

Natural infections of aposymbiotic *Cassiopea xamachana* scyphistomae from environmental pools of *Symbiodinium*

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Abstract

The ability to acquire different types of the symbiotic dinoflagellate *Symbiodinium* (zooxanthellae) from the environment was investigated using aposymbiotic scyphistomae of the jellyfish *Cassiopea xamachana*. Non-symbiotic scyphistomae were placed on an offshore Florida patch reef and in Florida Bay during 3- and 5-day periods in March, and 5-day exposures in May, August and December of 2003. Scyphistomae were maintained in culture for several months, after which members of clades A, B, C and D *Symbiodinium* were detected in these hosts by denaturing gradient gel electrophoresis (DGGE) analyses. These findings contrast with naturally collected *C. xamachana* medusa from Florida Bay where all specimens possessed only *Symbiodinium* type A1. Furthermore, the polyps did not acquire the symbionts found in nearby cnidarian colonies, suggesting that a diverse pool of symbiont lineages exists in the environment. These results support previous laboratory studies where aposymbiotic hosts were initially non-selective and capable of acquiring many kinds of *Symbiodinium*. The specificity seen in adult hosts is likely a result of post-infection processes due to competitive exclusion or other mechanisms. A higher percentage of polyps became infected after 5 days of exposure, compared to 3 days, and no infections were observed in laboratory controls held in filtered seawater. Infections were lowest (50% at both sites) in March of 2003, when seawater temperatures were at their annual minima. Infection was 100% in scyphistomae exposed for 5 days during the months of May, August and December of 2003. These findings suggest that this host system, in addition to addressing questions of host-symbiont selectivity, can be employed to monitor and define the abundance and distribution of natural pools of *Symbiodinium*.

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1. Introduction

Symbiotic cnidarians often harbor genetically uniform populations of symbiotic dinoflagellates in the genus *Symbiodinium* (e.g. LaJeunesse, 2002; Goulet and Coffroth, 2003; LaJeunesse et al., 2003; Santos et al.,

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2003; Thornhill et al., 2006). However, many host species are capable of symbiosis with more than one *Symbiodinium* spp.; these different symbiotic associations are typically partitioned by the external physical environment or geographic location (i.e. irradiance, depth and temperature related to latitude) (Rowan and Knowlton, 1995; Rodriguez-Lanetty et al., 2001; van Oppen et al., 2001; LaJeunesse, 2002; LaJeunesse et al., 2004a,b).

Partner recombination, where an alternate symbiont population replaces the resident population, is a hypothetical mechanism by which symbiotic cnidarians adjust to changes in their surrounding environment (Buddemeier and Fautin, 1993; Baker, 2001). The larvae of most broadcast spawning corals, many species of Rhizostome scyphozoans, and numerous other cnidarians depend on “horizontal” acquisition from environmental pools of *Symbiodinium* to establish their symbioses (Fitt, 1984; Szmant, 1986; Richmond, 1997). In contrast, brooded larvae usually acquire their symbionts from their maternal parent prior to their release, a process referred to as “vertical” transmission (Richmond, 1997). Previous work on “horizontal” acquisition indicates that chemical cues play a role in bringing symbionts and hosts together (Fitt, 1985b) and that entry and persistence of healthy symbionts somehow blocks fusion of endosomes with lysosomes or phagosomes (Hohman et al., 1982; Fitt and Trench, 1983). Cnidarians exhibiting such broadcast spawning life histories tend to show greater variability in associating with particular symbionts over environmental and geographic gradients (Rowan et al., 1997; LaJeunesse et al., 2004b).

Despite the constancy and stability observed for many adult coral–algal symbioses (Goulet and Coffroth, 2003; Stat et al., 2004; Thornhill et al., 2006), experiments have demonstrated that many aposymbiotic cnidarians are capable of infection with multiple types of symbiotic algae, often exceeding the symbiotic diversity found in adult hosts. For instance, numerous forms of *Chlorella* spp. are readily endocytosed by freshwater *Hydra viridis* (e.g. McAuley and Smith, 1982; Rahat and Sugiyama, 1993). Similar patterns are well documented for the initial uptake of various *Symbiodinium* spp. in scyphistomae (polyp-stage) of the jellyfish *Cassiopea xamachana* (Colley and Trench, 1983; Fitt, 1984, 1985a), sea anemones (Kinzie and Chee, 1979; Schoenberg and Trench, 1980; Davy et al., 1997; Belda-Baillie et al., 2002), tridacnid clams *Hippopus hippopus* (Fitt, 1985a), gorgonians (Kinzie, 1974; Benayahu et al., 1989; Coffroth et al., 2001), and scleractinian corals (Schwartz et al., 1999; Weis et al., 2001; Little et al., 2004; Rodriguez-Lanetty et al., 2004). However, following

