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Mixing it up: Multiple symbiont acquisition strategies as an adaptive mechanism in the coral Stylophora pistillata

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MIXING IT UP: MULTIPLE SYMBIONT ACQUISITION STRATEGIES AS AN ADAPTIVE MECHANISM IN THE CORAL *STYLOPHORA PISTILLATA*

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ABSTRACT

In obligate symbioses, the host's survival relies on the successful acquisition and maintenance of symbionts, which can be transferred from parent to offspring via direct inheritance (vertical transmission) or acquired anew each generation from the environment (horizontal transmission). Vertical transmission ensures progeny acquire their obligate symbionts, but progeny encountering an environment that differs from that of their parent may be disadvantaged by hosting a suboptimal symbiont. Conversely, horizontal symbiont acquisition provides hosts the benefit of acquiring symbionts well suited to the prevailing environment, but progeny may fail to acquire their obligate symbionts. Here I show that the coral *Stylophora pistillata* may garner the benefits of both transmission modes by releasing progeny with maternally derived symbionts that are also capable of subsequent horizontal symbiont acquisition. The algal symbionts (*Symbiodinium*) present in *S. pistillata* adults, juveniles, and larvae (planulae) were identified using denaturing-gradient gel electrophoresis (DGGE) and real-time PCR. DGGE confirmed previous reports that in the Gulf of Eilat, Red Sea, shallow water (2-6m) *S. pistillata* adult coral colonies host clade A *Symbiodinium*, while deep-water (24-26m) colonies host clade C. Real-time PCR uncovered previously undetected *Symbiodinium* present at low-levels in some deep, but no shallow water adult colonies. Planulae only inherited the dominant symbiont clade from their maternal colony. While most shallow water juveniles hosted only clade A *Symbiodinium*, deep-water juveniles either hosted clade C, clade A, or a mixture of both clades. As all planulae analyzed hosted only one symbiont clade, while some juvenile colonies

hosted either multiple symbionts, or symbionts not characteristic of the depth in which they occurred, these data support environmental *Symbiodinium* acquisition, in addition to vertical symbiont inheritance, in the coral *S. pistillata*. Reciprocal depth transplant experiments of juvenile *S. pistillata* colonies were executed to monitor potential changes in the *Symbiodinium*. Hosting physiologically distinct *Symbiodinium* may allow coral host survival under varied environmental conditions. Therefore, horizontal symbiont acquisition may enable coral species with vertical transmission to acquire advantageous symbionts. In turn, this may provide genetic variation in the symbiosis on which natural selection can act, providing a mechanism for coral adaption to global climate change.

DEDICATION

To the Byler Clan and Stan, for always giving me the strength to finish what I start.

LIST OF ABBREVIATIONS AND SYMBOLS

C_T	Cycle Threshold
DGGE	Denaturing-Gradient Gel Electrophoresis
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
ITS2	Internal Transcribed Spacer Region 2
IUI	Interuniversity Institute for Marine Sciences in Eilat
NaCl	Sodium Chloride
PCR	Polymerase Chain Reaction
rDNA	Ribosomal Deoxyribonucleic Acid
R_n	Normalized Reporter
RNase	Ribonuclease
SCUBA	Self Contained Underwater Breathing Apparatus
TE Buffer	Tris & Ethylenediaminetetraacetic Acid

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Introduction

Obligate Symbioses

In obligate symbioses, the host's survival relies on the successful acquisition and maintenance of symbionts. Symbionts can either be transferred from parent to offspring via direct inheritance (vertical transmission) or acquired anew each generation from the environment (horizontal transmission). Vertical transmission should promote obligate mutualisms because the fitness of the symbiont is intrinsically correlated to the successful reproduction of the host (Wilkinson & Sherratt 2001; Sachs *et al.* 2011). Additionally, partner fidelity eliminates the risk of cheating (Leigh 2010). In turn, the host benefits because vertical transmission ensures offspring inherit their obligate symbionts, eliminating the risk of lack of symbiont acquisition (Douglas 1998; Wilkinson & Sherratt 2001).

Horizontal symbiont transmission seems counterintuitive in obligate symbioses because host survival hinges on a potentially unpredictable symbiont source and the risk of cheating is high (Leigh 2010; Douglas 1998). Despite these risks, obligate symbioses are maintained via horizontal transmission in a variety of species (Bright & Bulgheresi 2010; Wilkinson & Sherratt

2001; Leigh 2010; Sachs *et al.* 2011), including most scleractinian corals (Baird *et al.* 2009). As partner fidelity is not absolute in horizontal transmission, strong partner choice can allow mutualisms to persist in systems with horizontal transmission (Ruby & Lee 1998; Leigh 2010; Sachs *et al.* 2011). Partner sanctions can also promote stable mutualism through the "punishment" of cheaters or nonperforming symbionts (Leigh 2010; Sachs *et al.* 2011). Corals with horizontal transmission may use both partner choice and partner sanctions to maintain stable symbioses with dinoflagellate algae (Leigh 2010).

Partner choice creates the opportunity for the host to acquire symbionts that may improve host fitness and allow for adaptation to environmental changes (Douglas 1998; Wilkinson & Sherratt 2001). Vertical symbiont transmission, on the other hand, does not provide the opportunity to acquire non-parental symbionts. Consequently, hosts may be disadvantaged in changing environments by hosting a suboptimal symbiont. Host species may therefore benefit from employing both symbiont transmission modes. Phylogenetic analyses have revealed predominant vertical transmission punctuated by relatively infrequent horizontal symbiont acquisition in some obligate prokaryote-insect (Baldo *et al.* 2006; Allen *et al.* 2007; Kikuchi & Fukatsu 2003; Haine *et al.* 2005), prokaryote-marine invertebrate (Lim-Fong *et al.* 2008; Schmitt *et al.* 2008; Stewart *et al.* 2008; Yang *et al.* 2011), prokaryote-earthworm (Schramm *et al.* 2003), and eukaryotic lichen symbioses (Dal Grande *et al.* 2012). As scleractinian corals host obligate algal symbionts, it is possible that corals may also utilize both symbiont transmission strategies.

Symbiodinium *Diversity and its Ramifications for Corals*

Dinoflagellate algae in the genus *Symbiodinium* form mutualistic symbioses with a wide array of marine organisms including cnidarian hosts (e.g. scleractinian corals, octocorals, sea

anemones) (Pochon *et al.* 2007). In the obligatory relationship scleractinian corals form with *Symbiodinium*, the host provides its unicellular tenants with protection and nutrients that aid photosynthesis in oligotrophic tropical waters. In return, *Symbiodinium* translocate up to 95% of their photosynthate, providing a nutritional foundation for host metabolism (Muscatine 1990) and calcification (Allemand *et al.* 2011).

Symbiodinium are divided into nine distinct phylogenetic clades named A-I (Pochon & Gates 2010). Within clades, *Symbiodinium* exhibits substantial diversity, but as species limits remain largely unresolved, subclades are referred to as types (LaJeunesse 2001; Baker 2003; van Oppen 2004; LaJeunesse *et al.* 2005; Correa & Baker 2009a; Stat *et al.* 2011). Specific *Symbiodinium* types exhibit distinct physiologies and respond differently to variation in light and temperature (Warner *et al.* 1999; Iglesias-Prieto *et al.* 2004; Rowan 2004; Tchernov *et al.* 2004; Loram *et al.* 2007; Reynolds *et al.* 2008; Sampayo *et al.* 2008).

Given the genetic and physiological diversity of the *Symbiodinium* genus, and the rapidly declining health of coral reef ecosystems worldwide, there is a great deal of interest in understanding whether corals are able to host multiple *Symbiodinium* types, either sequentially or simultaneously. Stress events that lead to a reduction in *Symbiodinium* numbers may provide an opportunity for coral hosts to acquire novel symbionts from the environment (Buddemeier & Fautin 1993; Buddemeier *et al.* 2004). Corals may be able to rapidly adapt to changing environmental conditions by sequentially altering their symbionts such that *Symbiodinium* types mal-adapted to current stressors are replaced with types well suited to the prevailing environment (Buddemeier *et al.* 2004). Following stress events, the acquisition of novel *Symbiodinium* has been documented in numerous coral species, but the relationship appears transient, as novel

symbionts do not persist after recovery (Baker 2004; Lewis & Coffroth 2004; Thornhill *et al.* 2006b; Sampayo *et al.* 2008; LaJeunesse *et al.* 2009b; Coffroth *et al.* 2010).

In adult coral colonies, although not common, some species host multiple *Symbiodinium* types within the same colony. Subsequent to a stress event, background, or low-level, *Symbiodinium* populations may increase in abundance (Buddemeier *et al.* 2004; Silverstein *et al.* 2012). While some species do host multiple clades, at abundant or background levels, in the vast majority of coral species, adults feature extremely stable and highly specific symbioses with a single *Symbiodinium* clade (Goulet 2006), even during stress events (LaJeunesse *et al.* 2010; Stat *et al.* 2009a; Thornhill *et al.* 2006a,b; Thornhill *et al.* 2009). In general, coral species that horizontally acquire symbionts may exhibit a greater capacity to host multiple *Symbiodinium* types at either abundant or low-levels (Stat *et al.* 2008a). Even still, low-level *Symbiodinium* have been detected in adult colonies of several coral species with vertical symbiont transmission (Mieog *et al.* 2007; LaJeunesse *et al.* 2009b; Silverstein *et al.* 2012). In these vertical systems, if a single adult colony hosts multiple *Symbiodinium*, progeny may inherit all or any one of the *Symbiodinium* types present in the maternal colony.

Initial Symbiont Acquisition in Corals

Deciphering symbiont acquisition strategies utilized by corals throughout ontogenesis is key to understanding corals' ability, or lack thereof, to alter their symbionts based on the environmental conditions of the habitat in which they grow. Scleractinian corals initially acquire *Symbiodinium* through either horizontal or vertical symbiont transmission and, in general, each symbiont transmission mode is associated with one of two distinct coral reproductive modes. The majority of coral species (~84-89%) broadcast spawn, whereby eggs and sperm are released

into the water column where external fertilization and larval development take place (Baird *et al.* 2009; Harrison 2011). The remaining 11-16% of coral species brood larvae (planulae), in which fertilization and planula development occur internally (Baird *et al.* 2009; Harrison 2011). The mode of symbiont transmission is not known for many species, but in general, ~90% of brooding species display vertical symbiont transmission (Baird *et al.* 2009). In contrast, horizontal acquisition is the predominant transmission mode in broadcast spawning coral species, with only ~25% of all spawners exhibiting vertical symbiont inheritance (Baird *et al.* 2009).

Vertical transmission of symbionts results in stable, long-lasting symbioses that are well adapted to the prevailing environment (Wilkinson & Sherratt 2001). Thus, vertical transmission ensures that coral planulae inherit an advantageous *Symbiodinium* type while eliminating the risk of death due to a lack of symbiont acquisition (Douglas 1998; Weis *et al.* 2001). Symbiotic planulae also benefit from energy resources of the photosynthetically derived nutrients provided by *Symbiodinium* (Richmond 1987; Isomura & Nishihira 2001; Baird *et al.* 2009; Harii *et al.* 2010). Most symbiotic planulae are competent to settle within hours to days of release (Fadlallah 1983; Nishikawa *et al.* 2003), but long competency periods (from 35-103 days) have been documented in several brooding species with vertical transmission (Richmond 1987; Rinkevich & Loya 1979a; Harii *et al.* 2002). Long competency periods coupled with nutritional provisioning from *Symbiodinium*, may allow for occasional long-distance dispersal, which increases the probability of successful settlement in a favorable habitat (Isomura & Nishihira 2001; Harii *et al.* 2010) and allows for the colonization of new habitats (Richmond 1987; Baird *et al.* 2009).

