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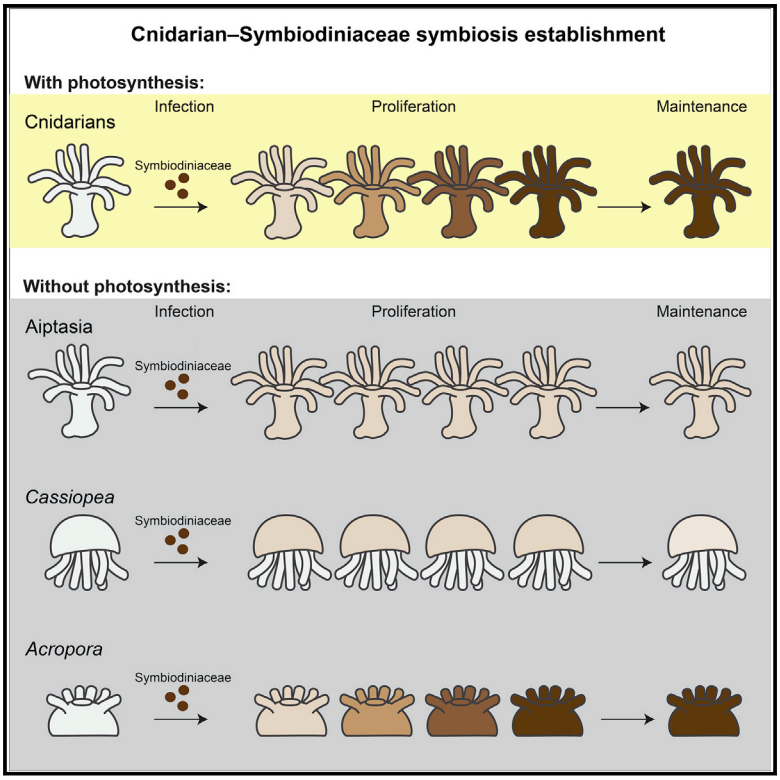
Cnidarian-Symbiodiniaceae symbiosis establishment is independent of photosynthesis

Graphical abstract



Highlights

- Cnidarian-Symbiodiniaceae symbiosis establishment can proceed without photosynthesis
- Infection proceeds independent of photosynthesis in the 31 symbiotic pairs evaluated
- Photosynthesis is required for proliferation in Aiptasia but not in *Acropora* polyps
- *Breviolum minutum* photosynthetic mutants were created to investigate symbiosis

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In brief

Photosynthesis shapes the symbiotic relationships between cnidarian hosts and Symbiodiniaceae algae. Jinkerson et al. show that infection, proliferation, and maintenance can proceed without photosynthesis during symbiosis establishment, but the ability to do so depends on specific host-symbiont pairs.

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Cnidarian-Symbiodiniaceae symbiosis establishment is independent of photosynthesis

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SUMMARY

Photosynthesis shapes the symbiotic relationships between cnidarians and Symbiodiniaceae algae—with many cnidarian hosts requiring symbiont photosynthate for survival—but little is known about how photosynthesis impacts symbiosis establishment. Here, we show that during symbiosis establishment, infection, proliferation, and maintenance can proceed without photosynthesis, but the ability to do so is dependent on specific cnidarian-Symbiodiniaceae relationships. The evaluation of 31 pairs of symbiotic relationships (five species of Symbiodiniaceae in sea anemone, coral, and jellyfish hosts) revealed that infection can occur without photosynthesis. A UV mutagenesis method for Symbiodiniaceae was established and used to generate six photosynthetic mutants that can infect these hosts. Without photosynthesis, Symbiodiniaceae cannot proliferate in the sea anemone Aiptasia or jellyfish *Cassiopea* but can proliferate in the juvenile polyps of the coral *Acropora*. After 6 months of darkness, *Breviolum minutum* is maintained within Aiptasia, indicating that Symbiodiniaceae maintenance can be independent of photosynthesis. Manipulating photosynthesis provides insights into cnidarian-Symbiodiniaceae symbiosis.

INTRODUCTION

Energy entering the biosphere through photosynthesis shapes biotic interactions as it flows between trophic levels. This is thought to be especially true for cnidarian-Symbiodiniaceae symbiosis, such as those found in some species of corals, sea anemones, and jellyfish. Algae from the family Symbiodiniaceae use photosynthesis to convert light energy into chemical energy stored in the form of carbohydrates, lipids, and amino acids. These photosynthetic products can be transferred to hosts that harbor Symbiodiniaceae, providing up to 100% of the energy requirements of some cnidarians.[1–3] The need to capture sunlight has even led some corals to evolve forms that resemble terrestrial plants, which are wholly dependent on photosynthesis.[4–6] Some motile cnidarians, like the golden jellyfish and the sea anemone Aiptasia, have evolved behaviors to keep their symbionts in the sunlight by avoiding shade[7] or moving toward light.[8] In return, for providing the products of photosynthesis, it is thought that Symbiodiniaceae are allowed to shelter within their hosts where they receive nutrients such as inorganic carbon, nitrogen, sulfur, and phosphorus.[9,10]

Cnidarian-algal symbiosis establishment proceeds through three stages that photosynthesis may impact: (1) infection, (2) proliferation, and (3) maintenance. The first stage of symbiont acquisition, referred to as infection throughout this study, proceeds with an initial contact and recognition, followed by engulfment via phagocytosis and a dynamic sorting of symbionts that leads to acceptance or rejection.[9] During infection, the process of symbiont selection has been described as a winnowing process that could involve a number of algal properties and recognition events with the host.[9,11] Photosynthetic ability may be one of these properties evaluated. Studies of the *Hydra-Chlorella* symbiosis suggest that products of photosynthesis, such as maltose, may serve as microbe-associated molecular patterns (MAMPs) that are recognized by cnidarian hosts.[12] In cnidarian-Symbiodiniaceae symbiosis, we previously showed that the *Breviolum minutum* strain SSB01 cultured in media containing glucose lose photosynthetic function in continuous (24 h) light.

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Interestingly, these cells have a reduced ability to infect their host sea anemone. However, it is difficult to establish a causal relationship because many other aspects of algal physiology are also changed, such as cell surface complexity.[13] Additionally, nonphotosynthetic objects, such as food particles or even polystyrene beads, or nonsymbiosis-forming algae, such as *Nannochloropsis oculata*, which are photosynthetic but not expected to exchange photosynthate with the host cells, can be taken up by cnidarians such as the sea anemone Aiptasia.[14–16] However, it is unclear if these undergo the same processes as a symbiont does during infection, leaving an open question about the impact of Symbiodiniaceae photosynthesis on infection.

After infection, the algae proliferate through cell division within the host tissue. The rate at which algae proliferate *in hospite* may be linked to photosynthesis. Studies have shown that algal symbionts that provide less photosynthetic carbon exhibit a slower rate of proliferation in Aiptasia hosts and often achieve lower symbiont densities in the host tissue.[17]

Maintenance occurs after infection and proliferation when a steady-state algal population within the host is reached for a given environmental condition. Some evidence suggests that photosynthesis may be involved in the maintenance of a steady symbiont population level. When treated with the photosynthesis inhibitors 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) or menthol, fully populated corals and sea anemones begin to lose symbionts,[18–21] indicating that photosynthesis may be required for symbiont maintenance. Some studies however have suggested the opposite—that photosynthesis may not be required for maintenance—because fully populated coral and sea anemones kept in the dark for long periods of time may be able to retain some level of symbionts.[22,23]

Despite the importance of photosynthesis in cnidarian-algal symbiosis, there has not been a systematic evaluation of the impact of photosynthesis on the different stages of symbiosis establishment. Much of the data available on this topic are difficult to interpret because there are often confounding variables associated with the experiments, and an evaluation of the effect of photosynthesis on symbiosis was not typically the primary focus of the research.

In this study, we investigate how photosynthesis affects infection, proliferation, and maintenance during cnidarian-Symbiodiniaceae symbiosis establishment by using darkness, a chemical inhibitor of photosynthesis, and photosynthetic mutants. Systematic evaluation of 31 pairs of cnidarian-algal relationships using five symbiotic species of Symbiodiniaceae and three cnidarian hosts (sea anemone, coral, and jellyfish) reveal that infection can occur in the dark. Moreover, we have established a UV mutagenesis method for Symbiodiniaceae and generated six mutants deficient in photosynthesis, all of which can infect the host cells. However, proliferation and maintenance within the host tissue is dependent upon the specific cnidarian-Symbiodiniaceae relationship. Symbiodiniaceae can proliferate in a coral without photosynthesis and also be maintained within sea anemones in continuous darkness for more than 6 months. Taken together, our results indicate that photosynthesis is not required for symbiont infection, whereas proliferation and maintenance without photosynthesis depends upon the specific cnidarian-Symbiodiniaceae relationships.

RESULTS

Symbiont infection occurs in the presence of a photosynthesis inhibitor in the sea anemone Aiptasia

To investigate how photosynthesis affects the three stages of cnidarian-algal symbiosis establishment (infection, proliferation, and maintenance), we used *Breviolum minutum* SSB01[24] and the sea anemone host *Exaiptasia pallida* (referred to as Aiptasia)[25,26] as a model system. *B. minutum* SSB01 was originally isolated from the Aiptasia strain H2. Aiptasia can be made aposymbiotic and live without algal symbionts, which can be added back to allow for the assessment of symbiont infection, proliferation, and maintenance. We first evaluated how the photosynthesis inhibitor DCMU affects the stages of symbiosis establishment using Aiptasia and *B. minutum*. DCMU is a photosystem II (PSII) inhibitor that blocks electron flow from PSII to plastoquinone[27] and is widely used for photosynthesis studies in algae. In Symbiodiniaceae, DCMU has been shown to block PSII activity as indicated by a marked decrease in the F_v/F_m[28,29] and induce the breakdown of symbiosis between *B. minutum* and its cnidarian host, Aiptasia.[30] We inoculated aposymbiotic Aiptasia by adding Symbiodiniaceae algae into seawater containing DCMU and evaluated symbiotic establishment using fluorescence imaging to quantify algal cells within individual anemones. In the presence of DCMU, algal cells were visible 1 day post-inoculation (dpi) within Aiptasia tissue, including the tentacles, indicating that infection of the host had occurred despite disruption in PSII activity (Figures S1A and S1B). No significant difference was observed in the algal cell numbers infected at 1 dpi between control and DCMU (Figure S1C; $p = 0.307$, two-sided Mann-Whitney U). However, tracking and quantification of algal cells within individual anemones revealed that the algae exhibited exponential proliferation without inhibitor but did not proliferate inside the host in seawater supplemented with DCMU (Figures S1B and S1D). Algae were maintained within the tissue of Aiptasia in the presence of DCMU for the duration of the experiment, which was 10 days (Figures S1B and S1D).

