued development over time of gastrodermal cells lining the coelenteron. Hirose et al. (2008a) reported that the cilia of the gastrodermal cells are elongated and sometimes trap symbionts in the coelenteron of acroporid coral primary polyps. Similarly, larvae of the soft coral *Xenia umbellate* also have cilia in the coelenteron (Benayahu et al. 1988). With the passage of time after fertilization, the larval coelenteron may become enlarged and lined with well-developed gastrodermal cells. This enlargement and the current produced by well-developed, ciliated gastrodermal cells may enhance acquisition of symbionts by late developmental stage larvae. Once symbionts are retained in the coelenteron, the hosts (larvae) recognize and phagocytose them. Some lectins in the octocoral *Sinularia lochmodes* have been proposed as mediators of interactions between host and symbionts (Jimbo et al. 2000; Koike et al. 2004). Lectin/glycan interactions have recently been implicated as components of the inter-partner signalling mechanisms of symbiosis in the coral *Fungia scutaria* larvae (Wood-Charlson et al. 2006). It is likely that well-developed larvae have mechanisms to recognize symbionts for subsequent phagocytose.

The larvae of *A. digitifera* acquired more symbionts in the presence of *Artemia* homogenate than in its absence. This is consistent with the findings that larvae of *F. scutaria* under laboratory conditions acquire symbionts though ingestion while feeding (Schwarz et al. 1999; Rodriguez-Lanetty et al. 2004, 2006). Larvae of the sea anemone *Anthopleura elegantissima* also ingest dinoflagellates while feeding (Schwarz et al. 2002). Once an oral pore and coelenteron have developed, the larvae may obtain nutrients via ingestion. These observations suggest that the *Artemia* homogenate is effective in stimulating the establishment of planula larva symbiosis.

We demonstrated that larvae of many coral species have the potential to acquire symbionts during the planktonic phase. Although little is known about the distribution and

