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Symbiont Identity Influences Patterns of Symbiosis Establishment, Host Growth, and Asexual Reproduction in a Model Cnidarian-Dinoflagellate Symbiosis

YASMIN GABAY[1], VIRGINIA M. WEIS[2], AND SIMON K. DAVY[1,*]

[1]*School of Biological Sciences, Victoria University of Wellington, Kelburn Parade, Wellington 6140, New Zealand; and* [2]*Department of Integrative Biology, Oregon State University, Corvallis, Oregon 97331*

Abstract. The genus *Symbiodinium* is physiologically diverse and so may differentially influence symbiosis establishment and function. To explore this, we inoculated aposymbiotic individuals of the sea anemone *Exaiptasia pallida* (commonly referred to as "Aiptasia"), a model for coral symbiosis, with one of five *Symbiodinium* species or types (*S. microadriaticum*, *S. minutum*, phylotype C3, *S. trenchii*, or *S. voratum*). The spatial pattern of colonization was monitored over time *via* confocal microscopy, and various physiological parameters were measured to assess symbiosis functionality. Anemones rapidly formed a symbiosis with the homologous symbiont, *S. minutum*, but struggled or failed to form a long-lasting symbiosis with *Symbiodinium* C3 or *S. voratum*, respectively. *Symbiodinium microadriaticum* and *S. trenchii* were successful but reached their peak density two weeks after *S. minutum*. The spatial pattern of colonization was identical for all *Symbiodinium* taxa that were ultimately successful, starting in the oral disk and progressing to the tentacles, before invading the column and, finally, the pedal disk. In all cases, proliferation through the anemone's tentacles was patchy, suggesting that symbionts were being expelled into the gastrovascular cavity and re-phagocytosed by the host. However, the timing of these various spatial events differed between the different *Symbiodinium* taxa. Furthermore, *S. microadriaticum* and *S. trenchii* were less beneficial to the host, as indicated by lower rates of photosynthesis, anemone growth, and pedal laceration. This study enhances our understanding of the link between symbiont identity and the performance of the overall symbiosis, which is important for understanding the potential establishment and persistence of novel host-symbiont pairings. Importantly, we also provide a baseline for further studies on this topic with the globally adopted "Aiptasia" model system.

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* To whom correspondence should be addressed. E-mail: simon.davy@vuw.ac.nz.

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; P : R, ratio of hourly gross photosynthesis to respiration.

Introduction

Among the most significant marine mutualisms are those between cnidarians and their photosynthetic dinoflagellate symbionts (Roth, 2014). These interactions, in particular, between anthozoan cnidarians (*e.g.*, corals and sea anemones) and dinoflagellates of the genus *Symbiodinium*, underpin the existence and success of coral reef ecosystems (Little *et al.*, 2004; Wang *et al.*, 2012; Lesser *et al.*, 2013).

The genus *Symbiodinium* is very diverse, consisting of nine clades (A–I) and numerous subclades (types) and species (LaJeunesse, 2002; Pochon and Gates, 2010) that display considerable physiological diversity (Schoenberg and Trench, 1980a; Fitt *et al.*, 1981; Chang *et al.*, 1983; Iglesias-Prieto *et al.*, 2004; Robison and Warner, 2006; Hennige *et al.*, 2009). Given this, different symbiont taxa can differentially affect host and, hence, holobiont (*i.e.*, the entire symbiotic entity) performance. For example, in corals, some members of clades A and D can be less beneficial to their hosts than members of clade C, with lower rates of photosynthetic carbon fixation and translocation to the host and reduced host fitness (Mieog *et al.*, 2009; Leal *et al.*, 2015). Alongside host phylotype and the prevailing environmental conditions, symbiont identity may therefore determine the fate of the symbiosis (Loram *et al.*, 2007; Yuyama *et al.*, 2016).

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Such observations have led to the proposal that several *Symbiodinium* taxa might exhibit more opportunistic, and even parasitic, traits than others (Stat and Gates, 2011; Lesser *et al.*, 2013; Pettay *et al.*, 2015; Silverstein *et al.*, 2015). Starzak *et al.* (2014) tested this idea more broadly by modeling carbon fluxes in the model symbiotic anemone Aiptasia when colonized by a range of different *Symbiodinium* taxa. These models suggested that the usual homologous symbiont (*S. minutum*, internal transcribed spacer region 2 [ITS2] type B1) forms a more beneficial symbiosis with Aiptasia than do several nonnative heterologous symbiont types, such as A1.4, E, and F5.2; *Symbiodinium* E and F5.2 were, on occasion, even observed to cause host mortality (Starzak *et al.*, 2014). However, direct measures of host growth and reproductive output, to corroborate the models of Starzak and coworkers, have not been performed. Indeed, we still know relatively little about the behavior and physiology of homologous and heterologous *Symbiodinium* taxa, in general, during symbiosis establishment, including whether they exhibit similar spatial patterns of uptake and host colonization. Such information is important for understanding the events that might occur during the establishment of novel host-symbiont pairings, such as after a coral bleaching event. Furthermore, such details are important for understanding the drivers of host-symbiont specificity, that is, the reasons why many hosts are found in association with only one or a few *Symbiodinium* taxa and *vice versa* (Baker, 2003; LaJeunesse *et al.*, 2004; Coffroth *et al.*, 2010).