Symbiodiniaceae infect and are maintained by Aiptasia in continuous darkness

Although symbiont infection and maintenance can occur in the presence of DCMU, this chemical, in addition to impacting photosynthesis, may also affect other aspects of algal or host biology, such as inhibiting mitochondrial electron transport.[31] To validate the results observed with DCMU, we sought alternative approaches to modulate photosynthesis. Some studies have suggested that darkness can interfere with coral-algal symbiosis;[32–34] however, systematic evaluations of how darkness affects symbiosis establishment are limited. To address this question, aposymbiotic Aiptasia were inoculated with *B. minutum* in the dark and kept in continuous darkness while control anemones were inoculated with *B. minutum* in the light and maintained on a diurnal light-dark cycle, with both samples fed with Artemia twice a week. *B. minutum* cells were found in tentacles at 1 dpi both in light (Figure 1A) and in dark (Figure 1B), indicating that infection can occur without light. No significant difference was observed in the algal cell numbers infected at 1 dpi between light and dark (Figure S1C; $p = 0.131$, two-sided Mann-Whitney U).

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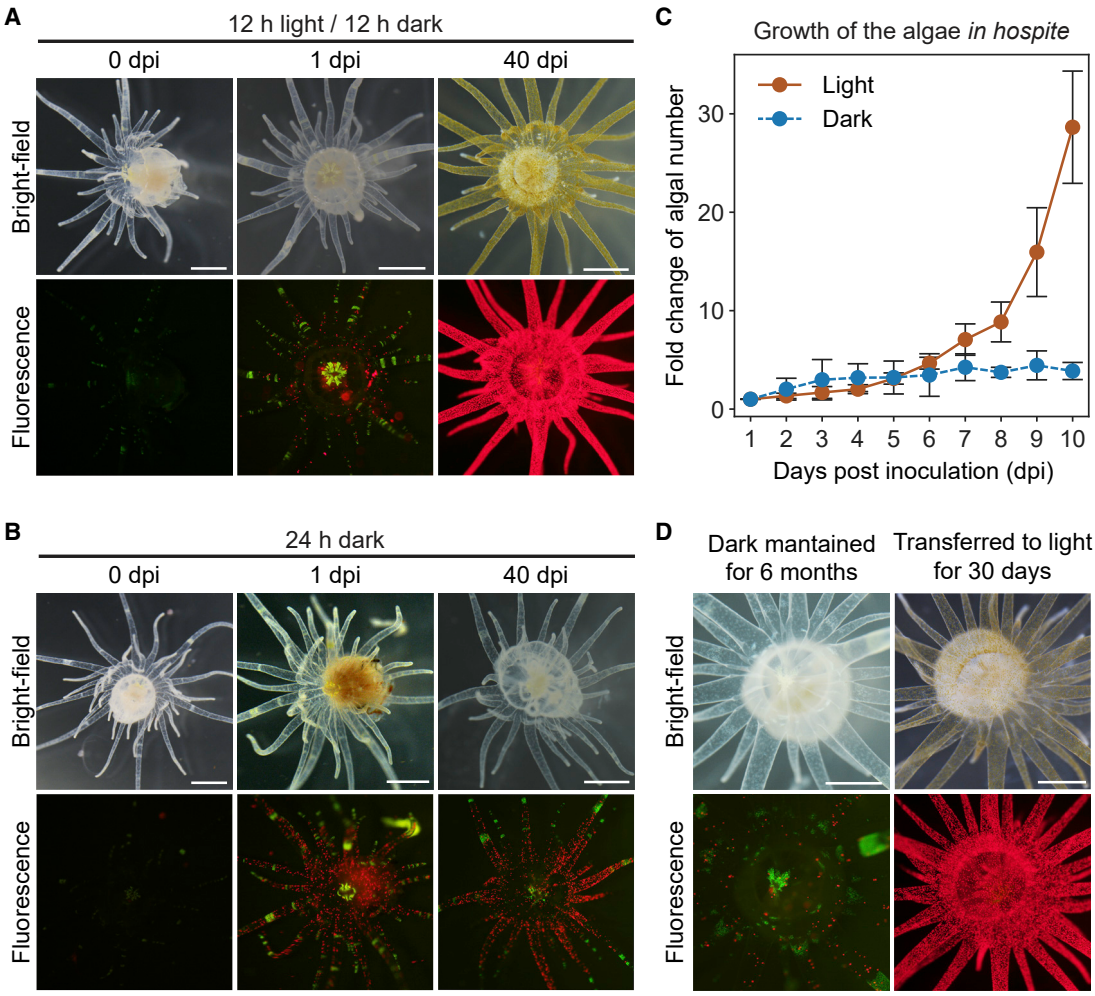

Figure 1. *Breviolum minutum* infect and are maintained within sea anemones kept in dark

(A and B) Representative microscopic images of aposymbiotic sea anemones Aiptasia (*Exaiptasia pallida* CC7) inoculated with algal cells (*B. minutum* SSB01) in (A) light and (B) dark. Animals were then kept in (A) 12 h light/12 h dark and (B) continuous darkness. A top-down view of animals in bright field and fluorescence is shown. Chlorophyll autofluorescence (red) from algal cells is visible 1 day and 40 days post-inoculation (dpi). Scale bars, 1 mm. See also Table S1.

(C) The fold change of algal cell number *in hospite* after initial infection. Error bars represent standard deviation from at least 3 biological replicates. See also Figure S1.

(D) Representative microscopic images of Aiptasia inoculated with *B. minutum* in the dark and then kept in continuous darkness. After 6 months, animals were imaged (left), transferred to 12 h light/12 h dark, and imaged again after 30 days (right). Scale bars, 1 mm.

Infected algal cells were tracked and quantified inside the tissue of individual anemones to evaluate their ability to proliferate. In the light, the algal cells grew exponentially in the host tissue for over 10 days, after which quantification of individual cells through fluorescence imaging was no longer possible due to high algal cell density (Figures 1A and 1C). In contrast, the number of algal cells in the dark remained relatively constant during the entire 40-day experiment (Figures 1B and 1C). In the dark, the algal cells were not able to proliferate and increase their numbers within the host but were able to be maintained at a constant level during the 40 days.

To determine if the products of photosynthesis stored within Symbiodiniaceae contribute to the infection process, *B. minutum* cells were cultured in the IMK minimal medium in the dark for 21 days prior to the inoculation of aposymbiotic Aiptasia in an attempt to deplete them of any stored photosynthate. Even with this dark pretreatment without photosynthesis, these cells were still capable of infecting Aiptasia either in dark or in light (Figures S1E and S1F).

To investigate if algae could be maintained in host tissue without photosynthesis for longer periods of time, we kept dark-infected animals in continuous darkness for 6 months. Algal symbionts were still visible within the Aiptasia tentacles after 6 months of darkness (Figure 1D). Animals were then transferred to the light to determine if the visible algae were viable. Once in the light, the algae began to proliferate and reached high density in the host tissue (Figure 1D), confirming that the cells remaining in the tissue in the dark were still viable. Similar to the results seen with the DCMU treatment, algae were able to infect and be maintained within Aiptasia in the dark; however, in both cases, there was limited or no proliferation.

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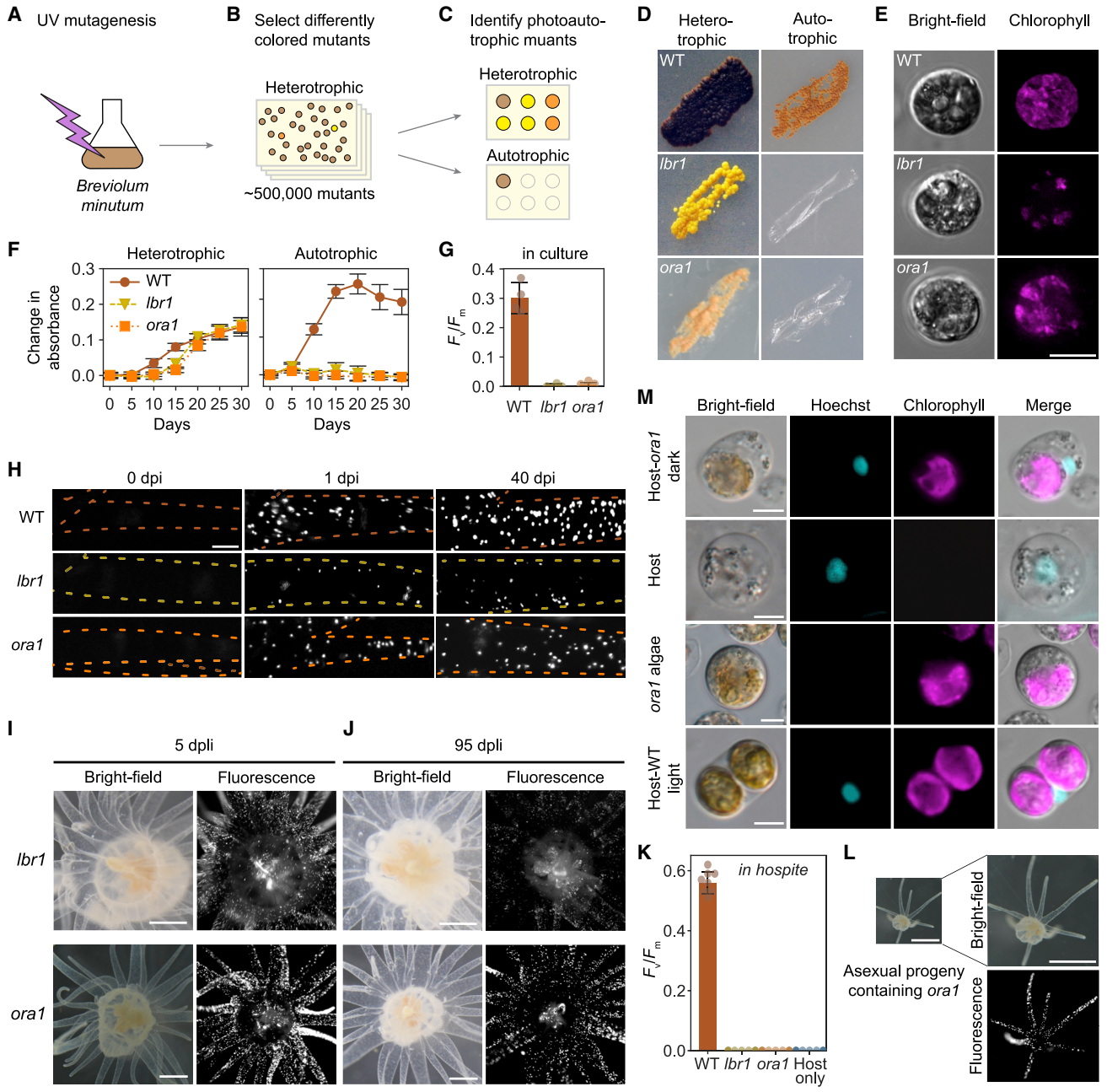

Figure 2. *Breviolum minutum* photosynthetic mutants infect and are maintained within sea anemones

(A–C) Schematic overview for generating *B. minutum* SSB01 photosynthetic mutants. (A) *B. minutum* cultures were subjected to UV mutagenesis and plated, (B) mutants with altered colors were identified, and (C) replica plated to autotrophic (MB in the light) and heterotrophic (MB with glucose in continuous darkness) conditions to identify photosynthetic mutants.