endocytosis of *Symbiodinium* spp., most of these studies show symbiont specificity early in the ontogeny of the host, with preference for homologous algae (those originating from same host) over heterologous algae (those originating from a different host). Homologous algae have superior growth rates and competitive ability in many hosts (e.g. Fitt, 1985a; Belda-Baillie et al., 2002).

The recent availability of molecular methods to investigate *Symbiodinium* diversity and associations has provided additional information on the development of host–symbiont specificity. Coffroth et al. (2001) documented non-specific uptake of *Symbiodinium* spp. by aposymbiotic juvenile gorgonians, however, specificity was eventually achieved, as clade B *Symbiodinium* became the only detectable symbiont lineage. A similar pattern occurred in another gorgonian species (Santos et al., 2003). The scleractinian coral *Fungia scutaria* similarly demonstrated specific selection for one *Symbiodinium* ITS type from clade C both during and after infection (Rodriguez-Lanetty et al., 2004).

Symbiodinium spp. are continuously released from their hosts (e.g. Steele, 1977; Hoegh-Guldberg et al., 1987; Stimson and Kinzie, 1991), yet little is known about the actual abundance or diversity of *Symbiodinium* living in the external environment. Free-living dinoflagellates matching the description of *Symbiodinium* have been found and cultured from the external environment (e.g., Loeblich and Sherley, 1979; Carlos et al., 1999; LaJeunesse, 2001; Schwarz et al., 2002). Field infection experiments utilizing aposymbiotic gorgonian hosts indicate that viable symbiont cells are readily available from the surrounding water (Coffroth et al., 2001). Longer exposure time to waters of the reef ecosystem and/or to dilute solutions containing cultured *Symbiodinium* spp. in the laboratory increased the percentage of animals infected. The rate of spread of a symbiont population within host tissues appears to also depend on the initial dose received during the exposure/treatment phase (Coffroth et al., 2001).

The initial lack of specificity by aposymbiotic scyphistomae of *C. xamachana* for a particular *Symbiodinium* spp. during acquisition (Colley and Trench, 1983; Fitt, 1984, 1985a) provides a mechanism to investigate the abundance and diversity of free-living pools of *Symbiodinium*. The current study investigates infection from environmental pools of *Symbiodinium* spp. off Key Largo, FL. We document temporal aspects of infection and identify various types of *Symbiodinium* taken into symbiosis with scyphistomae of *C. xamachana*, and that specificity leading to clade A1 found in adult jellyfish occurs later.

2. Methods

Prior to this experiment, one clonal line of azooxanthellate scyphistomae of *C. xamachana* was grown and maintained in Petri dishes containing 35 ppt Instant Ocean™ in a 26 °C incubator with 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ illumination for approximately 14 h of illumination per day. Animals were fed *Artemia* nauplii twice weekly and the water changed after each feeding.

Scyphistomae were placed in 10-ml centrifuge tubes with modified, open ends covered by 100 μm mesh plankton netting. These tubes were placed in 1-l Nalgene bottles containing 0.45 μm -filtered seawater for transportation to various habitats in the waters around Key Largo, FL. Twelve replicate tubes, each containing 3–5 aposymbiotic scyphistomae, were placed on Admiral Patch Reef off Key Largo. Six tubes were stationed on six colonies (one tube per colony) of the scleractinian coral *Montastraea faveolata* and six other tubes were tethered to three cement bricks (two tubes per brick) in a small sand patch surrounded by a diversity of symbiotic anthozoans and octocorals containing various *Symbiodinium* ITS types (LaJeunesse, 2002). Additional, similarly prepared, tubes of scyphistomae were placed in Florida Bay, with four tubes each on a colony (one tube per colony) of the coral *Siderastrea radians*, four tubes next to the anemone *Aiptasia pallida* (each tube surrounded by a separate cluster of anemones), four tubes attached to four different heads of the symbiotic sponge *Anthosigmella varians*, and four tubes tethered to four cement bricks (one tube per brick) in beds of *Thalassia* sea grass. Half of the tubes at each location were collected after 3 days of exposure, the other half after 5 days. The tubes were placed in 1-l Nalgene bottles and transported to the laboratory where the polyps were rinsed with 0.45 μm -filtered seawater into clean Petri dishes (one dish per polyp) and placed in a 26 °C incubator with 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ illumination. Animals were fed *Artemia* nauplii twice weekly and the water changed after each feeding. Mortality rates were low (<5%) for all locations. Control scyphistomae ($n=20$) were maintained under similar conditions, but without field exposure.