This study addressed these knowledge gaps using the sea anemone *Exaiptasia pallida* (Agassiz in Verrill, 1864), commonly referred to as "Aiptasia," a widely adopted model system for the study of reef-building corals and the cnidarian-dinoflagellate symbiosis (Weis *et al.*, 2008; Baumgarten *et al.*, 2015). The current study measured the influence of symbiont diversity on host colonization dynamics, as well as host performance. We employed five different *Symbiodinium* species or types, representing a wide range of clades (A–E) and physiologies, including the free-living and relatively heterotrophic *S. voratum* (type E; Jeong *et al.*, 2012) and the thermally tolerant but opportunistic *S. trenchii* (type D1a; Pettay and Lajeunesse, 2009). Specifically, we asked whether symbiont identity affects (1) symbiont cell proliferation, (2) colonization pattern inside the host, (3) photosynthetic performance, and (4) host fitness. In doing so, we provide not only further insight into the cellular events that occur during host colonization by symbiotic dinoflagellates and the physiological implications of symbiont diversity but also a baseline from which to conduct more detailed studies of symbiosis establishment and function with the Aiptasia model system.

Materials and Methods

Experimental organisms

A clonal culture of the symbiotic sea anemone Aiptasia (culture ID: NZ1), originally from the Indo-Pacific region, was grown in the lab at 25 °C and an irradiance of 100 μmol photons m^{-2} s^{-1} (light/dark cycle of 12 h : 12 h) and fed twice weekly with freshly hatched *Artemia* nauplii. To generate aposymbiotic (*i.e.*, symbiont-free) anemones, animals were incubated in a solution of 0.27 mmol l^{-1} menthol in filtered seawater (FSW; 0.22 μm-FSW) for 8 h, after which the menthol in FSW was removed and the anemones were incubated in 5 μmol l^{-1} 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) in FSW overnight, as described by Matthews *et al.* (2015). The treatment was repeated daily for four weeks or until no symbionts were present, as determined by confocal microscopy (IX81, Olympus, Auckland, New Zealand; 635 nm laser, 655–755-nm emission filter).

Cultured *Symbiodinium* from five clades were used as inoculates (Table 1). Species names of these dinoflagellates are used where available; otherwise, the ITS2 type is given. The algae were subcultured from long-term (>5 y) laboratory stocks and grown in silica-free f/2 medium (Sigma-Aldrich, Auckland, New Zealand) at 25 °C and an irradiance of 100 μmol photons m^{-2} s^{-1} on a light/dark cycle of 12 h : 12 h. All cultures were sampled for experimental use during the log phase of growth.

Inoculation of aposymbiotic anemones with symbionts

Following 1 wk of starvation, a total of 312 aposymbiotic anemones (n = 60 anemones for each *Symbiodinium* type and/or species; n = 12 uninoculated anemones as a negative control) of similar size (2–3-mm oral disk diameter) were transferred to 400-ml clear plastic jars (one anemone per jar) filled with FSW and allowed to settle for 3 days. Each algal culture was diluted in 25 ml FSW and 1 drop of *Artemia* nauplii suspension added, to give a final concentration of ~1×10^6 cells ml^{-1}. The anemones were inoculated with one of the five different symbiont cultures by pipetting with a glass pipette 1 ml of this dinoflagellate suspension onto the oral disk of each anemone. Colonization was then monitored over a period of 8 weeks, with sampling at 1, 2, 4, 6, and 8 weeks after inoculation. During this time, the anemones were fed twice weekly with *Artemia* nauplii, with each feeding followed by a water change. Seawater temperature was maintained at 25 °C in a controlled-temperature room, and irradiance was held at ~100 μmol photons m^{-2} s^{-1} on a light/dark cycle of 12 h : 12 h.

Symbiont proliferation

Spatial pattern of colonization. To determine the effect of symbiont identity on the spatial pattern of host colonization, 10 randomly selected anemones from each treatment were chosen at each sampling point and assessed *via* chlorophyll autofluorescence measured with a confocal microscope (details in Experimental organisms, above). A drop of relaxation solution (50% 0.37 mol l^{-1} $MgCl_2$ in dH_2O and 50% FSW) containing 1.5% of agar was added to a subset of the sampled

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Table 1

Identity and geographical origin of Symbiodinium *cultures and the original host species if known*

Culture ID	Original host source	Geographical location	*Symbiodinium* species	Subclade
FlAp2	*Exaiptasia pallida*	Long Key, Florida	*S. minutum*	B1
CCMP2467	*Stylophora pistillata*	Gulf of Aqaba	*S. microadriaticum*	A1
Mp	*Mastigias papua*	Palau	*Symbiodinium* sp.	C3
Ap2	Unknown anemone sp.	Okinawa	*S. trenchii*	D1a
CCMP421	Free living (host, if any, unknown)	Wellington Harbour, New Zealand	*S. voratum*	E

anemones on a fluorodish (World Precision Instruments, Sarasota, FL) ($n = 5$ per treatment), and z-stack images were generated (section thickness < 10 μm). The multiple z-stack sections were then summed into a single photo, regions of interest were marked, and fluorescence was measured with ImageJ software (ver. 1.48; National Institutes of Health, Bethesda, MD). The following equation was used to calculate fluorescence intensity as a proxy for algal number:

$$Correct\ Fluorescence = IntDen_S - (Area_S - Mean_B),$$

where $IntDen$ = fluorescence measured using ImageJ, S = sample, and B = background.