Although hosting *Symbiodinium* confers benefits to planulae, there are costs. First, having symbionts at the planula stage can generate high levels of anti-oxidant defense activity

and increased oxidative cellular damage (Yakovleva *et al.* 2009). Further, high intensities of photosynthetically active radiation (PAR, 400-700nm) and UV radiation (280-400nm) can reduce chlorophyll concentration and consequently survivorship in symbiotic planulae (Gleason & Wellington 1995). The negative effects associated with hosting *Symbiodinium* in the larval stage can apply to species with either symbiont transmission mode, since even in some species with horizontal symbiont transmission, *Symbiodinium* acquisition can occur prior to settlement (Harii *et al.* 2009).

Second, due to the fidelity of symbiont transfer, vertical symbiont transmission may preclude coral hosts from associating with novel *Symbiodinium* during times of environmental change (Benayahu & Schleyer 1998; Douglas 1998; Loh *et al.* 2001; Weis *et al.* 2001; Thornhill *et al.* 2006a; Harii *et al.* 2009). In contrast to vertical transmission, horizontal *Symbiodinium* acquisition may predominate in corals because it allows planulae or juveniles to acquire new symbionts each generation, increasing the probability of acquiring *Symbiodinium* well suited to the environment in which the planulae settle (Rowan & Knowlton 1995). While planulae that inherit their *Symbiodinium* vertically may be incapable of acquiring symbionts from the environment, planulae may still inherit a diverse *Symbiodinium* population if maternal colonies host multiple *Symbiodinium* types. To my knowledge, no studies have assessed the *Symbiodinium* types present in planulae that inherit symbionts directly from their maternal colony, and therefore it remains unclear whether planulae with vertically transmitted symbionts can host multiple types of *Symbiodinium*.

Symbiont Promiscuity in Juvenile Corals

The *Symbiodinium* specificity exhibited by adult corals is thought to limit the possibility of symbiont altering in the adult life stage (LaJeunesse 2002; Goulet & Coffroth 2003; Goulet 2006). But, some coral species with horizontal transmission are capable of acquiring novel *Symbiodinium* during the juvenile life stage (Coffroth *et al.* 2001; Gomez-Cabrera *et al.* 2008; Abrego *et al.* 2009a,b; Weis *et al.* 2001; Little *et al.* 2004; Rodriguez-Lanetty *et al.* 2006). While the juvenile stage may be key in establishing novel symbioses, no studies to date have demonstrated juveniles capable of successfully maintaining an exogenously acquired novel symbiont into adulthood. The onset of *Symbiodinium* specificity has been shown to range from as little as 21 hours in *Fungia scutaria* planulae (Rodriguez-Lanetty *et al.* 2006) to up to 3.5 years in the case of *Acropora tenuis* juveniles (Abrego *et al.* 2009b), but the mechanisms of specificity are not well understood (Hirose *et al.* 2008; Schnitzler and Weis 2010). Initial uptake of symbionts by planulae and juveniles may be non-selective (Little *et al.* 2004; Harii *et al.* 2009), and infection may be dominated by opportunistic symbionts that are subsequently removed from symbiosis (Abrego *et al.* 2009a). Furthermore, it is unclear whether selection occurs at the holobiont (host and symbiont) level, whereby hosts with certain symbionts perish, the host level, whereby either the host retains or excludes certain algal types (Little *et al.* 2004), or the symbiont level, where algal types compete for dominance within the host (Gomez-Cabrera *et al.* 2008).

Vertical transmission is often regarded as a "closed" system that limits symbiont diversity in all life stages (Benayahu & Schleyer 1998; Douglas 1998; Loh *et al.* 2001; Weis *et al.* 2001; Thornhill *et al.* 2006a; Harii *et al.* 2009; but see van Oppen 2004). As such, corals with vertical symbiont transmission may not acquire *Symbiodinium* in early ontogeny because they are already

equipped with maternally derived symbionts and may be incapable of additional horizontal acquisition. Studies to date have consequently focused on juvenile corals that horizontally acquire symbionts. Although not tested, it has been suggested that horizontal acquisition may occur in coral species with vertical symbiont transmission (van Oppen 2004; Megalon *et al.* 2006; Stat *et al.* 2008a). To investigate this hypothesis, the present study sought to determine the *Symbiodinium* identity in the planulae and juveniles of a species with vertical symbiont transmission.

Stylophora pistillata *in the Red Sea*

The brooding coral *Stylophora pistillata* (family Pocilloporidae) is widely distributed throughout the Indo-Pacific and Red Sea (Veron 2000), and is among the most abundant frame building corals in the Gulf of Eilat, Red Sea (Figure 1; Rinkevich & Loya 1979a; Loya 1976b). As a sequential hermaphrodite, the development of *S. pistillata* in the Red Sea is categorized into four distinct life stages: 1) young, non-reproductive, 2) first year reproductive colonies (commonly male), 3) hermaphroditic mature colonies, and 4) hermaphroditic old colonies (Rinkevich & Loya 1979b). Hermaphroditic mature and old colonies are distinct stages because old colonies produce more female gonads per polyp compared to mature colonies (Rinkevich & Loya 1979b). Reproductively mature hermaphroditic colonies have a long reproductive season in shallow water, releasing planulae from December through July (Loya 1976a, Rinkevich & Loya 1979b; Zakai *et al.* 2006). The reproductive season is about two or three months shorter in deep as compared to shallow water colonies (Rinkevich & Loya 1987).

Figure 1. Shallow water adult *Stylophora pistillata* colony. Photograph taken on the reef in front of the Interuniversity Institute for Marine Science in Eilat, Eilat, Israel.

Adult *S. pistillata* in the Gulf of Eilat, host two distinct *Symbiodinium* clades. Shallow water colonies (<17m) associate with clade A *Symbiodinium* (Lampert-Karako *et al.* 2008; Winters *et al.* 2009), specifically type A1 (LaJeunesse 2001; Daniel 2006; LaJeunesse *et al.* 2009a). Deep-water congeners harbor symbiont types in clade C (Winters *et al.* 2009). Deep-water colonies ranging from 20-30m host *Symbiodinium* type C72 (LaJeunesse *et al.* 2005; Daniel 2006). Mesophotic colonies (>30m) host several *Symbiodinium* clade C types: C1s, C21s, C21t, and C72s (Daniel 2006). Depth mediated patterns of symbiont variability are seen in coral species around the world at both the cladal and subcladal level (Sampayo *et al.* 2007; Bongaerts *et al.* 2010a,b; Frade *et al.* 2008; Copper *et al.* 2011; Rowan & Knowlton 1995). To my knowledge, no studies have assessed the potential presence of low-level *Symbiodinium* in a species that exhibits depth mediated symbiont variability.

Research Objectives

Given that *S. pistillata* in the Gulf of Eilat hosts two different *Symbiodinium* clades as a function of depth, I determined if shallow and deep-water adult colonies hosted previously undetected *Symbiodinium* at low-levels. Since *S. pistillata* vertically transmits its symbionts, I further analyzed planulae released from both shallow and deep-water maternal colonies to determine the *Symbiodinium* clade(s) inherited. Which symbiont(s) progeny inherit may affect their survivorship in different habitats due to physiological differences in *S. pistillata* symbionts (Winters *et al.* 2009). Finally, by examining the *Symbiodinium* genetic identity at both depths in juvenile colonies, I determined whether juveniles are capable of acquiring Symbiodinium from the environment. Understanding the *Symbiodinium* present throughout *S. pistillata* ontogenesis may lend insight into the symbiont depth distribution observed in adult colonies and whether it is a consequence of host differential mortality or symbiont succession. Additionally, deciphering whether *S. pistillata* juvenile colonies are capable of both vertical and horizontal symbiont transmission has important implications concerning corals' ability to adapt to global climate change.

Methods

Sample Collection

Samples were collected from a reef in front of the Interuniversity Institute for Marine Sciences in Eilat (IUI), Gulf of Eilat (Aqaba), Red Sea (29º 30' N, 34º 56' E). *Stylophora pistillata* colonies were haphazardly collected from both shallow (2-6m) and deep (24-26m) water habitats in May, June, and/or July of 2009-2011 using SCUBA. *S. pistillata* were collected from three distinct age classes: adult colonies (~15-30cm width), juveniles colonies

(~0.5-2.8cm width), and pelagic planulae. All samples were immediately frozen at -80ºC or preserved in 95-100% ethanol for DNA analysis. From each adult colony, a branch piece of approximately 2cm in length was collected. In total, samples were collected from 14 shallow and 21 deep-water colonies in July of 2009; from 10 shallow and 11 deep-water colonies in May-July of 2010; and from 10 shallow and 14 deep-water adult colonies in June of 2011.

From each adult colony sampled in 2009 and 2010, spawned planulae were also collected using planula collection nets as described by Zakai *et al.* (2006; Figure 2). No planulae were collected in 2011. Planula collecting nets consisted of a mesh plankton net topped with a positively buoyant plastic collection container and a drawstring that secured the net around the base of the maternal colony. As planulae are positively buoyant, upon release, they swam into the collection container. The collection nets were placed at dusk and left on the colonies overnight for approximately 12 hours. The following morning, the nets were collected and immediately moved to an outdoor flowing seawater table, and the contents of each collection container were emptied into a bucket. Planulae were then collected with a Pasteur pipette and preserved in 95-100% ethanol. *S. pistillata* colonies were less fecund in 2010 compared to the 2009 sampling period. Therefore, while an adequate number of planulae were collected from all adult colonies over a single collection night in 2009, adult colonies were repeatedly sampled in 2010 in order to obtain a sufficient sample size.

Figure 2. Planula collection net. Net is placed over a shallow water adult *Stylophora pistillata* colony.

Juvenile *S. pistillata* colonies were haphazardly collected from both depths only in July of 2010. Entire juvenile colonies were removed from both natural and artificial substrates using a hammer and chisel. Juveniles were immediately transferred to an outdoor flowing seawater table under shading equivalent to the irradiance levels present at the depth from which they were collected. All collected colonies appeared healthy with no visible signs of bleaching. As *S. pistillata* colonies are approximately spherical, the length (l), width (w), and height (h) of all juvenile colonies were recorded. The geometric mean radius ($\bar{r}$) was then calculated (Loya 1976a, Loya 1976b) using the following formula:

$$\bar{r} = ((l * w * h)^{1/3})/2.$$

An approximate age was then extrapolated for each juvenile colony based on growth rate estimates for shallow water *S. pistillata* colonies in the Gulf of Eilat (Loya 1976a, Loya 1976b). A small chip (~4-7mm) was removed from each juvenile and preserved in 100% ethanol for subsequent DNA analysis.

