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RESEARCH ARTICLE

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Temporal and spatial infection dynamics indicate recognition events in the early hours of a dinoflagellate/coral symbiosis

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Abstract The obligate symbiotic relationship between dinoflagellates, *Symbiodinium* spp. and reef building corals is re-established each host generation. The solitary coral *Fungia scutaria* Lamarck 1801 harbors a single algal strain, *Symbiodinium* ITS2 type C1f (homologous strain) during adulthood. Previous studies have shown that distinct algal ITS2 types in clade C correlate with *F. scutaria*—*Symbiodinium* specificity during the onset of symbiosis in the larval stage. The present study examined the early specificity events in the onset of symbiosis between *F. scutaria* larvae and *Symbiodinium* spp., by looking at the temporal and spatial infection dynamics of larvae challenged with different symbiont types. The results show that specificity at the onset of symbiosis was mediated by recognition events during the initial symbiont—host physical contact before phagocytosis, and by subsequent cellular events after the symbionts were incorporated into host cells. Moreover, homologous and heterologous *Symbiodinium* sp. strains did not exhibit the same pattern of localization within larvae. When larvae were infected with homologous symbionts (C1f), ~70% of the total acquired algae were found in the equatorial area of the larvae, between the oral and aboral ends, 21 h after inoculation. In contrast, no spatial difference in algal localization was observed in larvae infected with heterologous symbionts. This result provides evidence of functional differences among gastrodermal cells, during development of the larvae. The cells in the larval equator function as nutritive phagocytes, and also appear to function as a region of enhanced symbiont acquisition in *F. scutaria*.

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Introduction

Mutualistic endosymbiosis between two organisms includes a stage where the larger host first acquires its smaller symbiont. Symbionts may be transmitted vertically where the symbiont is passed directly from host parent to offspring, or horizontally where host sexual progeny must acquire symbionts from the environment (Douglas 1994). In horizontally transmitted associations, the onset of symbiosis has been shown for several well-studied symbioses, such as squid/luminous bacteria and legume/nitrogen fixing bacteria mutualisms, to include a complex series of steps, recently referred to by Nyholm and McFall-Ngai (2004) as "the winnowing". These steps range from molecular signaling to inter-microbe ecological interactions, all of which are necessary but none of which are sufficient alone to establish a successful, specific symbiosis (e.g., Nyholm and McFall-Ngai 2004; Somers et al. 2004). Moreover, this series of specificity mechanisms occurs over a considerable time, starting minutes after first contact and extending from weeks to months into host development and maturity.

The onset of symbiosis in the highly diverse array of cnidarian/algal associations has been studied in a broad range of partnerships. These include symbioses between (1) the freshwater hydroid *Hydra viridis* and a chlorophyte (Muscatine et al. 1975, reviewed by Jolley and Smith 1980), (2) the scyphozoan *Cassiopeia xamachana* and the dinoflagellate *Symbiodinium* spp.

(Colley and Trench 1983; Fitt and Trench 1983; Colley and Trench 1985) and (3) a variety of anthozoans and *Symbiodinium* spp. (e.g. Kinzie 1974; Schoenberg and Trench 1980; Davy et al. 1997; Coffroth et al. 2001; Belda-Baillie et al. 2002; Rodriguez-Lanetty et al. 2003). Taken together, these studies suggest an analogous winnowing process in cnidarian/algal symbioses, which were first discussed 25 years ago (see Jolley and Smith 1980; McAuley and Smith 1982). Evidence for the steps in this process includes the presence of putative molecular signals on the surfaces of symbionts and in hosts (Meints and Pardy 1980; McNeil et al. 1981; Markell et al. 1992; Lin et al. 2000; Koike et al. 2004), discrimination by the host early in infection between its own symbionts (homologous symbionts) and those from other hosts (heterologous symbionts) (e.g. Pool 1979; Jolley and Smith 1980; Schoenberg and Trench 1980; Weis et al. 2001; Belda-Baillie et al. 2002; Rodriguez-Lanetty et al. 2004), and a succession of different algal phylotypes later in host development (Rahat 1991; Coffroth et al. 2001; Little et al. 2004). Nonetheless, the information we have is far from complete.

