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THE EFFECT OF *SYMBIODINIUM* SPP. SYMBIONT DIVERSITY ON CORAL
HOST PERFORMANCE

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Abstract

Scleractinian corals such as *Acropora palmata*, a threatened Caribbean coral, form obligate symbioses with single-celled algae of the genus *Symbiodinium*. Differences in the symbiont clade harbored have been linked to coral performance variation. The objective of this research was to measure variations in the survival and growth of larval settlers dependant on the clade harbored. We hypothesized that endogenous *A. palmata* symbiont exposure would result in the greatest settler growth. *Acropora palmata* larvae, collected in the field, were sent to Aquaria (n=6) across the US and settled on tiles. Larvae were either exposed to the native *A. palmata* symbiont (clade A3), isolated from adults, or left to take up algal symbionts from Caribbean and Pacific corals in their rearing tanks (n= 4-8 tanks per aquaria). Settlement tiles (n =44) were sent from aquaria at two time points (1 and 4 months) and DNA was extracted from all settlers (n=160). The internal transcribed spacer 1 (ITS1) regions of the large ribosomal subunit rDNA of zooxanthellae was isolated, run on acrylamide denaturing gels, and sequenced to differentiate between *Symbiodinium* clades. Settlers were also photographed for size. Performance of the coral-algal symbiosis was analyzed by correlating the symbiont clades present in the juveniles with polyp lengths measured. Our results revealed an aquarium effect on settlers at one month and four months with Omaha aquarium leading all others in settler performance. Interestingly, at four months, we found that a dominance of the native clade A3 did not result in better larval performance. Opportunistic clade D, and mixed A/C/D symbioses resulted in settlers with the largest sizes. Most importantly, the

low specificity of initial symbiont acquisition seen in juvenile *A. palmata* may be adaptive, allowing for the establishment of an advantageous multi-clade symbioses during early juvenile growth that can then be further specified when requirements change during adulthood.

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(a) At one month, settler size varied significantly among aquaria, with Omaha aquarium having the largest lengths in comparison to settlers reared at all other aquaria. Settler performance did not vary with clade diversity harbored (x-axis). No interaction effect is seen between clade diversity and aquaria. **(b)** At four months, settler fitness was significantly different among aquaria, and among the 3 clade diversity levels. Again, Omaha aquarium has the largest lengths among aquaria, and successively increasing levels of clade diversity (number of clades harbored by settlers) appeared to have larger mean lengths. An aquarium-clade diversity interaction effect is seen. See text for details on statistical testing.

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Chapter 1

1. Introduction

Significance of Research

Coral reefs are key ecological and commercial resources; they harbor much of the ocean's biodiversity, offer coastal protection, and generate approximately \$30 billion each year through fishing and tourism (Hoegh-Guldberg 2009). Despite this value, reefs worldwide are in decline (Wilkinson 2000). As global sea surface temperatures continue to rise, there have been increasing numbers of climate-related mortality events, such as bleaching and disease outbreaks (Green and Bruckner 2000, Mayor et al. 2006). These events have led to a severe reduction in Caribbean coral populations, particularly the Elkhorn coral, *Acropora palmata*. This species features large, broad branches that provide complex structure and support diverse vertebrate and invertebrate life (Bruckner and Hourigan 2002). It has historically served as the primary hermatypic (reef-building) coral in the Caribbean, Florida, and the Bahamas (Mayor et al. 2006, OPR NOAA Fisheries), but now the species is listed as 'threatened' under the US Endangered Species Act, ESA (Precht and Aronson 2004, Hogarth 2006).

Current efforts are underway to restore and protect populations of *Acropora palmata*. Restoration research has focused on factors that affect the survival and recruitment of *A. palmata* in the wild and in captivity (Herlan and Lirman 2008).

The Coral-Algal symbioses and Climate change

Reef corals such as *Acropora palmata* enter a mutualistic association with algal symbionts as a means of nutrient acquisition (Muscatine 1976, Barnes and Chalker 1990). Photosynthetic dinoflagellates of the genus *Symbiodinium* (also called 'zooxanthellae') are the dominant eukaryotic symbiont found in hermatypic corals (LaJeunesse 2004). Previous research has demonstrated the importance of algal symbiont types ("clades") in determining survival of the holobiont (that is, the coral animal, its algal symbionts, and associated microbial community) (Iglesias-Prieto and Trench 1994). Without the symbiotic algae, the coral's ability to proliferate and maintain dense populations is significantly reduced (LaJeunesse et al. 2009). The dinoflagellates live inside the endodermal tissue of the coral and use photosynthesis to fix carbon, creating organic substances such as glycerol that serve as respiratory substrates for the coral and allow for accelerated calcification; in return, the coral provides inorganic substances, shelter, and a well lit environment for the zooxanthellae (reviewed in Muscatine 1976, Barnes and Chalker 1990).

Under normal conditions, zooxanthellae are found at high concentrations in the tissue of coral. However, under stressful conditions, such as elevated sea surface temperature, this association breaks down, and the coral may eject some or most of its symbionts, resulting in a process called "bleaching" (Brown 1997, Kinzie et al. 2001). Although this process is potentially reversible, if left without its symbionts for too long, the host will starve (Glynn and D'Croz 1990).

Current rates of climate change pose a major threat to corals worldwide; bleaching events are becoming more frequent as ocean temperatures warm at an unprecedented rate (Kinzie et al. 2001). Some corals do display some ability to tolerate higher temperatures, however. Berkelmans and Van Oppen (2006) demonstrated variable tolerance for bleaching conditions among related coral species, with an increased tolerance being the direct result of a change in the zooxanthellae clade (or taxonomic lineage) dominating host tissues. This indicates that the *Symbiodinium* clade harbored by the coral affects the host's susceptibility to environmental changes.

Symbiosis can be established anew each generation in one of two ways: vertically, through maternal inheritance, or horizontally, through environmental acquisition (Trench 1987). Numerous genetically distinct strains of zooxanthellae co-occur in the water column (Baker 2003). During early ontology, coral larvae with horizontal acquisition mode can potentially acquire multiple clades (Van Oppen et al. 2001). However, the mature symbiosis may be relatively specific; the diversity of *Symbiodinium* clades and strains decreases over time resulting in a dominant clade, and several background clades (Van Oppen et al. 2001, Baker 2003). In most cases, symbiosis onset results in an association between a specific host coral and a specific strain of algal symbiont (Weis et al. 2001).

Whether a species' juveniles acquire zooxanthellae vertically or horizontally is somewhat independent of the species' mode of sexual reproduction. Two main modes exist in coral: brooding and broadcast spawning (Fig. 1). In brooding corals, fertilization and development into larvae occur within the coral polyps.

Conversely, broadcast spawning coral species release gametes into the water column in a synchronous annual event; fertilization subsequently occurs. The focal species of our study, *A. palmata*, is a spawning coral. Individual colonies of *A. palmata* are hermaphroditic and therefore can release both sperm and egg bundles. This release is timed with the lunar cycle and in the case of *A. palmata*, typically occurs 2-3 days after the August full moon.

A. palmata eggs do not contain zooxanthellae. Instead larvae take up symbionts from the environment once they settle (Abrego et al. 2009, van Oppen et al. 2001, Little et al. 2004, Weis et al. 2001). Adult *Acropora palmata* associates predominantly with *Symbiodinium* from clade A3 (LaJeunesse 2002) and this association is stable over time (Baums and LaJeunesse unpubl data). It is unclear when this specificity is established and if it depends on the availability of symbiont clades in the environment.

Discerning Symbiodinium at clade level

Coral host susceptibility to environmental changes may depend on the algal symbiont harbored (Iglesias-Prieto and Trench 1994). Therefore, knowing which strains of *Symbiodinium* spp. maximize the fitness of the symbiosis could in turn increase successful captive breeding and potentially help restore reefs.

Molecular analyses of the small and large ribosomal subunit have been used to distinguish between different *Symbiodinium* clades (LaJeunesse 2001).

However, the internal transcribed spacer region (ITS) provides a higher level of resolution for *Symbiodinium*, and even minor differences in ITS sequence identify

distinctive symbionts, as related to their distribution and host specificity in nature (Hunter et al. 1997, LaJeunesse 2002). Sequences of a region *within* the ITS region, the nuclear ribosomal DNA internal transcribed spacer 1 (rDNA ITS1) region, were used here to differentiate between *Symbiodinium* at the clade level. While nuclear ribosomal DNA genes are strongly conserved, ITS regions have faster substitution rates and therefore contain enough sequence variation to distinguish *Symbiodinium* at the intraspecies level and above (Van Oppen et al. 2001).

Denaturing Gradient Gel Electrophoresis (DGGE), a technique that detects differences at the single base level was used to discriminate between different ITS sequences. Using this process in conjunction with polymerase chain reaction (PCR) allows for the production of a fingerprint pattern showing dominant sequences of dinoflagellates present in the coral (LaJeunesse 2004).