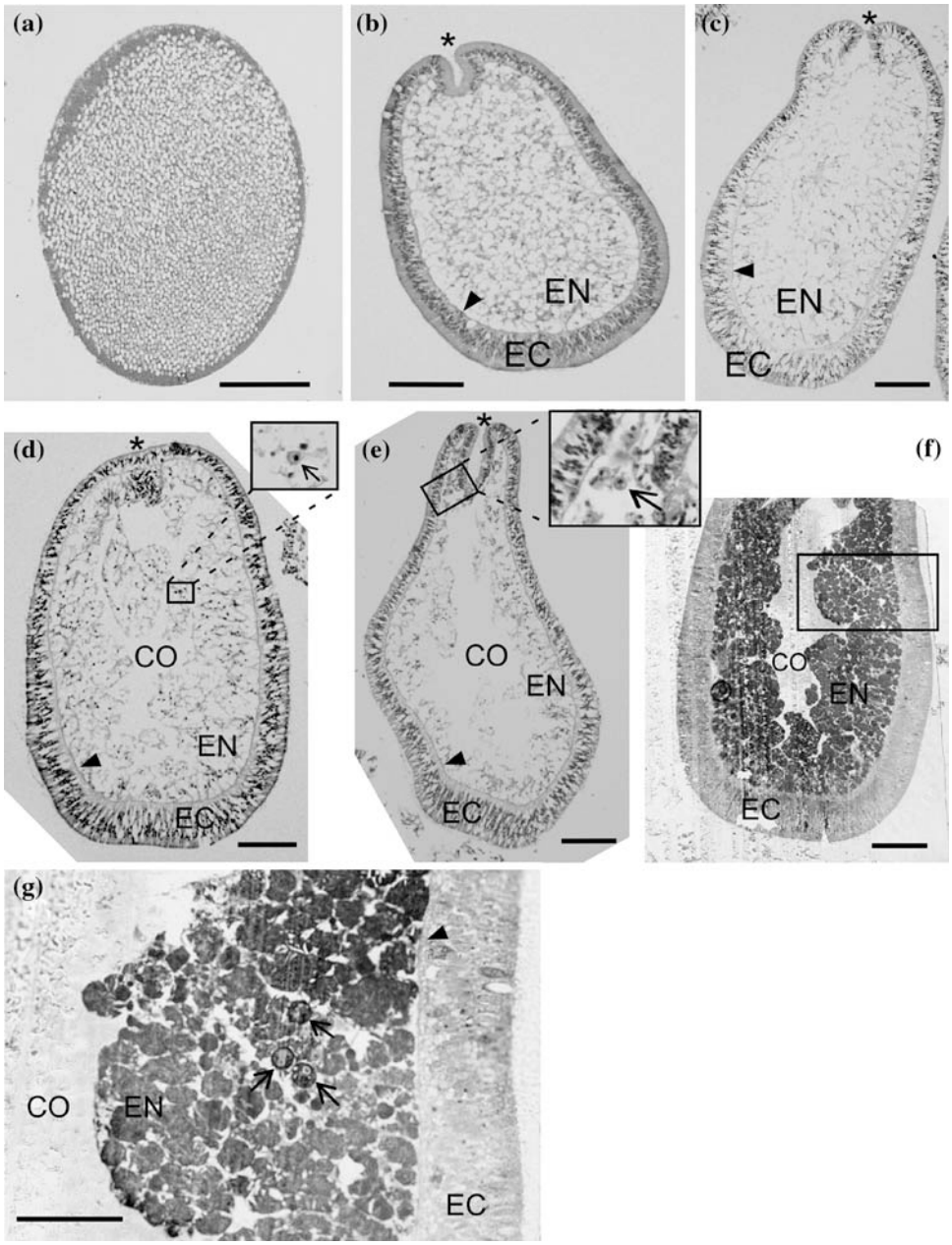

Fig. 3 *Acropora digitifera*. Histological sections of the egg and larvae. **a** Egg without *Symbiodinium* symbionts; **b** 4-day-old larvae with observable oral pore and developed endoderm and ectoderm; **c** 5-day-old larvae with open oral pore; **d** 6-day-old larvae with coelenteron and symbionts present within the endodermal cells; **e** 8-day-old larvae; **f** 10-day-old larvae with a well-developed coelenteron; **g** higher magnification of boxed area in (f); ectoderm (EC); endoderm (EN); coelenteron (CO); *Symbiodinium* spp. (*arrows*); mesoglea (*arrow heads*); oral pores (*). *Scale bars* = 200 μm (**a**–**e**) and 20 μm (**f**)

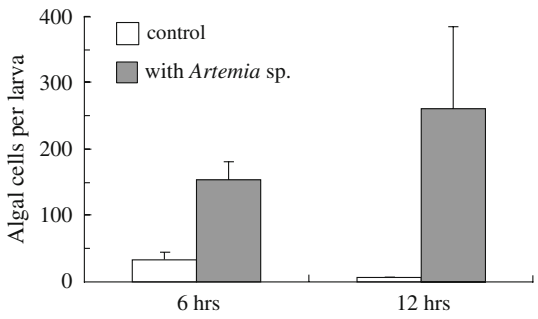

Fig. 4 *Acropora digitifera*. Number of symbiotic dinoflagellates in 14-day-old larvae with and without *Artemia* sp. conditions. *Error bars* indicate ± SE, $n = 4$

abundance of *Symbiodinium* spp. in natural environments, "free-living" *Symbiodinium* spp. (i.e., outside a host) have been found in sand, substrates, on seagrasses, and water columns in coral reefs (Coffroth et al. 2006; Hirose et al. 2008b; Manning and Gates 2008; Porto et al. 2008; Littman et al. 2009). A proportion of the larva produced by *Favites chinensis* and *Goniastrea aspera* cultured in outside-running seawater tanks at the Sesoko Station acquired symbionts by horizontal transmittance (Nozawa and Harrison 2005, Nozawa, personal communication). Because it is likely that the concentration of *Symbiodinium* spp. in the ocean water column is similar to that in aquaria provided with unfiltered running seawater, the planula larvae in

seawater tanks may have had an opportunity to acquire symbionts under "natural" conditions.

Symbiotic larvae have the advantage of obtaining energy from symbiotic dinoflagellate cells (Richmond 1987; van Oppen 2001; Harii et al. 2002), though they are also exposed to elevated risk of oxidative damage under stressful conditions (Yakovleva et al. 2009). Symbiotic larvae of *Pocillopora damicornis*, which is a brooder and vertical transmitter, have a long larval dispersal period (>100 days), which may be related to the additional energy provided by the photosynthetic algal cells (Richmond 1987; Harii et al. 2002). If aposymbiotic larvae do not find a suitable place to settle during the competent period (i.e., 4 days after fertilization; Babcock and Heyward 1986; Harii et al. 2007), they may extend their planktonic life (Nishikawa et al. 2003; Harii et al. 2007; Graham et al. 2008). During this time the larvae have the potential to acquire symbiotic dinoflagellates, which may increase larval survivorship and/or settlement-competency periods and thus play an important role in larval dispersal time (van Oppen 2001). On the other hand, Yakovleva et al. (2009) reported that symbiotic larvae suffer a high level of oxidative cellular damage under high temperature, resulting in lower survivorship of symbiotic larvae in comparison with aposymbiotic larvae. They suggest that symbionts are a costly burden for larval stages under stressful environmental conditions. Determination of when and how larvae acquire symbionts in the natural environment is key in understanding advantages and disadvantages of coral–algal relationship.

Acknowledgments We thank the staff of the Sesoko Station of the Tropical Biosphere Research Center, University of the Ryukyus, for providing research facilities. We also thank Kenji Iwao, Masaya Morita, Akira Iguchi, Yossi Loya, Takeshi Hayashibara, Naoko Isomura, Hironobu Fukami, Nami Okubo, Makoto Kitamura, Kenji Iwai and staff of Okinawa Churaumi Aquarium for providing samples. We are also grateful to Mamiko Hirose for valuable suggestions regarding histological observations and the reviewers who helped to improve this manuscript. This research was funded by the 21st Century Center of Excellence (COE) program of the University of the Ryukyus, a Ministry of Education, Culture, Sports, Science and Technology Grant-in-Aid for Young Scientists (B) No. 20770017 (SH), and the Australian Research Council Centre of Excellence for Coral Reef Studies at the University of Queensland.

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