(D) Images of *B. minutum* wild type (WT), *LESS BROWN 1* (*lbr1*), and *ORANGE 1* (*ora1*) grown on agar plates under heterotrophic or autotrophic conditions for 30 days.

(E) Representative microscopic images of WT, *lbr1*, and ora*1* cells. Left image is bright field; right image is chlorophyll autofluorescence. Scale bars, 5 μm.

(F) Growth curve of WT, *lbr1*, and *ora1* in liquid culture in heterotrophic or autotrophic conditions. Error bars represent SD from at least 5 biological replicates. See also Table S2.

(G) Maximum PSII efficiencies F_v/F_m of WT, *lbr1*, and *ora1* cultures grown in the dark with 5 $g \cdot L^{-1}$ glucose. Error bars represent SD from at least 3 biological replicates.

(H) Representative fluorescence microscopy images of infected sea anemones Aiptasia (*Exaiptasia pallida* CC7) tentacles. Aposymbiotic Aiptasia were inoculated with WT, *lbr1*, and *ora1* cells in dark and then kept in continuous darkness. Chlorophyll autofluorescence (white dots) from algal cells are visible 1 day and 40 days post-inoculation (dpi) in tentacles. Edges of the tentacles are indicated by dashed lines. Scale bars, 100 μm. See also Figure S2 and Table S1.

(legend continued on next page)

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The nature of a host-symbiont relationship can be very species specific;[24,35,36] hence, we evaluated how the lack of photosynthesis impacts symbiosis establishment in four other Symbiodiniaceae species previously shown to form symbiosis with Aiptasia. *Symbiodinium linucheae* (strain SSA01), *Cladocopium goreaui* (strain rt-152 and LHI-33), *Durusdinium trenchii* (strain CCMP2556), and *Symbiodinium necroappetens* (strain MAC-225 and SSA02) were all able to infect Aiptasia in the dark but were not able to proliferate within the host tissue (Table S1). However, differences in the symbionts' ability to be maintained within the host tissue were observed. Some symbionts like *B. minutum* or *S. linucheae* were able to be maintained without noticeable change or with only slight decreases. Other strains such as *C. goreaui* LHI-33, *C. goreaui* rt152, and *D. trenchii* CCMP2556 were gradually lost in continuous darkness, but they were still able to be maintained a minimum of 4 times longer than inert fluorescent beads (Table S1). Symbionts from native host-symbiont pairs seemed to be maintained longer than heterologous pairs that were lost at a faster rate. This suggests that symbiont maintenance was dependent on the specific host-symbiont relationship in Aiptasia.

Breviolum minutum SSB01 photosynthetic mutants were generated

In the previous dark experiments, every effort was made to keep the experiments in the dark, but imaging and feeding the animals exposed them for very short periods of time to low levels of light. To eliminate the possibility that this incident light exposure was influencing these experiments and to further investigate the role of photosynthesis in symbiosis establishment, we sought to generate *B. minutum* mutants deficient in photosynthesis. Our previous work has shown that *B. minutum* can grow in the dark heterotrophically on glucose,[13] suggesting that it is not an obligate photoautotroph. We hypothesized that this metabolic flexibility could be exploited for the creation of photosynthetic mutants that require heterotrophic sources of nutrients. The limited availability of genetic tools in Symbiodiniaceae,[37] however, forced us to explore classical approaches for mutant generation. UV mutagenesis has been broadly used in many model organisms, including other species of algae.[38–41] We exposed *B. minutum* cultures to different doses of UV radiation and determined their survival rates (Figures S2A and S2B). We selected a target survival rate of less than 1% and used the corresponding UV dosages to generate a mutant library (Figure 2A), which was subsequently plated on a glucose-containing medium to allow for heterotrophic growth.

As a primary screen of the mutant library, we sought mutant colonies that had an altered pigment phenotype (Figure 2B). A similar approach to screen for colorless or less colored phenotypes in the green alga *Chlamydomonas reinhardtii* has identified many photosynthesis-deficient mutants.[41] Following our primary screen, putative mutants were transferred to a medium without glucose where photosynthesis-deficient mutants would not be able to grow (Figure 2C). We identified a mutant that was less brown in color, *LESS BROWN 1* (*lbr1*), and a mutant that was orange in color, *ORANGE 1* (*ora1*). Both of these mutants could not be grown photoautotrophically as colonies on agar medium plates (Figure 2D) or in a liquid medium (Figure 2F). Fluorescent microscopy of *lbr1* and *ora1* showed less chlorophyll fluorescence compared with *B. minutum* wild type (WT) (Figure 2E). To evaluate the photosynthetic function of the two mutants, we measured the maximum quantum efficiency of PSII as indicated by F_v/F_m. The F_v/F_m of *lbr1* and *ora1* decreased to zero, indicating the loss of PSII function in these mutants (Figure 2G). These mutants represent the first photosynthetic mutants isolated in Symbiodiniaceae.

B. minutum photosynthetic mutants can infect and be maintained in Aiptasia

We next assessed the ability of the photosynthetic mutants to establish symbiosis with Aiptasia. Aposymbiotic Aiptasia were inoculated with photosynthetic mutants in the dark. Observation at 1 dpi identified mutant *lbr1* and *ora1* cells in the tentacles, indicating that algae incapable of photosynthesis can infect Aiptasia (Figure 2H). Tracking of the infected mutants in individual anemones indicated that they were not able to proliferate inside of Aiptasia but were able to be maintained at a steady-state population during the course of the entire 40-day experiment (Figure 2H). To validate the symbiosis establishment results observed with *lbr1* and *ora1*, four additional *B. minutum* photosynthetic mutants were isolated. These mutants were yellow in color and thus were named *YELLOW 7* (*yel7*), *YELLOW 8* (*yel8*), *YELLOW 9* (*yel9*), and *YELLOW 12* (*yel12*) (Figures S2C and S2D). Mutants *yel7*, *yel8*, *yel9*, and *yel12* cells were observed within Aiptasia tentacles 3 dpi (Figure S2C), and tracking tentacles of individual infected anemones over time showed that these mutant algal cells cannot proliferate but can be maintained in the host over long periods of time (Figure S2C), similar to what was observed for *lbr1* and *ora1*.

To evaluate if there was a limit to the number of nonphotosynthetic algal cells that could be maintained in the host tissue, aposymbiotic Aiptasia were inoculated multiple times with the photosynthetic mutants. This allowed high densities of *lbr1* and

(I and J) Aiptasia can host high densities of photosynthetic mutants after multiple inoculations. Representative microscopy images of aposymbiotic Aiptasia H2 inoculated multiple times with algal photosynthetic mutants *lbr1* or *ora1* in the dark. Animals were then kept in continuous darkness for more than 3 months without additional inoculations and then imaged. In the top-down view of animals, chlorophyll autofluorescence (white) from algal cells is visible in tentacles 5 (I) and 95 (J) days post-last inoculation (dpli). Scale bars, 1 mm.

(K) Aiptasia inoculated with *B. minutum* WT, *lbr1*, or *ora1* in the dark and then kept in continuous darkness were evaluated for maximum PSII efficiencies F_v/F_m of the *in hospite* algae. Aposymbiotic Aiptasia (host only) was used as a control. Error bars represent SD from at least 5 biological replicates.

(L) During the 3 months of darkness without algae inoculation, a number of anemones (from J) propagated asexually by pedal laceration. These offspring contained photosynthetic mutants, indicating that nonphotosynthetic symbionts can be transmitted vertically. Small image is to scale with *ora1*-infected animals in (J), whereas the larger image is zoomed in for clarity. Scale bars, 1 mm.

(M) Representative microscopy images of a dissociated Aiptasia CC7 cell enclosing an *B. minutum ora1* mutant cell from an animal kept in the dark (Host-*ora1* dark), aposymbiotic Aiptasia host cell only (Host), *B. minutum ora1* cells only (*ora1* algae), and an Aiptasia cell enclosing two *B. minutum* WT cells from an animal kept in the light (Host-WT light). Cyan, cell-permeable Hoechst 33342 fluorescent nucleic acid stain; magenta, algal chlorophyll fluorescence. Scale bars, 5 μm.

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ora1 photosynthetic mutants to be observed in host tissue (Figure 2I). More than three months after the last inoculation, high densities of algae remained (Figure 2J) that maintained the visual differences between the *B. minutum* WT and mutants (Figures S2E and S2F) and also lacked the ability to perform photosynthesis (Figure 2K). No obvious deleterious phenotypes were observed for these animals, such as host death or formation of "tissue balls." After the last inoculation, several anemones propagated by pedal laceration. These offspring contained photosynthetic mutants (Figure 2L), indicating that nonphotosynthetic symbionts can be transmitted vertically in Aiptasia through pedal laceration.

Finally, to determine if the photosynthetic mutant cells observed through fluorescent microscopy are maintained intracellularly without photosynthesis, Aiptasia tentacles from anemones infected with *ora1* and kept in the dark for more than 3 months were dissociated into single cells. Within some intact dissociated host cells, as indicated by the presence of a stained nucleus, *ora1* cells were also observed (Figure 2M). These observations indicate that *ora1* cells are intracellularly localized because in controls of just algae, stained nuclei were not seen, and when algae were added to dissociated cells from aposymbiotic animals, there was no colocalization (Figure 2M). Together, these data indicate that symbiont infection and maintenance are independent of photosynthesis in Aiptasia.

Symbiosis establishment occurs in the coral *Acropora* without photosynthesis

Symbiodiniaceae can form symbiotic relationships with many species of cnidaria; hence, we next assessed if the results using the Aiptasia-Symbiodiniaceae system were similar to what was observed for other cnidarian-Symbiodiniaceae symbioses. The stony coral *Acropora tenuis* (referred to as *Acropora*), natively hosting *Symbiodinium tridacnidorum* (ITS2 type A3) as the dominant symbiont type in the juvenile stage,[42] has also been shown to form a symbiotic relationship with *B. minutum* in the light;[14] hence, we used aposymbiotic coral larvae and polyps from this species to evaluate the impact that photosynthesis has on symbiosis establishment in this host. We first inoculated *Acropora* larvae by adding to the seawater *B. minutum* WT in the light and *B. minutum* WT and *ora1* in the dark. Microscopic observation 3 dpi revealed that the algae were present within the larvae in all treatment conditions (Figure 3A). Larvae in the light infected at levels similar to what has been reported previously,[14] but in continuous darkness, the levels may be slightly lower (Figure 3B; $p = 0.043$, one-tailed t test), which may be due to differences in larvae behavior in the dark.[43] In the light, algae proliferated during a 7-day period of observation, whereas levels of *B. minutum* WT and *ora1* in continuous darkness did not appear to change after the initial infection (Figure 3A). To determine if algal symbionts are maintained during the transition from larva to polyps independent of photosynthesis, we induced metamorphosis of dark-infected larvae in the dark and found that *B. minutum* WT were retained despite a lack of photosynthesis (Figure 3C).