We have been studying the onset of symbiosis in the planula larva of the solitary scleractinian coral *Fungia scutaria* and its dinoflagellate symbiont *Symbiodinium* clade C1f (nomenclature sensu LaJeunesse et al. 2004). This association is an ideal model for studying the onset of symbiosis due to the predictable availability of larvae and the ability to establish the symbiosis experimentally in the laboratory. Previous work has demonstrated that planulae acquire symbionts during feeding. Algae are phagocytosed by host gastrodermal cells and persist within host vacuoles inside these cells (Schwarz et al. 1999). Other studies have shown that this process is specific. For example, when host larvae are challenged with closely related heterologous *Symbiodinium* clade C phylotypes isolated from other host corals, the symbiosis is less robust than that between *F. scutaria* and its homologous *Symbiodinium* C1f algae, as measured by the percentage of larvae infected and the density of algae in larvae 21 h after inoculation (Weis et al. 2001; Rodriguez-Lanetty et al. 2004). It is still unknown whether the development of this specific association is governed by processes occurring before and/or after the symbionts enter host cells.

In the present study, we examined the early specificity events in the onset of symbiosis between *F. scutaria* larvae and *Symbiodinium* spp., after the initial contact of larvae challenged with different symbiont types. First, we determined whether differential infection by symbionts was due to recognition during the initial contact between gastrodermal cells and symbionts, or later within the host cells after algal symbionts were phagocytosed. Second, we determined if initial infection occurred in a specific region of the larval gastroderm, and whether there were spatial differences in algal uptake depending on symbiont type.

Material and methods

Collection and maintenance of coral larvae

Adult *F. scutaria* Lamarck 1801 corals were collected from several different patch reefs in Kaneohe Bay, Hawaii, USA in July 2003 and 2004, and placed in seawater tables at the Hawaii Institute of Marine Biology 4 day before full moon. This dioecious coral species (Kramarsky-Winter and Loya 1998) generally spawns between 1700 and 1900 hours, 2–4 days after the full moon (Krupp 1983). Collection and fertilization of gametes, which lack symbionts (aposymbiotic), were performed as previously described (Rodriguez-Lanetty et al. 2004). The experiments described below were conducted in non-settled larvae.

Preparation of *Symbiodinium* isolates, genetic typing and infection experiments

Infection experiments were conducted in July–August of 2003 and 2004. For all experiments, 4-day-old aposymbiotic *F. scutaria* larvae were kept in bowls at ambient temperature (~26°C), each containing an average of 10^5 larvae in 1 l of 0.25-µm filtered seawater. Algae were extracted from adult coral colonies, and before inoculation were mixed with homogenized *Artemia* sp. to stimulate a feeding response in the larvae (Schwarz et al. 1999). Each bowl was provided with approximately the same number of symbionts (~10^8 cells). After 3 h, larvae were washed, and any remaining algae not ingested by the larvae were removed by concentrating the larvae onto a 60-µm mesh filter and placing them in clean bowls. Larvae were collected at various sampling times, fixed in 4% paraformaldehyde fixative in phosphate buffered saline (PBS: 2 mM NaH_2PO_4, 7.7 mM Na_2HPO_4, 0.14 M NaCl) and rinsed in PBS before examination with light and confocal microscopy. The genetic typing of the symbiotic dinoflagellates was determined as described by Rodriguez-Lanetty et al. (2004) from sequences of ITS-2 nrDNA amplified from genomic DNA extracts of the algal preparations used for the infection experiments.

In 2003, an experiment documented the uptake of homologous symbionts (C1f extracted from *Fungia scutaria*) and heterologous symbionts (C31 extracted from *Montipora capitata* Dana 1846) in the early hours following inoculation. We chose to use C31 symbionts based on previous studies comparing infectivity of a variety of symbiont C strains (Weis et al. 2001; Rodriguez-Lanetty et al. 2004). In these studies, C31 exhibited the lowest level of infectivity at 24 h after infection. Larvae were collected randomly from each treatment and fixed at 1, 5, 21, and 93 h after the inoculation period.

