Similar specificities of symbiont uptake by adults and larvae in an anemone model system for coral biology

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SUMMARY

Reef-building corals depend for much of their energy on photosynthesis by symbiotic
dinoflagellate algae (genus *Symbiodinium*) that live within their gastrodermal cells. However, the
cellular mechanisms underpinning this ecologically critical symbiosis, including those governing
the specificity of symbiont uptake by the host, remain poorly understood, in part because of the
difficulties of working with corals in the laboratory. Here we use the small symbiotic sea
anemone *Aiptasia* as an experimentally tractable model system to analyze the specificity and
timing of symbiosis onset in larval and adult animals under controlled laboratory conditions.

Using four clonal, axenic *Symbiodinium* strains, we found no difference in uptake specificity
between larvae (even when very young) and adults. Although both compatible and incompatible
algal strains were found within the larval guts, only the former appeared to be internalized by
gastrodermal cells, and they (but not incompatible algae) proliferated rapidly within the larvae in
the absence of detectable exchange with other larvae. Older larvae showed reduced ingestion of
both compatible and incompatible algae, and the addition of food failed to promote the uptake of
an incompatible algal strain. Thus, *Aiptasia* adults and larvae appear to have similar mechanisms
for discriminating between compatible and incompatible dinoflagellate types prior to phagocytosis
by host gastrodermal cells. Whether a particular algal strain is compatible or incompatible appears
to be stable during years of axenic culture in the absence of a host. These studies provide a
foundation for future analyses of the mechanisms of symbiont-uptake specificity in this emerging
model system.
INTRODUCTION

Endosymbiosis is a driving factor in evolution that weaves together diverse lineages and is fundamental to the lives of many thousands of species (Wernegreen, 2012). Perhaps the best-known marine endosymbiosis is that of corals and other cnidarians with the dinoflagellate algae (genus *Symbiodinium*) that live within their gastrodermal cells. This symbiosis is critical for the survival and growth of coral reefs because algal photosynthesis provides most of the energy for the coral animals (Yellowlees et al., 2008). Coral reefs are deteriorating rapidly worldwide (Hoegh-Guldberg et al., 2007), in large part because the coral-algal symbiosis can break down under environmental stress ("coral bleaching"). However, the cellular and molecular mechanisms that govern this symbiosis and its breakdown remain largely unknown (Davy et al., 2012).

*Symbiodinium* strains have been categorized into nine major lineages, known as clades A through I (Pochon and Gates, 2010), and the relationships between the many different algal strains and their hosts are governed by complex specificities. Many corals can associate with multiple *Symbiodinium* strains (van Oppen et al., 2001; Apprill and Gates, 2007; Silverstein et al., 2012), and many *Symbiodinium* strains can infect some cnidarian species but not others (LaJeunesse et al., 2004; Coffroth et al., 2010). Most species of reef-building corals produce larvae without symbionts (aposymbiotic) and must acquire them anew from the environment in each generation (Baird et al., 2009). In addition, bleached adult corals may need to re-acquire symbionts from the surrounding seawater to survive (Lewis and Coffroth, 2004; Coffroth et al., 2006). There is considerable evidence that the stress resistance of a particular coral is determined, at least in part, by the intrinsic stress resistance of the *Symbiodinium* type that it contains (Baker et al., 2004; Sampayo et al., 2008). Thus, understanding the specificity patterns and mechanisms of symbiosis establishment and maintenance is critical to both basic coral biology and efforts at coral conservation.