Next, we examined the impact that photosynthesis has on symbiosis establishment in *Acropora* polyps. Aposymbiotic polyps were inoculated in the dark by adding to the seawater various Symbiodiniaceae species (*B. minutum* SSB01, *S. linucheae* SSA01, *C. goreaui* LHI-33, *D. trenchii* CCMP2556, and *S. necroappetens* MAC-225 and SSA02) and also with the *B. minutum* photosynthetic mutants (*ora1*, *yel7*, *yel8*, *yel9*, and *yel12*). All inoculations resulted in infections in which algae could be observed in the coral tentacles (Figures 3D and S3A). To evaluate the specificity of the initial infection, we compared the retention time of algal cells with fluorescent beads in the coral host tissue. Less than 50% of polyps had internalized the beads post-inoculation, which were subsequently lost/expelled after a few days (Figure S3A), whereas algal cells without photosynthesis were retained (Figure 3E). Tracking individual polyps infected with *B. minutum*, *C. goreaui* LHI-33, *S. necroappetens* MAC-225, or *S. necroappetens* SSA02 in continuous darkness over several days revealed that these algae were able to proliferate *in hospite* (Figures 3D and S3A). The *B. minutum* photosynthetic mutants (*ora1, yel7, yel8, yel9*, and *yel12*) were also able to proliferate (Figures 3D and S3A), eliminating the possibility of light contamination leading to the proliferation observed with the WT strains. The algae from these polyps inoculated with mutants had the same visual differences (Figure S3B) as previously observed between *B. minutum* WT and mutants (Figure 2), suggesting that these algae were not WT Symbiodiniaceae. Quantification of algal proliferation in individual polyps revealed that the proliferation rate of *B. minutum* WT and *ora1* in continuous darkness is lower than that of *B. minutum* WT in the light (Figure 3F). To verify that the algae observed in the coral polyps are in fact intracellular, polyps infected with *ora1* in the dark were dissociated into single cells. Within some intact dissociated coral cells, as indicated by the presence of a stained nucleus, individual or multiple *ora1* cells were also observed (Figure 3G). Proliferation, however, was not observed for all Symbiodiniaceae species, indicating that it may depend on the host-symbiont relationships. For example, *S. linucheae* were found in tentacles, but proliferation was not observed, and algal levels remained constant during the observation period (Figure 3D). For symbiont maintenance, *B. minutum*, *C. goreaui*, *S. necroappetens*, and all five *B. minutum* photosynthetic mutants were able to be maintained within coral polyps in continuous darkness, without polyps being fed, for at least 60 dpi without noticeable decrease (Table S1). During this time, no visible changes in polyp size were observed for polyps kept with or without photosynthesis (Figures S3C and S3D). Taken together, these data indicate that symbiosis establishment in *Acropora* can be completely independent of photosynthesis, with proliferation of symbionts even possible *in hospite* without photosynthesis.

Symbiont infection occurs without photosynthesis in the jellyfish *Cassiopea*

The upside-down jellyfish *Cassiopea xamachana* (referred to as *Cassiopea*) is another cnidarian that hosts Symbiodiniaceae and is an emerging model for evaluating cnidarian symbiosis.[44] We isolated *Cassiopea* from the Florida Keys that harbor native symbiont *Symbiodinium microadriaticum* and used the strain in the same genus *Symbiodinium linucheae* SSA01 to evaluate symbiosis establishment. *Cassiopea* and *S. linucheae* have not previously been used together for experiments; hence, we first determined if they can form a stable symbiosis under standard conditions in the light. *S. linucheae* was able to infect and

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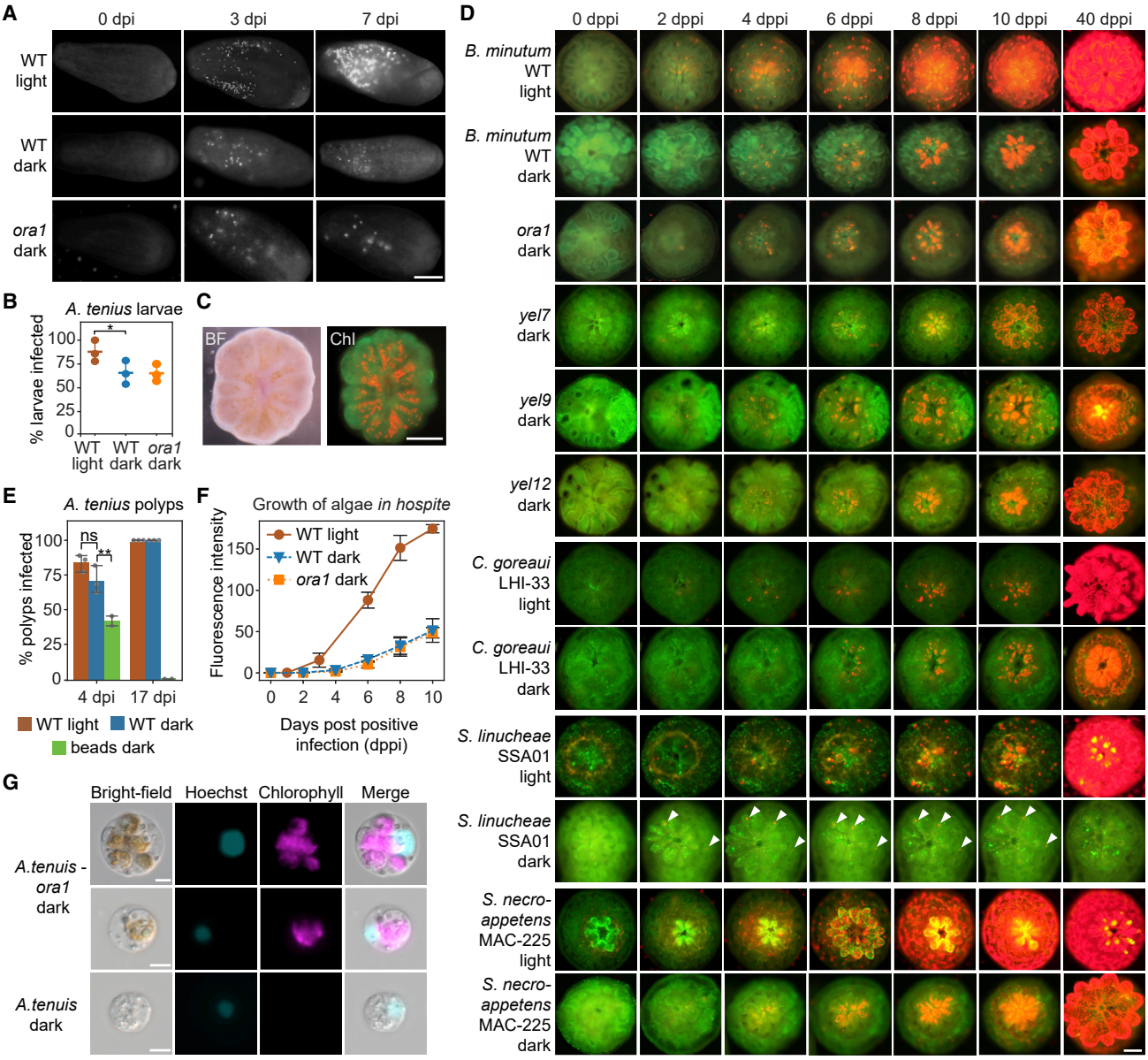

Figure 3. Symbiodiniaceae infect, proliferate, and are maintained within coral polyps without photosynthesis

(A) Representative fluorescence microscopy images of aposymbiotic *Acropora tenuis* (*Acropora*) larvae inoculated with *Breviolum minutum* SSB01 wild type (WT) in 12 h light/12 h dark (WT light) or in the dark (WT dark), or *ORANGE 1* (*ora1*) in the dark. Chlorophyll autofluorescence (white dots) from algal cells are visible 3 and 7 days post-inoculation (dpi) of the larvae. For WT light larvae were then kept in 12 h light/12 h dark. WT dark and *ora1* dark were then kept in continuous darkness. Scale bars, 200 μm.

(B) Percentage of infected *Acropora* larvae with WT in the light, WT in the dark, and the *ora1* mutant in the dark. Horizontal bar indicates the average and the vertical line the data range. For each replicate, n ≥ 10. p value (one-tailed t test) for the significance of WT light versus WT dark differences is indicated (*p = 0.043).

(C) Representative images of an *Acropora* polyp that recently metamorphosed from a larval planula that was inoculated with WT while kept in continuous darkness. Left image is bright field (BF); right image is fluorescence with algal chlorophyll (Chl) autofluorescence in red. Scale bars, 200 μm.

(D) Representative fluorescence microscopy time course images of individual aposymbiotic juvenile *Acropora* polyps that were inoculated with different Symbiodiniaceae species and photosynthetic mutants. Polyps were then kept in 12 h light/12 h dark (light) or in continuous darkness (dark). Algal cells are visible in the top-down view of polyps by their chlorophyll autofluorescence (red) 2 days post-positive infection (dppi). White-colored arrows indicate the positions of *S. linucheae* SSA01 alga in the polyps. Scale bars, 200 μm. See also Figure S3A and Table S1.

(E) Percentage of infected *Acropora* polyps with WT or fluorescent beads 4 and 17 dpi. Data are from 2 or 3 biological replicates with over 34 polyps per replicate. Error bars represent standard deviation with the p values (two-sided t test) indicated (**p < 0.01; ns, p > 0.05).

(F) Growth of algal cell population in *Acropora* polyps quantified by algal chlorophyll fluorescence. Error bars represent standard deviation from at least 3 biological replicates. See also Table S2.

(G) Representative microscopy images of dissociated coral *A. tenuis* polyp cells containing *ora1* cells from animals kept in the dark (top and middle panels) and an aposymbiotic coral cell without algae. Cyan, cell-permeable Hoechst 33342 fluorescent nucleic acid stain; magenta, algal chlorophyll fluorescence. Scale bars, 5 μm.