The goal of this project was to determine how different clades of *Symbiodinium* spp. affect the performance of their coral host, *Acropora palmata*. It was hypothesized that the type of *Symbiodinium* spp. used for symbiosis, at a clade level, would significantly affect *A. palmata*'s growth, with the native zooxanthellae leading to better performance during the coral's development. This hypothesis was tested by exposing *A. palmata* larvae to native and non-native zooxanthellae strains in a captive environment. Settlers were reared and their growth performance and clade content measured over time.

2. Methods

Field collection and Aquarium rearing

In 2008, during summer mass spawning events in Puerto Rico, *Acropora palmata* gametes were collected from previously genotyped adult colonies and sperm and eggs from distinct colonies were crossed (following Baums et al. 2005). Spawn was collected from two Puerto Rican reefs: Bajo Gullardo (18.003° N, 67.33169° W) and Tres Palmas (18.35° N 67.263° W) (Fig. 2). Endogenous zooxanthellae from select parent colonies were also sampled. The crosses yielded hundreds of thousands of aposymbiotic larvae, which were reared in on-site tanks using natural, filtered seawater for three days. On day four, larvae were placed in 1L clear transport bottles, kept cold, and shipped along with preconditioned settlement tiles to six locations: Shedd Aquarium, Baltimore Zoo and Aquarium, Omaha Zoo and Aquarium, Dallas Zoo and Aquarium, Columbus Zoo and Aquarium, and the Smithsonian Institute for settlement and rearing. Each aquarium received between 8,000 and 12,000 larvae. Along with the larvae, each aquarium received a suspension of zooxanthellae isolated from *A. palmata* parents.

All aquaria followed largely the same protocol when exposing the settlers to zooxanthellae (Table 2). Prior to the arrival of the larvae, eight 10-gallon tanks were setup, and filled with 5 gallons of artificial seawater, and brought to 36‰ salinity and a temperature of 26°C. Transport bottles with larvae suitable for use were selected through visual examination; bottles with milky or rotten smelling

water were not used. To separate aggregated larvae, bottles were shaken gently. Larvae from good quality bottles were distributed equally among four 10-liter plastic bowls creating a well-homogenized larval suspension. As a control, five 10-mL samples of the suspension were taken from each bowl and transferred into small plastic cups to calculate the larval concentration per liter. Over the course of two hours, two liters of tank water were slowly added to each bowl to acclimate the larvae to the tank temperature.

After acclimation, all larval suspension bowls were mixed with each other and divided equally among tanks. Previous to the addition of the larvae, settlement tiles were inserted into 6-well by 9-well plastic egg crate, and then placed in the tanks. Tile shapes varied across aquaria, with three main tile types “plugs,” and large and small SECORE tiles. Four egg crates, all labeled either “no zoox” or “zoox,” were placed in each tank. These two groups represented settlers exposed to their native *A. palmata Symbiodinium* spp. symbiont (“zoox” group), and settlers left to obtain *Symbiodinium* spp. from their surrounding environment (“no zoox” group). The provided *A. palmata* zooxanthellae extract was divided equally among the “zoox” tanks,

Water source and lighting for the settlement tanks differed across aquaria (Table 2). Exposure to the zooxanthellae lasted five to seven days, with the water being exchanged several times over that period. After the first day, food was added to the tanks. Tiles were checked for settlement after six days by randomly choosing an egg crate and counting all settlers per tile (Fig. 3). After checking the tiles, all settlers were transferred to a separate holding tank.

Settlement tiles were preserved in 80-100% non-denatured ethanol and shipped to Pennsylvania State University at one month (Baltimore, Columbus, Dallas, Omaha, Shedd) and four months (Omaha, Smithsonian). Baltimore Zoo also provided a sample of an Indonesian adult coral from which they obtained the zooxanthellae for their “no zoox” settlement tiles.

Extraction and Settler measurements

At one month (time point one) *A. palmata* settlement tiles were obtained from Dallas (n=12), Baltimore (n=8), Omaha (n=4), Columbus (n=10), and Shedd (n=10) Zoos/Aquaria. One coral settler was removed from each tile received (n=44) and DNA was obtained using a spin-column protocol described in DNEasy animal tissue kit (Qiagen, Valencia, Calif.).

Remaining coral spat (settlers) from the first time point were examined and photographed with a dissection microscope before removal from tiles.

Photographs were taken along side a ruler and the program Axiovision v. 4.8.1.0 (Carl Zeiss Microimaging Inc, Thornwood, NY) was used to scale pictures according to the magnification used. The number of polyps per settler was counted and individual metamorphosed settlers were measured along the longest diameter as a fitness metric. If the settler had already divided into multiple polyps, several polyp diameters were measured as well. The general location of each settler on the tile was noted as well, using a diagram labeling different areas of the tile.

At four months (time point two), *A. palmata* settlement tiles were obtained from Smithsonian (n=13), and Omaha (n=3) Zoos/Aquaria. The same procedure of photographing and removing settlers was followed for settlement tiles received at this time point. DNA was extracted from the remaining time point one settlers (n=77), and all time point two settlers (n=40) using the Qiagen DNEasy animal tissue kit.

ITS1 amplification and DGGE

Optimization of the amplification process resulted in the use of a combination, or nesting, of two polymerase chain reactions (PCRs), the first targeting the entire ITS region, and the second targeting the ITS1 region. Nested-PCRs are up to 1000 times more sensitive than single-reaction PCRs (Grote 2002) and involve using the product from an initial PCR as the starting product for the second. DNA preparations (n=161) were first used as a template in a PCR in which the entire internal transcribed spacer (ITS) region was selectively amplified. Coral and symbiont-specific ITS primers “ZITSup,” (CACGACGTTGTAACGAC CCGGTGAATTATTCGGACTGACGCAGTGCT) and “ZITSDn” (TGTTTAGTTCCTTTTCCTCCGC) were used for this PCR amplification (Santos et al. 2001). PCR amplification recipe used is as listed in Table 1a. The reaction was carried out on an Eppendorf thermocycler (Mastercycler EP Gradient, Eppendorf, Hauppauge, NY) under the following conditions: an initial denaturing step at 94°C for 2 minutes, was followed by 35 cycles of: 30 seconds at 94°C, an annealing step at 60°C for 30 seconds, and 45 seconds of 72°C. Lastly, the annealing temperature was maintained at 72°C for 7 minutes for a single cycle,

and then held at a temperature of 4°C. Products were separated on an agarose gel (1.5%) together with Bioline's 3λ Hyperladder IV (Boston, MA) as a size standard. Gels were run at a constant voltage of 100 volts, for 30 minutes or a constant voltage of 115 volts for 25 minutes. They were visualized in a Bio-Rad Universal Hood II (Bio-Rad Laboratories, Milan, Italy). For samples showing a distinct band, products were diluted 1:50 with deionized water before proceeding with the next PCR, all other samples were left at full concentration.

The "ITS1forCLAMP" (CGCCCGCCGCGCCCGCGCCCGTCCCGCCGCCCC
CGCCCGGGATCCGTTTCCGTAGGTGAACCTGC) G-C clamp forward primer and the "ITSintrev2" (TTCACGGAGTTCTGCAAT) reverse primer, which flank the ITS1 region, were used for the second portion of the nested-PCR amplification (La Jeunesse et al 2002). PCR amplification recipe used is as listed in Table 1b. The reaction was carried out on an Eppendorf thermocycler (Mastercycler EP Gradient, Eppendorf, Hauppauge, NY) under the following conditions: initially, with a denaturing step at 94°C for 3.5 minutes, followed by 20 cycles of 30 seconds of 94°C, an annealing step at 60°C for 45 seconds, and 30 seconds of 72°C. Every cycle, the annealing temperature (60°C) was decreased by 1°C. In a second set of 20 cycles, the temperature was increased to 94°C for 30 seconds, reduced to 52°C for 45 seconds as an annealing step, and then again increased for 30 seconds of 72°C, conditions remained the same for all 20 cycles. Lastly, the annealing temperature was maintained at 72°C for 10 minutes and then held at a temperature of 4°C.

PCR amplification products were confirmed using gel electrophoresis. The PCR product bands on each gel were sized using Bioline's 3 λ Hyperladder IV (Boston, MA). Gels were all run at a constant voltage of 100 volts, for 30 minutes, or at a constant voltage of 115 volts, for 25 minutes.

Denaturing gradient gel electrophoresis (DGGE) was then performed on nested ITS1 PCR reaction samples that had correctly amplified ITS1 regions, as determined via gel electrophoresis. The LaJeunesse PCR-DGGE protocol was used for this process (LaJeunesse 2001). DGGE is a way of separating DNA fragments based on sequences rather than equal size. A denaturing acrylamide gradient that increases in concentration from top of gel to bottom, is used to detect single-base polymorphisms in PCR amplified DNA regions of equal size. The GC-rich primer used in the DGGE-PCR prevents the entire band from denaturing, but because the amplified bands vary in sequence, they melt (stopping migration) at different points along the gradient. All samples were loaded with a neutral loading dye onto a gel containing a denaturing acrylamide gradient of 50% to 90%, and separated by electrophoresis for 16 hours at a constant voltage of 115 V and a constant temperature of 60°C. All gels were run using the DGGE System, Model DGGE-2001 (C.B.S Scientific Co., Del Mar, Calif.). For visualization, gels were stained with Syber Green (Applied Biosystems) for 20 minutes, photographed, and images inverted.