In 2004, an experiment examined the uptake of homologous symbionts extracted from *F. scutaria*

compared to symbionts belonging to the same genetic strain (C1f), but extracted from another coral species, *Leptastrea* sp. This experiment addressed the effect that potential residual host symbiosome membrane around freshly isolated cells might have on symbiont re-uptake. Symbionts (*Symbiodinium* C1) extracted from *Cyphastrea* sp. were also included in the experiment as the closest phylogenetic heterologous symbionts to C1f (sensu LaJeunesse et al. 2004). Nine bowls of larvae were inoculated with freshly isolated symbionts: three with symbionts (C1f) extracted from *F. scutaria*, three with symbionts (C1f) extracted from *Leptastrea* sp., and three with the symbionts (C1) extracted from *Cyphastrea* sp. We quantified percent infection and symbiont density at 21 h after inoculation in 100 larvae per bowl for each treatment. In addition, to compare the uptake of inert particles to that of symbionts by host cells, we provided a separate group of larvae with synthetic fluorescent latex beads (0.4 μm) (505/515, Molecular Probes, Invitrogen).

Microscopy and data analysis

Larvae from the experiment in 2004 were rinsed in PBS and visualized under light microscopy to obtain infection rates and average density of symbionts. ANOVA analyses were conducted to detect differences in percent of larval infection and algal cell number per larva among larvae infected with homologous symbionts C1f (extracted from two different corals species) and heterologous (C1) symbionts.

Larvae from the experiment conducted in 2003 were rinsed in PBS and stained with the fluorescent DNA dye Hoechst 33258 to visualize the host cells in infected coral larvae. Samples were mounted in well slides with glycerol (50%) and observed under confocal microscopy either on a Leica TCS 4D Laser Scanning or a Zeiss LSM 510 Meta. Samples were scanned with excitations of UV (Leica: 380 nm, Zeiss: 405 nm) and green (Leica: 500 nm, Zeiss: 488 nm) light, and emissions were collected at 450 nm to visualize stained host cells, 515 nm for latex beads, and 600 nm for the autofluorescence of algal symbionts. In larvae where spatial localization of algae was examined, 20-μm optical sections of a larva were scanned using green excitation to collect full reflected light (Leica) or Differential Interphase Contrast, (DIC, Zeiss) to visualize the inner gastrodermal host cells. This imaging technique allowed for unequivocal scoring of algae as either in host gastrodermal cells or outside of host cells but within the gastric cavity. ANOVA analyses were conducted to detect (1) differences in the proportion of symbionts in distinct sections within the larvae and whether it changed with time, and (2) differences in the proportion of total symbionts in larval gastroderm cells with time between larvae infected with homologous C1f and heterologous C31 symbionts. The multiple comparison test, Tukey HSD, was conducted after ANOVA analyses to determine significant differences between the treatments. Data expressed as percentages were arcsine-transformed, and data for algal density were log-transformed when heteroscedasticity in the original data was encountered. All statistical analyses were performed using SPSS v. 9.0.

Results

The density of symbionts in *F. scutaria* larvae infected with *Symbiodinium* C1f did not change with time from 1 h following algal inoculation ($F_{3,60}$: 1.87, $P=0.144$) (Fig. 1). However, the density of symbionts in larvae infected with *Symbiodinium* C31 decreased with time until no algal symbionts were present after 21 h. While an initial uptake of C31 symbionts from gastrodermal host cells was observed at 1 h after inoculation, the uptake was significantly lower than in larvae containing homologous C1f symbionts (Algal density: $C31=4.01\pm3.2$; $C1f=16.7\pm9.7$; t_{32}: 4.68, $P<0.001$). The difference in infections between homologous C1f and heterologous C31 symbionts could be attributed to an antagonistic reaction by the larvae to foreign coral host tissue in the heterologous C31 symbiont extract. However, this possibility is unlikely because no difference in percent infection and algal cells per larva was detected when larvae were infected with the same C1f symbiont extracted from different corals species (Fig. 2; Tukey's HSD test, percentage of infection: $P=0.41$; algal density: $P=0.48$). Moreover, larvae infected with the more closely related symbiont (C1) also showed significantly lower percent infection ($F_{2,8}$: 19.97, Tukey's HSD test, $P<0.01$) and algal density ($F_{2,8}$: 7.84, Tukey's HSD test, $P<0.05$) at 21 h, when compared to larvae infected with the C1f symbiont irrespective of host (Fig. 2).