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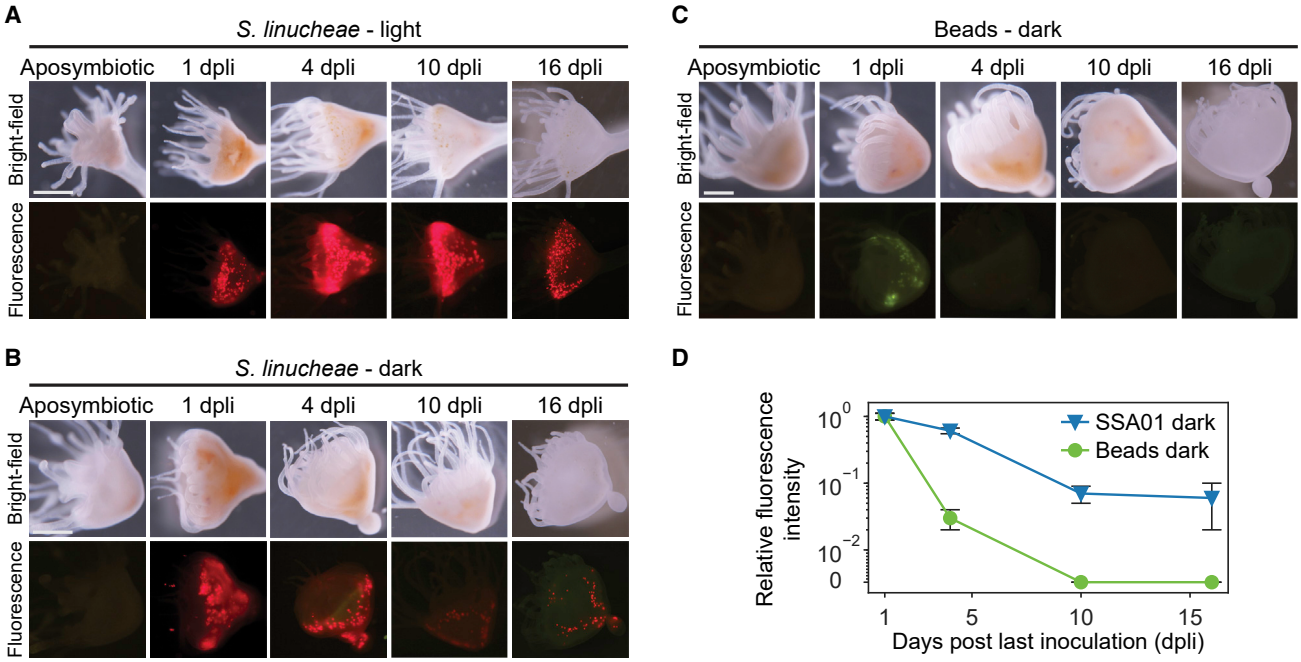

Figure 4. *Symbiodinium linucheae* infects and maintains within jellyfish polyps without photosynthesis

(A–C) Representative microscopic images of aposymbiotic upside-down jellyfish *Cassiopea* (*Cassiopea xamachana*) polyps inoculated with *S. linucheae* SSA01 or green fluorescent beads in the (A) light and (B and C) dark. Animals were then kept in (A) 12 h light/12 h dark and (B and C) continuous darkness. The calyx portion of the polyps were imaged, and algal cells are visible by their chlorophyll autofluorescence (red) 1 day post-last inoculation (dpli). Scale bars, 0.5 mm. See also Figure S4 and Table S1.

(D) Algal cell population and bead levels in *Cassiopea* polyps quantified by chlorophyll and bead fluorescences. Error bars represent standard deviation from at least 3 biological replicates.

proliferate within aposymbiotic *Cassiopea* and was found in buds constricted off from the calyx of the polyp (Figure S4A). *S. linucheae* also induced *Cassiopea* to undergo strobilation (Figure S4A), a metamorphic change from polyp to ephyra that requires symbionts in this species.[44] These results demonstrate that *S. linucheae* can form a stable symbiosis with *Cassiopea*. To determine how the lack of photosynthesis affects symbiosis establishment for this host-symbiont pair, aposymbiotic *Cassiopea* polyps were inoculated by adding to the seawater *S. linucheae* either in the light and kept in 12 h light/12 h dark (Figure 4A) or in the dark and kept in continuous darkness (Figure 4B). Polyps were also inoculated with fluorescent beads as a control (Figure 4C). In the dark without photosynthesis, *S. linucheae* was able to infect and be maintained within *Cassiopea* tissue longer than the beads (Figure 4D; Table S1). Eventually, without photosynthesis, the algae were gradually lost.

We next evaluated how photosynthesis affects symbiosis establishment between *B. minutum* and *Cassiopea*. First, we demonstrated that *B. minutum* can form a stable symbiosis with *Cassiopea*. Like *S. linucheae*, *B. minutum* was able to infect, proliferate, and induce strobilation (Figure S4B); however, in order to achieve a robust infection, *B. minutum* required more rounds of inoculation than *S. linucheae*, which may be due to *B. minutum* not being a native symbiont. Nevertheless, without photosynthesis, *B. minutum* WT, *lbr1*, and *ora1* were all able to infect and be maintained within *Cassiopea* polyps longer than beads (Figures S4C–S4F; Table S1); however, no strobilation was observed. Taken together, these results indicate that photosynthesis is not required for Symbiodiniaceae to infect and maintain in *Cassiopea*.

DISCUSSION

In this work, we determined how symbionts that lack the ability to perform photosynthesis impact infection, proliferation, and maintenance during cnidarian-Symbiodiniaceae symbiosis establishment (Figure 5). We discovered that each step of symbiosis establishment can proceed without photosynthesis, but the ability to do so is dependent on specific cnidarian-Symbiodiniaceae relationships. For example, in the absence of photosynthesis, species of *Breviolum* and *Symbiodinium* can infect, proliferate, and be maintained in the coral *Acropora*, but in the jellyfish *Cassiopea*, they can only infect, do not proliferate, and are gradually lost. These results give insights into the nature of specific cnidarian-Symbiodiniaceae relationships, highlighting the diversity of interactions that are not universal across symbiont-host pairs and into the mechanisms of symbiosis establishment.

Infection proceeds independent of photosynthesis in 31 cnidarian-Symbiodiniaceae relationships evaluated

Previous studies have shown that the initial uptake of would-be symbionts by cnidarian hosts can be nonspecific, exemplified by the uptake of beads, incompatible species, or even heat-killed algae.[16,45–48] However, these are quickly lost by expulsion. We discovered that Symbiodiniaceae without photosynthesis

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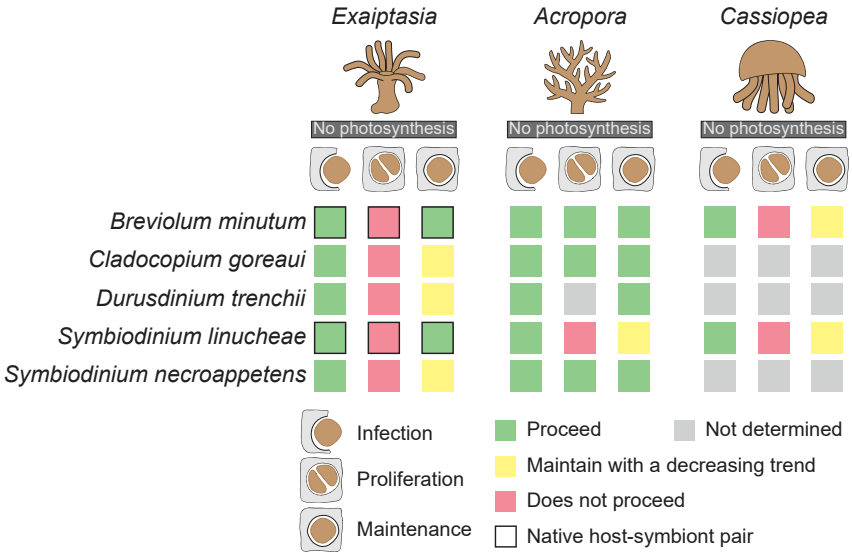

Figure 5. Symbiont infection, proliferation, and maintenance are independent of photosynthesis in select cnidarian-Symbiodiniaceae relationships

Summary of results. With photosynthesis cnidarian-Symbiodiniaceae symbiosis, establishment proceeds through infection, proliferation, and maintenance stages (not shown). Without photosynthesis from either a lack of light or from mutants incapable of photosynthesis, infection still occurs in the sea anemone Aiptasia (*Exaiptasia pallida*), the coral *Acropora* (*Acropora tenuis*), and the jellyfish *Cassiopea* (*Cassiopea xamachana*). Proliferation of Symbiodiniaceae within Aiptasia or *Cassiopea* is extremely limited without photosynthesis, but it can occur in the coral *Acropora*. Maintenance of Symbiodiniaceae within host tissue can occur without photosynthesis but may depend on the specific host-symbiont relationship. For example, *Breviolum minutum* can remain at relatively constant levels in Aiptasia for more than 6 months (green), whereas in the jellyfish *Cassiopea*, the algae are retained longer than beads but are gradually lost (yellow).

can be taken up but are not quickly lost, lasting at least 4 times longer than beads or incompatible species (Table S1). Our results suggest that the specific interpartner molecular signaling that occurs during infection between Symbiodiniaceae and the host that is necessary for symbiosis establishment is independent of photosynthesis or products of photosynthesis. These findings allow for insights to be made into some of the leading theories regarding infection. In one model of infection, it is thought that recognition of carbohydrates on the surface of Symbiodiniaceae cells (symbiont-associated MAMPs) by specific host receptors (pattern recognition receptors) play a critical role in the early stages of symbiosis establishment.[9,49] Our data suggest that photosynthesis does not affect this recognition, and production of these carbohydrates is not dependent on photosynthesis. These carbohydrates can persist without photosynthesis and may not be affected by recent photosynthetic status, as *B. minutum* kept in the dark in an autotrophic medium for 3 weeks were still able to successfully infect Aiptasia (Figures S1E and S1F). It has also been hypothesized that the transfer of products of photosynthesis may serve as interpartner signaling molecules to help establish symbiosis specificity. These may serve as a "token" that is transferred to the host in order to facilitate symbiosis establishment. Newly fixed carbon has been found in the sugar-alcohol *scyllo*-inositol, a leading metabolite thought to be involved in symbiosis recognition and signaling that has been found to be more abundant in native symbioses.[50–52] However, without photosynthesis, the ability to fix carbon to produce *scyllo*-inositol or other potential carbon-based interpartner signaling molecules is likely to be very limited, calling into question the role of newly fixed carbon metabolites in signaling specificity, especially if large concentrations are needed. Finally, light perception and signaling govern many aspects of biology and symbiosis[53,54] and may regulate important cnidarian-Symbiodiniaceae interactions.[8] Our results suggest that infection can proceed without light perception or light signaling, and molecular processes essential for infection, such as phagocytosis and sorting of symbionts, do not seem to require light or photosynthesis.

Photosynthesis is not required for proliferation in some cnidarian-Symbiodiniaceae relationships

Symbiodiniaceae have a flexible metabolism that allows them to grow and divide in a variety of environments.[24,55] Supplied only with inorganic nutrients and light, Symbiodiniaceae can grow autotrophically with photosynthesis. However, if given fixed carbon sources, they can grow heterotrophically.[13,15,56] When Symbiodiniaceae are residing *in hospite*, all of the nutrients required for either autotrophic or heterotrophic cell growth and division must be obtained from the host. By denying Symbiodiniaceae the ability to perform photosynthesis, insights about the host environment and the nature of cnidarian-Symbiodiniaceae relationships can be made.