Re-amplification and Sequencing

Examples of prominent (dominant) bands that appeared on the DGGE gels were sequenced to determine their clade identity by comparing them to known reference sequences (provided by Todd LaJeunesse). All DGGE bands of the same size were subsequently assumed to represent the same clade as the sequenced band. DGGE bands were excised and placed in 1.5 mL tubes along with 500µL DNase free H₂O. To break down the acrylamide, tubes were stored overnight at 4°C. The clamp-free “ITS1” forward primer and the “ITSintREV” reverse primer were later used in the PCR re-amplification of the dissolved bands. The reactions were carried out on an Eppendorf thermocycler (Mastercycler EP Gradient, Eppendorf, Hauppauge, NY) under the following conditions: initially, with an initial denaturing step at 94°C for 2.0 min was followed by 37 cycles of 94°C for 45 s, and an annealing step at 52°C for 45 s, 30 s of 72°C. A final extension at 72°C for 5.0 min followed and then temperature was held at 22°C. PCR amplification recipe used is as listed in Table 1c.

PCR amplification products that appeared as a single, strong band on a 1.5% agarose gel were prepared for sequencing. Sequencing preparation involved purification up the sample with exoSAP enzyme, a combination of exonuclease I (New England Biolabs, Ipswich, Mass.) and SAP enzyme (Roche, Indianapolis, Indiana). The exoSAP and sample combination (in a 1 to 5 ratio, respectively) were incubated in a thermocycler (Mastercycler EP Gradient, Eppendorf, Hauppauge, NY) machine (37°C for 15 minutes). This purification process

dissolves the primers added in the PCR amplification process, which must be removed prior to sequencing.

Samples were cycle-sequenced either at Penn State's Nucleic Acid Facility or in lab. For in-house cycle sequencing, the ABI Prism Big Dye Terminator Cycle Sequencing ready reaction kit (PE Applied Biosystems, Foster City, Calif.) was used, reagents and reaction conditions were as specified in kit. Sequencing reactions were analyzed with an ABI Hitachi 3730XL DNA Analyzer (PE Applied Biosystems, Foster City, Calif.).

Sequence analysis and Cladistics

Chromatograms were checked, edited, and sequences aligned with CodonCode Aligner software (CodonCode, Dedham, Maryland). Paup 4.0b10 (Swofford 2000) combined with the PaupUp graphical interface (Calendini & Martin, 2005) was used to perform maximum parsimony cladistic analyses on aligned data sets. Under maximum parsimony, sequence gaps were designated as a fifth character state. Bootstrap analyses were performed at 1000 replicates to assess branch support.

Statistical Analyses

A Goodman-Kruskal tau crosstabulation of the *Symbiodinium* clade/s and the treatment type was performed to assess the predictive power of the zooxanthellae treatment (i.e. was treatment indicative of the type of zooxanthellae the settlers eventually took up?). Treatment type refers to whether settlers were provided with endogenous *Symbiodinium* clade A, or left to pick up *Symbiodinium* clades from tanks (zoox or no zoox groups).

Two-way Analysis of Variance (ANOVAs, $\alpha = 0.05$) were used to test the effect of *Symbiodinium* type or *Symbiodinium* diversity, and aquarium on mean settler diameter length, a fitness metric. Tukey's HSD post-hoc tests were used to identify statistically significant differences between and within groups, where applicable. Data conformed to assumptions of normality and homogeneity of variances. PASW Statistics v. 18.0 (SPSS, Inc. an IBM company, Chicago, Illinois) was used for all statistical analyses.

3. Results

DGGE profiles

PCR-denaturing gradient gel electrophoresis profiles were typically characterized by either a single prominent band along with other faint bands located higher on the gel, (Lane 13) or several distinct bands (Lane 14, Fig. 4). Faint bands seen in combination with one prominent band (e.g. Lane 15 Fig. 4) were attributed to heteroduplexes, less stable strands formed during the denaturing and re-annealing of DNA strands during PCR amplification (LaJeunesse 2002, Myers, et al. 1989). Sequencing of heteroduplexes yielded the same clades as the prominent band. Profiles with more than one distinctive band represented a combination of symbiont clades.

Exposure to native symbiont

Inoculation did not have significant predictive power on the clade of zooxanthellae settlers acquired (*Goodman-Kruskal Tau crosstabulation*, $p > 0.05$, $n = 144$) in the two groups of settlers: those exposed to clade A3 (zoox), and those

not exposed (no zoox). Settlers exposed to zooxanthellae contained not only clade A3 (n=37) but also clades A/C (n=9), A/C/D (n=4), A/D (n=9), and B (n=1). This was comparable to the clades taken up by the untreated group (Clade A, n=29; A/C, n=9; A/D n=10; B, n=1; B/C, n=1; C, n=2; and D, n=5) This may indicate that “zoox” larvae have not been successfully infected with native *A. palmata* type A3 zooxanthellae after the 5-day exposure period, and uptake of zooxanthellae may have occurred later, *after* removal from exposure tanks. However, sequencing of the ITS 1 regions was not sensitive enough to distinguish whether the clade A symbionts present in the settlers were from the presented zooxanthellae (native A3 from *Acropora palmata*), or just taken up from the tanks (other A3 strains) (Fig .6a). Nevertheless, since the treatments “zoox or no zoox” as a factor did not significantly affect the presence of the types of *Symbiodinium* in the settler recruits, the two groups were pooled for the rest of the analyses.

Clade diversity

At one month and at four months, *Acropora palmata* settlers at different aquaria hosted a variety of combinations of *Symbiodinium* clades: A, B, C, and D (Fig. 5). A majority of settlers maintained symbioses with clade A symbionts, whether alone (53%), or in combination with clades B, C, and D (35.4%); only 17 (11.8%) settlers did not contain clade A as a part of their symbioses (Table 3, Fig. 5). At one month, combinations of clades included A (55%), A/C (14.9%), B (2.1%), B/C (1.2%), C (2.1%), D (10.6%), A/D (11.7%), and A/C/D (2.1%), while at four

months, combinations only included A (48%), A/C (8%), D (4%), A/D (32%), and A/C/D (8%) (Table 3, Fig. 5).

While the frequency of clade A (as dominant symbiont) remained the highest in one month to four month settler recruits, Clade A/D shifted from being present in only 12% of recruits at one month, to 32% at four months, the second highest clade combination frequency. Interestingly, while at one month, all

Symbiodinium clades (A, B, C, D), were present both as dominant, or “co-dominant” with other clades, by four months, most (96%) settlers harbored clade A, or clades in combination with A, and only clade D was present as another single dominant clade (4%, the lowest frequency). Clade D had previously (one month) been the fourth most prevalent clade. Also, at four months, clade B was no longer a part of any of the symbioses.

Phylogenetic analysis of representative DGGE band sequences (n= 78) showed 18 *Symbiodinium* spp. distinctions. ITS1 rDNA sequences could not be aligned between major *Symbiodinium* clades (A, B, C, and D); individual maximum parsimony bootstrap consensus trees were made for each clade separately. Two A types (Fig. 6a), 4 B types (Fig. 6b), 9 C types (Fig. 7), and 3 D types (Fig. 6c) were observed. Reference sequences used in the analyses were provided by Todd C. LaJeunesse (samples labeled with black font in Fig 6 and 7). Not all grouped samples had reference sequences for their specific clade subtype in the LaJeunesse reference library and may represent unknown clade subtypes (n=11). There was no clear association between aquarium and subclades in *A. palmata* settlers.

Aquaria, Clade, Clade diversity, and Growth performance

One month

At one month, settlers were obtained from five aquaria: Dallas, Shedd, Smithsonian, Baltimore, and Omaha. After one month, coral settler fitness, as measured by the longest diameter, was significantly different among aquaria (2-way ANOVA with factors aquaria and clade, $F=14.705$, $p=0.00$, $n=94$), with Omaha aquarium having the largest diameter values in comparison to settlers reared at all other aquaria sampled at this time point (Fig. 8a, 9a). Settler performance did not appear to vary with clade diversity, e.g. the number of symbiont clades harbored by the settlers (2-way ANOVA with factors aquaria and clade diversity, $F=0.359$, $p=0.70$, $n=94$ Fig. 9a), or with clade ($p=0.414$, Fig.8a). The relationship between aquaria and settler performance was not mediated by the clade harbored by the settlers (2-way ANOVA with factors clade and aquaria, $F=0.328$, $p=0.858$, $n=94$), or by symbiont clade diversity (2-way ANOVA with factors clade diversity and aquaria, $F=0.035$, $p=0.991$, $n=94$).