The specificity and dynamics of *Symbiodinium* uptake have been studied in larvae of the temperate anemone *Anthopleura elegantissima* (Schwarz et al., 2002) and of the corals *Fungia* (Schwarz et al., 1999; Weis et al., 2001; Rodriguez-Lanetty et al., 2004; Rodriguez-Lanetty et al.,
2006) and Acropora (Harii et al., 2009; Bay et al., 2011). In all cases, larvae readily took up some
Symbiodinium types but not others, and, in at least some cases, they could take up a wider variety
of algae than were found in adult conspecifics found nearby (Bay et al., 2011). Similarly,
comparisons of the naturally occurring symbiont populations in newly settled juvenile polyps and
nearby conspecific adults have found a greater variety of algae in the juveniles than the adults
(Coffroth et al., 2001; Little et al., 2004; Gómez-Cabrera et al., 2008; Abrego et al., 2009). Taken
together, these data have led to the prevailing hypothesis that juvenile hosts are typically more
permissive than their adult counterparts.

Studies of symbiont specificity in corals face several major limitations that include (i) the
intrinsic difficulties both of field studies and of studies of corals in the laboratory, (ii) the typical
availability of larvae only during the once-yearly spawning season, and (iii) the difficulty or
impossibility of keeping adult colonies alive while they are rendered fully aposymbiotic in
preparation for subsequent re-infection experiments (Coffroth et al., 2010). In addition, the use of
wild populations introduces an unavoidable element of genetic heterogeneity that is problematic
for detailed studies of mechanisms.

The small sea anemone Aiptasia provides a way to avoid these limitations, as it is closely
related to corals and houses similar symbionts, yet is far more tractable in the laboratory (Weis et
al., 2008). Aiptasia also has a growing array of resources available, including large clonal
populations of both symbiotic and aposymbiotic adults (Sunagawa et al., 2009; Lehnert et al.,
2012; Xiang et al., 2013), transcriptomes for both aposymbiotic (Lehnert et al., 2012) and
symbiotic (Lehnert et al., 2013) animals, metabolomic information (Burriesci et al., 2012), and a
method for obtaining regular spawning in the laboratory throughout the year (Perez and Pringle,
2013). Aiptasia has been used previously to study symbiosis specificity in aposymbiotic adult
polyps (Schoenberg and Trench, 1980; Belda-Baillie et al., 2002); as with corals, the animals
selectively took up certain Symbiodinium strains but not others.

In this study, we used aposymbiotic adult polyps from a clonal Aiptasia line, the naturally
aposymbiotic larvae derived by mating this line (male) to any of several clonal female lines, and
four clonal, axenic strains of *Symbiodinium* (Xiang et al., 2013) to perform a tightly controlled comparison of symbiont specificity and accumulation in larvae and adults. For the *Symbiodinium* strains tested, we could observe no differences in specificity between larvae and adults, and compatible strains accumulated rapidly in both larvae and adults even in the absence of further exposure. These studies provide a foundation for future mechanistic studies of these phenomena.

**RESULTS**

**Symbiosis specificity in *Aiptasia* adults**

To examine the specificity of clonal CC7 *Aiptasia* adults for different *Symbiodinium* types, we exposed aposymbiotic animals to equal concentrations of four clonal, axenic algal strains (see Materials and Methods). Strains SSA02 and SSB01 were taken up efficiently by the anemones, whereas strains SSA03 and SSE01 were not (Fig. 1A). Quantification of the algal populations of the anemones after 31 d of exposure showed that the compatible strains were present at concentrations similar to those of the endogenous Clade A algae of stably infected CC7 adults that had been maintained for years under the same environmental conditions (Fig. 1B). The anemones were not fed during these experiments, and those containing compatible algae were larger and appeared healthier (stalks and tentacles more extended; more responsive to stimuli) than animals exposed only to incompatible algae (Fig. 1A); thus, it appears that the compatible algae begin contributing to the host's nutrition soon after uptake.