We found that without photosynthesis Symbiodiniaceae proliferation was not observed in the sea anemone Aiptasia, the jellyfish *Cassiopea*, or larvae of the coral *Acropora*, suggesting several possibilities: (1) Without photosynthesis, Symbiodiniaceae cannot grow autotrophically, and because proliferation is not observed *in hospite*, this suggests that these hosts do not provide enough fixed carbon or energy to support the heterotrophic growth of symbionts. Even when Aiptasia and *Cassiopea* are fed, no algal proliferation is observed, suggesting that no or very limited fixed carbon are provided to Symbiodiniaceae by these hosts, even when it is available. This would indicate that under these environmental conditions, fixed carbon and energy can only flow in one direction—from Symbiodiniaceae to the host. Additionally, it seems to suggest that the Symbiodiniaceae evaluated cannot parasitize these hosts in these environmental conditions because if this symbiosis mode were possible, algal growth would be observed but is not. (2) An alternative hypothesis is that fixed carbon is provided by the host, but lack of another key nutrient(s) is limiting Symbiodiniaceae proliferation. Without the production of photosynthate by the symbiont, the typical nutrient exchange between host and symbiont may be disrupted, limiting the flow of inorganic nutrients to the symbiont that could limit or stop its proliferation, even if a fixed carbon source from the host is available for heterotrophic growth.

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Symbiodiniaceae from three species were unexpectedly observed proliferating in *Acropora* polyps without photosynthesis. Several possibilities may explain the heterotrophic growth of Symbiodiniaceae observed: (1) The coral host provides both inorganic and fixed carbon nutrients to the algae to promote algal proliferation. During the course of our experiment, the coral were not fed; hence, all sustenance provided to the algae had to come internally from the polyps themselves. This allowed *B. minutum* to grow heterotrophically *in hospite* in the dark at a rate 3.5-fold faster than growing *ex hospite* autotrophically with photosynthesis (Table S2). *Acropora* polyps after metamorphosis and settlement are usually aposymbiotic in nature.[57] Newly settled polyps can survive without algal symbionts for up to 2 months and even can undergo budding without symbionts (Figure S3E). This suggests that nutrients are stored in the metamorphosed polyp, presumably originating from the egg yolk. However, as *Acropora* ages and this nutrient source is depleted, it becomes dependent on symbiotic algae, with adult colonies dying quickly after symbiont loss during bleaching.[58,59] Symbiodiniaceae exist at low densities in the water column,[60] suggesting that incidental capture of small numbers of algae may be a rare event; hence, it may be critical to allow for rapid proliferation of algal cells throughout the polyp. Infant polyps may invest fixed carbon from their yolk into Symbiodiniaceae proliferation in order to help facilitate symbiosis establishment during the recruit phase of the coral life cycle. (2) The coral host may provide fixed carbon and other nutrients in exchange for essential nutrients synthesized by the algae. A hallmark of the mutualistic exchange between coral and Symbiodiniaceae is the transfer of photosynthate to the former and inorganic nutrients to the latter. Without photosynthesis, the production of photosynthate is not possible; however, a mutualistic exchange could still exist. The algae may obtain fixed carbon and inorganic nutrients from the host and use them to proliferate but also synthesize and translocate back essential nutrients to the coral host. For example, *Acropora* appears to lack an enzyme essential for cysteine biosynthesis,[61] which could be synthesized by Symbiodiniaceae and transferred to the host, a process known to occur for amino acids in some cnidarian-Symbiodiniaceae relationships.[62,63] Previous studies have shown that when transferred to darkness, some adult corals can maintain their symbionts for long periods of time,[23] which may suggest that heterotrophic transfer of carbon from the coral to the symbiont may be occurring in adults as well and may warrant further study. (3) Lastly, the algae may behave as a parasite on the coral host. In order to sustain heterotrophic growth, Symbiodiniaceae may extract the fixed carbon and other nutrients from *Acropora*, imposing a negative burden. Examples of parasitism have been reported in some cnidarians during heat or other environmental stresses;[64,65] however, our polyps were grown under standard conditions. No negative impacts were observed for polyps infected with Symbiodiniaceae without photosynthesis. Measurement of polyp sizes showed no burden related to harboring Symbiodiniaceae without photosynthesis (Figures S3C and S3D). If parasitism was the case, our results would indicate that at least three genera of Symbiodiniaceae (*Breviolum*, *Cladocopium*, and *Symbiodinium*) are capable of parasitizing *Acropora*. Not all Symbiodiniaceae evaluated were able to proliferate in *Acropora*. Post-inoculation, *Symbiodinium linucheae* SSA01 were observed in tentacles, but their population level never increased. The inability of *S. linucheae* to proliferate suggests that there is a difference in this alga compared with the other species evaluated that results in a different type of symbiotic relationship. Further comparative analysis of these differences may give insights into the specificity of coral-Symbiodiniaceae symbiosis.

The maintenance of Symbiodiniaceae within cnidarian hosts does not require photosynthesis

In order to have a sustained symbiosis between cnidarian hosts and Symbiodiniaceae, it must last beyond the initial inoculation. Symbiodiniaceae algae were maintained without photosynthesis in the host tissue longer than beads in 31 cnidarian-Symbiodiniaceae relationship evaluated, although the retention time varies depending on host-symbiont combinations (Table S1). How symbionts are maintained without photosynthesis can be used to gain insights into the nature of the cnidarian-Symbiodiniaceae relationship evaluated because Symbiodiniaceae density within a host is a function of algal infection, proliferation, expulsion, and death. In Aiptasia without photosynthesis, *B. minutum* WT and mutants can remain at relatively constant levels without noticeable changes for at least 180 days. In our experiments, no algae are available for infection after the initial inoculation, and proliferation has not been observed in this host without photosynthesis. Because *B. minutum* WT and mutants reach a steady-state population in these conditions for 180 days, it indicates that expulsion and digestion by the host are minimal. These symbionts remain viable without photosynthesis, and *B. minutum* WT can quickly proliferate in the host when brought into the light (Figure 1D). This finding is similar to early studies that showed Aiptasia kept in prolonged darkness could still harbor Symbiodiniaceae that proliferate when brought into the light,[22] although it is unclear if they were periodically exposed to light during dark treatment for culture upkeep. Our results with *B. minutum* WT in the dark and photosynthetic mutants perfectly match, implying that any incident light exposure during dark treatment did not impact our findings.

However, not all Symbiodiniaceae species were maintained at a constant level in Aiptasia without photosynthesis, with some being gradually lost over time (Table S1). This may be due to the varying ability of different Symbiodiniaceae species to survive in the dark. Interestingly, the species maintained, *B. minutum* and *S. linucheae*, are native symbionts of Aiptasia, whereas the species that were lost are non-native species. Previously, it has been shown that there are metabolic incompatibilities between Aiptasia and non-native symbionts,[66] which may explain why non-native symbionts are lost. Similar decreasing trends of Symbiodiniaceae were also observed in *Cassiopea* for *B. minutum* and *S. linucheae*. This indicates that within these hosts, some species of Symbiodiniaceae without photosynthesis are either expelled, digested, or lose viability. The losses observed in these host-symbiont pairs may represent a basal level of symbiont loss that is always occurring but not apparent under photosynthetic conditions because proliferation is equal to or greater than this loss, resulting in a stable or increasing symbiont population. Without photosynthesis and subsequent proliferation, this loss would result in the diminution of the symbiont population.

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In *Acropora*, representatives from *Breviolum*, *Cladocopium*, and *Symbiodinium* can be maintained at a relatively constant level in continuous darkness without photosynthesis for more than 60 days, presumably because the rate of symbiont proliferation is greater than expulsion or digestion. For *S. linucheae* and *D. trenchii*, no proliferation is observed without photosynthesis that correlates to a shorter period of algal maintenance than the other species. This suggests that symbiont loss through expulsion or digestion does occur in *Acropora*, but the rate of loss is lower than the rate of heterotrophic proliferation, resulting in symbiont accumulation and maintenance of *Breviolum*, *Cladocopium*, and *Symbiodinium*.

Phagosome membranes may be hijacked allowing for Symbiodiniaceae to be maintained inside the host cell without the need to transfer photosynthate

It is thought that Symbiodiniaceae enters the host cell initially via a mechanism that resembles phagocytosis. Several theories have emerged to explain how these cells avoid being fused with lysosomes for degradation. One of these, known as the "arrested phagosome hypothesis," suggests that to avoid digestion or expulsion, the symbiont enclosed within a host-derived symbiosome mimics a phagosome digesting prey by releasing photosynthate.[67,68] Our results, however, suggest that it is unlikely that release of photosynthate would be a universal determining factor that allows for symbiont escape from digestion or expulsion. The photosynthetic mutants are unable to fix inorganic carbon into compounds such as glucose and glycerol,[69] but they are still maintained as endosymbionts *in hospite*. Interestingly though, emerging evidence suggests that the symbiosome may indeed have a surface composition that resembles a phagosome, evidenced by the identification of phagosomal markers on the symbiosome membranes like LAMP1 or Rab5.[16,70]

If the surface of the symbiosome resembles a phagosome, this brings up an intriguing possibility that all that may be necessary for maintenance is to be outwardly disguised as a phagosome. A hijacked phagosome membrane may serve as cover allowing the algae to be maintained inside a host cell, even without the need to transfer photosynthate. In this "hijacked phagosome theory," the fixed carbon released through photosynthesis in the light benefits the host but would not be required for the algae to remain inside the host cells. The ability of a symbiont to hijack the host phagosome membrane may lead to differences in symbiont maintenance that results in host-symbiont specificities seen in many cnidarian-Symbiodiniaceae symbiosis. A similar "hijacking" approach is seen in various intracellular symbionts, like Rhizobium bacteria and apicomplexan parasites, that are able to avoid the fusion of the symbiosome or pathogen-containing compartment with lysosomes to allow for their maintenance and proliferation by altering the phagosome membrane.[71,72]

In summary, our results suggest that diverse mechanisms associated with different host-symbiont interactions and that mechanisms independent of photosynthesis may govern and are likely more critical for symbiosis establishment in cnidarian-Symbiodiniaceae than those associated with photosynthesis.

The creation of Symbiodiniaceae mutants and forward genetic approaches can be transformative for the study of cnidarian-Symbiodiniaceae symbiosis

Mutagenesis strategies that disrupt gene function have been transformative for biological discovery in many organisms. With unique three-dimensional genome organizations[73] and some genes residing in tandem arrays,[74–76] genetic tools, however, have been very limited in Symbiodiniaceae,[37] slowing the ability to directly answer questions about the molecular and cellular mechanisms of cnidarian-Symbiodiniaceae symbiosis. Our study is the first to apply a UV mutagenesis approach coupled with high-throughput screening methods to discover *B. minutum* mutants with disruptions in photosynthetic ability. This is an important step in advancing forward genetic approaches in this system that can be used to understand and explore coral-algal symbiosis. These photosynthetic mutants created in this study demonstrate the power of generating mutants with a specific dysfunction phenotype and using that to probe symbiosis. Given that *B. minutum* is haploid,[77] mutations in genes not found in tandem arrays are more likely to show a phenotype. Future mutagenesis strategies may enable researchers to address fundamental questions in symbiosis, such as discovering essential genes for symbiosis establishment by screening mutant libraries to identify mutants that fail to establish symbiosis. Beyond assessing how photosynthesis affects cnidarian-Symbiodiniaceae symbiosis, further study of photosynthetic mutants in Symbiodiniaceae may provide insights into some of the unique photosynthetic features of dinoflagellates such as accessory pigment composition and the reduced plastid genome size.[78,79] Our study highlights the power of UV mutagenesis to probe cnidarian-Symbiodiniaceae symbiosis and provides a promising platform to answer key questions in symbiosis.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2022.04.021.