Colley and Trench (1983) found that all strains of zooxanthellae rapidly declined in density to undetectable levels after 4 to 12 days before increasing in successful strains. Therefore, the presence of zooxanthellae was determined visually using a microscope 21 days post exposure for all scyphistomae, to ensure that only successful associations were measured. To test the hypothesis that length of exposure had an effect on zooxanthellae infection rate, data were analyzed using a paired *t*-test for independence by groups ($\alpha=0.05$) with the analytical software Statistica™ (Statsoft, 1999).

Scyphistomae were similarly exposed during May, August, and November of 2003, except that exposure time was 5 days during each season and tubes of scyphistomae were placed only in Florida Bay next to the same species as in the March samples. To test the hypothesis that time of year has an effect on zooxanthellae infection rate, data were analyzed Contingency Table Analysis with the analytical software StatXact 5.0™ (Cytel, 2002).

Symbiodinium were identified from the scyphistomae and strobilated ephyra collected in March 2003 after 3 to 5 months of laboratory maintenance when densities of symbionts were in the 10,000–50,000 per polyp range, as estimated from previous hemocytometer counts of infected polyps. Nucleic acids were extracted using the Wizard DNA preparation protocol (Promega). Symbiotic individuals were macerated with a 2-ml tissue grinder in 600 μl of nuclei lysis buffer (Promega). The sample was transferred into a 2-ml centrifuge tube containing 200 μl of 0.5 mm glass beads, then was bead beaten for 120 s at 4.8×10^3 rpm in a Biospec Mini-Beadbeater. 2.5 μl of 0.1 mg ml^{-1} proteinase K was added and the sample was incubated at 65 °C for 1 h, followed by incubation at 37 °C for 30 min with 3 μl of 100 mg/ml RNAse A. 250 μl of protein precipitation solution (Promega) was mixed with each sample and the samples were placed on ice for 30 min. The precipitate was pelleted by centrifugation for 10 min at $13\,000 \times g$. 600 μl of supernatant nucleic acids were precipitated by transfer to a new 1.5-ml microcentrifuge tube containing 700 μl 100% isopropanol and 50 μl of 3 M NaAc (pH 5.6) and incubated on ice for a minimum of 15 min. The tube was then centrifuged for 10 min at $13\,000 \times g$, the supernatant removed, and the pellet washed with 70% ethanol. The DNA was centrifuged again for 5 min, dried and resuspended in 95 μl of water and 5 μl of 400 M Tris, 10 mM EDTA solution.

The internal transcribed spacer 2 region (ITS 2) of nuclear ribosomal RNA was used to discriminate molecular types of *Symbiodinium* (LaJeunesse, 2001, 2002). This region was amplified from 0.5 to 3 μl of DNA extract for denaturing gradient gel electrophoresis (DGGE) using primers “ITS 2 clamp” and “ITSintfor 2” (LaJeunesse and Trench, 2000). PCR amplification followed the “touchdown” thermal cycle profile described by LaJeunesse (2002). Products of these PCR reactions were checked by electrophoresis on agarose gels (0.8% agarose in 40 mM Tris–acetate, 1 mM EDTA solution). Successfully amplified PCR products were subsequently electrophoresed in denaturing gradient gels (45–80% formamide, 8% acrylamide denaturing gradient gels; 100% consists of 7 M urea and 40% deionized formamide) following the protocol described

Table 1
Symbiodinium ITS 2 types found in *Cassiopea xamachana* following exposure seawater of Florida Bay and Admiral Reef off of Key Largo, Florida, in March 2003