Four months

Data at four months included settlers from only two aquaria: Omaha and Smithsonian. At four months, 2-way ANOVA analysis indicated that the effect of aquarium, clade, and the aquarium by clade interaction on mean settler length differences were significant ($F=41.826$, $p=0.00$; $F=2.660$, $p=0.046$; and $F=4.842$, $p=0.013$, respectively, Fig. 8b). Again, settlers reared at Omaha aquarium displayed the larger mean settler lengths. Tukey's post-hoc test revealed that

settlers harboring the clade combination A/C/D were significantly different in their performance, in that they had larger mean lengths, from settlers harboring clade A ($p=0.031$), clade A/C ($p=0.006$), and clade A/D ($p=0.027$). Clade D harboring settlers performed equally well as A/C/D harboring settlers ($p=0.971$).

ANOVA results of aquarium and symbiont clade diversity effects on mean lengths, again showed a significant aquarium effect (2-way ANOVA with factors aquaria and clade diversity, $F=46.863$, $p=0.00$, $n=50$), with Omaha exhibiting the largest mean lengths (Fig. 9b). Clade diversity also had a significant effect ($p=0.007$) on settler performance with settlers harboring three clades displaying larger mean diameters in comparison to those harboring only one ($p=0.002$), or two ($p=0.013$) clades (Fig. 9b). Lastly, a significant interaction effect between symbiont diversity and aquarium ($p=0.011$) was seen.

4. Conclusions and Discussion

The successful establishment of a symbiosis between Symbiodinium dinoflagellates and coral settlers is a crucial step in the coral's life history. Adults of species *Acropora palmata*, a threatened Caribbean coral, show high specificity for A3 Symbiodinium. Here, we show that this specificity develops only over time with larvae initially taking up a wide variety of symbionts, including symbionts from the Pacific (clade D) that they have not been in contact with for millions of years.

Rearing conditions affect growth rate

The aquarium at which settlers were raised clearly influenced settler size, with Omaha aquarium having the largest or most fit settlers, indicating a discrepancy in care or holding tank conditions between the various sites. Variable conditions that could affect the growth, settlement, and survivorship of the juvenile settlers include variant microbial communities on tiles and in tanks, settlement tile condition and shape, and light conditions (Negri et al. 2001, Harrington et al. 2004). Tile shapes may have differential priming levels and therefore vary in crustose coralline algae (CCA) cover, a known settlement cue (Negri et al. 2001, Erwin et al. 2008). Rates of larval metamorphosis also vary in response to different CCA species (Negri et al. 2001, Erwin et al. 2008, Ritson-Williams et al. 2010), which were not controlled for in this experiment. Recently published research has found that CCA species *T. prototypum* and *H. boergesenii* seem to facilitate the larval settlement and post-settlement survival of *Acropora palmata* settlers (Ritson-Williams et al. 2010); these species may improve settlement and survival in ongoing *A. palmata* sexual recruitment studies.

Acquisition and persistence of symbionts in juvenile A.palmata

The majority of settlers contained A3 symbionts in both the treated and untreated *A. palmata* settlers. We could not distinguish between settlers that took up native A3 *Symbiodinium* and those that obtained A3 from other sources after (or even during) the 5-day inoculation period. Clearly, inoculation protocols could be improved. Previous literature has revealed a large range of conditions that may lead to successful symbiont establishment. Coffroth et al. (2001) demonstrated

successful infection of gorgonian larvae with short exposure times of less than 1 hour, and an initial density of 100 cells per mL. Kinzie et al. (2001) reported a dose-dependent infection rate: exposure to higher concentrations of zooxanthellae accelerated the rate at which the symbiosis was established. Further research on juvenile coral *Symbiodinium* infection in an aquarium setting is necessary to determine the efficacy of controlled infection in *A. palmata*.

Based on the variety of symbionts present in our aquarium-reared larvae, initial acquisition of zooxanthellae does not coincide with adult host specificity; new *A. palmata* settlers acquired multiple major *Symbiodinium* clades early in development, in various combinations. Our results are consistent with previous studies, which have found *initial* symbiont specificity in the horizontal mode of algal acquisition to be broad or weak, with many distantly related algae within the genus *Symbiodinium* possibly entering the host (Coffroth et al. 2001, Little et al. 2004, Van Oppen et al. 2001, del C. Gómez-Cabrera 2008). This initial diversity may reflect the differences in physiological requirements at different stages of ontogeny, with a selection for clades or clade combinations that provide the most benefit to the coral settler at a specific time (Little et al. 2004). Del C. Gómez-Cabrera (2008) also suggested a contribution of the differences in the physical environment associated with juveniles versus adult colonies in nature; this conclusion supports the current clade diversity present across the aquaria. The low specificity of initial symbiont acquisition, seen here, may also represent an adaptive measure that supplies the coral juvenile host with an assortment of established symbionts that it may select for as it matures (Little et al. 2001).

Initial acquisition of symbionts by settlers is coupled with changes in host gene expression (Voolstra et al. 2009). Voolstra et al. (2009) and DeSalvo et al. (2010) found an inverse relationship between the number of transcriptional changes and the degree of coral-algal compatibility. *Symbiodinium* that cause a large transcriptional response in the host are said to have failed at establishing a symbioses; infection represents an invasion of *Symbiodinium* into the coral cells, which is then followed by a host immune response (the higher levels of transcriptional change) during which the coral then rejects the unstable symbionts. This represents an additional explanation for low initial larval specificity.

Although accompanied by other clades, the presence of Clade A *Symbiodinium* seen in a majority of the settlers, whether in combination, or in isolation, follows the symbiont pattern seen in “natural reef ecosystems” in which *Acropora palmata* colonies are known to primarily harbor *Symbiodinium* clade A3 (LaJeunesse 2002, Oppen et al. 2001). The high frequencies (96%) of this symbiont in the settler recruits were expected because of the adult coral specificity to the clade. Previous literature suggests that clade D1a (*Symbiodinium trenchi*) is an opportunist which dominates initial infection and whose physiology allows for growth and persistence in hosts from stressful environments (Abrego et al. 2009a, LaJeunesse 2002, LaJeunesse et al. 2009, Baker 2003). The identification and the unique persistence of *Symbiodinium trenchi* as a dominant symbiont in settlers from one month to four months supports these conclusions; our settlers may well have been under stress from

handling and transport as larvae and being reared in aquaria. The absence of *Symbiodinium* clade B in recruits by four months recruits may represent a low symbiotic success with the juvenile *A. palmata* hosts, (Knowlton and Rohwer 2003).

Effects of symbiont clade on juvenile performance

Settlers with combinations of clades A/C/D and D were significantly longer than settlers harboring clades A, A/C, and A/D. This clade effect is consistent with research showing differential growth rates associated with the clade harbored by coral juveniles experimentally infected with symbionts (Little et al. 2004).

Previous studies have demonstrated unequal contributions to host growth (Loram et al. 2007). *Acropora millepora* juveniles harboring *Symbiodinium* clade C1 grew 2-3 times larger than those harboring clade D due to a doubling of photosynthetic carbon incorporation (Cantin et al. 2009, Stat et al. 2008). Additional studies suggest that clade A symbionts may not provide as much carbon to the host as clade C symbionts (Stat et al. 2008).

With these findings in mind, symbioses with clade D *Symbiodinium* may have resulted in higher settler fitness because clade D symbionts rapidly and effectively infected settlers, e.g. at high densities, allotting these recruits with plentiful energy earlier than other recruits and therefore allowing earlier growth. Settlers harboring a combination of clades A/C/D may have had the benefit of increased energy (and thus growth rate) from clade C *Symbiodinium*, as well as the early infection by clade D. The additive benefits of harboring a diverse

combination of symbionts may also be why settlers performed better with higher levels of *clade diversity*.

Settler performance variation could also be attributed to the time course of algal symbiont uptake. For example, settlers harboring the A/C/D mix may have possibly picked up each of these clades at different times and therefore did not have the benefits of a diverse clade combination during the whole rearing process. This may explain why clade D dominated symbioses had similar performance levels to clade A/C/D dominated symbioses and why the other symbioses (clade A, A/C, and A/D) resulted in smaller settler lengths.

Summary and Conclusions

The symbiosis between coral species and dinoflagellates is a necessary relationship in which each organism benefits from the other. Dinoflagellates of the genus *Symbiodinium* are extremely diverse (Baker, 2003), and correlations between coral survival, growth rates, and symbiont type are extremely important in the context of today's changing global environment. Rising sea surface temperature has already caused massive losses of coral both directly and indirectly, and oceanic climate is only continuing to change towards less and less hospitable temperatures for coral growth (Wilkinson 2000, Green and Bruckner 2000, Mayor et al. 2006).