Quantification of symbionts during colonization. Following confocal microscopy, each anemone was homogenized separately in 500 μl of FSW (IKA T-10 tissue lyser, ThermoFisher Scientific) and the homogenate centrifuged (Sigma 3-16k) for 5 min at 400 × g to separate algal cells from anemone tissue. A 100-μl aliquot was removed from the supernatant (host fraction) for protein determination and the remaining supernatant discarded. The pellet containing the dinoflagellate cells was re-suspended in 200 μl FSW. A 50-μl aliquot was added to 50 μl of dimethyl sulfoxide buffer for DNA sequencing (see DNA sequencing, below), and the remaining 150 μl was used for algal cell counts. The samples were stored at −20 °C until further analysis. Host protein content was determined *via* the Bradford assay (Bradford, 1976), and cell counting was performed with a hemocytometer (Improved Neubauer) with six replicate counts per sample. Cell density (symbionts per milligram protein) was then calculated.

Photophysiology and host performance

Photosynthesis and respiration of the host-symbiont partnership. Maximum gross photosynthetic and dark respiratory O_2 fluxes were measured for all host-symbiont partnerships at the end of the 8-wk experiment ($n = 4$ for each treatment). Individual anemones were placed at 25 °C in a 10-ml glass chamber, fitted with a magnetic stir bar protected by nylon mesh, filled with FSW, and sealed by a glass lid with a rubber O-ring, into which an oxygen electrode (FIBOX 3 fiber-optic oxygen meter; PreSens, Regensburg, Germany) was inserted. Animals were allowed to settle for 30 min prior to the beginning of the experiment, with the chamber remaining unsealed and hence open to the air during this time. The respiration rate (ml O_2 h^{-1}) was measured in darkness for an hour, followed by 1 h of illumination by a 150-W Thorn parabolic aluminized reflector lamp 38 at 400 μmol photons m^{-2} s^{-1}. Each anemone was then homogenized in 500 μl of FSW and host protein content determined as described before. The rates of gross photosynthesis and respiration were normalized to host protein, and the ratio of hourly gross photosynthesis to respiration (P : R) was calculated as a proxy for the autotrophic potential of the symbiosis.

Host growth and asexual reproduction. Host growth between the start and the end of the experiment (*i.e.*, 8 weeks after inoculation) was estimated *via* the change in oral disk diameter, assuming a starting diameter of 2–3 mm ($n = 10$ for each treatment). At the final time point, anemones were incubated in their containers in $MgCl_2$ relaxation solution (see Spatial pattern of colonization, above) until they were not responsive to disturbance, and then the containers were placed onto millimetric graph paper for measurement of the oral disk diameter. Throughout the duration of the experiment, asexual reproduction was measured as the number of pedal lacerates generated by each anemone between the start of the experiment and the sampling point ($n = 10$ for each treatment and time point).

DNA sequencing

To confirm that symbiont populations in animals matched the populations used for inoculation, symbiont identity was verified for each culture and a subset of inoculated anemone samples ($n = 3$ samples per culture or sample). *Symbiodinium* samples containing dimethyl sulfoxide (see Quantification of symbionts during colonization, above) were re-suspended, and 10 μl of each sample was added to a new tube containing 50 mg of glass beads (G1152-10G, Sigma-Aldrich) and placed in a TissueLyser (Qiagen, Bio-Strategy, Auckland, New Zealand) bead beater for 1 min at 50 Hz. Ninety microliters of ddH_2O was added, and lysed cell material was pelleted at 16,100 × g for 10 min at 4 °C. A 50-μl aliquot of the resulting supernatant was transferred to a new tube containing 50 μl of cold molecular-grade isopropanol, and samples were re-

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pelleted as described above. Samples were washed twice in 200 μl of 70% ethanol, centrifuged, and then dried at room temperature. Fifty microliters of 1 mol l^{-1} Tris (pH 8) were added to each tube, and samples were further extracted by bead beating at 30 Hz for 1 min and then stored at −20 °C until further analysis. Polymerase chain reaction (PCR) was performed using the following *Symbiodinium*-specific ITS2 primers: forward primer (ITS2infor) 5′-GAATTGCAGA AC TCCGTG-3′ and reverse primer (ITS2CLAMP) 5′-CGCCC GCCGC GCCCCGCGCC CGTCCCGCCG CCCCCGCCC GGGATCCATA TGCTTAAGTT CAGCGGGT-3′. Reactions were performed using the MyTaq Mix (Bioline, London) in a total volume of 25 μl with an amplification profile consisting of 1 cycle of 3 min at 95 °C; 40 cycles of 15 s at 95 °C, 15 s at 56 °C, and 10 s at 72 °C; and a final hold temperature of 4 °C. PCR products were sequenced by Macrogen (Seoul, South Korea). Sequences were aligned using Geneious Pro, version 4.8.5 (Biomatters, Auckland, New Zealand), and a BLAST search was performed in the National Centre for Biotechnology Information (NCBI) database to identify the sequences. In all cases, the expected genotypes were present at both the beginning and the end of the experiment. Multiple genotypes were never detected in any sample.

Statistical analysis

ANOVA followed by either Tukey or Bonferroni *post hoc* tests was performed on the data by using STATISTICA, version 10 (StatSoft, Tulsa, OK), and SPSS (IBM, Armonk, NY), except in the following cases. Log transformation was conducted on symbiont density data to achieve a normal distribution, followed by Welch's ANOVA, because constant variance could not be assumed. Welch's ANOVA was also performed on dark respiration data for the same reason. This test was followed by Games-Howell *post hoc* analysis. A chi-square test was conducted on asexual reproduction data to test the effect of time × algal identity. Results are expressed as mean ± standard error (SE).