**Symbiosis specificity in *Aiptasia* larvae**

We next examined the specificity of the naturally aposymbiotic planula larvae (Fig. 2A, day 0) for the same *Symbiodinium* types; in the initial experiments, fresh algae were added with every water change (see Materials and Methods). As in the adults, strains SSA02 and SSB01 were taken up readily: both the percentage of larvae containing algae and the number of algal cells per larva increased steadily over time (Fig. 2A-C). In contrast, strains SSA03 and SSE01 were never found in more than a small percentage of the larvae (Fig. 2B) or as more than one or two algal cells per larva (Fig. 2C). The data in Fig. 2 show that larvae discriminated strongly between compatible
and incompatible algae by 8 days after fertilization, and even younger larvae also appeared capable of such discrimination. In experiments similar to but distinct from those of Fig. 2, we examined larvae at either 5 days or 4 days. At 5 days, 57 of 127 larvae (45%) exposed to the compatible strains appeared to contain algae, in contrast to only 13 of 84 larvae (15%) exposed to the incompatible strains. At 4 days, the corresponding numbers were 28 of 88 (32%) and 2 of 47 (4%). The differences were significant on both days with \( p < 0.01 \) (binomial exact tests).

In unstained samples, compatible algal cells generally appeared to be either within the gastrodermal cells or apposed to the apical surfaces of these cells (Fig. 2A), whereas the incompatible algae generally appeared to be simply within the gut. To examine this question more closely, we stained larvae both with a DNA stain (to reveal the host-cell nuclei) and with fluorescently tagged phalloidin (to stain actin and thus reveal the host-cell cytoplasms). At both early times (Fig. 3A) and late (Fig. 3B), the compatible algae were almost always close to or within the gastrodermal cells: note the bright rims of phalloidin-stained host cytoplasm and/or plasma membrane surrounding the algal cells, as seen particularly clearly around the nucleus and the pair of algal cells – or dividing algal cell – in the region of the inset (Fig. 3B). In contrast, in 64 larvae observed that contained cells of incompatible algal strain SSA03 (37 stained with DAPI plus phalloidin, 27 stained with DAPI only), no examples were found of algal cells that appeared to be within the gastrodermal cells (Fig. 3C and data not shown).

10-15% of young larvae exposed to the incompatible *Symbiodinium* strains contained one or two algal cells (Fig. 2B, 2-day time-point), but larvae containing incompatible algae were rarely seen at later times despite the presence of algae in the environment throughout these experiments. These data initially suggested that older larvae might have an increased ability to discriminate between compatible and incompatible algae and to avoid ingesting the latter (or evict them more quickly). Indeed, when we allowed larvae to age in the absence of algae and then exposed them for 24 h to an incompatible algal type, fewer of them contained algae than when young larvae were given a similar exposure (Fig. 4A). However, in a similar experiment performed with a compatible algal strain, the older larvae also appeared to take up the algae more slowly (Fig. 4B),
suggesting that the observations with the incompatible algae may reflect a general slowdown in algal ingestion in older larvae rather than an improved discrimination against incompatible algal types.

Food particles appear to enhance symbiont uptake in larvae of the corals *Fungia scutaria* and *Acropora digitifera* (e.g., Schwarz et al., 1999), presumably by stimulating a general feeding behavior. To test for a similar effect in *Aiptasia* larvae, and to ask if feeding might promote the uptake (at least transiently) of otherwise incompatible algal types, we exposed larvae to compatible and incompatible *Symbiodinium* strains together with homogenized brine shrimp. Surprisingly, the presence of food appeared to reduce the uptake of both of the algal types tested (Fig. 4C; cf. Fig. 2B).

**Algal proliferation in *Aiptasia* larvae**

The results above suggested that compatible algae proliferated within larvae after they had been taken up, but this conclusion was uncertain because of the addition of fresh algae with each water change. Thus, we performed a wash-out experiment in which young larvae were exposed to compatible algal cells for 8 d and then washed and incubated further in the absence of added algae. After the wash-out, the percentage of larvae containing algae remained essentially constant (Fig. 5A), suggesting that there was little or no exchange of algae with the environment. However, the number of algae per infected larva increased steadily (Fig. 5B), showing that compatible algae indeed proliferate actively in the larvae. Similar proliferation in the absence of added algae is observed in re-infected adults (our unpublished results).