In our study, we found a low specificity of initial symbiont acquisition in juvenile coral recruits that contrasts with the high specificity observed in adults, and have suggested that this may represent an adaptive measure allowing for the

establishment of an advantageous multi-clade symbioses that can later be adjusted. Also, a dominance of clade A, the dominant clade found in *Acropora palmata* adults, did not result in better settler performance. Instead, opportunistic clade D, and mixed A/C/D symbioses resulted in settlers with the highest fitness, probably due to the combination of the advantageous traits of different *Symbiodinium* clades. Previous research has suggested that biogeographical factors may play a part in the specificity of *Acropora-Symbiodinium* associations (Van Oppen et al. 2001). The improved fitness of *A. palmata* juveniles with clades mixtures may be a product of rearing larvae in aquarium conditions rather than in a natural environment.

Continuation of this research could lead to the eventual discovery of optimal *Symbiodinium* clades, or clade combinations, for *A. palmata* at differing stages of development. Further experimentation similar to the research in this report is therefore imperative; elucidation of the selective events involved in the acquisition of a particular symbiont clade would allow for a better understanding of the coral-algal symbioses and its variation and improve our efforts at restorative captive breeding of *A. palmata* and other threatened coral species. This knowledge could also shed light on the changes endosymbiont populations may possibly encounter due to climate change, and thus has implications for the potential restoration of *A. palmata*, and other coral species in nature.

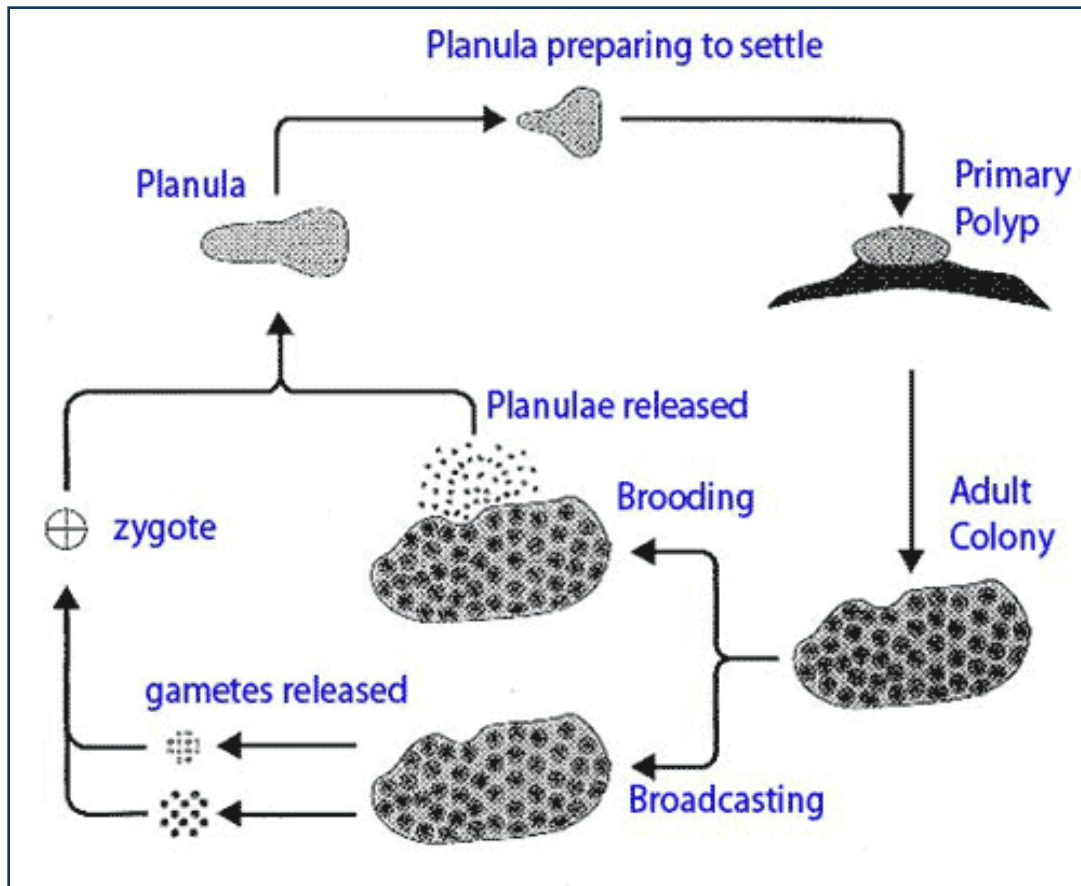


Figure 1. The coral life cycle showing two alternative forms of releasing gametes, broadcast spawning and brooding. *Acropora palmata* releases its gametes through broadcast spawning. Fertilization of eggs occurs in the water column. Here, zygotes undergo larval development until they become mature planula larvae, a process that takes about 3 days. Eventually, planulae sink towards the benthos where they search out appropriate settlement locations and metamorphose into a primary polyp. Colony growth is achieved by budding of the polyps. (www.bios.edu/research/CRRcycle.html).

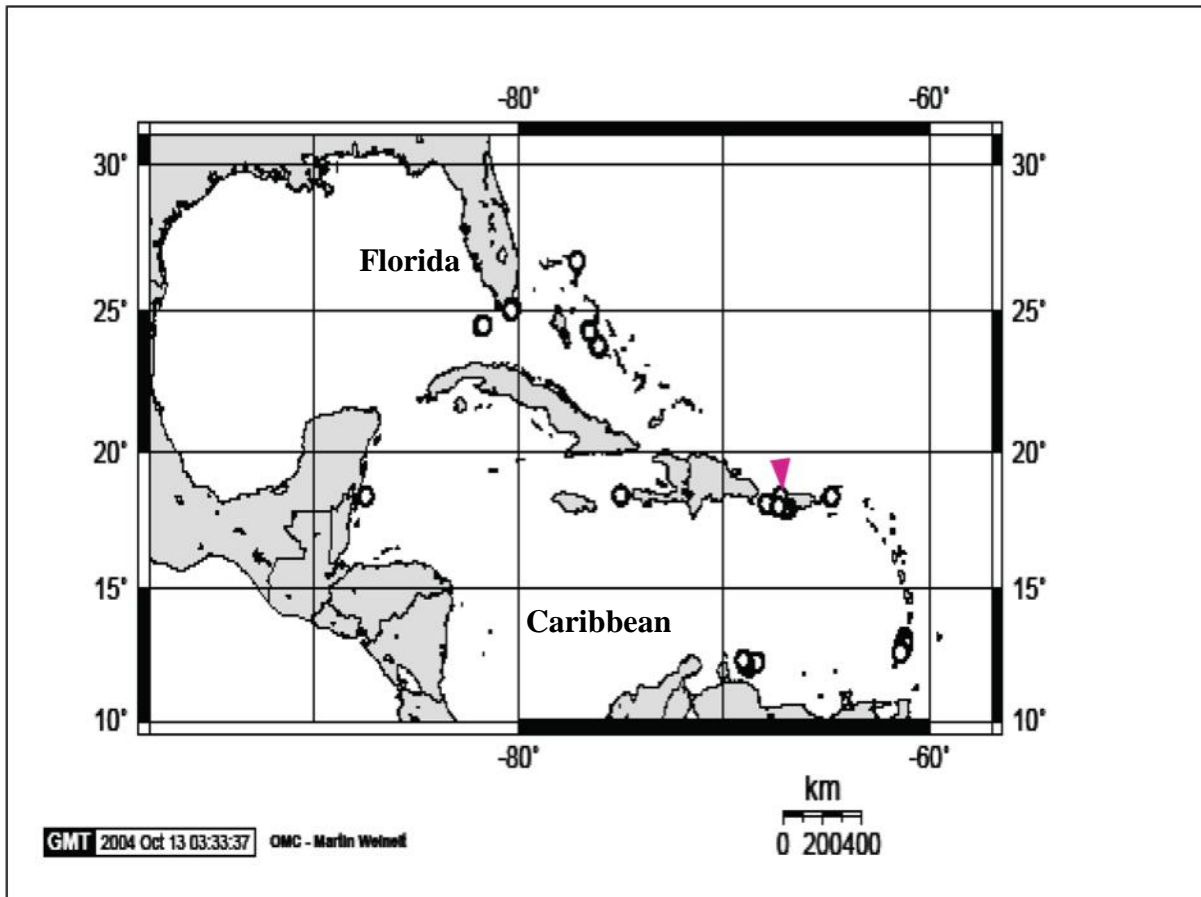


Figure 2. Coral spawn was collected at Rincon, Puerto Rico. *Acropora palmata* occurs throughout the Caribbean, Florida, and the Bahamas. Red arrow indicates Puerto Rico.