Results

All *Symbiodinium* taxa initially colonized Aiptasia. There was no cross-contamination of symbionts between treatments (data not shown), and aposymbiotic anemones remained symbiont-free throughout the experiment.

Symbiont proliferation in hosts

Symbiont cell density (algal cells per mg host protein) was significantly different between anemones inoculated with different *Symbiodinium* taxa (Fig. 1; Welch's ANOVA, $F_{19,58}$ = 130.087, $P < 0.0001$). The homologous *S. minutum* was the most successful at colonizing hosts, reaching its peak density 2 weeks after inoculation and achieving a maximum density that was 13-fold higher than that of *S. trenchii*, the second

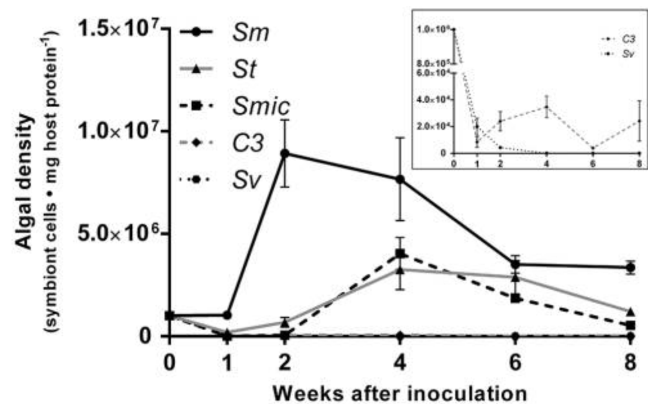

Figure 1. Colonization success in host Aiptasia varied with symbiont identity. Symbiont density (symbiont cells per mg host protein) in host anemones was quantified for 8 weeks after inoculation ($n = 10$ anemones per treatment per time point). Points are means ± SE. C3, *Symbiodinium* phylotype C3; *Sm*, *S. minutum*; *Smic*, *S. microadriaticum*; *St*, *S. trenchii*; *Sv*, *S. voratum*.

most successful symbiont, at that same time point (Games-Howell *post hoc*, $P < 0.0001$). Between weeks 2 and 8, the density of *S. minutum* decreased by 62% to $3.35 \times 10^6 \pm 3.18 \times 10^5$ cells per mg protein, which was still significantly higher than that of all other symbionts (Games-Howell *post hoc*, $P < 0.006$). *Symbiodinium microadriaticum* and *S. trenchii* populations exhibited a slower initial increase in density than *S. minutum*, but by week 4 their densities peaked at levels similar to those of *S. minutum* (Games-Howell *post hoc*, $P > 0.05$ for both comparisons; Fig. 1); their densities then declined during the remainder of the experiment by 71% and 58%, respectively, so that they were again significantly lower than the density of *S. minutum* (Games-Howell *post hoc*, $P < 0.001$). *Symbiodinium* C3 and *S. voratum* were the least successful at colonization. The population density of C3 was significantly less than that of *S. minutum* and *S. trenchii* (Games-Howell *post hoc*, $P < 0.05$) throughout the experiment and significantly less than *S. microadriaticum* from week 4 onward. In comparison, *S. voratum* initially colonized the host, but after 2 weeks its density declined dramatically, such that by week 4 and thereafter, no symbionts were observed (Fig. 1).

The different *Symbiodinium* taxa showed similar spatial patterns of colonization through host tissues, albeit at different temporal rates (Fig. 2A–C). Colonization in the tentacles did not progress smoothly from the tentacle base to the tip; rather, it was patchier, with punctuated bursts of algal growth in spatially disparate portions of the tentacle (Fig. 2A, B). Furthermore, the timing of these various spatial events differed substantially between the different *Symbiodinium* (Fig. 2C), consistent with the strength of overall colonization, as shown in Figure 1. Of particular note, the relatively unsuccessful *Symbiodinium* C3 only ever colonized the oral disk and tentacles and by week 8 appeared in the oral disk alone, suggesting that colonization was failing.