Holobiont	Nearest host	Location	n	ITS 2 type
Scyphistomae	<i>Aiptasia pallida</i> (A4)	Florida Bay	3	C3
	<i>Anthostigmella</i> (B11)	Florida Bay	3	C3
	<i>Siderastrea radians</i> (B5a)	Florida Bay	2	A1
	<i>Siderastrea radians</i> (B5a)	Florida Bay	1	C3
	None, sea grass	Florida Bay	3	C3
	None, sea grass	Florida Bay	1	A1
	<i>Montastraea faveolata</i> (B1)	Admiral Reef	4	D1a
	None, reef	Admiral Reef	6	B1
	None, reef	Admiral Reef	6	B1
Ephyra	<i>Aiptasia pallida</i> (A4)	Florida Bay	1	C3
	<i>Montastraea faveolata</i> (B1)	Admiral Reef	1	D1a
	None, sea grass	Florida Bay	1	C3
Adult	–	Florida Bay	6	A1
Medusae	–	–	–	–

Symbiodinium types were identified by DGGE analysis from scyphistomae and newly strobilated medusae (ephyra) and are listed using the alphanumeric nomenclature of LaJeunesse (2001). Parentheses following nearest-host denotes the symbiont type found to dominate that host. Adult medusae of *C. xamachana* (n=6) collected from Florida Bay in March 2003 all contain the symbiont identified as type A1.

by LaJeunesse and Trench (2000), with the modifications of LaJeunesse et al. (2003).

3. Results

Representatives of virtually all clades of *Symbiodinium* (clades A, B, C, and D) known to reside in cnidarians were identified from symbiotic scyphistomae and ephyra cultured from March of 2003, including ITS 2 types A1, B1, C3, and D1a (Table 1). A sample diagnostic PCR–DGGE profile is shown for all types detected in *C. xamachana* in this study (Fig. 1). Adult *C. xamachana* medusae from Florida Bay were found exclusively with *Symbiodinium* type A1 (Table 1). For scyphistomae immediately adjacent to another symbiotic organism, the symbiont type harbored in that neighboring host colony was also identified (Table 1). There was no apparent relationship between where the tubes were placed and which type of *Symbiodinium* was taken into symbiosis (Table 1).

Percentages of scyphistomae containing zooxanthellae 21 days following a 5-day field exposure were 50% in March 2003 (n=17 out of 34 samples), and increased to 100% in May (n=54), August (n=36) and November 2003 (n=36) (Fig. 1). Infectivity rates in March 2003

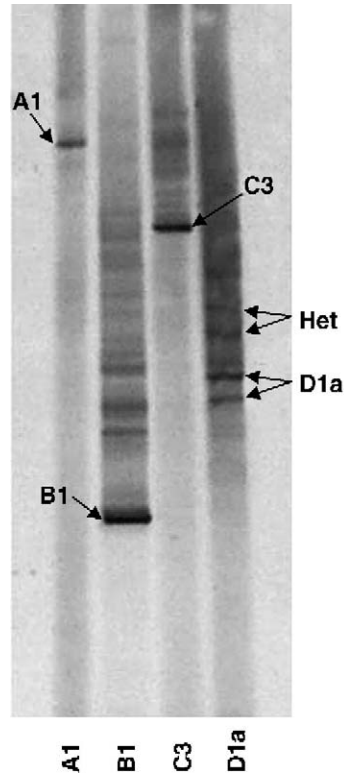


Fig. 1. Example PCR–DGGE profile of the *Symbiodinium* ITS 2 region for all *Symbiodinium* types detected in symbiosis with *Cassiopea xamachana* scyphistomae. Diagnostic bands are labeled for types A1, B1, C3 and D1a. Faint, repeated bands in each profile are possibly rare intragenomic variants and heteroduplexes (Het) created dominant sequences.

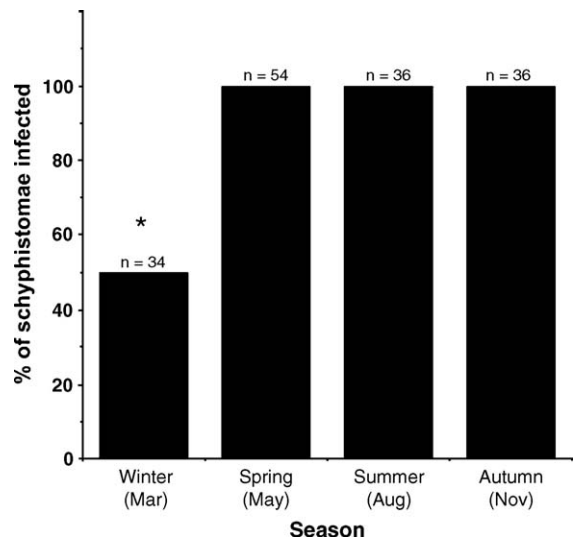


Fig. 2. Percentage of scyphistomae of *Cassiopea xamachana* containing zooxanthellae following a 5-day exposure in Florida Bay through seasons of 2003. The number of samples (number of polyps) is listed above each histogram. *Significant ($p < 0.0001$) effect of season when compared to all other treatments.