There was a distinct spatial pattern of algal acquisition in *F. scutaria* larvae (Fig. 3a). The proportion of algal symbionts was quantified in the gastric cavity and in three cross sections orthogonal to the oral–aboral axis

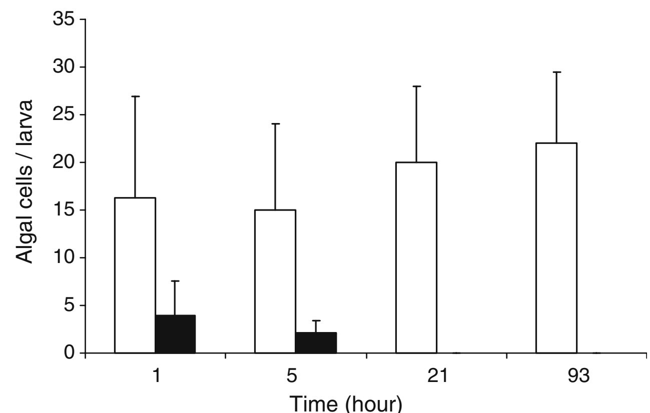

Fig. 1 *Fungia scutaria*. Algal density in larvae with time after symbiont inoculation period. Inoculations were carried out with *Symbiodinium* sp. C1f (from *F. scutaria*) (*white bar*), and C31 (from *Montipora capitata*) (*black bar*). *Bars* represent means ± SD [*n* (larvae) = 15 and 19]

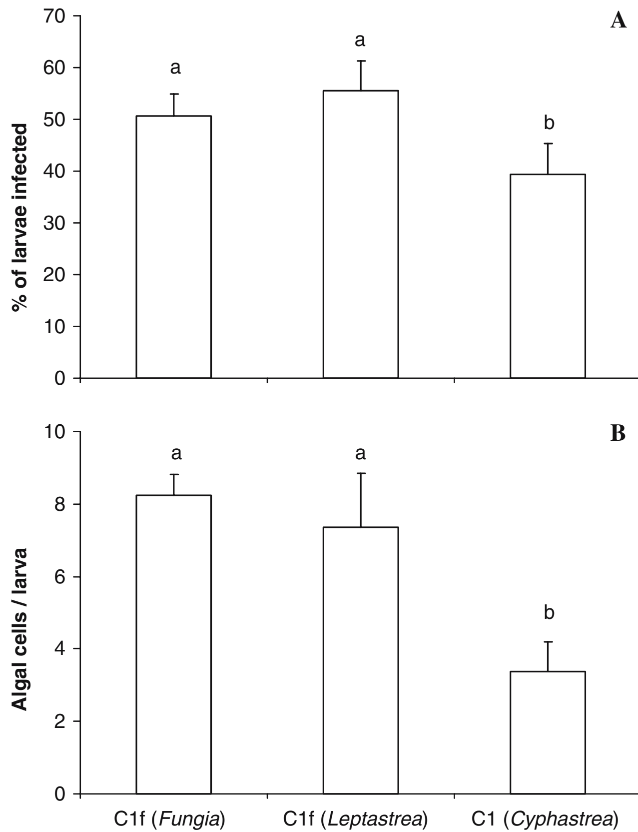

Fig. 2 *Fungia scutaria*. Infection dynamics of larvae inoculated with different strains of *Symbiodinium* spp. Inoculations were performed with *Symbiodinium* clade C1f from *F. scutaria* and *Leptastrea* sp., and C1 from *Cyphastrea* sp. Data were collected 21 h after the symbiont inoculation period. **a** Percentage of larvae infected, **b** Algal cells per larva. *Bars* represent means ± SD (*n* [bowls] = 9). *Different letters* above bars indicate significant differences between treatments ($P < 0.05$)