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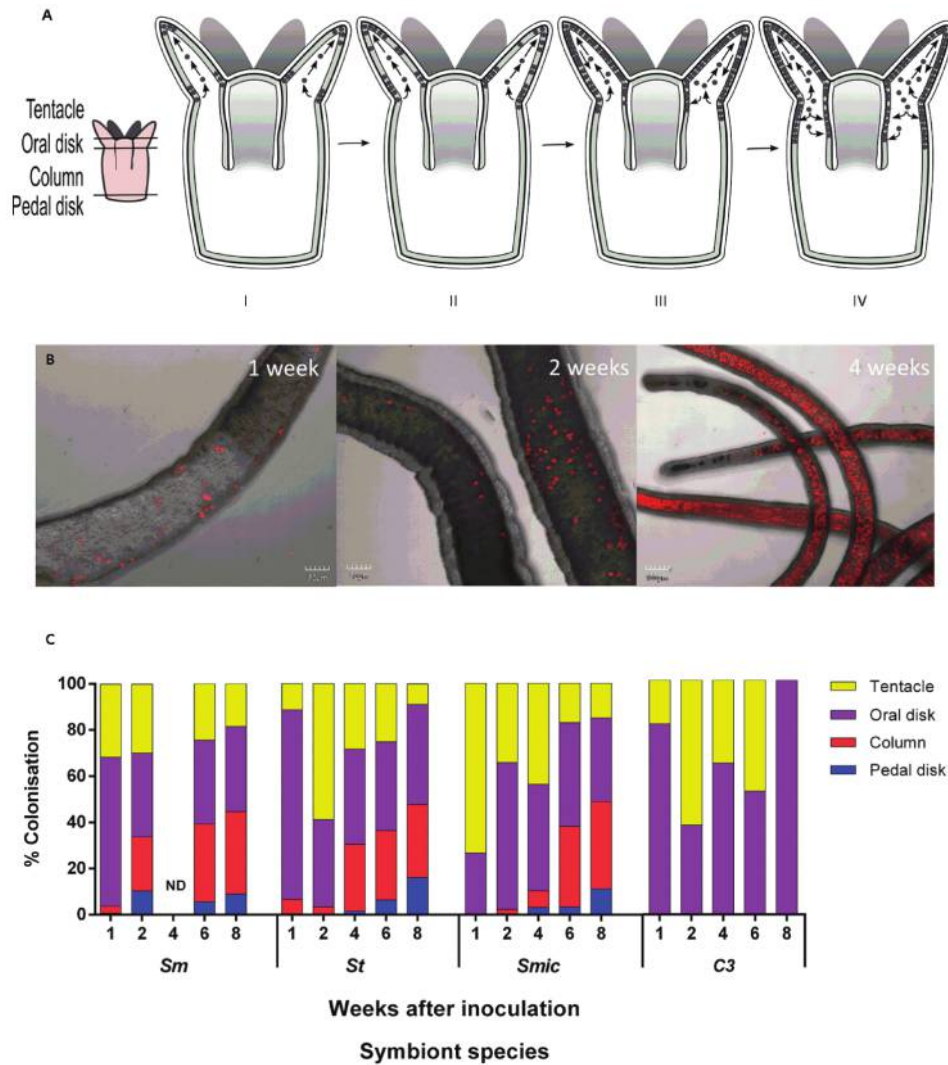

Figure 2. The spatial pattern of symbiont colonization in Aiptasia is similar for different symbionts. Spatial pattern and colonization across four body sections in the anemone host—tentacles, oral disk, column, and pedal disk—quantified for 8 weeks after inoculation (n = 2–6 anemones per treatment and time point). (A) Schematic representation: I. Symbionts taken up by the anemone and concentrated in the oral disk. Proliferation continues to the tentacles and exhibits a patchy pattern, suggesting symbiont migration *via* the gastrovascular cavity. II. Colonization of the tentacles continues through mitotic division and gastrovascular migration. III. Tentacles are fully colonized, and the symbiont cells begin to colonize the upper part of the column. IV. The symbionts continue to colonize the column from top to bottom, ultimately reaching the pedal disk. (B) Confocal images of tentacle colonization by *Symbiodinium trenchii* over 4 weeks. *Symbiodinium trenchii* rather than *S. minutum* is shown here, since *S. trenchii* was slower to colonize the host, and hence the spatial patterns are clearer across time. (C) Spatial pattern of colonization by different symbionts, presented as the percentage of total symbiont chlorophyll fluorescence occurring in the four different body regions of the anemone over time. C3, *Symbiodinium* phylotype C3; *Sm*, *S. minutum*; *Smic*, *S. microadriaticum*; *St*, *S. trenchii*; *Sv*, *S. voratum*.

Photophysiology and host performance

Photosynthesis and respiration of the host-symbiont partnership. Gross photosynthesis (mg O_2 per mg protein h^{-1}) was significantly different when Aiptasia formed a symbiosis with the different *Symbiodinium* species or types (one-way ANOVA, $F_{4,13} = 12.214$, $P < 0.001$; Fig. 3A). At the end of the experiment, anemones containing *S. minutum* or *S. trenchii* photosynthesized at a similar rate, as did those anemones containing *S. microadriaticum* or type C3; however, the photosynthetic rate with the former two symbionts was faster than that with the latter two (Tukey *post hoc*, $P < 0.01$ for all comparisons). In contrast, dark respiration rate (mg O_2 per mg protein h^{-1}) was not affected by symbiont identity (Fig. 3A; Welch's