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AUTHOR CONTRIBUTIONS

R.E.J. and T.X. conceived and planned the research and analyzed the data. J.A.R., R.E.J., A.L.K., and T.X. generated the mutant library and photosynthetic mutants, measured the mutant growth, and performed experiments with Aiptasia. R.E.J., T.X., and M.H. performed experiments with coral. C.R.N., M.Q.M., and T.X. performed experiments with *Cassiopea*. R.J.C. assisted with the fluorescence imaging of the algal cells. R.E.J., T.X., A.R.G., and J.A.R. wrote the manuscript with contributions and edits from all authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
Clonal axenic *Breviolum minutum* (Clade B) strain SSB01	The Aiptasia Symbiosis Resource	SSB01
Cladocopium goreaui (Clade C) strains LHI-33	The Aiptasia Symbiosis Resource	LHI-33
Cladocopium goreaui (Clade C) strain rt-152	The Aiptasia Symbiosis Resource	rt-152
Durusdinium trenchii (Clade D) strain CCMP2556	The Aiptasia Symbiosis Resource	CCMP2556
Clonal axenic *Symbiodinium linucheae* (Clade A) strain SSA01	The Aiptasia Symbiosis Resource	SSA01
Clonal axenic *Symbiodinium necroappetens* (Clade A) strain SSA02	The Aiptasia Symbiosis Resource	SSA02
Symbiodinium necroappetens (Clade A) strain MAC-225	The Aiptasia Symbiosis Resource	MAC-225
Exaiptasia pallida strain CC7	The Aiptasia Symbiosis Resource	CC7
Exaiptasia pallida strain H2	The Aiptasia Symbiosis Resource	H2
Cassiopea xamachana	This study	N/A
Acropora tenuis	This study	N/A
Breviolum minutum lbr1	This study	*lbr1*
Breviolum minutum ora1	This study	*ora1*
Breviolum minutum yel7	This study	*yel7*
Breviolum minutum yel8	This study	*yel8*
Breviolum minutum yel9	This study	*yel9*
Breviolum minutum yel12	This study	*yel12*
Software and algorithms		
ImageJ	NIH – public domain	https://imagej.nih.gov/ij/
Python (2.7.11)	Python Software Foundation	https://www.python.org
SciPy (0.17.0)		https://www.scipy.org
Pandas (0.18.1)		https://pandas.pydata.org/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagent should be directed to and will be fulfilled by the lead contact, Robert Jinkerson (robert.jinkerson@ucr.edu)

Materials availability

Materials generated in this study are available from the lead contact upon request. Distribution of lines is governed by the appropriate material transfer agreements (MTAs) and availability of algae material is dependent on provision of appropriate import permits acquired by the receiver.

Data and code availability

All data generated and analyzed for this study are included in the manuscript and Supplementary files. No original code was created for this work. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Symbiodiniaceae strains and growth conditions

Clonal and axenic *Breviolum minutum* (Clade B) strain SSB01, *Cladocopium goreaui* (Clade C) strains rt-152 and LHI-33, *Durusdinium trenchii* (Clade D) strain CCMP2556, clonal and axenic *Symbiodinium linucheae* (Clade A) strain SSA01,[80] clonal and axenic *Symbiodinium necroappetens* (Clade A) strain SSA02,[24] and *Symbiodinium necroappetens* (Clade A) strain MAC-225 were used in this study. *B. minutum*, SSA01, and SSA02 liquid cultures were grown either in Daigo's IMK minimal medium for marine microalgae (Wako Pure Chemicals, Osaka, Japan) made in artificial seawater, 37.4 $g \cdot L^{-1}$ marine broth (MB) (Millipore-Sigma 76448) medium, or MB supplemented with 10 $g \cdot L^{-1}$ glucose (Millipore-Sigma G8270). *S. necroappetens* MAC-225, *C. goreaui* (rt-152 and LHI-33), and *D. trenchii*

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CCMP2556 were grown in F/2 media. Cultures were incubated without agitation at 27 °C on a 12 hr-light/12 hr-dark cycle with an irradiance of ~10 μmol photons m^{-2} s^{-1} of photosynthetically active radiation (PAR) provided by Percival SciWhite LED tiles.

Clonal *Exaiptasia pallida* strains

Clonal *Exaiptasia pallida* (referred to as Aiptasia) strain CC7 and H2 were used in this study. Anemones were rendered fully aposymbiotic (cleared of their endogenous Symbiodiniaceae algae) using a short-term cold shock method[30] and menthol bleaching.[81] Aposymbiotic animals were kept in artificial seawater (referred to as ASW for the rest of the paper) in polycarbonate tanks at 27 °C on a 12 hr-light/12 hr-dark cycle (~10 μmol photons m^{-2} s^{-1} of PAR from Percival SciWhite LED tiles) with feeding and water changes every 2 days except where noted. Before use, all aposymbiotic anemones were verified with fluorescent microscopy to be free of algae. To validate the elimination of symbionts in aposymbiotic animals, fluorescence imaging was conducted three times on the whole animal at high magnification to detect any potential algal chlorophyll fluorescence and before infection assays aposymbiotic animals were kept under 12 hr-light/12 hr-dark cycle (~10 μmol photons m^{-2} s^{-1} of PAR from Percival SciWhite LED tiles) for a minimum of three weeks and monitored every three days by fluorescence microscopy to allow for the detection of any residual symbionts that began proliferating. Only anemones with no detectable chlorophyll were considered aposymbiotic and used for subsequent experiments.

Jellyfish *Cassiopea xamachana*

Adult *Cassiopea xamachana* (referred to as *Cassiopea*) medusa were collected from the Florida Keys and maintained in laboratory aquaria on a 13 h light / 11 h dark cycle with feeding Artemia three times a week. Polyps found on the walls of the aquarium, grown up from larvae released into the water column, were collected and used for *Cassiopea* infection studies. Collected polyps were rendered aposymbiotic through high-temperature bleaching at 35.5 °C and confirmed as aposymbiotic using a Zeiss 710 confocal microscope.[82] Aposymbiotic polyps were kept in 6-well plates with 0.2 μm-filtered artificial seawater (FASW) in the dark at 27 °C and fed with Artemia three times a week. A week before the inoculation experiments, the aposymbiotic polyps were brought to light and validated under the fluorescence microscope to be free of algae.

Coral *Acropora tenuis*

Acropora tenuis (referred to as *Acropora*) colonies were collected from Sesoko Island (26°37′41″N, 127°51′38″E, Okinawa, Japan) under collecting permit #30–78 and #2–57 by Okinawa prefecture and maintained as described previously.[14] After coral spawning occurred on June 12, 2019 and on May 22, 2021, bundles of symbiont-free gametes from those colonies were mixed to form planula larvae. In 2021, larvae were transported from Japan to the USA under CITES permission 21JP100506/TE.

METHOD DETAILS

Creation of *B. minutum* SSB01 photosynthesis deficient mutants

To create photosynthesis deficient mutants in the *B. minutum* SSB01 background, we first assessed the survival rate of *B. minutum* WT cells to a series of UV exposures (0, 15, 30, 60, 120, 180, and 240 s) produced by a germicidal lamp (NuAire X999432). Exposed cells were then spread on MB with glucose agar plates, allowed to grow, and colonies were counted to determine the survival rate. To create a mutant library, UV exposures of 180 and 240 s were used which give less than 1% survival. Liquid cultures of $2x10^7$ *B. minutum* cells at a concentration of $1x10^6$ cells·mL^{-1} were exposed with UV light while being stirred slowly to achieve homogeneous exposure. Algal cells were collected by centrifugation at 500 × *g* for 5 minutes at room temperature and spread on solid MB with glucose agar plates. Plates were kept at room temperature in 24 hour darkness for a period of 1 to 3 months and then exposed to light (~10 μmol photons m^{-2} s^{-1} of PAR) for up to a week to ensure the majority of colonies reached a brown color. Plates were then screened for mutant colonies that had an altered color compared to the brown color observed in wild type *B. minutum*. Single colonies with a putative altered color phenotype were picked and transferred to autotrophic (MB in the light) and heterotrophic (MB with glucose in the dark) conditions for a secondary screen to identify mutants deficient in photoautotrophic growth. Mutants unable to grow in autotrophic conditions were identified as photosynthesis deficient mutants. In this study, mutants *lbr1*, *ora1*, *yel7*, *yel8*, *yel9, and yel12* were identified and cultured in MB with glucose in the dark to minimize potential light damage to the mutant cells.

Analyses of photosynthetic function

To measure photosynthetic function of cultured algal cells, approximately 10^6 algal cells were used. To measure photosynthetic function of algae *in hospite* in sea anemones, individual H2 anemones infected with algae or aposymbiotic anemones (as a negative control) were used and placed in 1 ml ASW. Maximum quantum yields of photosystem II (PSII), $F_v/F_m = (F_m–F_0)/F_m$ were determined for all algal samples using a JTS-10 spectrophotometer (Bio-Logic)[83] after 30 minutes of dark adaptation.

Growth studies

An assay comparing the growth of *B. minutum* WT, *lbr1*, and *ora1* in autotrophic and heterotrophic conditions was conducted. Strains were pre-cultured in liquid MB with glucose media in 27 °C in continuous darkness. Cells were isolated from pre-cultures by centrifugation at 500 × *g* for 5 mins, washed in their respective treatment media, and resuspended in either MB or MB with glucose. Cells were grown in 96-well plates with an initial concentration of $6.67x10^5$ cells·mL^{-1}, volume of 150 μl per well, and with six replicates.

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Plates for autotrophic (MB) growth were incubated in continuous white light at an intensity of 50 μmol photons m^{-2} s^{-1} of PAR at 27 °C and heterotrophic (MB with glucose) growth were incubated in continuous darkness at 27 °C. Absorbance at 678 nm was collected after a shaking step on the linear setting at 1096 cpm for 5 minutes every 5 days using the Synergy H1 plate reader from BioTek held at 27 °C.

Inoculation of Aiptasia with Symbiodiniaceae

For inoculation of Aiptasia CC7 or H2, on day 0 individual aposymbiotic anemones were placed in each well of a 6-well polypropylene plate and Symbiodiniaceae or green fluorescent beads (7 μm diameter, Thermo Fisher Scientific C36950) washed in sterile ASW were added to reach a final concentration of $1x10^4$ cells·mL^{-1} or $1x10^4$ beads·ml^{-1}. After 24 hours individual anemones were washed with ASW and moved to a clean well of a 6-well plate. The infection of each sea anemone was tracked individually. For DCMU treatment in Figure S1, individual aposymbiotic anemones were incubated in ASW containing 25 μM DCMU (Millipore-Sigma D2425) and inoculated with *B. minutum* at $1x10^4$ cells·mL^{-1}. For inoculation in the dark, all procedures were carried out in near-darkness and animals were kept in continuous darkness throughout the course of the experiment.