**DISCUSSION**

We exposed aposymbiotic adult and larval *Aiptasia* to four clonal *Symbiodinium* strains representing three of the nine major clades; the algal strains had been grown in axenic culture for ≥1.5 years. The adult *Aiptasia* were from a clonal strain, and the larvae resulted from crosses between this strain and several maternal lines of unknown degrees of relatedness to the male line and to each other. Two of the *Symbiodinium* strains reproducibly infected both the adults and the
larvae, whereas the other two did not. Thus, the compatibility of a particular *Symbiodinium* strain with a particular host is (or at least can be) a genetically determined characteristic that can survive many generations of growth apart from any host. Although the determinants of compatibility are not yet clear, the available evidence suggests that interaction of *Symbiodinium* cell-surface glycans with host lectins may be important (Wood-Charlson et al., 2006; Davy et al., 2012), and the glycan profiles of several *Symbiodinium* strains indeed appear to be stable over multiple generations (Logan et al. 2010). It remains to be determined whether recent prior growth in a particular host can affect the subsequent compatibility of an algal strain with the same or other hosts.

Importantly, the compatibility of a strain did not closely track its cladal identity, as the two compatible strains were from Clades A and B, whereas the other Clade A strain tested was incompatible with this host. This observation is not surprising given the substantial phylogenetic diversity within the major *Symbiodinium* clades (LaJeunesse et al., 2012).

The identical specificities of uptake observed for larvae (even very young larvae) and adults indicate that these life-cycle stages possess similar machineries for distinguishing among algal types; determining whether these machineries are identical or not will require testing a wider range of *Symbiodinium* strains and/or actually elucidating the molecular mechanisms involved in discrimination. Although our initial observations suggested that older larvae might be more discriminating than younger ones at the stage of initial algal ingestion (see below), further investigation suggested that the older larvae were simply less active in ingesting algae of any type. Our results contrast with several previous reports suggesting that coral larvae and juvenile polyps are less specific than adults in *Symbiodinium* uptake (Coffroth et al., 2001; Little et al., 2004; Rodriguez-Lanetty et al., 2004; Gómez-Cabrera et al., 2008; Abrego et al., 2009; Bay et al., 2011). This discrepancy may simply reflect differences in the behavior of the different host species or the limited variety of *Symbiodinium* strains that we have tested to date. However, it may also reflect the differences in experimental protocols. In the previous studies, the infecting algae either were those present naturally in the environment or were freshly isolated from a host animal. Thus, the results obtained may have reflected the presence of a variety of *Symbiodinium* types in the
environment (perhaps including types with marginal compatibility with the hosts tested),

environmental factors (such as the recent presence in a host) that conferred limited compatibility
on an otherwise incompatible algal type, and/or other factors. Such complicating factors may, of

course, play roles in governing *Symbiodinium* uptake by hosts in nature. However, our simplified

protocol, using pairwise combinations of genetically homogeneous algae and hosts, should

provide a more tractable system for elucidating the molecular mechanisms underlying the
discrimination between compatible and incompatible algal strains.

In stable cnidarian-dinoflagellate symbioses, the algae reside within the gastrodermal cells of

the host (Colley and Trench, 1983; Schwarz et al., 1999). Accordingly, we observed that cells of

the two compatible algal strains were found within the gastrodermal cells of *Aiptasia* larvae.

Moreover, these algae were observed to proliferate within the larvae after the removal of

exogenous algae, although it remains unclear whether this proliferation involves division of algae

within host cells accompanied or followed by division of those host cells, release of algal progeny

into the gastric cavity followed by rapid uptake by other gastrodermal cells, or both. In contrast,
incompatible algal cells that had been ingested were always found within the larval gastric cavity, but never within the gastrodermal cells. Although we cannot rule out the possibility that such cells

are phagocytosed but then rapidly recognized as incompatible and evicted by exocytosis, these

observations suggest that the "winnowing" step (Nyholm and McFall-Ngai, 2004) of this

symbiosis occurs at the level of phagocytosis, with only compatible cells actually being taken up

by the host gastrodermal cells. We also found that the gastrodermal cells containing algae

appeared to be concentrated in the aboral half of the gastric cavity (see Figs 2A, 3A,B). These

observations are similar to those made with coral larvae, where incompatible algae were scattered
throughout the gastric cavity, but compatible algae were largely found in the larval equatorial
plane (Rodriguez-Lanetty et al., 2006). Taken together, the data suggest that the gastrodermal

cells in specific regions may be specialized for phagocytosis of *Symbiodinium*.