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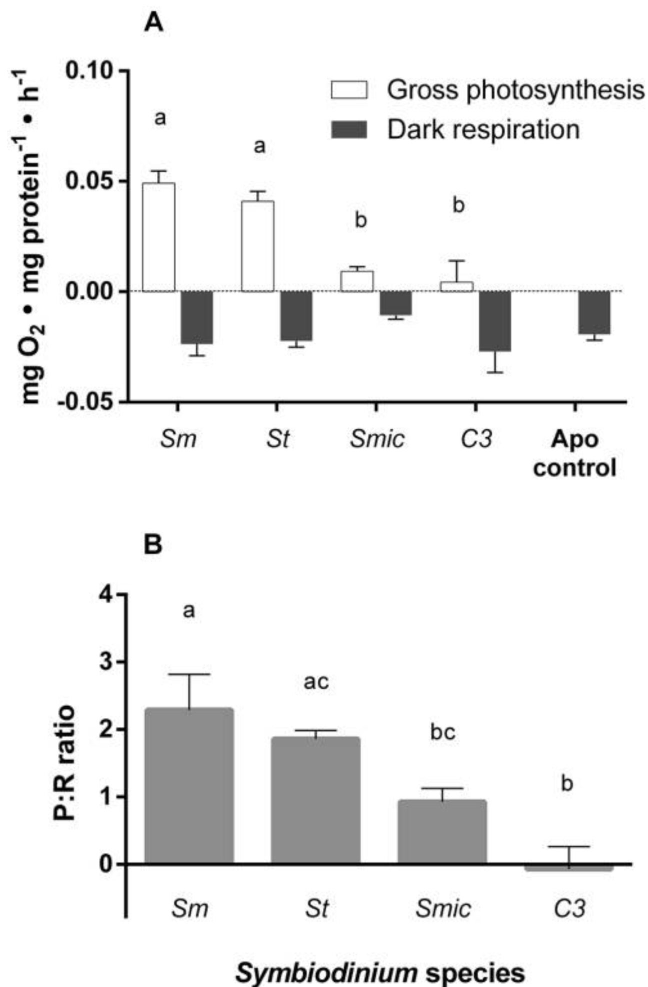

Figure 3. Gross photosynthesis and dark respiration of the holobiont varied with symbiont identity. Photophysiological performance (mg O_2 per mg protein h^{-1}) of the holobiont was quantified 8 weeks after inoculation. (A) Gross photosynthesis (white bars) and total symbiosis dark respiration (gray bars). (B) Ratio of hourly gross photosynthesis to respiration (P : R ratio). Bars represent means ± SE; $n = 3$–4 anemones per treatment. Different letters above bars indicate significant differences between treatments ($P < 0.05$). Apo, aposymbiotic; C3, *Symbiodinium* phylotype C3; *Sm*, *S. minutum*; *Smic*, *S. microadriaticum*; *St*, *S. trenchii*; *Sv*, *S. voratum*.

ANOVA, $F_{4, 5.5} = 3.043$, $P > 0.05$). As a result, there was a significant difference in the P : R ratio between anemones hosting the different symbionts at the end of the experiment (1-way ANOVA, $F_{3, 10} = 11.420$, $P < 0.05$), with *S. minutum*-colonized anemones having a significantly higher ratio than those containing *S. microadriaticum* or C3 (Tukey *post hoc*, $P < 0.05$ for both comparisons) but not *S. trenchii* (Fig. 3B).

Host growth and asexual reproduction. Host growth, estimated at the end of the experiment from the oral disk diameter, was significantly impacted by *Symbiodinium* identity (1-way ANOVA, $F_{5, 44} = 19.820$, $P < 0.0001$). In particular, anemones colonized by *S. minutum* were significantly larger at week 8 than anemones colonized by any of the heterologous symbionts (Tukey *post hoc*, $P < 0.0001$ for all comparisons; Fig. 4A), while anemones colonized by heterologous symbionts were no larger than aposymbiotic anemones (Tukey *post hoc*, $P > 0.9$ for all comparisons). Anemones containing *S. minutum* grew by an average of 9.22% ± 0.68% between the beginning and the end of the experiment, while anemones containing *S. trenchii* and *S. microadriaticum* grew by just 1.9% ± 1.06% and 1.54% ± 1.05%, respectively. Anemones containing C3 and *S. voratum* showed, on average, negative growth of (−1.91%) ± 0.65% and (−1.71%) ± 1.08%, respec-