DNA Extraction

Genomic DNA was extracted from each adult and juvenile coral fragment and individual planula using either a phenol:chloroform protocol or a Wizard Genomic DNA Purification Kit (Promega). Most of the 2009 adult samples were extracted using a phenol:chloroform extraction protocol as described by Goulet *et al.* (2003). An adult coral branch piece, approximately 4mm by 4mm, was added to a 1.5ml microcentrifuge tube with 600µl of digestion buffer (100mM Tris, 5mM EDTA, 200mM NaCl, 0.2% sodium dodecyl sulfate (SDS)). Coral tissue was disrupted with a plastic pestle before 3.6µl of proteinase K (20mg/ml) was added. The tubes were then incubated at 65°C for one hour. 600µl of chloroform-isoamyl alcohol (24:1) was added and tubes were left on a shaker for five minutes. Samples were then centrifuged for five minutes. The top aqueous phase was removed and placed into a fresh 1.5ml microcentrifuge tube with 600µl of phenol:chloroform:isoamyl alcohol (25:24:1). Once again, the samples were left to shake for five minutes prior to a five-minute centrifugation. The top aqueous phase was removed and placed into a fresh 1.5ml microcentrifuge tube with 1ml of 95% ethanol. Samples were then left to precipitate overnight at -20°C. Next, samples were centrifuged for 30 minutes and the resulting supernatant was decanted. The pellet was then washed in 500µl of 70% ethanol and centrifuged for five minutes, two separate times. After the second wash and centrifugation, the ethanol was removed and the pellet was dried prior to re-suspension in 20-30µl of TE buffer. This protocol was also used for most of the 2009 planula samples, but reagent volumes were reduced ten-fold according to Coffroth *et al.* (2001). As multiple planulae released from a single parent were stored in the same microcentrifuge tube, clumps of multiple planulae were separated using a dissecting microscope such that entire individual planulae were transferred to new microcentrifuge tubes for DNA extraction.

In an effort to eliminate the extra source of error associated with the use of lab-made reagents in the phenol:chloroform extraction protocol (an important consideration for real-time PCR analysis, S. Baerson pers. comm.), I extracted some of the 2009 and all of the 2010 adult and planula samples using the Wizard DNA (Promega) extraction protocol according to LaJeunesse *et al.* (2003). All juveniles and 2011 adult colonies were extracted using the Wizard DNA protocol.

Following LaJeunesse *et al.* (2003), an approximately 4mm by 4mm adult or juvenile coral fragment was placed in a 1.5ml microcentrifuge tube with 600µl of nuclei lysis buffer (Promega) and 100-200µl volume of glass disruption beads (0.25-0.05mm). Samples were incubated at 65°C with 3µl of proteinase K (20mg/ml) for one hour and briefly vortexed every 20 minutes. The samples were next incubated for 20 minutes at 37°C with 1µl of RNAase (4mg/ml; Promega). After incubation, 260µl of protein precipitation solution (Promega) was added, gently mixed, and the tubes were placed on ice for 15 minutes. The samples were centrifuged for five minutes (12,000rpm) and 600µl of the resulting supernatant was placed into a new 1.5ml microcentrifuge tube with 700µl of 100% isopropanol and 25µl of sodium acetate (3M). The precipitate was then placed on ice for an additional 20 minutes. The DNA was centrifuged for five minutes (12,500rpm) and the resulting pellet washed in 500µl of 70% ethanol. After a final centrifugation, the supernatant was decanted and the pellet was dried before re-suspension in 10-20µl of DNA rehydration solution (Promega).

In order to extract planula DNA, I modified the LaJeunesse *et al.* (2003) Wizard DNA protocol to accommodate the small sample volume. Planula samples were processed by the same protocol as the adults and juveniles, but all reagent volumes were reduced 10-fold (with the exception of final DNA pellets, which were re-suspended in 10µl of DNA rehydration solution

(Promega)). As planulae have no calcified structures, no glass beads were used and instead a planula was crushed against the side of the microcentrifuge tube using a pipette tip.

Amplification of the Hypervariable Internal Transcribed Spacer 2 (ITS2) Region of the Ribosomal Array

The rapidly evolving internal transcribed spacer 2 (ITS2) region of the ribosomal array (Figure 3) is the most extensively utilized marker for differentiating *Symbiodinium* due to its ability to resolve sub-cladal diversity. Owing to the widespread use of this marker, there is extensive DNA sequence data available, allowing for the rapid characterization of *Symbiodinium* types present within a host. While other markers are available and still more are being developed (LaJeunesse & Thornhill 2011; Pochon *et al.* 2012), to date, ITS2 is the best available marker for type level resolution in the *Symbiodinium* genus.

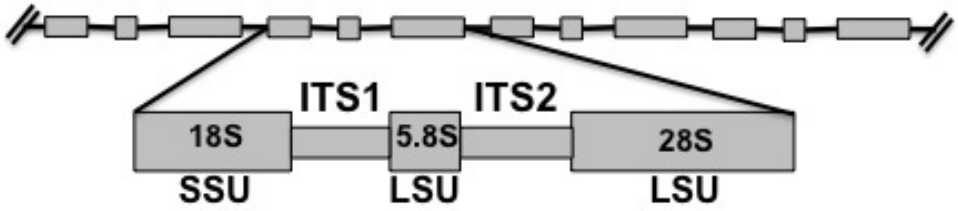

Figure 3. Schematic of the ribosomal subunit consisting of tandemly repeated copies of the rDNA operon. Each cistron consists of the highly variable ITS1 and ITS2 regions surrounded by the highly conserved 18S, 5.8S, and 28S regions of the large (LSU) and small (SSU) ribosomal subunits.

The ITS2 region of the ribosomal gene was amplified using the *Symbiodinium* specific primer set developed by LaJeunesse and Trench (2000; Table 1). The forward primer (ITSintfor2) anneals to a highly conserved region of the 5.8S ribosomal gene. The reverse primer (ITS2CLAMP) is equipped with a 39bp GC-clamp on the 5' end and anneals to the conserved 28S region (Figure 3).

To ensure PCR specificity, a "touchdown" amplification protocol was used as described by LaJeunesse (2002). Following an initial denaturation step of 92°C for three minutes, samples were run for two sets of 20 cycles with the following conditions: 92°C for 30 seconds, 60°C for 45 seconds, and 72°C for 30 seconds. For the first round of 20 cycles, annealing conditions were set at 60ºC and were dropped by 0.5ºC each cycle until a final annealing temperature of 50ºC was reached. With a constant annealing temperature of 52ºC, another 20 cycles were run. A final 10 minute annealing step was run at 72°C.

Denaturing-Gradient Gel Electrophoresis (DGGE)

Denaturing-gradient gel electrophoresis (DGGE) of ITS2 has been used extensively to investigate *Symbiodinium* diversity within coral hosts. DGGE fingerprinting of ITS2, coupled with the sequencing of diagnostic bands, has resulted in the identification of hundreds of unique *Symbiodinium* types (LaJeunesse *et al.* 2002; Correa & Baker 2009a; Stat *et al.* 2011).

DGGE takes advantage of the fact that double stranded DNA exhibits unique melting temperatures based on the length and G/C content of the sequence. Therefore, as double-stranded DNA migrates through a gradient of increasing denaturant chemicals, diagnostic banding profiles are created through differences in the dissociation, or melting point of unique DNA sequences. Even a single base-pair change will alter the dissociation point such that DNA fragments with low melting temperatures will quickly denature and migrate only a short distance into the gel relative to fragments with high melting temperatures (LaJeunesse 2002, Sampayo *et al.* 2009).

The PCR amplified ITS2 product was electrophoresed on an 8% polyacrylamide denaturing gradient gel (45-80% urea-formamide gradient) at a constant temperature (60ºC) for

13 hours at 120V (CBS Scientific DGGE system; Thornhill *et al.* 2006b). Gels were stained with SYBR Green I nucleic acid gel stain (Invitrogen) for at least 20 minutes. The most distinct and dominant bands were excised and placed into individual 1.5ml microcentrifuge tubes with 500µl of nuclease-free water. The tubes were vortexed for two minutes and left to incubate at 4ºC for at least two days before the elute was PCR amplified for direct sequencing using the ITSintfor2 primer and the ITS2Rev primer without the GC-clamp (LaJeunesse 2002, Sampayo *et al.* 2007). The PCR reaction products were sequenced, in the forward direction only, on an Applied Biosystems 3730 capillary sequencer (Core DNA Laboratory at Arizona State University, Tempe, AZ). Sequence chromatographs were analyzed manually using Geneious (version 5.3.6) and compared to GenBank submissions for *Symbiodinium* type identification.

Adult corals were run on the same gel with a maximum of 17 of their released planulae (some colonies released more than 17 planulae, but due to well restrictions, no more than 17 planulae were run per adult colony). In total, from the 2009 samples, 11 shallow and 19 deep-water colonies were run in addition to 131 shallow and 143 deep-water planulae. From the 2010 samples, nine shallow and 10 deep-water adults were run along with 115 shallow and 96 deep-water planulae. Of the juveniles collected in 2010, 26 shallow and 22 deep-water colonies were analyzed with DGGE.

Several limitations inherent to the ITS2 marker and DGGE can confound the interpretation of *Symbiodinium* diversity. While the ITS2 primers used are *Symbiodinium* specific, they are capable of occasionally amplifying host coral DNA (LaJeunesse 2002), resulting in additional, confounding bands in the DGGE profile. In addition, ribosomal genes in eukaryotic organisms consist of multiple tandem repeats, or cistrons (Figure 3). *Symbiodinium* and other dinoflagellate algae are estimated to contain anywhere from 200 to 1200 tandemly

repeated copies of the rDNA operon (Maroteaux *et al.* 1985; Galluzzi *et al.* 2004). Through concerted evolution rDNA cistrons are homogenized, resulting in intragenomic sequence variation (reviewed in Sampayo *et al.* 2009). The multi-copy nature of the ITS2 region has made it difficult to differentiate intragenomic from intergenomic variation and has contributed to the large number of *Symbiodinium* types that are characterized as unique based on single base pair changes in the ITS2 region. In other words, when multiple bands are seen on a DGGE gel, it can be difficult to determine if each band represents a distinct *Symbiodinium* type or multiple, rare, intragenomic variants of the same *Symbiodinium* type.

DGGE often produces multi-band profiles making it challenging to differentiate diagnostic bands. Many of these additional bands represent heteroduplexes, which are PCR artifacts formed when heterologous DNA strands anneal (LaJeunesse 2002). Heteroduplexes, as well as pseudogenes, result in relatively unstable DNA strands that denature quickly, leaving multiple uninformative bands that migrate only a short distance into the gel (LaJeunesse 2002). Additionally, *Symbiodinium* types with unique DNA sequences will co-migrate to the same distance in the gel if they happen to have the same melting temperature (Pochon *et al.* 2007; Sampayo *et al.* 2009). Consequently, to validate DGGE analysis, one must start by sequencing all bands in a given profile to differentiate diagnostic from erroneous bands (D. Thornhill pers. comm.). Further, even after validating a given DGGE profile, any unique bands observed must be excised and sequenced.

Despite these limitations, DGGE is still favored over cloning, which is the other most widely used technique for determining *Symbiodinium* diversity with the ITS2 marker (Apprill & Gates 2007; Winters *et al.* 2009; Stat *et al.* 2009b; Pochon and Gates 2010; Stat *et al.* 2011). Studies have shown that DGGE, in conjunction with direct sequencing of the dominant bands,

provides a more accurate representation of *Symbiodinium* diversity compared to cloning (Thornhill *et al.* 2007, Sampayo *et al.* 2009). Distinct *Symbiodinium* types contain fixed sequence variation that allows for a diagnostic, and repeatable DGGE profile in which the dominant band corresponds to the most dominant intragenomic variant (LaJeunesse 2002, Thornhill *et al.* 2007, Sampayo *et al.* 2009). As such, excising and sequencing the dominant DGGE band(s) allows for the accurate characterization of the *Symbiodinium* type(s) present in a given sample.