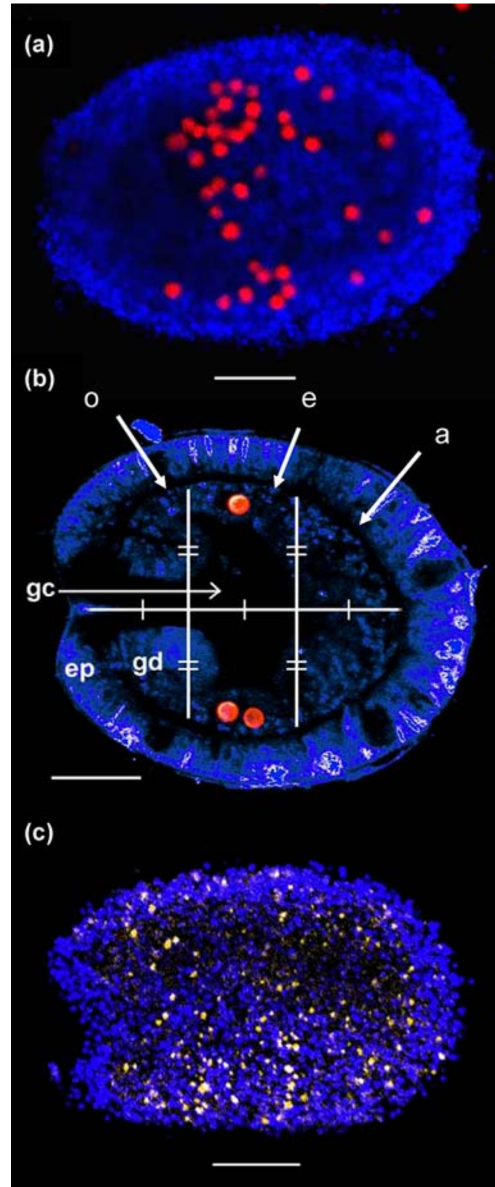

Fig. 3 *Fungia scutaria*. Localization of algae and fluorescent latex beads within larvae. **a** 3-D confocal reconstruction of a larva infected with C1f algae from *F. scutaria* adults. **b** Twenty-micro meter optical reflected light section through a larva showing the four different scored regions of the larva. Symbionts (*red spheres*) are present inside the larval gastroderm (*gd*). *o* Oral, *e* equator, *a* aboral, *gc* gastric cavity, *ep*, epiderm. **c** Larva infected with inert fluorescent latex beads, which are a proxy for food. *Red* algal autofluorescence (488/600 nm). *Blue* Hoechst DNA stain (405/450 nm). *Yellow* latex beads (405/515 nm). *Scale bar* 50 µm

of the larvae, corresponding to the oral, equatorial, and aboral areas (Fig. 3b). The majority of the homologous (C1f) symbionts were acquired in the larval equator ($F_{15,196}$: 26.37, Tukey's HSD test, $P < 0.001$; Figs. 3a, 4a). This spatial pattern of acquisition was detected in the early hours following inoculation and persisted throughout the sampling period. In addition, symbionts in the gastric cavity decreased with time, while those in the equatorial gastrodermal area increased, from 42 ± 25% at 1 h to 85 ± 16% at 93 h. This pattern was also detected in larvae infected with the same type of symbionts (C1f) extracted from *Leptastrea* sp. These larvae had 79.1% of total algae in the equatorial area at 21 h after inoculation. However, this spatial pattern of algal acquisition was not observed in larvae infected with the heterologous symbionts C31 ($F_{6,69}$: 1.519, $P = 0.185$; Fig. 4b), where initial symbiont acquisition was not different among the three areas of the larva. The relative abundance of symbionts (C31) 1 h after inoculation was 12 ± 33, 25 ± 34, and 5 ± 10% for oral, equatorial, and aboral areas, respectively.

To test whether the observed spatial pattern of phagocytosis of homologous symbionts in *F. scutaria* larvae could be attributed to an area specialized for algal phagocytosis, we inoculated coral larvae with inert fluorescent latex beads and observed them in the host cells after phagocytosis. If non-specific phagocytosis were more active in the equatorial area of the larvae, we expected to observe a significantly higher proportion of