In a surprising contrast to several previous studies (Schwarz et al., 2002; Harii et al., 2009),

we found that addition of food slowed, rather than accelerated, the acquisition of symbionts.
Although the reasons for this discrepancy remain unclear, it seems likely that the presence of food (particularly at high concentrations) affects symbiosis establishment in complex ways, so that it is a complicating factor probably best avoided in future studies of the mechanisms underlying symbiosis establishment in cnidarians.

The model system described here should facilitate future studies of the specificity and dynamics of symbiont uptake and thus help to define the timing of host-symbiont signaling events, an important step in the molecular dissection of these signals. From an ecological perspective, it should also help to clarify the biological consequences of symbiosis establishment in larval stages, for example by comparing life-span and vitality parameters (such as protein content or growth rate) between larvae with and without compatible symbionts. The availability of two genetically distant but host-compatible *Symbiodinium* strains for the *Aiptasia* system should allow more precise evaluation than has previously been possible of the metabolic costs and benefits of harboring different symbiont types. Obtaining a deeper understanding of the parameters governing successful symbiosis establishment by cnidarian larvae has important implications for efforts to promote coral survival in the increasingly volatile global oceans.

**MATERIALS AND METHODS**

**Organisms and culture conditions**

Experiments with adult *Aiptasia* used animals of clonal line CC7 (Sunagawa et al., 2009) that were maintained in artificial sea water (ASW) as described previously (Xiang et al., 2013). Animals either contained their endogenous Clade A *Symbiodinium* or had been rendered aposymbiotic as described previously (Xiang et al., 2013). Larvae were obtained using the spawning protocol described previously (Perez and Pringle, 2013); each small tub contained one large CC7 animal (male) and one large female (from one of several clonal lines) (Perez and Pringle, 2013). Spawning occurred during the night, and the aposymbiotic planula larvae (~100 \( \mu \text{m} \) in diameter) were collected in the morning, cleaned by rinsing with filter-sterilized ASW (FASW) on a cell strainer with a 40-\( \mu \text{m} \) mesh (BD Falcon, Franklin Lakes, NJ, USA), placed into
sterile 6-well cell-culture plates (BD Falcon) with 200-300 larvae in 5 ml FASW per well, and maintained at 27°C and 25 μmol photons m⁻²s⁻¹ light on a 12L:12D cycle.

Clonal, axenic cultures of *Symbiodinium* strains SSA02, SSA03, SSB01, and SSE01 (in clades A, A, B, and E, respectively; see Xiang et al., 2013) were maintained in IMK medium (Ishikura et al., 2004) at 27°C and 25 μmol photons m⁻²s⁻² light on a 12L:12D cycle, as described previously (Xiang et al., 2013).

**Determinations of algal cell numbers**

For adjustment of inoculum sizes for infection experiments, we determined approximate algal numbers with a hemocytometer. For experiments with adults, we used a Guava flow cytometer (Millipore, Billerica, MA, USA) as described previously (Xiang et al., 2013). For experiments with larvae, we used direct microscopic counts (see below) of red-fluorescent cells. Protein concentrations of homogenates were determined with the BCA Assay (Thermo Fisher, Waltham, MA, USA) according to the manufacturer’s protocol; absorbance readings of the reactions were taken on an Infinite Pro spectrophotometer (Tecan, Männedorf, Switzerland).

**Infection experiments**

Prior to infection experiments, algae were gently rinsed by centrifugation three times at 1000 x g in FASW and quantified (see above). Both adult and larval *Aiptasia* were exposed to algae at a final concentration of ~10⁴ algal cells ml⁻¹, and FASW was used as a negative control.