Table 1. Primer pairs used for denaturing-gradient gel electrophoresis and real-time PCR. The ITSinfor2 and ITS2Rev primers target the ITS2 ribosomal region while the clade A and C specific real-time PCR primers target the 28S ribosomal region. *A GC clamp is added to the 5' end of the reverse primer for use in DGGE; the GC clamp sequence is 5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC -3'.

Clade	Primer	Sequence	Amplicon Length	Tm (°C)	Use	Reference
Universal	ITS2intfor	5'- GAA TTG CAG AAC TCC GTG -3'	~350bp		DGGE	LaJeunesse & Trench 2000
	ITS2Rev*	5'- GGG ATC CAT ATG CTT AAG TTC AGC GGG T -3'			DGGE	LaJeunesse & Trench 2000
A	SymA28S-IF	5'- GAT TGT GGC CTT TAG ACA TAC TAC C -3'	126bp	56.3	Real-time	Yamashita e*t al.* 2011
	SymA28S-IR	5'- CTC TGA GAG CAA GTA CCG TGC -3'		56.5	Real-time	Yamashita e*t al.* 2011
C	SymC28S-IF	5'- TTG CTG AGA TTG CTG TAG GCT -3'	124bp	56.5	Real-time	Yamashita e*t al.* 2011
	SymC28S-IR	5'- TCC TCA AAC AGG TGT GGC -3'		53.5	Real-time	Yamashita e*t al.* 2011

Real-time PCR

Most coral species maintain stable symbioses with a single *Symbiodinium* type (LaJeunesse 2002; Goulet & Coffroth 2003; Goulet 2006; Thornhill *et al.* 2006a,b; Stat *et al.* 2009a; Thornhill *et al.* 2009; LaJeunesse *et al.* 2010). However, *Symbiodinium* types present in proportions below five to ten percent of the total symbiont population cannot be detected using DGGE and other gel techniques (Thornhill *et al.* 2006b; Mieog *et al.* 2007). This has led some to hypothesize that while most corals dominantly associate with a single symbiont type, coral species may host a highly diverse symbiont population that cannot be detected with traditional gel techniques (LaJeunesse 2002; Ulstrup & van Oppen 2003; Buddemeier *et al.* 2004; van Oppen & Gates 2006; Silverstein *et al.* 2012). In response, there has been a relatively recent increase in the number of studies utilizing real-time PCR technology (Ulstrup & van Oppen 2003; Loram *et al.* 2007; Mieog *et al.* 2007; Ulstrup *et al.* 2007; Smith 2008; Correa *et al.* 2009b; LaJeunesse *et al.* 2009b; Mieog *et al.* 2009; Coffroth *et al.* 2010; Yamashita *et al.* 2011; Silverstein *et al.* 2012). Real-time PCR provides a nearly 100-fold increase in detection sensitivity compared to DGGE (Mieog *et al.* 2007; Loram *et al.* 2007), and has been successful in identifying previously undetected *Symbiodinium* types present at low-levels within a variety of host species (Mieog *et al.* 2007, LaJeunesse *et al.* 2009; Silverstein *et al.* 2012).

Unlike traditional end-point PCR, real-time PCR monitors target amplification through the detection of a fluorescent reporter molecule. The fluorescence generated is directly proportional to the amount of PCR product present in a given PCR cycle. Differences in the initial DNA template concentration will be reflected in the number of cycles needed for sample fluorescence to reach a set threshold value, which is referred to as the threshold cycle or C_T value (Kubista *et al.* 2006; Smith 2008).

As real-time PCR monitors the fluorescent signal generated during each PCR cycle, software accompanying the real-time instrument creates amplification plots (Figure 4). During the initial stages of PCR (e.g. cycles 1-16 in Figure 4) very little fluorescence is detected, as amplicon copy numbers remain low; these initial stages are referred to as the baseline (Kubista *et al.* 2006). Included in each PCR reaction are a reporter dye and an internal passive reference dye. The reporter dye produces the fluorescent signal from the target DNA. The passive reference dye generates an internal fluorescence upon which the reporter dye can be normalized. Therefore, fluorescence fluctuations are corrected in the normalized reporter (R_n) value, which is defined as the ratio between the fluorescence intensities of the reporter dye to the passive reference dye. The delta R_n (ΔR_n) value can then be calculated based on the signal magnitude generated following the formula:

$$\Delta R_n = R_n - baseline.$$

Real-time PCR software creates amplification plots of either R_n or ΔR_n versus the PCR cycle number (Figure 4) and allows for the monitoring of PCR amplification in real-time. In order to either quantify samples or determine positive versus negative reactions qualitatively, a threshold value must be set to determine the C_T value (Figure 4). Samples with low C_T values indicate the target DNA is of higher abundance compared to samples with high C_T values. The threshold can be manually set or automatically determined using real-time instrument software. Regardless, it is important that threshold values are set above the baseline but as low as possible within the exponential growth phase of the amplification curve (Larionov *et al.* 2005; Figure 4).

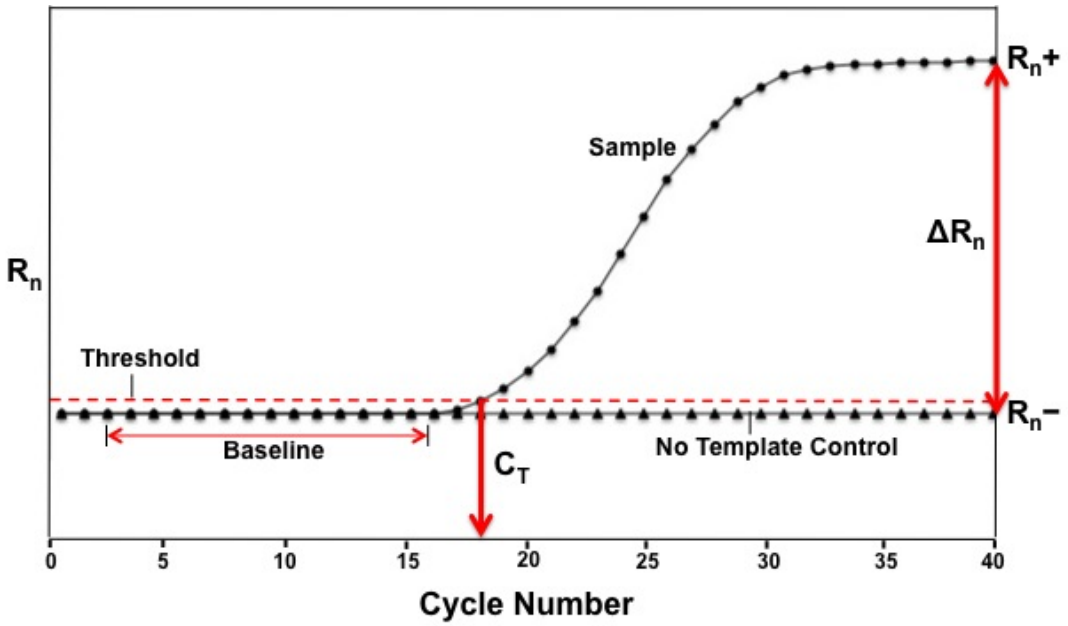

Figure 4. Annotated example of a real-time PCR amplification plot. Each point within the amplification curves represents the fluorescence signal generated during a PCR cycle (40 cycles) as measured by R_n. Circle points represent sample amplification while triangle points represent a no template negative control. The dotted red line represents a set threshold value that is placed above the baseline, but as low as possible within the exponential growth phase of the sample's amplification curve. The C_T value indicates the cycle number at which sample fluorescence exceeds the threshold; in this example, the sample has a C_T value of 18. (Image modeled after the Applied Biosystems Real-time SDS software (see "Absolute Quantification…").)

There are several different chemistries used for the detection of PCR products by real-time PCR instruments including intercalating dyes, duel labeled probes, minor groove binding probes, molecular beacons, fluorescence energy transfer, and fluorescently labeled primers (Gunson *et al.* 2006; Kubista *et al.* 2006; VanGuilder *et al.* 2008). In the present study I used the SYBR green (Applied Biosystems) detection chemistry, which is an intercalating dye that is widely used for many real-time PCR applications. The SYBR green dye indiscriminately binds to all double-stranded DNA (VanGuilder *et al.* 2008; Kubista *et al.* 2006). During PCR, the denaturing step creates single stranded DNA fragments that do not fluoresce, as the dye cannot properly bind. However, upon PCR extension, the SYBR green dye binds to the newly created double-stranded DNA fragments and fluoresces. Consequently, as the number of PCR fragments

increases with each PCR cycle, the net fluorescence detected by the real-time instrument proportionally increases. The chief limitation of SYBR green chemistry is that all double-stranded DNA, even non-target DNA, creates a fluorescent signal (VanGuilder *et al.* 2008). This limitation can be circumvented with the use of a dissociation-curve analysis.

With SYBR green chemistry, real-time instruments record the decrease in fluorescence that occurs each cycle when double-stranded DNA denatures. As denaturation occurs at slightly different temperatures according to the unique DNA sequences of each target, and because deceased fluorescence only occurs after denaturation, dissociation-curve analysis can determine diagnostic melting temperatures for each target. Dissociation-curve analysis can easily identify primer dimers, which display characteristically low melting temperatures relative to target DNA. Dissociation curves are typically plotted as the "first derivative of the rate of change in fluorescence as a function of temperature" (see "Absolute Quantification…").

Real-time PCR Assay

Real-time PCR was used to evaluate possible low-levels of *Symbiodinium* that fell below the detection limit of DGGE. *S. pistillata* in the Red Sea has only been reported to associate with clade A in shallow water and clade C in deep water (Daniel 2006; Lampert-Karako *et al.* 2008; Winters *et al.* 2009). Consequently, only clade A (SymA28S) and C (SymC28S) *Symbiodinium* specific primer pairs, designed by Yamashita *et al.* (2011; see Table 1 for primer sequences), were used to target the 28S ribosomal region (Figure 3).

All of the adults, juveniles, and planulae collected in 2010 were analyzed with real-time PCR, while only a subset of samples collected in 2009 and 2011 were run. In total, nine shallow water adults and 134 of their released planulae were analyzed from the 2010 samples. From the

2010 deep-water samples, 10 adults and 107 planulae were run. Of the samples collected in 2009 and 2011, 10 adults from each depth and year were analyzed. Additionally, I ran 28 shallow and nine deep-water planulae released from adults in 2009. Finally, 25 juveniles from each depth were run with real-time PCR.