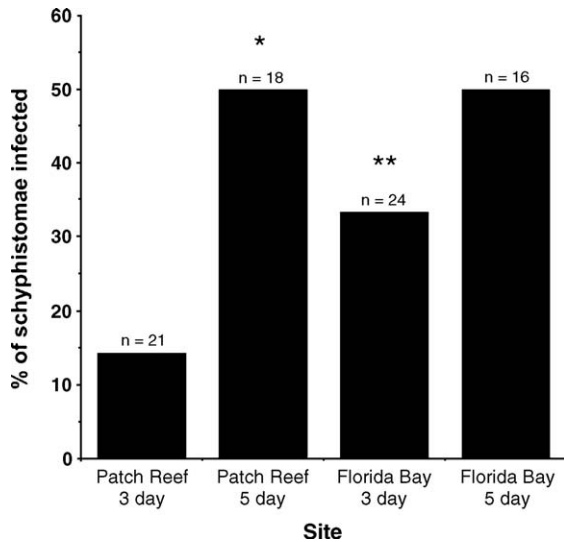


Fig. 3. Percentage of scyphistomae of *Cassiopea xamachana* containing zooxanthellae following a 3- or 5-day exposure in Florida Bay or on Admiral Patch Reef in the Atlantic Ocean off Key Largo in March 2003. The number of samples (number of polyps) is listed at the top of each histogram. *Significant ($p < 0.05$) effect of exposure time when compared to the lowest exposure duration from that location. **Significant ($p < 0.05$) difference in infection rates for that time of exposure between locations.

were significantly lower than that of all other groups (test of homogeneity of proportions, $\chi^2 = 70.4895$, $p < 0.0001$) (Fig. 2).

A higher percentage of polyps in March were infected when exposed to field conditions: 5 days compared to 3 days on both Florida Bay and Admiral Patch Reef. However, this difference between 3- and 5-day exposures was only significant on Admiral Reef ($p < 0.05$). About twice the percentage of animals were infected in Florida Bay after 3 days compared to those placed on the patch reef (Fig. 3), and this difference between habitats was significant ($p < 0.05$). No scyphistomae from the control group were infected during the experiment ($n = 20$).

4. Discussion

Aposymbiotic *C. xamachana* scyphistomae are open to infection by multiple types of symbiotic algae in natural environments. The results of this field study corroborate previous laboratory studies where *C. xamachana* formed successful symbioses with a number of *Symbiodinium* spp. (Schoenberg and Trench, 1980; Colley and Trench, 1983; Fitt, 1985b). Many aposymbiotic cnidarian hosts similarly endocytose a range of symbiotic algae (often with limited selectivity), however specificity for homologous algae is often apparent soon after infection with homologous algae exhibiting superior growth rates and

competitive ability (Kinzie, 1974; Kinzie and Chee, 1979; Schoenberg and Trench, 1980; McAuley and Smith, 1982; Colley and Trench, 1983; Fitt and Trench, 1983; Fitt, 1984, 1985a; Benayahu et al., 1989; Rahat and Sugiyama, 1993; Davy et al., 1997; Coffroth et al., 2001; van Oppen et al., 2001; Weis et al., 2001; Belda-Baillie et al., 2002; Rodriguez-Lanetty et al., 2004). In the experiments detailed here, scyphistomae of *C. xamachana* retained representatives of every major clade of *Symbiodinium* found in association with scleractinian corals, including clades A, B, C, and D (Table 1). Since adult *C. xamachana* medusae were detected exclusively in symbiosis with ITS type A1 symbionts in Florida (Table 1), selection for A1 in *C. xamachana* occurs post-endocytosis (see also Coffroth et al., 2001; Rodriguez-Lanetty et al., 2004), possibly by differential growth rates and competition within the host gastrodermal cell (e.g. Fitt, 1985b, Belda-Baillie et al., 2002) or by some other, currently unknown mechanism.