For infection of adults, small aposymbiotic polyps (1-2 mm in oral-disk diameter) were starved for 2 weeks, distributed into sterile 6-well cell-culture plates (~15 anemones in 5 ml FASW per well), and held at 27°C and 25 μmol photons m⁻²s⁻¹ light on a 12L:12D cycle. A few hours after transfer, rinsed algae (or FASW) were added and mixed with the animals by gentle agitation with a clean pipet. Water and algae were replaced every 7 d, with mixing as above, to maintain water quality and consistent algal exposure; no feeding was performed for the duration of the experiment. At intervals, six random anemones were removed from each well, rinsed gently with FASW, placed in FASW in a sterile Petri dish, photographed using the stereo-fluorescence
microscope (see below), and returned to the culture plates. After photography on Day 31, the animals from each well were pooled in 250 μl of 0.01% SDS (Sigma Aldrich, St. Louis, MO, USA) in distilled water, homogenized for 15 s with a hand-held homogenizer (PowerGen, Fisher Scientific, Waltham, MA, USA), and homogenized further by repeated passages through a sterile 25-gauge needle. Algal numbers and total protein were then determined as described above.

Except as noted, infection experiments with larvae were performed by adding rinsed algae (or FASW) to larvae in the six-well dishes (see above) on the day of collection (Day 0) and mixing by gentle agitation with a clean pipet. Water and algae were replaced every 7 d by transferring individual larvae into fresh wells using a plastic transfer pipet and the stereomicroscope (see below), with mixing as above, to maintain water quality and consistent algal exposure. For sampling, ≥30 random larvae were collected from each well and placed into 1 ml of 3.7% formaldehyde in FASW at 4°C.

In one experiment, we examined the effect of food on algal uptake by adding brine-shrimp homogenate (Lenhoff, 1983) to the wells with each addition of algae. Fresh Artemia nauplii were homogenized for 30 s with the hand-held homogenizer, and portions of the homogenate were added to each well. In another experiment, we tested the effect of larval age on algal uptake by holding larvae without algal exposure or feeding for various periods before adding algae. In a final experiment, we tested whether algal proliferation occurred in the larvae by adding compatible algae to larvae on Day 0 as usual, transferring the larvae after 8 d of exposure into fresh wells containing FASW without algae, and continuing to sample at intervals as in the other experiments.

Except where noted, statistical tests were Student's two-tailed t-tests.

**Microscopy**

Adult anemones were observed and photographed using a model MZ16FA stereo-fluorescence microscope and model DFC500 camera (Leica, Wetzlar, Germany). The same camera settings were used for all images, and exposure times were identical for all images taken on a given day.
To observe and photograph larvae, fixed larvae (see above) were adhered onto glass slides that had been coated with poly-L-lysine (Sigma Aldrich, St. Louis, MO, USA). The larvae were rinsed with Abdil (Tris-buffered saline, pH 7.4, containing 0.1% Triton-X100, 2% bovine serum albumin, and 0.1% sodium azide), and larval and algal nuclei were stained by incubation with Hoechst 33258 in Abdil (10 μg ml⁻¹) for 10 min at room temperature. In some cases, larval actin was also stained by incubating slides with 33 nM Alexa Fluor 488®-phalloidin (Invitrogen, Carlsbad, CA, USA) in Abdil for 1 h at room temperature. Larvae were covered with mounting medium (20 mM Tris, pH 8.8, 90% glycerol, 0.5% p-phenylenediamine) and sealed under a glass coverslip.