For real-time PCR, the amount of DNA in any given sample was quantified spectrophotometrically using a NanoDrop (Thermo Scientific, ND-1000 Spectrophotometer) and DNA concentrations were normalized to 0.1-2.0ng/µl. All samples were run in triplicate on an Applied Biosystems 7300 real-time PCR system. A total reaction volume of 25µl was used, but due to differences in primer concentrations, the reactions varied slightly between primer pairs. The clade A primer pair reaction contained 12.5µl 2x Power SYBR green PCR mastermix (Applied Biosystems), 7.75µl nuclease-free water, 450nM of each primer, and 2.5µl of genomic DNA at a concentration of 0.1-2ng/µl. The clade C primer pair reaction contained 12.5µl 2x Power SYBR green PCR mastermix (Applied Biosystems), 9.25µl nuclease-free water, 150nM of each primer, and 2.5µl of genomic DNA at a concentration of 0.1-2ng/µl. All plates were run under the same thermal conditions: 2 minutes at 50°C, 10 minutes at 95°C, and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. A dissociation curve was also run on each plate, which consisted of one cycle at 95°C for 15 seconds, 60°C for 30 seconds, and 95°C for 15 seconds. The dissociation curve was used to determine the melting temperature of each reaction to identify non-specific fluorescence characteristic of primer dimers. Additionally, clade A and C *Symbiodinium* exhibited diagnostic melting temperatures allowing for confirmation that the clade of interest was in fact amplified.

Since DGGE had already confirmed the presence of clade A in all shallow water adults, juveniles, and planulae, only the clade C primer pair was used to detect the presence or absence

of low-levels of clade C *Symbiodinium*. A subset of shallow samples was run on each plate with the clade A primer pair as a positive control. Likewise, all deep-water adults, juveniles, and planulae were run with the clade A primer pair to detect low-levels of clade A *Symbiodinium* and positive controls were run on each plate with the clade C primer pair. Due to ambiguous DGGE profiles for many of the juveniles collected in deep-water, all deep-water juveniles were run with both primer pairs. No template negative controls were run on each plate to detect non-specific fluorescence.

Primer Optimization and Standard Curves

Prior to running assays, primer optimizations and standard curves were run to confirm optimal primer and DNA concentrations. Both primer optimizations and standard curves were run in duplicate in 25µl reactions. Primer optimization reactions contained 12.5µl 2x Power SYBR green PCR mastermix (Applied Biosystems), 1ng DNA template, and either 0.25µl, 0.75µl, or 2.25µl of each forward and reverse primer to obtain final primer concentrations of 50nM, 150nM, or 450nM, respectively. The clade A and C specific primer pairs were run at all three primer concentrations using a DNA template from one shallow and one deep-water adult colony known to contain clade A and C *Symbiodinium*, respectively. With both primer pairs, amplifications run at a 450nM primer concentration only marginally outperformed amplifications run at 150nM; both the 450nM and 150nM concentrations proved more efficient than 50nM (Figure 5A). Therefore, 450nM was used as the primer concentration for all reactions run with the clade A primer pair. However, primer interactions in the clade C primers resulted in primer dimers that caused non-specific amplification at 450nM. The primer dimers were eliminated

when the primer concentration was dropped to 150nM and therefore, all clade C reactions were run with a 150nM primer concentration (Figure 5B).

Standard curves were initially run to determine an optimal DNA concentration. Standard curves run with the clade A primers were created using genomic DNA isolated from a shallow water adult colony known to contain clade A *Symbiodinium*. Similarly, standard curves run with the clade C primers were created from DNA isolated from a deep-water adult colony known to contain clade C *Symbiodinium*. Initial standard curves were run in duplicate over six, three-fold serial dilutions ranging from 1ng/µl to 0.004ng/µl. In both the clade A and C standard curves, 0.33ng/µl amplified more strongly than the 1ng/µl concentration indicating that 1ng/µl overwhelmed the PCR reaction and that lower DNA concentrations were required for efficient amplification. Consequently, standard curves were run in duplicate over five, three-fold serial dilution from 0.33ng/µl to 0.004ng/µl on all subsequent plates in order to monitor primer efficiency.

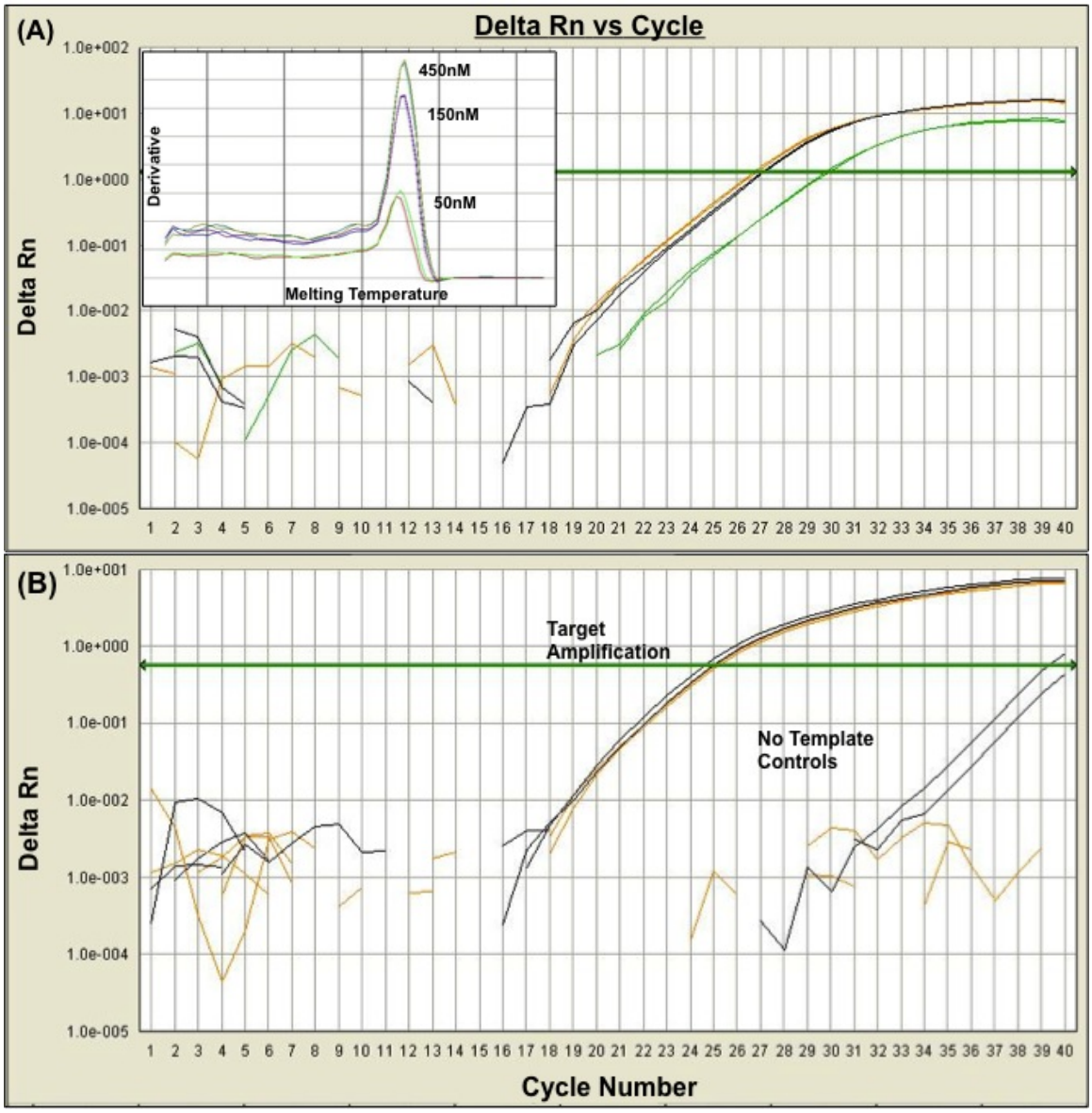

Figure 5. Real-time PCR results from primer optimization analysis. (A) At 450nM (black) and 150nM (orange) primer concentrations, the amplification efficiency was similar, but the 450nM concentration was considered optimal for this primer due to the higher derivative seen in the dissociation curve (inlay). Both concentrations performed better than 50nM (green). (B) Primer interactions with the clade C primer pair resulted in non-specific fluorescence in the no template control at 450nM primer concentration (black). At 150nM (orange) no non-specific fluorescence was detected. Target DNA amplified similarly at both concentrations for the clade C primer.

Data Analysis

Although real-time PCR has been used to quantify the number of algae present in coral samples (Ulstrup & van Oppen 2003; Mieog *et al.* 2007; Yamashita *et al.* 2011), the multi-copy nature of rDNA complicates the analysis. As I was unable to determine the copy number for the *Symbiodinium* types present in the samples, I chose a qualitative approach to detect the presence or absence of *Symbiodinium* clades not identified with DGGE (Smith 2008; LaJeunesse *et al.* 2009b; Coffroth *et al*. 2010). In order to accurately compare samples, strict values were set to consistently define a positive reaction.

Threshold Determination

The cycle-threshold (C_T) is a numerical value representing the PCR cycle at which sample fluorescence surpasses a set threshold limit (Figure 6). Therefore, in order to compare samples run on different plates, first a fixed threshold value had to be determined (Gunson *et al.* 2006; Smith 2008; Correa *et al.* 2009b). The exact placement of the threshold value is somewhat arbitrary as amplification curves are expected to be near parallel during the exponential, or growth phase, of the reaction (Kubista *et al.* 2006). Still, the selected threshold value should be set as low as possible within the exponential phase of growth and must produce an acceptable standard curve (r^2 values of 99%; Larionov *et al.* 2005). The Applied Biosystems SDS software uses machine set algorithms to automatically determine an optimal threshold value for each well. By comparing the standard curves run on each plate, I created a fixed threshold value based on the average automatic threshold setting for each primer pair. Each primer pair was considered individually and therefore, the clade C primer had a fixed threshold of 0.57 while the clade A primer pair had a fixed threshold of 1.30 (Figure 6A-D). Set threshold values were placed as low as possible within the exponential amplification phase (when plotted as R_n versus cycle number;

Figure 6C-D) and each standard curve was checked to determine if an acceptable coefficient of determination was produced when plotted as cycle number versus the logarithm of DNA concentration (Figure 6E-F). For both primer pairs, standard curves produced acceptable r^2 values indicating optimal threshold settings (clade A N=24, mean $r^2 = 0.9930$, SD = 0.0033; clade C N=27, mean $r^2 = 0.9928$, SD = 0.0031).