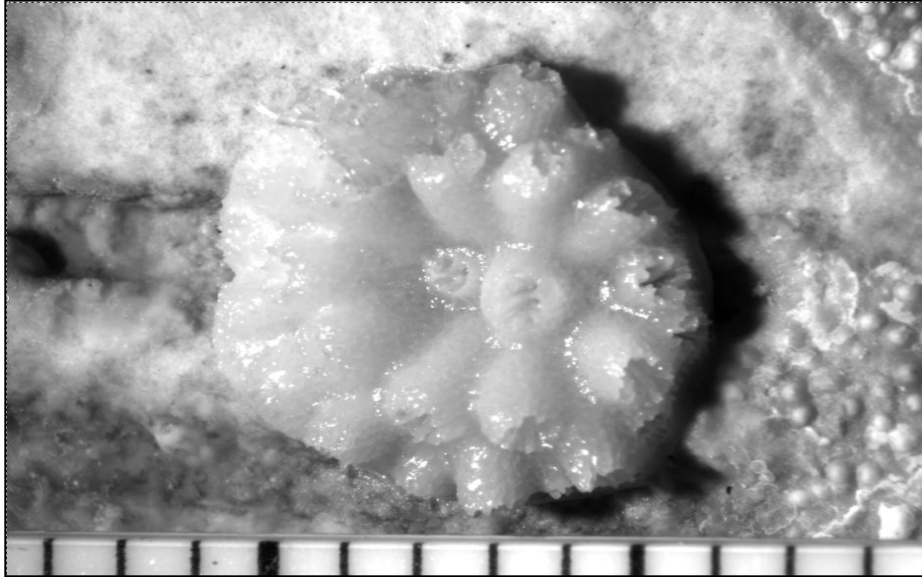


Figure 3. 4 months old *Acropora palmata* settler. Hatch marks in scale shown represent 1 mm.

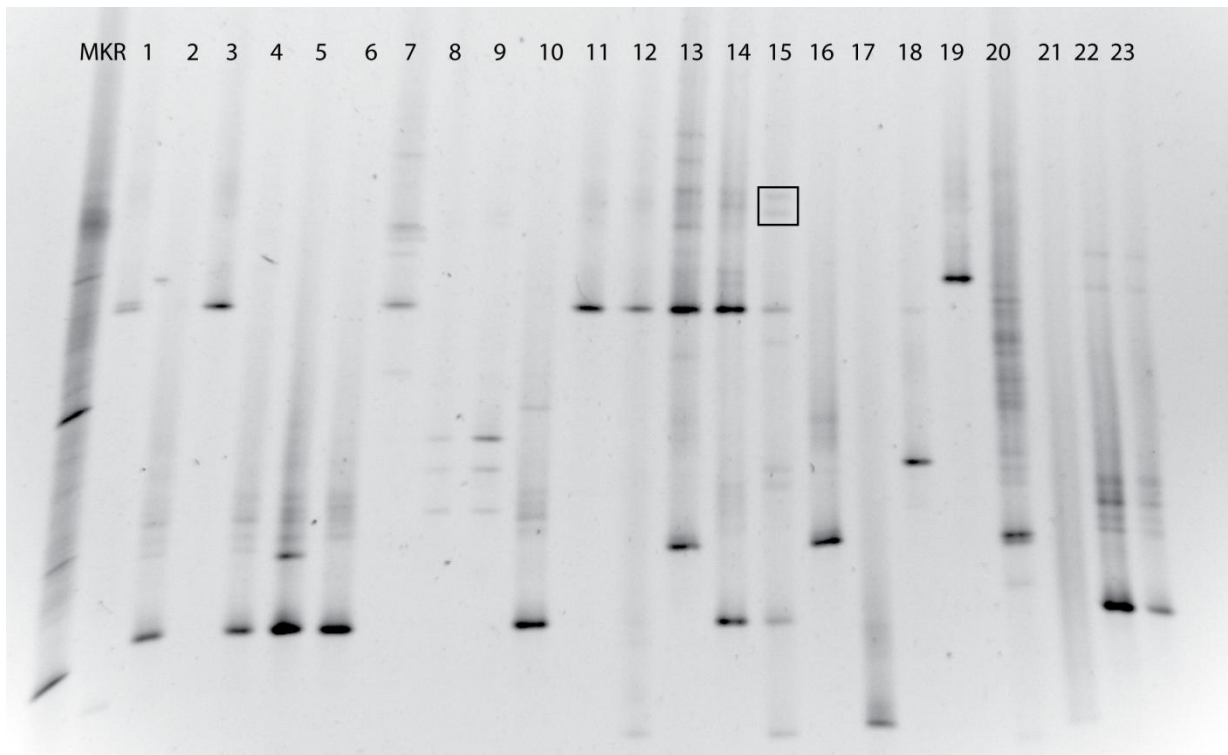


Figure 4. A negative image of ITS1 PCR-DGGE on *Symbiodinium* spp. showing positions of clades, A, B, C, D. The ladder in the left most lane (MKR) serves as a relative size standard between gels. Lane 13 is as an example of a single prominent band along with other faint bands located higher on the gel, and Lane 14 serves as an example of several dominant bands. Boxed-off bands in Lane 15 represent heteroduplex bands.

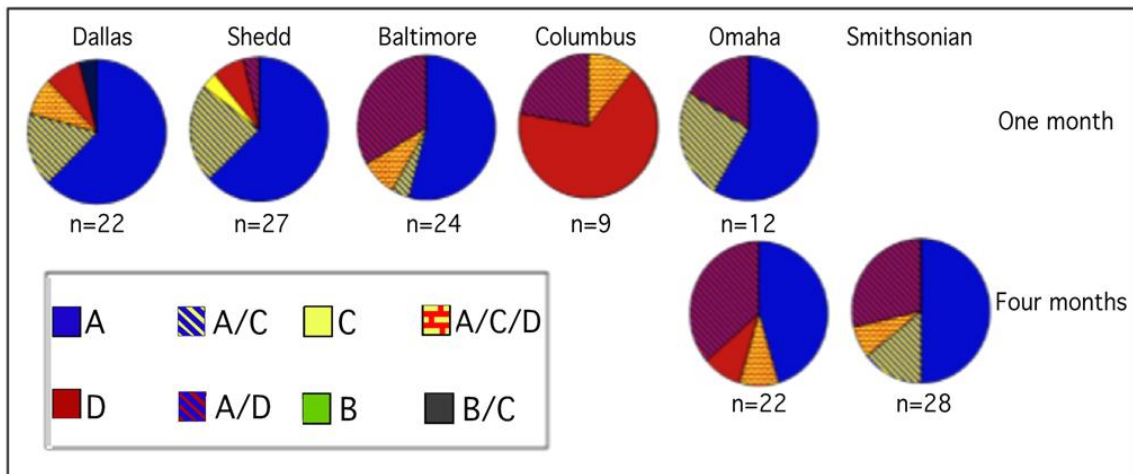


Figure 5. A summary of the distribution of zooxanthellae clades/clade combinations harbored by the *Acropora palmata* settlers at one month for Baltimore, Dallas, Shedd, Columbus and Omaha aquaria, and at four months for Omaha and Smithsonian aquaria. Numbers of settlers for each site and time point are listed (n). The figure shows a large variety of symbioses within and across aquaria.