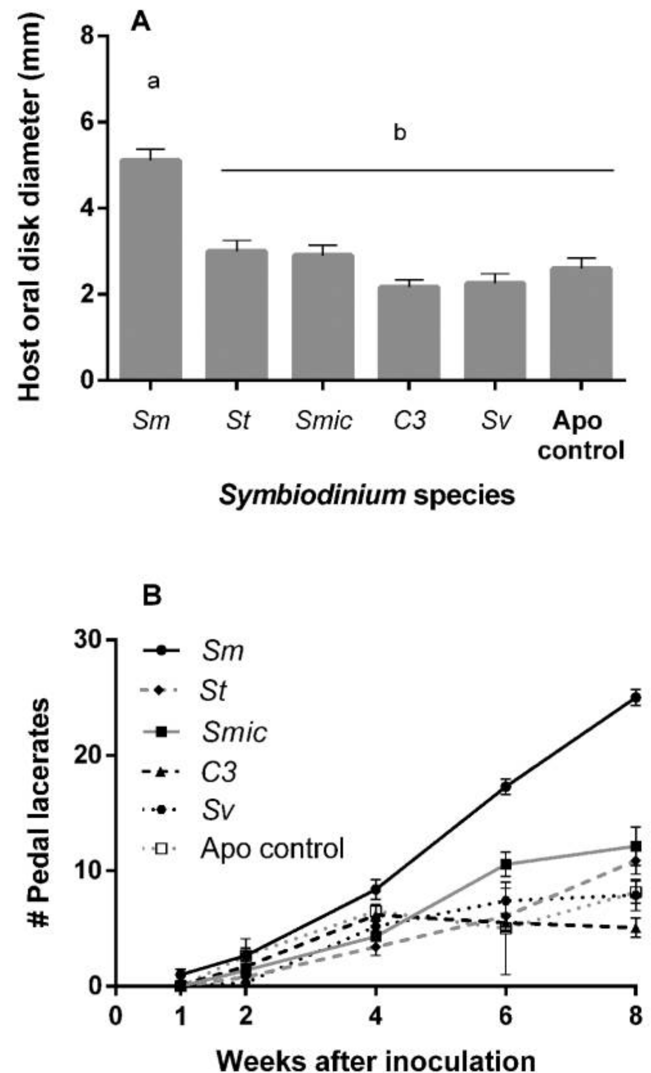

Figure 4. Host performance varied with symbiont identity. (A) Host growth estimated from the change in oral disk diameter (mm), quantified 8 weeks after colonization ($n = 10$ for each treatment). (B) Asexual reproduction *via* pedal laceration measured by counting pedal lacerates quantified over 8 weeks after inoculation ($n = 10$ anemones per treatment and time point). Values are means ± SE. Apo, aposymbiotic; C3, *Symbiodinium* phylotype C3; *Sm*, *S. minutum*; *Smic*, *S. microadriaticum*; *St*, *S. trenchii*; *Sv*, *S. voratum*.

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tively. By comparison, aposymbiotic anemones grew 0.28% ± 0.88%.

Consistent with the differences in size and growth, after 8 weeks anemones colonized by *S. minutum* had produced significantly more pedal lacerates than did anemones colonized by the other symbionts (chi-square, Bonferroni *post hoc*, $P < 0.0001$ for all comparisons; Fig. 4B). *Symbiodinium minutum*-colonized anemones produced an average of 25 ± 0.58 pedal lacerates per anemone between the start of the experiment and week 8, compared to 12 ± 1.48, 11 ± 1.20, 7 ± 1.11, and 5 ± 0.85 pedal lacerates when in symbiosis with *S. microadriaticum*, *S. trenchii*, *S. voratum*, and type C3, respectively. By week 8, *S. microadriaticum*-colonized anemones produced a number of pedal lacerates similar to that produced by *S. trenchii*-colonized anemones, and both produced a higher number of pedal lacerates than anemones inoculated with *S. voratum* or type C3 (chi-square, Bonferroni *post hoc*, $P < 0.05$). Aposymbiotic anemones produced 8 ± 0.76 pedal lacerates per anemone by week 8, a number similar to that produced by anemones inoculated with *S. microadriaticum*, *S. trenchii*, or *S. voratum* (chi-square, Bonferroni *post hoc*, $P > 0.05$ for all comparisons) but a significantly higher number than for anemones inoculated with C3 (chi-square, Bonferroni *post hoc*, $P < 0.05$).

Discussion

Colonization success varies with symbiont identity

Colonization success varied with symbiont identity, with the homologous *Symbiodinium minutum* being fastest to proliferate and invade host tissues. Consistent with our results, previous colonization studies have shown that symbiotic algae are more immediately successful when originally isolated from the same host species (Kinzie and Chee, 1979; Schoenberg and Trench, 1980b; Davy *et al.*, 1997; Belda-Baillie *et al.*, 2002; Harii *et al.*, 2009; Starzak *et al.*, 2014). For example, Weis *et al.* (2001) showed that homologous symbionts were able to colonize nearly all *Fungia scutaria* larvae inoculated and reach higher densities inside each larva than were heterologous symbionts, while some of these heterologous symbionts could not colonize the larvae at all.

In contrast, some of the other symbionts in our study failed or struggled to form a symbiosis with Aiptasia. In particular, C3 was taken up by the host, but there was no evidence of symbiont proliferation, while *S. voratum* did not persist inside the host beyond four weeks. Previous studies have likewise shown differential success of heterologous symbionts in Aiptasia. For example, Belda-Baillie *et al.* (2002) reported that adult Aiptasia could form only a limited symbiosis with cultured *Symbiodinium* A and was unable to form a symbiosis with a C-type and a free-living A-type, even after three months. Furthermore, *S. voratum* has previously been reported to be incapable of forming a symbiosis with both larval and adult Aiptasia, a finding that is consistent with our observations here (Xiang *et al.*, 2013; Wolfowicz *et al.*, 2016). Our results are, however, somewhat different from those of Starzak *et al.* (2014), who reported that *S. voratum* (culture ID CCMP421) was, on occasion, able to proliferate rapidly and reach high densities inside Aiptasia. The reason for the difference between this study and the current study is unclear—not least given that the same cultures of *Symbiodinium* (CCMP421) and Aiptasia (NZ1) were used in both studies—though it is important to note that Starzak and coworkers also reported a relatively low percentage of colonization success and a high incidence of host mortality when anemones were inoculated with *S. voratum*.

The failure of *Symbiodinium* C3 and *S. voratum* to proliferate and persist in Aiptasia, respectively, despite uptake from the external environment, highlights the role of post-phagocytotic events in the establishment of a successful symbiosis, including the initiation of a functional molecular cross-talk between the host and the symbiont. Exactly what this molecular cross-talk involves remains unclear, though it is likely that molecular signals and/or nutritional interplay have a role in the evasion of host immunity by the invading symbiont and the prevention of symbiont loss *via* events such as host expulsion or host-cell apoptosis (reviewed by Davy *et al.*, 2012). The failure of *S. voratum* to persist is not surprising given its free-living origin, as well as the fact that this species is relatively large compared to other symbiont species. We did not measure cell size here; however, a recent paper by Biquand *et al.* (2017) suggests that the colonization success of *Symbiodinium* in Aiptasia is negatively correlated with symbiont cell size. Our results, where different symbiont species or types had very different fates in symbiosis with Aiptasia, provide a baseline for further study of the molecular signals and events that dictate symbiosis success in this important model system.