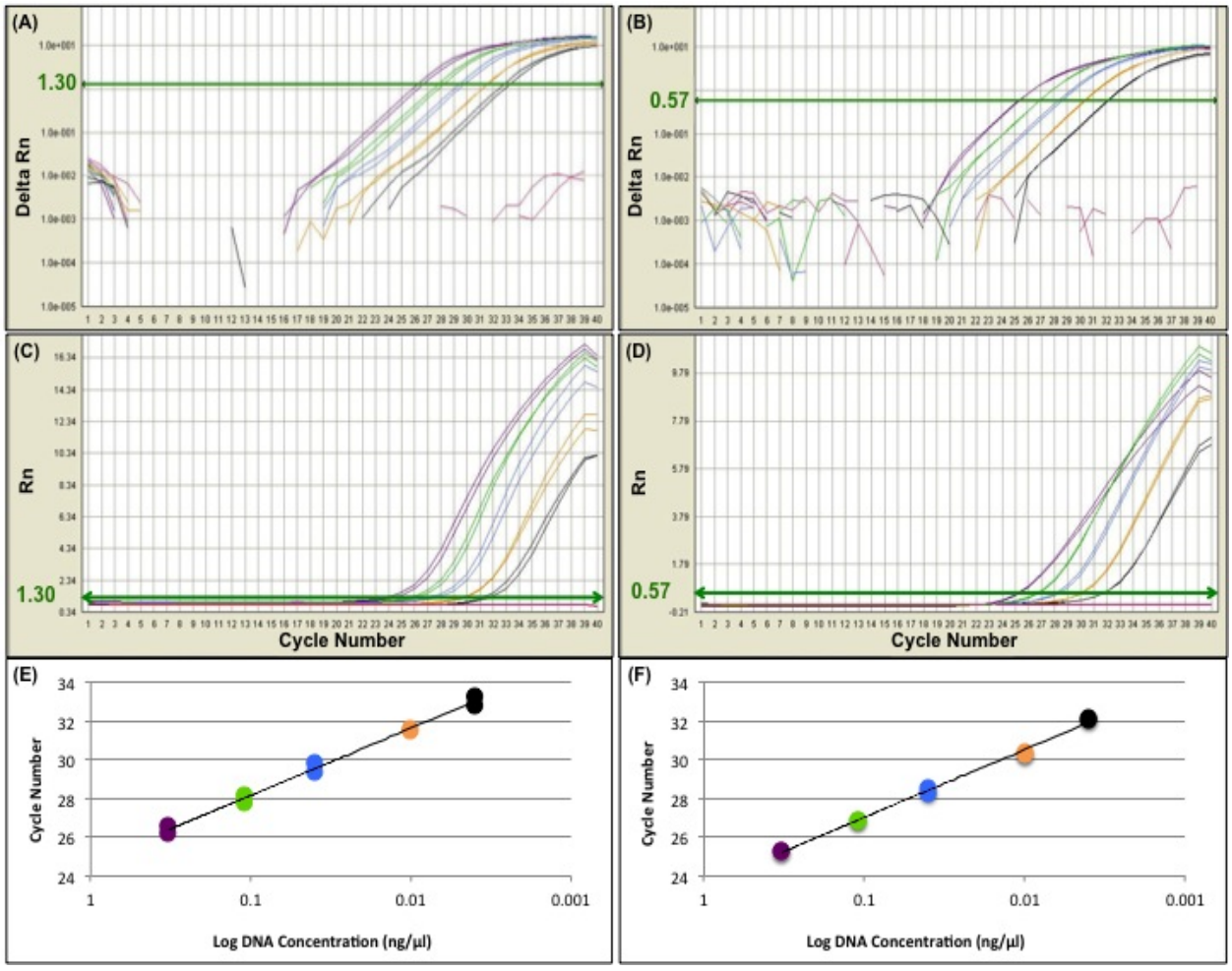

Figure 6. Real-time PCR standard curve analysis. All standard curves were run in duplicate over five, three-fold serial dilutions at the following concentrations: 0.33ng/µl (purple), 0.11ng/µl (green), 0.04ng/µl (blue), 0.01ng/µl (orange), and 0.004ng/µl (black). Amplificaton plots are show as ΔR_n versus cycle number for the clade A (A) and clade C (B) primers and as R_n versus cycle number for the clade A (C) and clade C (D) primers. The threshold value (green vertical line) was set as low as possible within the exponential phase of amplification (C-D). The clade A primer threshold was set a 1.30 (A,C) while the clade C primer threshold was set at 0.57 (B,D). These particular standard curves produced r^2 values of 0.993 for the clade A primer (E) and 0.996 for the clade C primer (F).

Cutoff C_T Determination

In addition to a fixed threshold, it is necessary to determine a cutoff C_T value to consistently define a positive versus negative reaction. I determined an appropriate cutoff C_T value for each primer pair through a series of competitive mixed clade amplifications. As described by Smith (2008), I created mixtures of clade A and C *Symbiodinium* in known proportions to determine an approximate detection limit. Samples were created using template DNA from one shallow and one deep-water planula. The planulae selected only contained the *Symbiodinium* clade characteristic of their depth of origin, *i.e.* the shallow water planula only contained clade A while the deep-water planula only contained clade C. This was confirmed through DGGE and real-time PCR. The planula DNA extract was combined such that the target clade represented 50, 9, 0.9, 0.1, and 0.01 percent of the total DNA template (Table 2). The mixtures were run three times, once in duplicate and twice in triplicate, in order to determine an average C_T value for each mixture proportion and to confirm amplification consistency between runs.

The target clade amplified consistently when it composed at least 0.9% of the total DNA template, indicating a conservative detection limit of approximately 1% (Table 2). Since field-collected samples were used to create the mixtures, by extension, any samples with a *Symbiodinium* clade comprising at least 1% of the total symbiont population can consistently be detected with this real-time PCR assay. Consequently, I set the cutoff C_T value at the average C_T value for the mixture where the target clade made up 0.9% of the template DNA. The cutoff C_T was set at 34 for the clade A primer pair and 32 for the clade C primer pair (Table 2; Figure 7). Only values equal to or lower than the set cutoff C_T were considered positive for the presence of low-level *Symbiodinium*. These estimates are conservative because amplification did occur at

higher C_T values, but, due to the consistency of amplification at 0.9%, this was determined to be the most appropriate cutoff C_T. Moreover, symbionts that comprise less than 1% of the symbiotic community may not be ecologically relevant.

Table 2. Mixed clade real-time PCR trials for threshold and cutoff C_T determination. Mixtures of clades A and C DNA were combined to determine approximate detection limits and C_T cutoff values. Boxed values indicate the detection limit of the real-time PCR assay for each primer pair as determined by the lowest DNA concentration where the target clade was consistently detected (*i.e.* crossed set threshold at any C_T value).

Clade A Primer Pair

DNA Conc. Clade C	DNA Conc. Clade A	Total DNA Conc.	Percent Clade A	No. Replicates Crossed	Average C_T ± SD
1ng	1ng	2ng	50.00%	8/8	26.61 ± 0.19
1ng	0.1ng	1.1ng	9.00%	8/8	29.95 ± 0.19
1ng	0.01ng	1.01ng	0.90%	8/8	33.54 ± 1.07
1ng	0.001ng	1.001ng	0.10%	7/8	36.44 ± 1.23
1ng	0.0001ng	1.0001ng	0.01%	2/8	37.21 ± 2.40

Clade C Primer Pair

DNA Conc. Clade A	DNA Conc. Clade C	Total DNA Conc.	Percent Clade C	No. Replicates Crossed	Average C_T ± SD
1ng	1ng	2ng	50.00%	8/8	25.40 ± 1.03
1ng	0.1ng	1.1ng	9.00%	8/8	28.54 ± 1.13
1ng	0.01ng	1.01ng	0.90%	8/8	32.13 ± 1.25
1ng	0.001ng	1.001ng	0.10%	5/8	31.46 ± 1.11
1ng	0.0001ng	1.0001ng	0.01%	2/8	36.44 ± 1.18

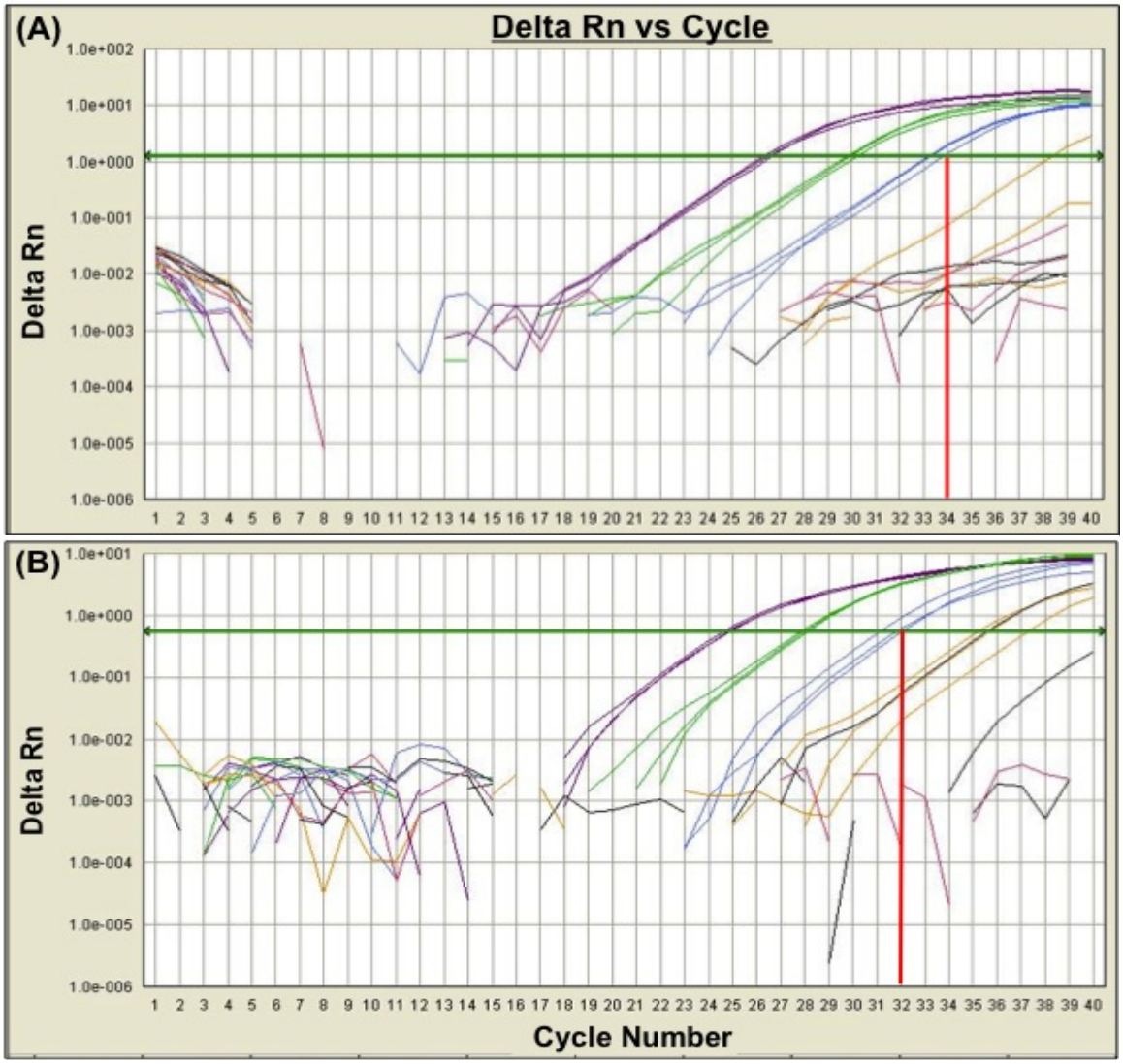

Figure 7. Mixed clade real-time PCR trials for threshold and cutoff C_T determination. Amplification plots of mixed clade trials for the clade A primers (A) and clade C primers (B) to determine the detection limit of the real-time PCR assay and to set cutoff C_T values. The green horizontal lines correspond to the set threshold values for the clade A (1.3) and clade C (0.57) primer pairs. Mixed clade amplifications were prepared in accordance with Table 2. Each color corresponds to triplicate runs in which the target clade composed 50% (purple), 9% (green), 0.9% (blue), 0.1% (orange), and 0.01% (black) of the DNA template. No template controls are shown in red. Note that for both primers, all replicates amplified the target clade consistently when it composed at least 0.9% of the DNA template. When the target DNA represented less than 0.9%, amplification was inconsistent. C_T is defined as the point at which sample fluorescence exceeds a fixed threshold value; the vertical red lines represent the chosen cutoff C_T value for the clade A primers (C_T 34) and clade C primers (C_T 32).