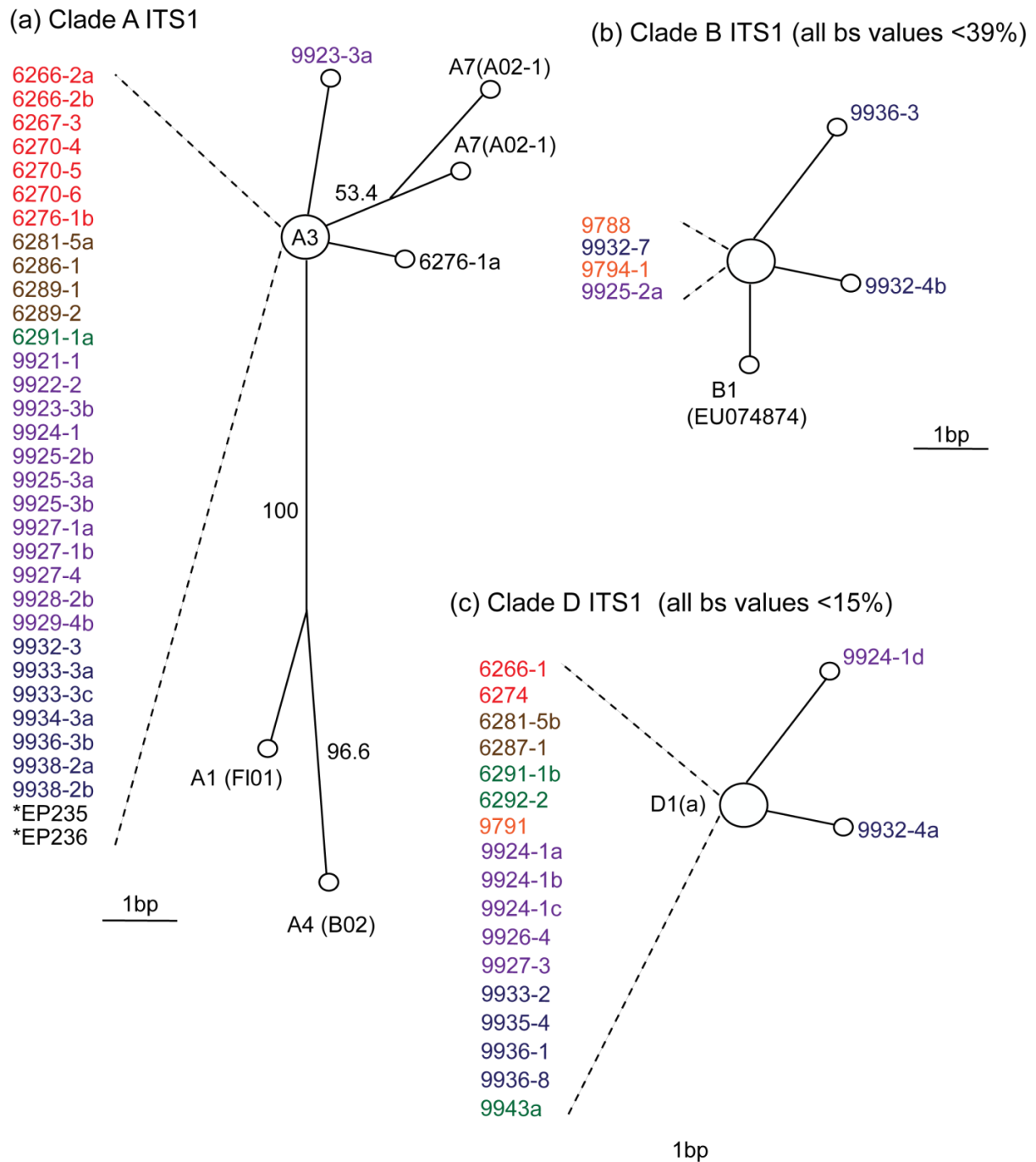


Figure 6. Maximum parsimony trees for *Symbiodinium* (a) Clade A (b) Clade B, and (c) Clade D. Numbers on the branches represent bootstrap values for 1000 replicates. Bootstrap values under 50% are not reported. Asterisk (*) indicates sequences from two donor colonies that provided zooxanthellae for inoculation of settlers. Two A types, 4 B types, and 3 D types are observed. No clear pattern of clade prevalence with aquarium is apparent.

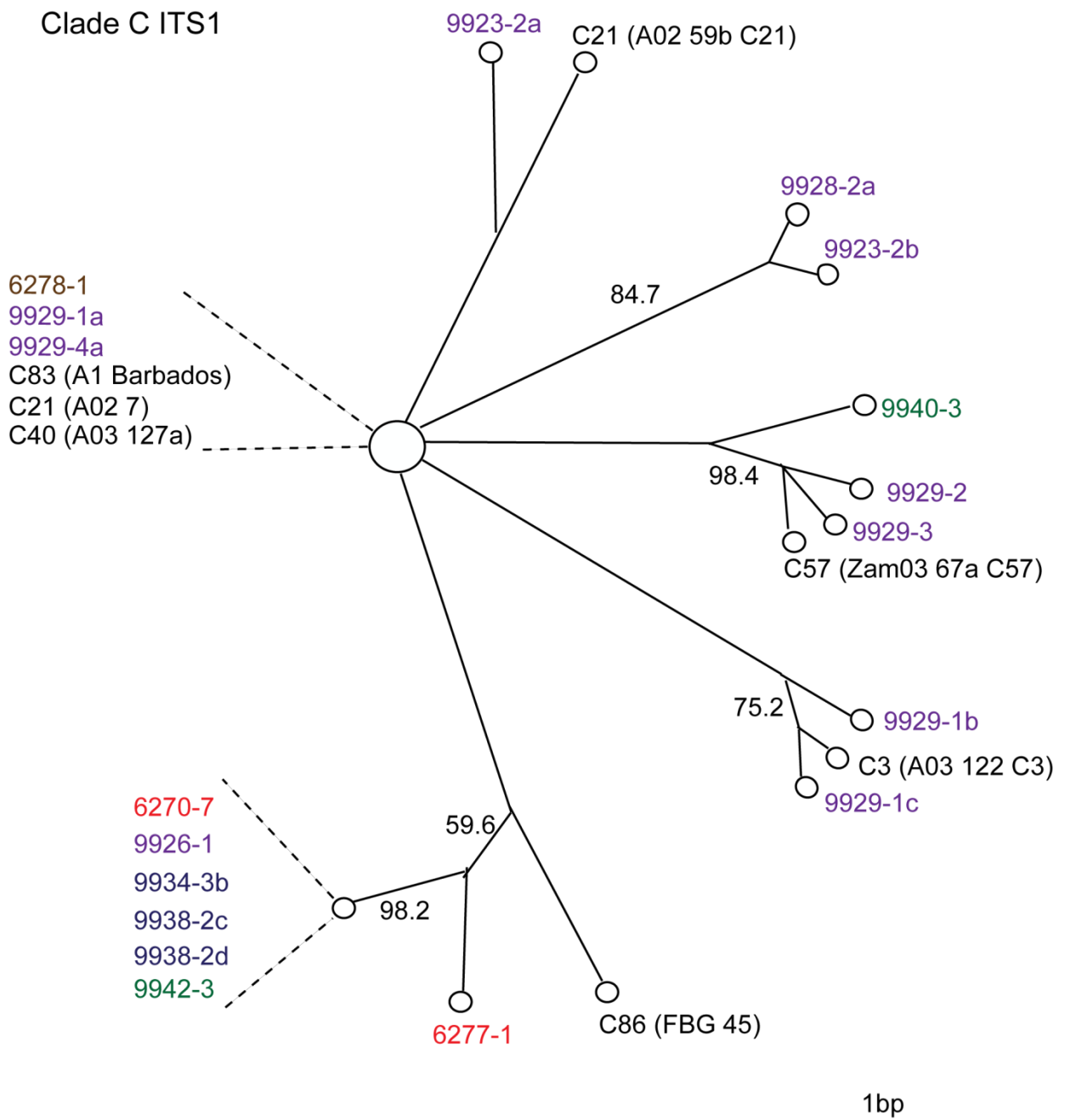


Figure 7. Maximum parsimony tree for *Symbiodinium* Clade C. Numbers on the branches represent bootstrap values for 1000 replicates. Bootstrap values under 50% are not reported. Nine C types are observed. No clear pattern of clade prevalence with aquarium is apparent.

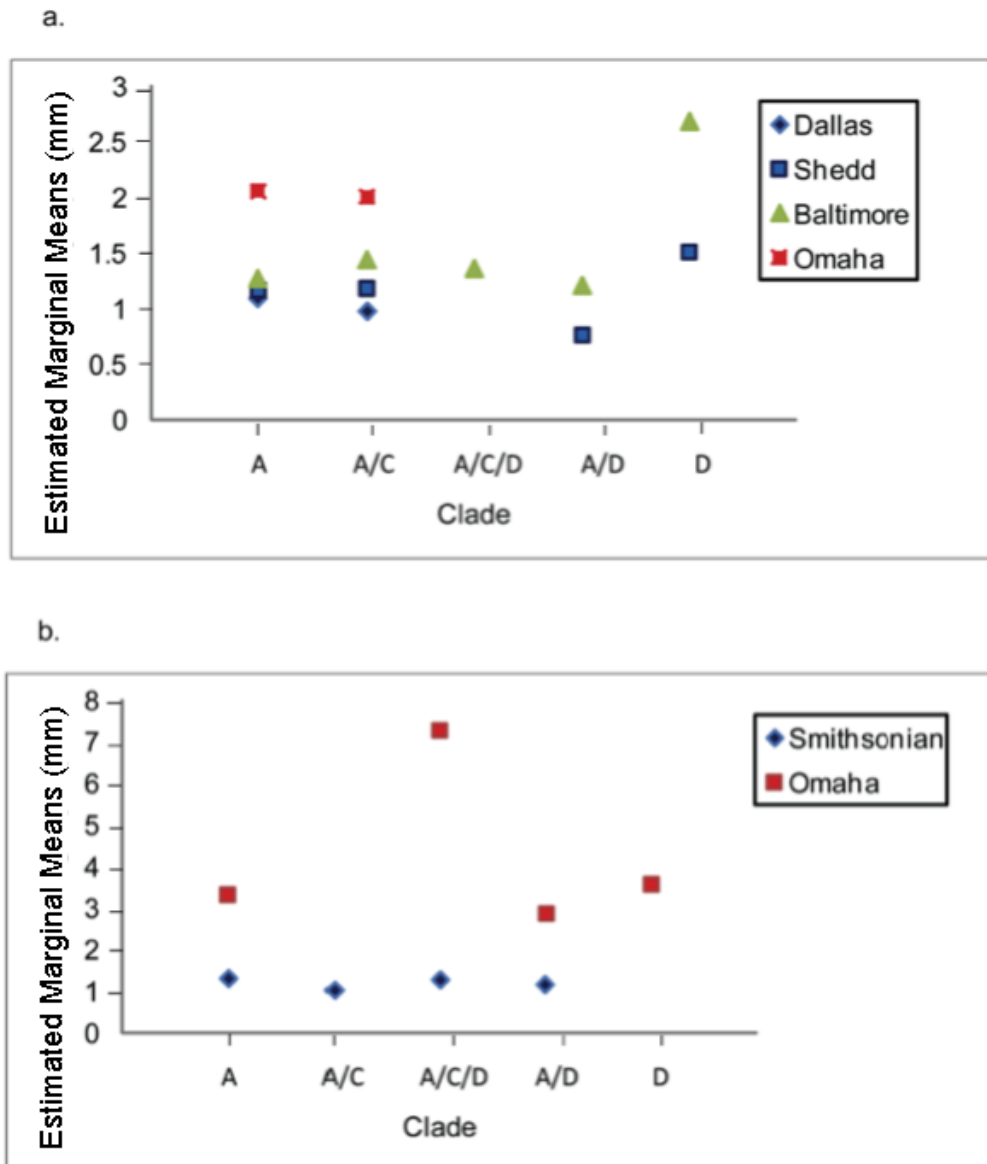


Figure 8. Estimated marginal means of settler size (y-axis, diameter in millimeters) among aquaria (color-coded) and clade harbored (x-axis) for settlers at **(a)** one month, and **(b)** four months.