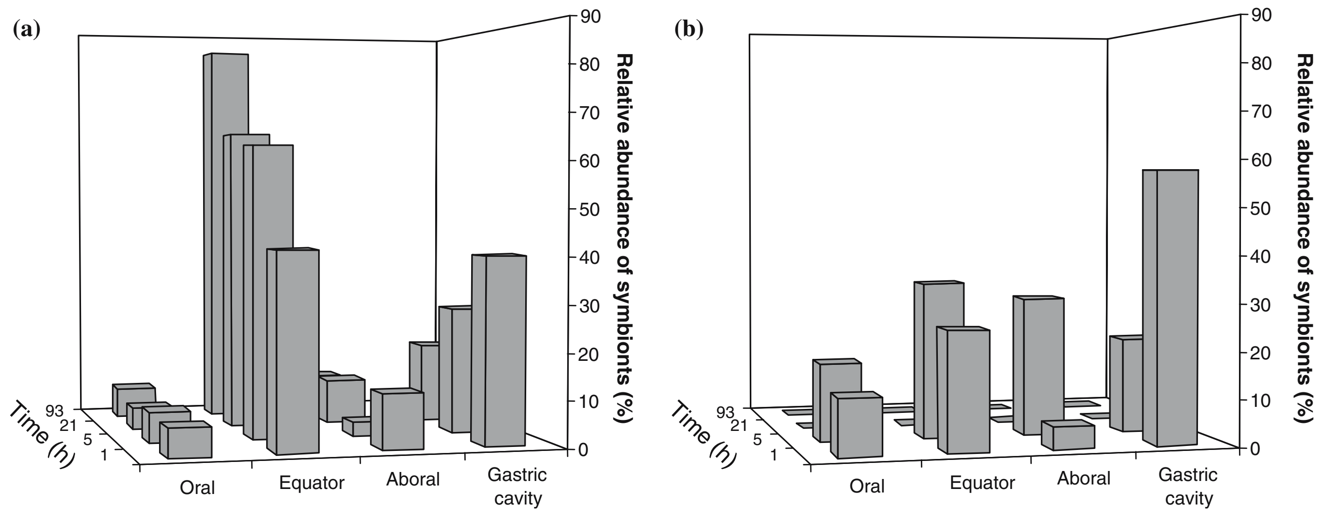

Fig. 4 *Fungia scutaria*. Spatial distribution of phagocytosed symbionts within larvae at different times after inoculation. Larvae were inoculated with **a** *Symbiodinium* C1f extracted from *F. scutaria*; or **b** *Symbiodinium* C31 extracted from *Montipora capitata*. Algae were scored as occurring in one of four regions through the larva: oral, equator, aboral or in the gastric cavity (see Fig. 3b). *Bars* represent means [*n* (larvae) = 14–19]

latex beads in the equatorial area as observed with C1f symbiont phagocytosis. However, the beads were phagocytosed uniformly by all gastrodermal host cells irrespective of larval section (Fig. 3c). It is possible that the smaller size of the latex bead compared to the actual size of the algal symbiont could have an effect on mobility in the host tissue, however this could not be determined.

Discussion

This study suggests that the specificity between the scleractinian coral *F. scutaria* and *Symbiodinium* sp. strain C1f during the initial hours of the onset of symbiosis is mediated both by recognition events before phagocytosis, and by subsequent cellular events after the symbionts are incorporated into host cells. We conclude that these findings were not affected by foreign host cellular contamination as an artefact of algal isolation. Furthermore, we document for the first time, spatial differences in algal uptake within the coral larva depending on symbiont type.

While both homologous C1f and heterologous C31 symbionts were initially incorporated into larval host cells within several hours of addition, significant differences in infection success between the two symbiont types were clearly detected. At 1 h after the inoculation period, larvae challenged with C1f symbionts from *F. scutaria* had phagocytosed three times more algae than those challenged with heterologous C31 algae from *Montipora capitata*. The observation that heterologous C31 symbionts are not differentially distributed in the larvae provides additional evidence for a recognition mechanism(s) occurring before phagocytosis. These observations of infectivity patterns in coral–algae symbioses have not been described before, although similar patterns have been documented for the well-studied scyphozoan *Cassiopeia xamachana* during initial acquisition of different strains of *Symbiodinium*, where homologous symbionts were acquired in higher densities than were heterologous ones within the first 2 h after inoculation (Trench et al. 1981).