Results

Denaturing-Gradient Gel Electrophoresis (DGGE)

Adults and planulae

Two distinct ITS2 DGGE fingerprints were identified from the 534 adult and planula samples processed. All shallow water (2-6m) samples analyzed (n=266) showed the same dominant two-band DGGE fingerprint (Figure 8). Sequencing revealed that the two dominant bands differed by 1bp in the ITS2 region. The top band in the shallow water DGGE fingerprint showed 100% similarity to ITS2 type A1, *Symbiodinium microadriaticum* subsp. *microadriaticum* (accession number: AF333505). *Symbiodinium* type A1 has been previously reported in shallow water *S. pistillata* adults in the Gulf of Eilat (LaJeunesse 2001; LaJeunesse *et al.* 2009a; Winters *et al.* 2009). The lower band in the shallow water DGGE profile reported here showed a consistent 1bp T/C transition compared to type A1. Daniel (2006) also reported a two-band DGGE profile for shallow water *S. pistillata* in the Gulf of Eilat. He described the second band as a novel *Symbiodinium* subtype named A1.2. Unfortunately, Daniel (2006) did not upload this sequence to GenBank, and therefore I was unable to determine if the second band detected here is the A1.2 type he reported. As both bands likely represent intragenomic variation, the two-band profile probably represents two co-dominant variants of the same *Symbiodinium* A1 variant.

All samples collected from deep-water (20-30m; n=268) exhibited a characteristic multi-band DGGE fingerprint (Figure 8). Upon sequencing, the dominant band aligned with ITS2 type C72 (accession number: AY765407). Type C72 has been previously reported for *S. pistillata* adult colonies from 20-30m depths in the Gulf of Eilat (LaJeunesse *et al*. 2005; Daniel 2006; Winters *et al.* 2009). In all cases, from both shallow and deep-water colonies, planulae displayed

the same DGGE fingerprint as their maternal colony, with no additional clades and/or types detected.

In addition to the dominant bands, multiple faint bands were also observed which probably represent rare intragenomic variants and/or heteroduplexes. Occasionally, additional bands appeared that migrated further in the gel than the characteristic *Symbiodinium* profiles. Sequencing revealed that these bands represented coral host DNA that aligned to *S. pistillata* or the closely related species, *Pocillopora damicornis*.

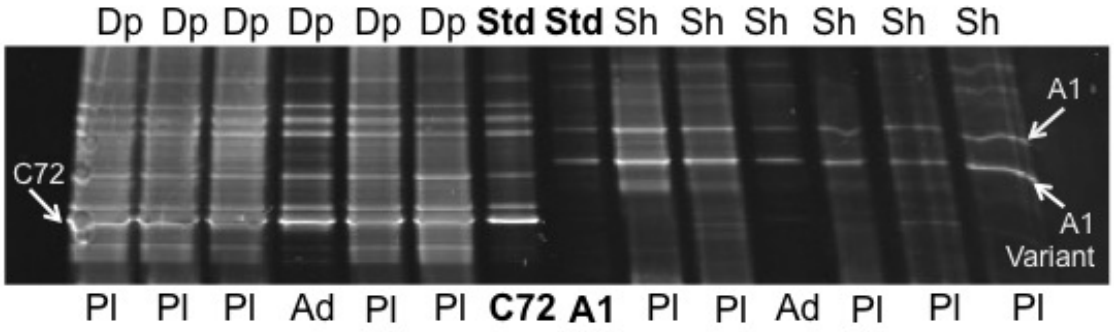

Figure 8. Denaturing-gradient gel electrophoresis image of the *Symbiodinium* hosted by adults and planulae. *Stylophora pistillata* adult colonies (Ad) and several of their released planulae (Pl) were collected from both shallow (Sh, 2-6m) and deep (Dp, 24-26m) water. In all cases, the planula DGGE fingerprints were identical to that of their maternal colony, demonstrating the vertical inheritance of the abundant *Symbiodinium* type. The sequence of the upper dominant band from the shallow water samples was identical to *Symbiodinium* type A1 (accession AF333505). The lower dominant band in the shallow water DGGE fingerprint showed a consistent 1bp difference compared to A1, indicating that *S. pistillata* hosts an A1 variant. All deep-water samples hosted type C72 (accession AY765407). *Symbiodinium* type A1 and C72 standards (Std) were run on every gel for reference.

Juveniles

Unlike the adults and planulae, which only contained one of two distinct DGGE fingerprints, the juvenile colonies analyzed displayed multiple profiles. All shallow water juvenile colonies, showed a two-band profile characteristic of *Symbiodinium* type A1, with the exception of one individual, which contained both types A1 and C72 (Figure 9). The juveniles collected in deep-water showed four different DGGE profiles. Of the 25 deep-water juveniles

analyzed, 11 contained type A1 *Symbiodinium*, nine contained type C72, four juveniles exhibited mixed profiles of both types A1 and C72, and one juvenile hosted *Symbiodinium* type C21 (Figure 9).

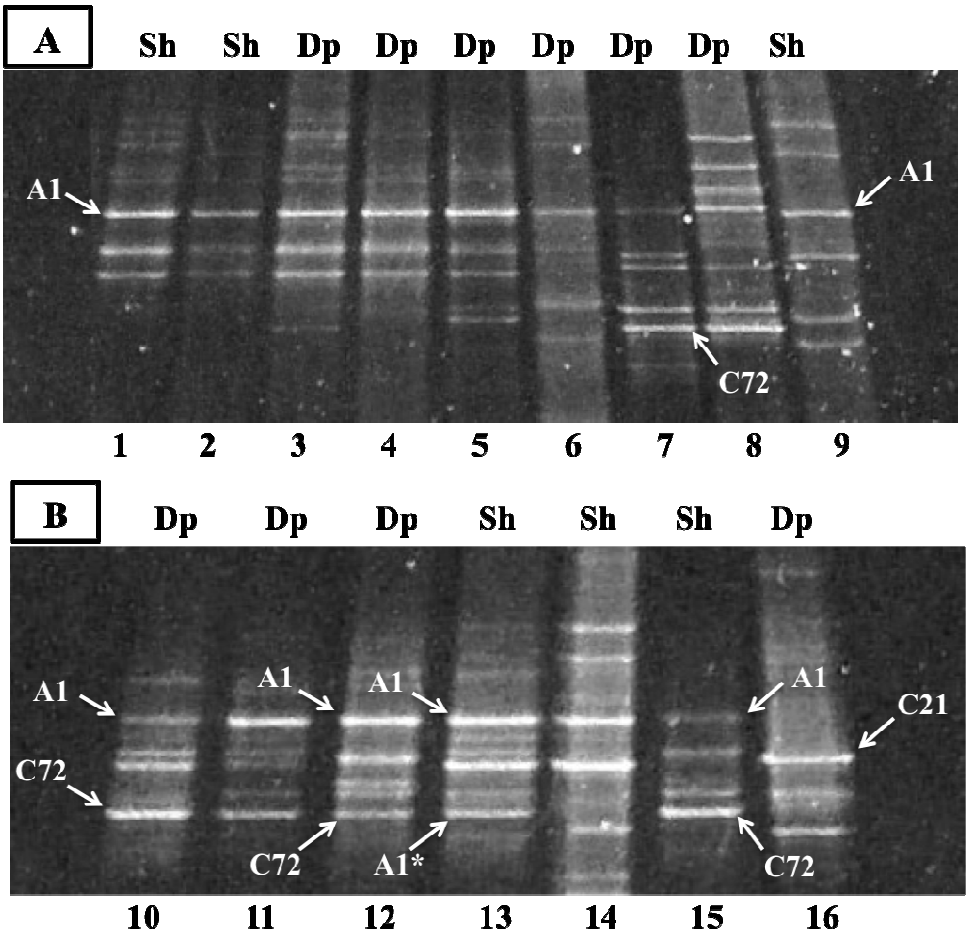

Figure 9. Denaturing-gradient gel electrophoresis image of the *Symbiodinium* hosted by juveniles. Juvenile *Stylophora pistillata* colonies collected from shallow (Sh, 2-6m) and deep (Dp, 24-26m) water. (A) Lanes 1-6 and 9 all contain type A1 *Symbiodinium* from both shallow and deep-water juveniles. Lanes 7-8 show *Symbiodinium* type C72 from two deep-water colonies. (B) Lanes 10-12 and 15 display mixed clade profiles consisting of *Symbiodinium* types A1 and C72 in shallow and deep-water juveniles. Lane 14 depicts only type A1 in a shallow water juvenile. Lane 16 exhibits a 3-band *Symbiodinium* type C21 profile from a deep-water juvenile. *While the shallow water colony in lane 13 appears to have the C72 band, sequences showed stronger similarity to type A1.

Real-time PCR

Adults

In adult *S. pistillata*, the presence of low-level symbionts varied with depth. Low-level *Symbiodinium* were not detected in any shallow water adult colonies, but low-levels of clade A *Symbiodinium* were detected in some of the deep-water adults analyzed (Table 3). In 2009, all adult deep-water colonies sampled hosted only clade C, but in 2010, the majority of deep-water adult colonies sampled contained low-levels of clade A in addition to the abundant clade C symbionts (Figure 10). In 2011, only one of the sampled deep-water colonies hosted clade A at low-levels (Table 3). In 2011, I re-sampled three colonies that were also sampled in 2010. Of those three colonies, two did not contain low-levels of clade A *Symbiodinium* in either year, while the third individual had detectable low-levels of clade A in 2010 but not in 2011.

Planulae

All planulae processed, from both depths, hosted a single *Symbiodinium* clade with no detectable low-levels of the second clade (Table 3). Even maternal colonies hosting both abundant and low-level *Symbiodinium* released planulae without low-level symbionts. The lack of detection of additional symbionts in planulae was not due to under-sampling. Using one-sample proportion tests, I tested a series of null hypotheses to determine the lowest proportion of released planulae that may contain low-level *Symbiodinium*. I was able to reject the null hypothesis that ≥2% of planulae released by shallow water adults contain low-levels of clade C *Symbiodinium* (n=161, df=1, χ^2=3.286, p-value=0.035). Similarly, I rejected the null hypothesis that ≥ 3% of planulae released from deep-water adults may contain low-levels of clade A *Symbiodinium* (n=116, df=1, χ^2=3.588, p-value=0.029).

Table 3. Summary of real-time PCR results. Real-time PCR results of the abundant and low-level *Symbiodinium* clades present in shallow (Sh) and deep (Dp) water *S. pistillata* in adults (Ad), planulae (Pl), and juveniles (Jv) analyzed (N). The numbers in parentheses denote the number of samples containing a given clade. * Samples that contained both A and C at levels detectable by DGGE, hence both clades were listed as abundant.