To determine the numbers of algae in larvae, samples were counted by eye using a Nikon Eclipse 80i microscope with a Nikon Plan Fluor 40X dry lens and Sedat Quad filter set (Chroma Technology, Bellows Falls, VT, USA) using the Texas Red channel. All larvae on each slide were scored, and algal cells were counted if they appeared to be anywhere inside the larva (i.e., either in the gut cavity or within the gastrodermal cells). When desired, images were also captured using a CoolSnapHQ charge-coupled-device camera (Photometrics, Tucson, AZ, USA). To distinguish algae within the gut cavity from those within the gastrodermal cells, larvae stained with Hoechst and phalloidin were imaged using an Olympus Apo/340 40X oil-immersion lens on an Olympus IX70 microscope equipped with a Deltavision Core system (Applied Precision, Issaquah, WA, USA), a Sedat Quad filter set (Semrock, Rochester, NY, USA), and a CoolSnapHQ camera. The softWoRx® 4.1.0 software package (Applied Precision) was used for microscope control and image deconvolution.

LIST OF ABBREVIATIONS

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ASW</td>
<td>artificial seawater</td>
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<td>FASW</td>
<td>filter-sterilized artificial seawater</td>
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COMPETING INTERESTS

The authors declare that they have no competing interests.
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FIGURE LEGENDS

Figure 1. Symbiosis specificity in *Aiptasia* adults. In each of three experiments, aposymbiotic adults from clonal anemone line CC7 were incubated for 31 d in the absence of algae or in the presence of one of four clonal, axenic *Symbiodinium* strains, as indicated (see Materials and Methods; 15 animals per treatment in each experiment). (A) At intervals, four randomly chosen anemones per treatment were photographed with the stereomicroscope using white light (Anemone) or a GFP filter set (to visualize the chlorophyll autofluorescence of any *Symbiodinium* present: Algae). Representative images are shown. (B) Quantification of *Symbiodinium* populations at Day 31. In each experiment, all 15 animals for each treatment were pooled and homogenized. Algal numbers and total protein amounts were then determined by Guava flow cytometry and the BCA assay (see Materials and Methods). Error bars, s.e.m. (n = 3). Separately, two individual CC7 animals stably populated with their endogenous algae were homogenized and analyzed in the same way, yielding 3,122 and 2,959 algal cells per µg protein.

Figure 2. Symbiosis specificity in *Aiptasia* larvae. In each of three experiments, naturally aposymbiotic *Aiptasia* larvae (<1 day post-fertilization) were incubated for 31 d in the absence of algae or in the presence of one of four clonal, axenic *Symbiodinium* strains, as indicated (see Materials and Methods). (A) Larvae exposed to strain SSA02 were sampled at intervals and imaged using an epifluorescence microscope to visualize overall structure (Phase) and endogenous chlorophyll autofluorescence (Algae; red in Merge). o, oral region; a, aboral region; gc, gastric cavity. Bar, 20 µm. (B-C) Quantification of *Symbiodinium* uptake as determined by epifluorescence microscopy as in a; for these counts, no attempt was made to distinguish algae in the gastric cavity from those within the gastrodermal cells. Means ± s.e.m. from the three experiments are shown. (B) Percentages of larvae that contained ≥1 algal cell. *t*-tests showed that the differences between compatible and incompatible strains at 8 d were significant (*p* values of 0.0005, 0.005, 0.007, and 0.03 for the four comparisons). Neither the difference between the two compatible strains nor that between the two incompatible strains was significant. (C) Numbers of...
algal cells per larva in the larvae containing one or more algal cells; larvae containing no visible
algal cells were not scored. Numbers of larvae scored: strain SSA03, 5-13 per time point; SSA02
and SSB01, 17-85 per strain per time point. ◊, no larvae containing algae were observed. t-tests
showed both that the numbers of algae per larva were significantly different for the compatible
and incompatible algae from 8 days onward (p-values for the indicated comparisons are a, 0.01; b,
0.025; c, 0.0001; and d, 0.0001) and that the numbers of compatible algae per larva increased
significantly over time (p-values for the indicated comparisons are e, 0.02 and f, 0.002).