Recent studies have described the molecular components putatively involved in cnidarian–algal recognition. Lin et al. (2000) have shown that cell surface modification of algal symbionts by glycosidases or lectin-masking resulted in decreased infection rates in *Aiptasia pulchella*. These results support previous findings showing the presence of glycoproteins associated with the cell walls of symbiotic dinoflagellates (Markell et al. 1992). These glycoproteins were assumed to be involved in cell signaling during recognition processes. The quantification of infection by Lin and colleagues, however, was performed several days after symbiont inoculation and did not directly address the effects of cell surface modification on the events occurring before phagocytosis. Future proteomic research on the chemical composition of the cell surface of *Symbiodinium* spp. will shed further light on this possibility. More recently, Koike et al. (2004) have suggested that a host-controlled physical environment in the gastrovascular cavity in cnidarians might play a role in symbiont selection. They provided evidence, from studies on cultured algae, that a D-galactosyl-binding lectin (SLL-2) chemically selects against some non-symbiotic dinoflagellates and certain *Symbiodinium* spp. types. They also showed that SLL-2 triggers a morphological change in the algae from a flagellated to a coccoid form, which may be favorable for the symbiosis between algae and host. This host-driven modification of the extracellular environment seems to mediate an ecological process of symbiont

selection, however, further evidence on mechanisms of action of this lectin on symbionts is needed before a final conclusion can be drawn.

After phagocytosis, *F. scutaria* larvae that took up homologous C1f symbionts maintained a stable association throughout the experimental period (93 h). In contrast, the few heterologous C31 symbionts initially phagocytosed by host cells disappeared over the first 21 h. It is not clear whether the heterologous symbionts were expelled (via exocytosis) by the host cells, digested (via phagosome–lysosome fusion), or both. Nevertheless, this observation clearly suggests that post-endocytotic events resulted in the elimination of the heterologous symbionts. Similar observations have also been documented in *Hydra–Chlorella* (Muscatine et al. 1975) and *Cassiopeia–Symbiodinium* (Colley and Trench 1983) systems where heterologous algae internalized by host cells were discarded 24–72 h after inoculation. In other cnidarian hosts, such as gorgonian octocorals, the time for heterologous symbiont disappearance was prolonged up to 3 month after symbiont infection (Coffroth et al. 2001), which suggests that other processes of symbiont selection must be mediating the high specificity outcome of these symbioses. However, it is important to mention that Coffroth et al.'s study only documented the presence of symbionts using molecular (nrDNA and cpDNA) detection techniques over a 3 month period. The density of homologous and heterologous symbionts was not documented, which is an important variable required to define successful symbiosis establishment.

The observation that most homologous symbionts were acquired by host cells around the larval equator was unexpected since it has been assumed that all nutritive gastrodermal cells have the same phagocytic competence (Gauthier 1963). We demonstrated that the increased incorporation of symbionts at the larval equator was not due to an intrinsically higher phagocytic capacity of this area because phagocytosis of inert latex beads, which simulate food particles, were not spatially different. This explanation assumes, however, that the smaller size of the latex beads did not cause a significant difference in particle mobility in the host tissue compared to phagocytosed algae. Our result provides evidence of functional differences among gastrodermal cells during development of the larvae. Although the cells at the larval equator function as nutritive phagocytes, they also appear to function as a symbiont-acquisition region, where symbiont incorporation is enhanced.

The existence of an apparent symbiont-acquisition region during early larval development in *F. scutaria* is a novel discovery in coral–dinoflagellate symbioses. Anthozoan (corals and sea anemones) morphogenesis from larva to a settled adult is unique within cnidarians because a pharynx is formed by an inversion of the body wall from the oral end (Martindale et al. 2004). Settlement also results in the shortening of the oral–aboral axis (Weis and Buss 1987; Schwarz et al. 1999). Therefore, tentacles are hypothesized to develop from the equatorial section of the larvae. This is consistent with the localization of homologous symbionts within the host larvae seen in this experiment and the observation that in adult polyps, most symbionts are housed in the tentacles where they have the greatest light exposure.

In summary, we have presented evidence that specificity in symbioses involving dinoflagellates results in part from a series of cellular events early in the onset of symbiosis that together influence the establishment and perpetuation of these associations. This does not exclude the existence of other factors that may also shape the final expression of specificity, such as ecological interactions among different types of symbionts (Rahat 1991; Coffroth et al. 2001; Little et al. 2004) and physiological alterations of one partner over the other (Koike et al. 2004).

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