For the multiple inoculations experiment in Figures 2I and 2J, inoculation of Aiptasia H2 using *lbr1* or *ora1* cultures were conducted weekly for approximately 6 months. Cells of each mutant culture were collected by centrifugation at 500 × *g* for 5 minutes at room temperature, cell pellets were resuspended in ASW, and mixed with saturating amounts of decapsulated brine shrimp (Artemia). The mixture was added to aposymbiotic anemones to a final algal concentration of at least $2x10^5$ cells·mL^{-1} and incubated until food was observed in the gut cavity, typically for 2 hours. Infected Aiptasia were kept in continuous darkness except during the reinoculation procedure and anemone care when they were briefly exposed to low levels of light. After completion of all inoculations, animals were then kept in continuous darkness for more than 3 months without additional inoculations. To further validate the identity of the algae after our infection experiments, marker genes were used to genotype the species of Symbiodiniaceae algae present in the host tissues. The domain V of chloroplast large subunit (cp23S)-ribosomal DNA sequence[24] and the nuclear internal transcribed spacer region 2 (ITS2)[24] markers were used. Only the expected Symbiodiniaceae species were ever detected from algae isolated from infected animals.

Inoculation of coral larvae and polyps with Symbiodiniaceae

For larva inoculation experiments, aposymbiotic larvae 6 to 9 days post-fertilization (dpf) were inoculated with Symbiodiniaceae cultures washed in 0.2 μm-filtered artificial seawater (FASW) at a final concentration of $1x10^4$ cells·mL^{-1} for two days with cleaning and fresh algae added at the same concentration as the initial inoculation after one day. FASW was exchanged daily thereafter. Larvae were kept at 27 °C in 12 hr-light/12 hr-dark (10 μmol photons m^{-2} s^{-1} of PAR) or in continuous darkness.

For polyp inoculation experiments, at 6 to 9 dpf larvae were induced to metamorphose and settle in 6-well plates by incubation overnight with 1 μM Hym-248 neuropeptide[84] in FASW at 27 °C. Polyps (~10–20 polyps per well) were verified with fluorescent microscopy to be free of algae and then inoculated with FASW-washed Symbiodiniaceae cultures at a final concentration of $1x10^4$ cells·mL^{-1} or $1x10^4$ fluorescent beads·ml^{-1} for two days. In the middle of the two-day inoculation, plates were cleaned and fresh FASW containing the same concentration of algae as the initial inoculation were added to the polyps. At the conclusion of the inoculation period, polyps were washed to remove algae, and the FASW was exchanged daily thereafter without feeding for 60 days. During and after the inoculation, plates were kept in 12 hr-light/12 hr-dark (10 μmol photons m^{-2} s^{-1} of PAR) or in continuous darkness. An average of 65 polyps were used per host/algae combination. Tracking of mutant *lbr1* was not possible given its weak chlorophyll fluorescence and the strong background autofluorescence from the coral polyps so was excluded from this study. FASW was used as a negative control without algae.

Inoculation of *Cassiopea* with Symbiodiniaceae

To identify the species of Symbiodiniaceae natively found in our collected *Cassiopea*, symbiotic polyps were homogenized using a bead mill homogenizer (Thermo Fisher Scientific) with zirconium ceramic oxide beads (1.4 mm in diameter, Thermo Fisher Scientific 15340153). Symbiodiniaceae algae were isolated through washing and centrifugation six times with sterile ASW. Genomic DNA was extracted from the isolated Symbiodiniaceae algae using the lysis buffer (1% SDS, 200 mM NaCl, 20 mM EDTA pH 8.0, 50 mM Tris-HCl pH 8.0) and purified using phenol and chloroform.[85] Sequencing of the *cp23S*[86] region (deposited at GenBank: MZ576210) confirmed that the Symbiodiniaceae symbiont is a member of Clade A and assigned to *Symbiodinium microadriaticum*.

For inoculation of aposymbiotic *Cassiopea* polyps, on day 0 individual polyps were placed in each well of a 48-well plate and were inoculated with FASW-washed Symbiodiniaceae cultures at a final concentration of $1x10^4$ cells·mL^{-1} or $1x10^4$ green fluorescent beads·ml^{-1}. Both algae and beads were mixed with Artemia. After 24 hours each individual polyp was washed with FASW and moved to a clean well of a 48-well plate. The infection of each polyp was tracked individually. Inoculations were conducted weekly 6 more times over the course of the 50 days. After each inoculation, polyps were fed with Artemia and cleaned three times a week. For inoculations in the light, polyps kept in 12 h light / 12 h dark (50 μmol photons m^{-2} s^{-1} of PAR). For inoculations in the dark, polyps were kept in continuous darkness throughout the course of the experiment. At least 10 polyps were used for each host and Symbiodiniaceae or bead combination.

Microscopy

For imaging Symbiodiniaceae colonies on agar plates, cells were streaked out on agar plates under either autotrophic (MB in the light) or heterotrophic (MB with glucose in the dark) conditions. Colonies were photographed using a stereomicroscope equipped with a

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Nikon 9500 digital camera. For fluorescence imaging of single algal cells, 3D image stacks were collected at 0.2-μm z increments on a DeltaVision Elite workstation (GE Healthcare) based on an inverted microscope (IX-70; Olympus) using a 60 × 1.42NA oil immersion lens. Images were captured at 24 °C with a 12-bit charge-coupled device camera (CoolSnap HQ; Photometrics) and deconvolved using the iterative-constrained algorithm and the measured point spread function. Algal autofluorescence was visualized using a FITC excitation filter paired with a polychroic CY5 emission filter.

To image Aiptasia, *Acropora* larvae and polyps, and *Cassiopea* polyps, we used a Nikon SMZ-25 fluorescence stereoscope either in bright-field mode or with blue-light excitation (GFP2 filter set; 480/40 nm excitation, 510 nm long-pass fluorescence emission) of the endogenous algal chlorophyll and captured the images using a Nikon DS-Ri2 Color CMOS Camera. Aiptasia infection images in Figure S2C were taken using the Keyence BZ-X710 automated fluorescence microscope. Mutant algal cells associated within Aiptasia tissue were visualized with CY5 fluorescence cube on high sensitivity settings. For fluorescence images in Figures 2H–2J, 2L, 3A, and S2C, because the algal mutants have less pigments and thus exhibit less fluorescence, the images were shown in grey scale for easier visualization. For the imaging of algae in Aiptasia tentacles in Figure S2E, individual sea anemones were allowed to relax for approximately five minutes after adding 5 drops of a 1:1 ASW and 0.37 M $MgCl_2$ solution. Relaxed sea anemones were imaged using a Leica DM6 fluorescence microscope either in bright-field mode or with a Texas Red filter set (534–59nm excitation and 630–69 nm emission). For the imaging of algae isolated from sea anemones in Figures S2F and S3B, two to five anemones or coral polyps (total wet weight, ~25 mg) were disrupted using a homogenizer (Fisher Scientific; Bead Mill 4) for ~30 s in ASW. The tissue homogenate was centrifuged at 3000 × *g* for 5 min at room temperature, and the pellet was washed 6X with ASW to remove most host tissue. The final pellet was resuspended in ASW, and algal cells were imaged using a Leica DM6 fluorescence microscope either in bright-field mode or with a Texas Red filter set (534–59nm excitation and 630–69 nm emission) or a Zeiss Axio Observer inverted microscope.

For the imaging of *Cassiopea* ephyra in Figures S4A and S4B, symbiotic ephyra were placed into a 35 × 10 mm petri dish with ASW and allowed to relax for approximately five minutes after adding 5 drops of a 1:1 ASW and 0.37 M $MgCl_2$ solution. Relaxed ephyra were imaged using Nikon SMZ-25 fluorescence stereoscope either in bright-field mode or with blue-light excitation (GFP2 filter set; 480/40 nm excitation, 510 nm long-pass fluorescence emission) of the endogenous algal chlorophyll and captured the images using a Nikon DS-Ri2 Color CMOS Camera.

Single-cell dissociation in adult Aiptasia tissue and coral polyps

To visualize photosynthesis mutants within dissociated Aiptasia host cells in Figure 2M or dissociated *Acropora* polyp host cells in Figure 3G, *ora1* infected Aiptasia, *B. minutum* WT infected Aiptasia, aposymbiotic Aiptasia, and *ora1* algae from culture or *ora1* infected coral polyps were washed in calcium- and magnesium-free artificial seawater (CMF-ASW) three times and then allowed to incubate in the CMF-ASW for 10 minutes. After incubation of Aiptasia, the tentacles were dissected off of the body of the anemone and diced into small pieces, which were subsequently centrifuged at 70 × *g* for 5 minutes to remove CMF-ASW. For the *ora1* infected coral, polyps were crushed gently to break-up the $CaCO_3$ skeletons and then centrifuged at 200 × *g* for 5 minutes to remove the CMF-ASW. To the anemone or coral tissue, 100 μl of 0.5% pronase from *Streptomyces griseus* (MilliporeSigma 10165921001) in CMF-ASW with EGTA (ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, MilliporeSigma 324626) was added, the solution was mixed by pipetting up and down eight times, allowed to incubate for 20 minutes with gentle agitation, and followed by mixing eight more times with a pipette. The dissociated tissue was centrifuged again at 70 × *g* for 5 minutes, and the pronase solution was removed. The dissociated anemone or coral tissue was gently resuspended in 50 μl of cell permeable Hoechst 33342 fluorescent nucleic acid stain at a concentration of 12 $\mu g \cdot ml^{-1}$ in CMF-ASW+EGTA, which stained the host nuclei. Dissociated tissue was incubated in the staining solution for 30 minutes in the dark at room temperature, followed by centrifugation at 70 × *g* for 5 minutes and the removal of 40 μl of staining solution. The dissociated tissue was gently suspended in the remaining staining solution and immediately imaged with a Zeiss Axio Observer inverted microscope either in bright-field mode or Texas Red filter set for chlorophyll fluorescence (546/12nm excitation, 590nm long-pass fluorescence emission), and fluorescence filter set for Hoechst 33342 fluorescent nucleic acid stain (365nm excitation, 445/50nm band pass fluorescence emission).

Algal cell quantification *in hospite*

For algal cell quantification after infection in Aiptasia, algae were identified by chlorophyll fluorescence imaging and counted in all tentacles of individual sea anemones during fluorescent microscopy on a Nikon SMZ-25 fluorescence stereoscope.

To track algal proliferation in *Acropora* polyps, GFP2 long-pass fluorescence images were taken with a 250 ms exposure in order to quantify algal chlorophyll fluorescence. Quantification was performed in ImageJ by measuring the red channel, adjusting the window center to 160 and the window width to 190 to eliminate the background, and measuring the mean intensity for a region of interest (ROI) that covers the polyp and/or tentacles.

QUANTIFICATION AND STATISTICAL ANALYSIS

All statistical tests were performed using Python (2.7.11; https://www.python.org), SciPy (0.17.0; https://www.scipy.org), Pandas (0.18.1; https://pandas.pydata.org/) packages integrated in Jupyter Notebook. Statistical tests used and number of repeats are indicated in the figure legends or in the text.