While there were marked differences between the colonization success of the different symbionts, the spatial patterns of colonization were very similar, with algae first appearing in the oral disk before spreading to the tentacles, then the column, and, finally, the pedal disk. This pattern is perhaps not surprising given that the oral disk is likely the first point of contact between host and symbiont. The patchy pattern of symbiont proliferation in host tentacles (Fig. 2A) suggests that algal clusters arise from mitotic division of a single invading cell, with the algal symbionts moving either through the anemone's tissue or *via* transport in the gastrovascular cavity. Symbionts have been shown to pass between host cellular compartments during host oogenesis or in larvae in a range of cnidarians (Benayahu *et al.*, 1992; Benayahu and Schleyer, 1998; Hirose *et al.*, 2000, 2001; Davy and Turner, 2003; Marlow and Martindale, 2007); however, the putative migration distances to generate the patterns seen in our study, even in small adult Aiptasia, would be much greater, and, hence, this mechanism of proliferation seems implausible here. It is more likely that the symbionts exit host cells, move through the gastrovascular cavity *via* ciliary water currents, and invade other host cells

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elsewhere in the animal. While as a result of the technical limitations of our confocal method we could not confirm the presence of symbiont cells in the gastrovascular cavity of the tentacles, Parrin *et al.* (2012, 2016) observed within-colony symbiont migration in soft corals as a response to thermal stress, providing support for this proposed mechanism. This topic warrants detailed investigation.

Host photophysiology and performance

Host photophysiology and performance were clearly influenced by *Symbiodinium* identity, consistent with previous observations of differential photosynthetic performance in different *Symbiodinium* types (Rowan, 2004; Goulet *et al.*, 2005; Berkelmans and van Oppen, 2006; Loram *et al.*, 2007; Cantin *et al.*, 2009). The homologous *S. minutum* was the most productive at the whole-symbiosis level and most beneficial with respect to host growth and pedal laceration, consistent with the carbon flux model of Starzak *et al.* (2014) for this same host-symbiont combination. In contrast, *S. microadriaticum* and *S. trenchii* successfully colonized Aiptasia, reaching relatively high population densities (though not as high as *S. minutum*), but did not contribute substantially to host growth and proliferation. As expected, given the inverse relationship between symbiont density and CO_2 availability for photosynthesis (Davy and Cook, 2001), the lower density of these two heterologous symbionts relative to the homologous *S. minutum* elevated their photosynthetic rates per cell (data not shown); but this partial (with *S. microadriaticum*) or total (with *S. trenchii*) offset of total symbiosis photosynthesis was insufficient to generate comparable rates of host growth and asexual reproduction. In the case of *S. microadriaticum*, the low symbiont density may partially explain the relatively low photosynthetic rate of anemones colonized by this species compared to that of those colonized by *S. minutum*. However, the situation in the *S. trenchii*-colonized anemones was different, as their photosynthetic rate was similar to that of the *S. minutum*-colonized anemones. It therefore seems likely that *S. trenchii* released less of its photosynthate to the host, consistent with previous observations for some members of clade D (Cantin *et al.*, 2009; Jones and Berkelmans, 2010). A further possibility is that *S. trenchii* is more energetically costly to maintain than *S. minutum*, though this is not supported by the respiration rates measured in our study, which were unaffected by symbiont identity. Previous evidence that some heterologous *Symbiodinium* taxa are energetically costly when in symbiosis with Aiptasia comes from Starzak *et al.* (2014), who found that *S. voratum* and type F5.1 induced high rates of host respiration that countered any photosynthetic benefits and ultimately led to a low P : R ratio and little scope for host growth and reproduction; these authors did not study the impacts of *S. trenchii*, however. The disadvantages of harboring some members of *Symbiodinium* clade D on host fitness have been reported previously. For example, when adult and juvenile *Acropora* sp. associated with *Symbiodinium* from clade D, they exhibited a decrease in lipid storage, egg size, and overall colony growth, compared to when they hosted *Symbiodinium* C2/1 (Little *et al.*, 2004; Jones and Berkelmans, 2011).

The behavior of *S. microadriaticum* and *S. trenchii* is interesting because it is consistent with the cheater strategy exhibited by many parasites, as well as strains of the typically mutualistic *Rhizobium* in plants, where colonization is not associated with any discernible benefit for the host (Herre *et al.*, 1999; Sachs and Simms, 2006; Sachs *et al.*, 2010, 2011). Previous studies have similarly suggested that several members of *Symbiodinium* clades A and D are less beneficial than those of, for example, clades B and C (Cantin *et al.*, 2009; Mieog *et al.*, 2009; Jones and Berkelmans, 2010; Stat and Gates, 2011; Lesser *et al.*, 2013). How these symbiont species persisted in Aiptasia is currently unknown, but they might somehow evade the host's immune responses and regulatory pathways and draw reserves from the host to support their metabolism and growth while providing little or no photosynthetically fixed carbon to the host in return. Further research on the differential regulation of these symbiont populations and the exchange of metabolites between the partners will help shed light on this matter. Ultimately, this information will enable us to better understand the long-term implications of harboring more *versus* fewer beneficial symbionts, especially in terms of the ability of coral reefs to recover from bleaching events and adapt, as our climate warms.

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