Figure 3. Localization of algae in Aiptasia larvae. Phase-contrast and deconvolved
fluorescence images (see Materials and Methods) of larvae exposed to compatible (A-B) or
incompatible (C) algal types as in Fig. 2. DNA, Hoechst staining of nuclei (blue in Merge);
Algae, chlorophyll autofluorescence of algal cells (red in Merge); Actin, staining of host cell
cytoplasmic actin with AlexaFluor 488-conjugated phalloidin (green in Merge). (A-B)
Accumulation of compatible algae within the gastrodermal cells of the host. Inset, a region in
which a host nucleus (arrowheads) and two algal cells (or one dividing cell) can be seen
surrounded by phalloidin-stained host cytoplasm. (C) Presence of incompatible algae within the
gastric cavity but not the gastrodermal cells. Bar (all panels), 20 μm.

Figure 4. Reduced uptake of algae in older larvae or in the presence of food. Except as
noted, all panels show the means ± s.e.m. from three replicate experiments. (A) Freshly collected
larvae (<1 d old) were held in the absence of algae for 2 d (to allow their mouths to develop) or 12
days, exposed to an incompatible algal strain for 24 h, and scored by epifluorescence microscopy for
the percentages that contained algal cells (as in Fig. 2). (B) Larvae that had been held in the
absence of algae for 12 d were exposed for the indicated periods to a compatible algal strain and
scored as in A for the percentages that contained algal cells; means ± s.d. are shown for two
replicate experiments. For comparison, similar data for freshly collected larvae (re-plotted from
Figure 2B) are also shown. The younger larvae appeared to have taken up more algae by 8 d than
the older larvae had by 9 d (p ≈ 0.07). (C) Freshly collected larvae were exposed to compatible
and incompatible algal types as in Fig. 2 but with food (homogenized brine shrimp) added
together with each addition of algae (see Materials and Methods). In contrast to the experiment of
Fig. 2B, the apparent difference in uptake of the compatible and incompatible strains was not
significant at 8 d ($p \approx 0.1$), and convincing differences did not appear until later (12 d, $p \approx 0.04$; 21
d, $p \approx 0.03$).

**Figure 5. Proliferation of compatible algae in larvae.** In each of three experiments, freshly
collected larvae (<1 day old) were exposed to compatible algal strains for 8 d, at which time larvae
were transferred to algae-free FASW (“algae wash-out”) and maintained without added algae for
the duration of the experiment. At the indicated times, the percentages of larvae containing ≥1
algal cell (A) and the numbers of algal cells in the larvae containing ≥1 algal cell were scored as in
Fig. 2. Means ± s.e.m. for the three experiments are shown; numbers of larvae scored in B were
21-63 per strain per time point. Significant increases in algal numbers could be seen for both algal
strains between 8 and 21 d (a, $p \approx 0.01$; b, $p \approx 0.02$).
A

<table>
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<tr>
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<th>Day 8</th>
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</tbody>
</table>

Below the images is a bar graph showing the algal cells/µg protein for different conditions:

- **No algae**: Low algal cells/µg protein.
- **SSA02**: Moderate increase in algal cells.
- **SSA03**: Significant increase in algal cells.
- **SSB01**: Further increase in algal cells.
- **SSE01**: Highest increase in algal cells.
- **CC7 endogenous**: Endogenous control with minimal algal cells.
After 24 h exposure to SSA03

% Larvae containing algae

Larval age
- 2-day old
- 12-day old

\( p \approx 0.026 \)

Duration of exposure to SSA02 (days)

% Larvae containing algae

Larval age
- <1-day old
- 12-day old

Age of larvae (days)

% Larvae containing algae

Algal type
- No algae
- SSA02
- SSA03
A

![Graph showing the number of algae per larva over age of larvae in days.](image)

- Algal type: No algae, SSA02, SSB01
- Age of larvae (days): 0, 10, 20, 30
- No. of algae per larva: 0, 5, 10, 15, 20, 25, 30

B

![Bar chart showing the percentage of larvae containing algae.](image)

- Algal type: No algae, SSA02, SSB01
- % Larvae containing algae: 0, 10, 20, 30
- Age of larvae: 2, 8, 12, 21, 31