(a) At one month, settler size was significantly different among aquaria, with Omaha aquarium having the largest diameters in comparison to settlers reared at all other aquaria. Settler size did not vary with clade harbored. No significant interaction was observed between clade and aquaria **(b)** At four months, settler fitness was significantly different among aquaria and among clades, with Omaha aquarium, and clade D and mixed clade A/C/D symbioses resulting in significantly larger settler diameters than other groups. A clade interaction effect is seen between aquarium and clade harbored. See text for details on statistical testing.

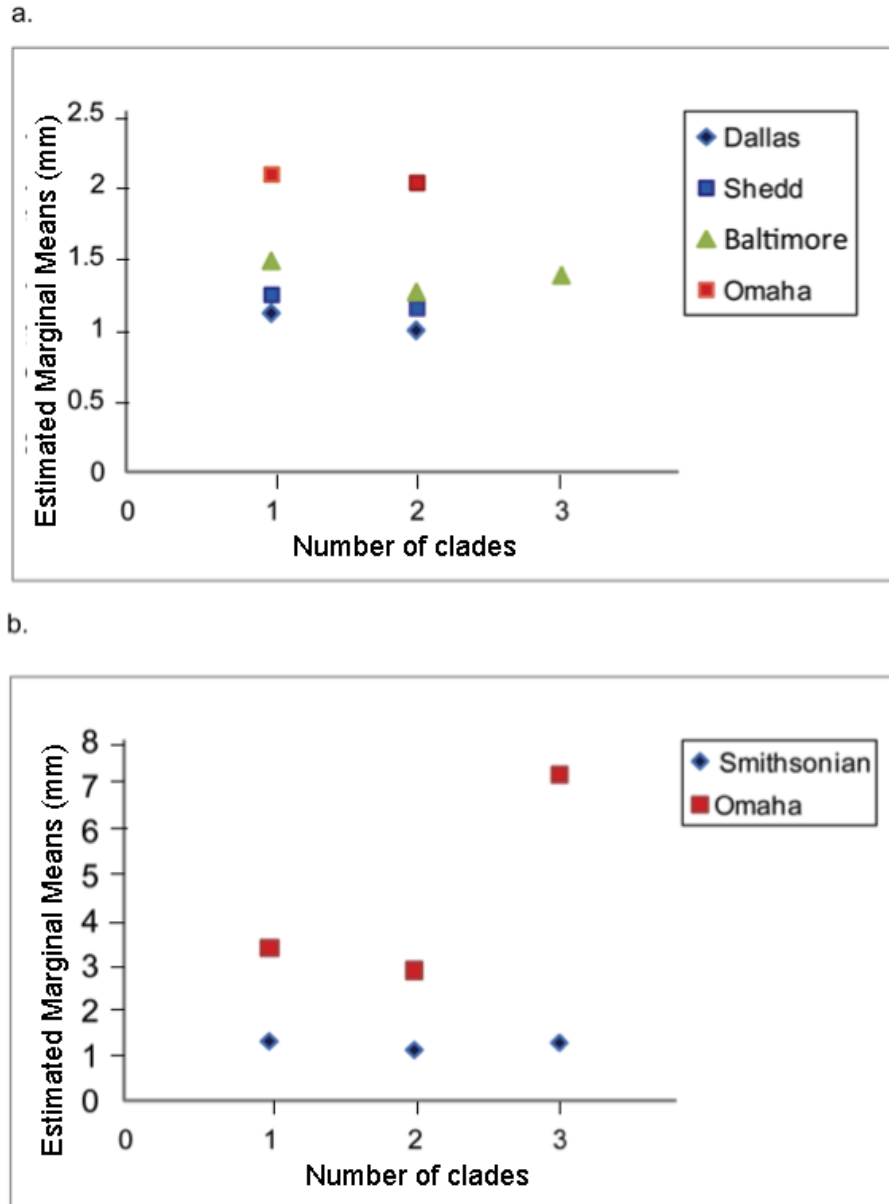


Figure 9. Estimated marginal means of settler size (y-axis, in millimeters) among aquaria (color-coded lines) and clade diversity harbored (x-axis) for settlers at **(a)** one month, and **(b)** four months.

(a) At one month, settler size varied significantly among aquaria, with Omaha aquarium having the largest lengths in comparison to settlers reared at all other aquaria. Settler performance did not vary with clade diversity harbored (x-axis). No interaction effect is seen between clade diversity and aquaria. **(b)** At four months, settler fitness was significantly different among aquaria, and among the 3 clade diversity levels. Again, Omaha aquarium has the largest lengths among aquaria, and successively increasing levels of clade diversity (number of clades harbored by settlers) appeared to have larger mean lengths. An aquarium-clade diversity interaction effect is seen. See text for details on statistical testing.

Table 1. PCR amplification recipes for *Symbiodinium* ITS PCR (a), ITS1 PCR (b), and the reamplification of bands cut out from DGGE gels (c).

a. ITS PCR

Reagent	Concentrations	Company	Master Mix (μL)
DNase free H ₂ O			5.4
Buffer	10x	NEB	1
MgCl	25mM	NEB	1
dNTPs	2.5 mM	TaKaRa	1
ZITSupM13	10mM		0.3
ZITSDnM13	10mM		0.3
Taq	5u/ul	NEB	0.05
DNA	1:01		1
Total:			10.05

b. ITS1 PCR

Reagent	Concentrations	Company	Master Mix (μL)
DNase free H ₂ O			18
Buffer	10x	NEB	2.5
MgCl	25mM	NEB	2.5
dNTPs	2.5 mM	TaKaRa	2.5
ITS1forCLAMP	10mM		0.75
ITSintRev2	10mM		0.25
Taq	5u/ul	NEB	0.13
DNA			1
Total:			27.63

c. Reamplification of DGGE bands, PCR

Reagent	Concentrations	Company	Master Mix (μL)
Dnase free H ₂ O			15.8
Buffer	10x	NEB	2.5
MgCl	25mM	NEB	2.5
dNTPs	2.5 mM	TaKaRa	2.5
ITS1forward	5mM		0.25
ITSintRev2	5mM		0.25
Taq	5u/ul	NEB	0.2
DNA			1
Total:			25

Table 2. Rearing conditions differed slightly among aquaria raising *Acropora palmata* settlers. Exposure length refers to the number of days larvae were exposed to extracted A3 Symbiodinium from an adult *Acropora palmata*.

Location	Exposure Length (days)	Temperature (°C)	Number of water changes	Water Source	Light conditions
Dallas Zoo	7	26	3	Coral propagation tanks with Indonesian coral	Indirect natural sunlight
Shedd Aquarium	5	27	2	Isolated tank with no coral	2- 40 watt fluorescent tubes
Omaha Zoo	5	27.8	2	Tank with <i>A. palmata</i> , <i>A. cervicornis</i> , and Indonesian <i>palmata</i>	2- 48 inch fluorescent tubes

Table 3. Clade distribution counts isolated from *Acropora palmata* settlers reared in different locations at (a) one month, and (b) two months.

a.

Location	Clade counts							
	A	A/C	A/C/D	A/D	B	B/C	C	D
Dallas	15	4	0	0	0	1	0	2
Shedd	17	6	0	1	0	0	1	2
Baltimore	13	1	2	8	0	0	0	0
Omaha	7	3	0	2	2	0	0	0
Columbus	0	0	0	0	0	0	1	6

b.

Location	Clade counts				
	A	A/C	A/C/D	A/D	D
Smithsonian	14	4	2	8	0
Omaha	10	0	2	8	2

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Related Experience:

Undergraduate Research, Baums Molecular Ecology Lab, 2008-current
Teaching Assistant, Biology Department, 2007-2008
University Learning Center Tutor, 2007-2008
EMT Basic Certification, 2008

Awards:

Biology Award
Mabel White Riker Scholarship
Dean's List, every semester
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Presentations:

Molecular Basis of Disease, HIV, 2007
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Activities:

Volunteer, ED Mount Nittany Medical Center, 2010
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Biology Club, Co-President, 2006-2008
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Disaster Relief in Mississippi, 2007
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