There was no apparent relationship between the nearest neighbor symbiont type and which type of *Symbiodinium* was taken into symbiosis in *C. xamachana* (Table 1). Most scyphistomae placed near long-term study colonies of *M. faveolata* became infected with D1a (Table 1). *Symbiodinium* B1 populated these colonies throughout the past 5 years of seasonal sampling (Thornhill et al., 2006), suggesting that proximity to a source of one kind of symbiont does not correlate with what successfully infects the scyphistomae. Similarly, in Florida Bay, *Symbiodinium* type C3 was identified from scyphistomae positioned near the anemone *A. pallida*, and the sponge *A. varians*, which contained B1 and B11 respectively. *Symbiodinium* A1 and C3 were identified from polyps adjacent to colonies of the coral *S. radians*, a small coral that associates with *Symbiodinium* B5a in backwater lagoonal environments (LaJeunesse, 2002). The symbionts acquired by these scyphistomae may have originated from free-existing environmental pools or they may have been released from other nearby hosts (e.g. Reimer, 1971; Steele, 1977; Lee et al., 1995). However, D1a is known to exist in some colonies of *M. faveolata* at concentrations that are not detectable by PCR–DGGE (LaJeunesse, unpublished). Therefore it is possible that the symbionts detected in experimental scyphistomae could originate from their nearest host at undetectable densities.

The four types of *Symbiodinium* detected in *C. xamachana* during this study are just a subset of types present in these environments (see LaJeunesse, 2002). Previous work in the laboratory also demonstrated that *C. xamachana* is capable of symbioses with numerous strains of algae, but did not form symbioses with all

available strains (Schoenberg and Trench, 1980; Colley and Trench, 1983; Fitt, 1985b). This selectivity may be a result of zooxanthellae size (Fitt, 1985b), competitive ability (Fitt, 1985b; Belda-Baillie et al., 2002), or some other unknown mechanism (e.g., host symbiont recognition mechanisms). Similar results have been documented for other hosts that form symbioses with multiple lineages of *Symbiodinium* (e.g., Davy et al., 1997; Belda-Baillie et al., 2002). The success of several *Symbiodinium* types in *C. xamachana*, is one example of an initially open symbiosis in the diversity of cnidarian–algal symbioses that exist.

Our experimental approach may have been biased by culturing time required to obtain sufficient material for DGGE analysis. Scyphistomae were maintained for 3 to 5 months following exposure to reef water. During this time, the artificial conditions possibly select for certain symbiont types to the exclusion of others. As a result, we cannot assume that the *Symbiodinium* types detected were the only symbionts to become infected during the 3- to 5-day exposure. Despite this limitation, it is still apparent that a diversity of *Symbiodinium* types (clades A–D) was readily available from the water column and they were capable of producing viable symbioses in *C. xamachana*.

Laboratory experiments show that infection rate is correlated to algal concentration (Montgomery and Kremer, 1995; Coffroth et al., 2001; Kinzie et al., 2001) and time of exposure (Fig. 2, but not Fig. 3 in Coffroth et al., 2001). Differences in infection rate vs. exposure time in the current experiments (Fig. 2) suggest that these same principles are acting in nature. Coffroth et al. (2001) found 100% infection of octocoral polyps placed in the field in Panama for 3–7 days, with time to infection much greater than that seen in laboratory experiments, where all polyps exposed to 500 cells ml⁻² density of *Symbiodinium* for 2–9 days became infected within 2–3 weeks. Backreef exposure took a shorter time course similar to that seen in laboratory experiments, implying higher densities of potential symbiont availability, whereas animals placed on the fore-reef and deep reef took 2–3 weeks longer to look visibly infected (Coffroth et al., 2001).

Differences in uptake rates in bay and patch-reef environments, as well as lower infection rates during the coldest season, suggest that availability of potential symbionts varies both temporally and spatially (Figs. 2 and 3). Lower infection rates of scyphistomae during the coldest season (March) indicate that low temperatures may inhibit the establishment of symbioses. Three-day exposures resulted in significantly lower infections on the patch reef (~15%) compared to scyphistomae placed in Florida Bay (~33%), indicating spatial variability of *Symbiodinium* available in the water column. Variability

in environmental concentrations of *Symbiodinium* may influence possible post-bleaching re-infection processes thereby affecting the ability of corals and other symbiotic cnidarians to survive periods of physiological stress (see Lewis and Coffroth, 2004).

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