Year	Depth	Life Stage	N	Abundant Clade	Low-level Clade
2009	Sh	Ad	10	A (10)	-
		Pl	28	A (28)	-
	Dp	Ad	10	C (10)	-
		Pl	9	C (9)	-
2010	Sh	Ad	9	A (9)	-
		Pl	134	A (134)	-
		Jv	25	A (25)	C (1)
	Dp	Ad	10	C (10)	A (7)
		Pl	107	C (107)	-
		Jv	25	C (5); A (11); A & C (9)*	-
2011	Sh	Ad	10	A (10)	-
	Dp	Ad	10	C (10)	A (1)

Juveniles

Similar to the results for adult colonies, the majority (24/25) of juveniles collected in shallow water hosted only clade A with no low-levels of clade C *Symbiodinium* (Table 3). One juvenile collected in shallow water, however, hosted clade A *Symbiodinium* in addition to low-levels of clade C. Of the 25 deep-water juveniles analyzed, only five individuals solely contained clade C *Symbiodinium.* Nine other juveniles collected in deep-water contained both clade C and clade A *Symbiodinium*. Unexpectedly, 11 of 25 deep-water juveniles analyzed solely contained clade A *Symbiodinium*, with no detectable traces of clade C (Table 3).

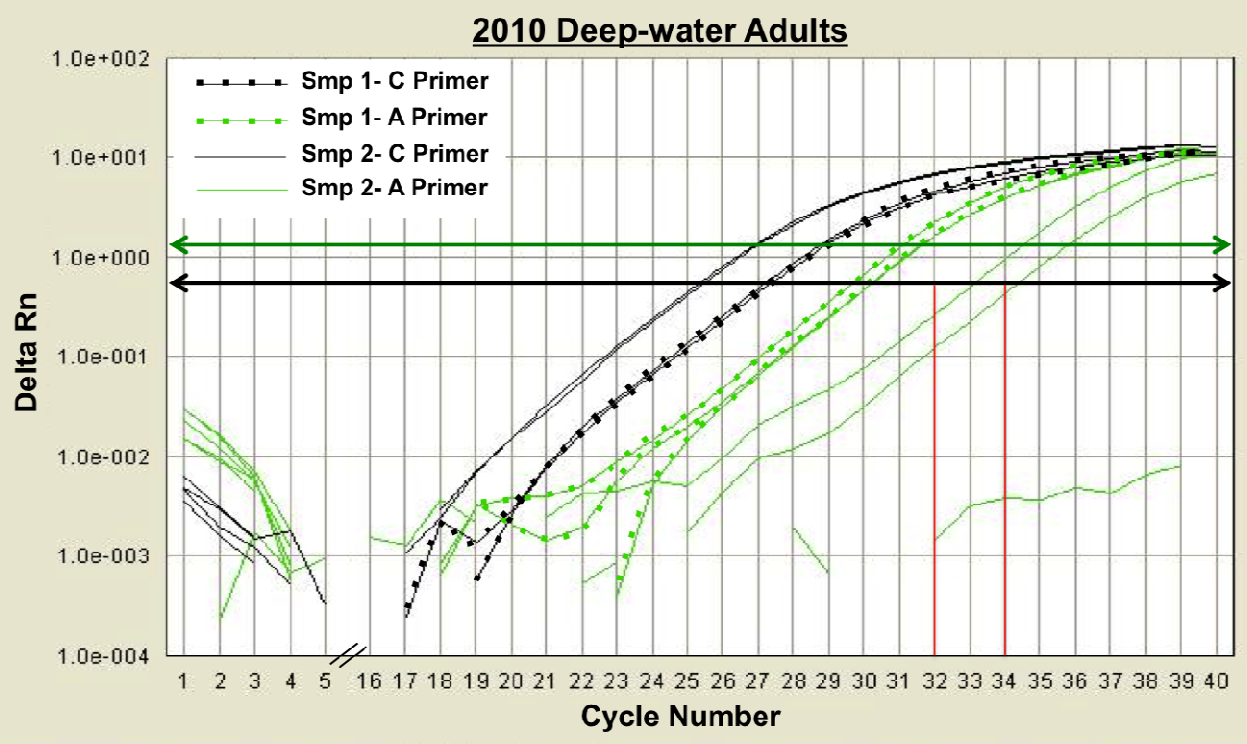

Figure 10. Real-time PCR amplification plot of *Symbiodinium* from two deep-water adult colonies. Each sample was run in triplicate with both the clade A primer (green) and the clade C primer (black). Horizontal green and black lines correspond to the threshold values set for the clade A (1.3) and C primers (0.57), respectively. Vertical red lines represent the cutoff CT values for the clade A (34) and clade C (32) primers. Note that sample 1 (dotted lines) amplified with both the clade A and C primer indicating the presence of both *Symbiodinium* clades. Sample 2 (solid lines) amplified with the clade C primers, but was negative for clade A *Symbiodinium*. Cycle numbers within the baseline are eliminated.

Juvenile Age Determination

The geometric mean radius ( ) of juvenile colonies collected in shallow water ranged from 0.342-1.229cm with an average of 0.794 ± 0.209 (Table 4). Deep-water juvenile colonies ranged in geometric mean radius from = 0.330 to 1.286cm with an average of 0.656 ± 0.263. Overall, the shallow water juvenile colonies analyzed had a significantly larger in comparison to the deep-water juvenile colonies collected (normal log transformed t-test, df = 48, p = 0.0252).

An approximate age range for juvenile colonies was determined based on a geometric mean radius growth rate estimate of 1.131 ± 0.158cm yr^{-1} (Loya 1976). Colonies ranged in age

from approximately 3 to 16 months (Table 4). As juvenile colonies were collected in July of 2010, age estimates indicate settlement likely occurred throughout 2009 and into early 2010. Consequently, the juveniles collected likely represent progeny from 2009 and 2010 spawning events.

The overall $\bar{r}$ was smaller for deep-water colonies, which, based on the growth rate estimate used, indicates that they are younger than the shallow water juveniles collected (Table 4). The smaller $\bar{r}$ recorded for deep-water juveniles, however, does not necessarily correlate to a younger age because the growth rate used here was based on estimates from shallow water (4-5m) colonies only (Loya 1976). Therefore, this growth rate may underestimate the actual age of the deep-water juveniles collected. Unfortunately, I was unable to recover any growth estimates for *S. pistillata* colonies present in the 20-30m depth range. As growth generally decreases with decreasing light (Dubinsky *et al.* 1990), the lower light levels present beyond 20m will likely lead to a decrease in the growth rate of deep-water juveniles. Therefore, while there was a significant difference in size between the shallow and deep-water juveniles collected, they may be of a similar age class.

Regardless of the exact age, all juvenile colonies collected were probably reproductively immature colonies. In *S. pistillata,* sperm production usually commences once colonies reach $\bar{r}$ = 1.51-2cm, although in extremely rare cases colonies as small as $\bar{r}$ = 0.5-1.5cm can produce sperm (based on analysis of shallow water colonies; Rinkevich & Loya 1979b). Ripe eggs are typically seen once colonies reach approximately $\bar{r}$ = 4-5cm (Loya 1976). Therefore, because all of the juveniles collected from both depths had a $\bar{r}$ below the 1.51cm size of reproductive maturation, these young colonies were classified as juveniles.

Table 4: Size and age ranges of juvenile colonies. Mean geometric radius and approximate age of each juvenile colony collected from either shallow (2-6m) or deep (24-26m) habitats. The mean geometric radius was calculated based on the length (l), width (w) and height (h) of colonies using the formula $\bar{r} = ((l * w * h)^{1/3})/2$. The average geometric mean radius of all colonies collected from each depth is recorded with the standard deviation. Age estimates were based on an average growth rate estimate of shallow water *S. pistillata* colonies in the Gulf of Eilat according to Loya (1976); growth rate estimate with standard deviation: $\Delta\bar{r}$ per year = 1.131 ± 0.158 cm yr^{-1}. The age range includes the standard deviation of the growth rate estimate.

Shallow Habitat (2-6m)			**Deep Habitat (24-26m)**		
Sample Name	**Geometric Mean Radius ($\bar{r}$)**	**Age Range (Months)**	**Sample Name**	**Geometric Mean Radius ($\bar{r}$)**	**Age Range (Months)**
RSh477	0.342	3-4	RD1	0.330	3-4
RSh393	0.453	4-6	RDY5	0.337	3-4
RSh21	0.466	4-6	RD45	0.396	4-5
RSh470	0.520	5-6	RD283	0.417	4-5
RSh0	0.637	6-8	RD5	0.437	4-5
RShY3	0.692	6-9	RD301	0.444	4-5
RShY4	0.712	7-9	RD372	0.455	4-6
RSh352	0.733	7-9	RD250	0.463	4-6
RShY7	0.744	7-9	RD221	0.467	4-6
RSh256	0.766	7-9	RD269	0.476	4-6
RShY6	0.783	7-10	RDY10	0.497	5-6
RSh340	0.788	7-10	RD254	0.514	5-6
RSh280	0.805	7-10	RD.2	0.543	5-7
RShY11	0.806	8-10	RD435	0.548	5-7
RShY38	0.819	8-10	RD227	0.694	6-9
RSh218	0.827	8-10	RD52	0.803	7-10
RSh415	0.855	8-11	RD259	0.842	8-10
RSh350	0.867	8-11	RD346	0.844	8-10
RSh55	0.917	9-11	RD24	0.862	8-11
RSh22.	0.941	9-12	RD268	0.877	8-11
RSh261	0.969	9-12	RD277	0.884	8-11
RSh272	1.011	9-12	RD257	0.896	8-11
RSh499	1.038	10-13	RD215	0.946	9-12
RSh347	1.138	11-14	RD70	1.147	11-14
RSh30	1.229	11-15	RD472	1.286	12-16
Average	**0.794 ± 0.209**	**-**	**Average**	**0.656 ± 0.263**	**-**

Discussion

Abundant and Low-level* Symbiodinium *in Adults and Planulae

This study determined the presence of previously unreported low-level *Symbiodinium* in *Stylophora pistillata* colonies in the Gulf of Eilat. Most likely, though, all individuals in a coral species do not host an ever-present resident population of low-level symbionts. As demonstrated here, even on the same reef, low-level *Symbiodinium* were not present in all *S. pistillata* individuals sampled. Further, the presence of low-levels symbionts may have varied over time, but since I did not repeatedly sample the same colonies, I cannot ascertain whether low-levels of clade A are hosted permanently or transiently in some deep-water colonies. Hosting transient symbionts at abundant and low-levels, even those not known to associate with a given host, has been documented in temporal studies in association with bleaching (LaJeunesse *et al.* 2009b; Coffroth *et al.* 2010). However, since no bleaching events were reported in the Gulf of Eilat during this three-year study, there is no evidence to support that the low-level *Symbiodinium* detected in some deep-water *S. pistillata* were acquired due to a stress event.

The role of low-level *Symbiodinium* in a host is still an unanswered question that has predominantly been discussed in conjunction with bleaching events. Resident populations of low-level *Symbiodinium* may increase in abundance following the expulsion of the dominant symbiont during a bleaching event (Berkelmans & van Oppen 2006; Mieog *et al.* 2007). Low-level symbionts may therefore provide functional redundancy by supplying the host with photosynthate during recovery, potentially preventing host mortality, until the previously abundant symbiont returns to dominance within the host (Silverstein *et al.* 2012). Conversely, opportunistic symbionts may increase in abundance during bleaching because they are able to

exploit the weakened health state of the holobiont (host and native symbiont; LaJeunesse *et al.* 2009b).

In the case of *S. pistillata* in the Gulf of Eilat, it seems unlikely that hosting low-level *Symbiodinium* acts as a buffer for occasional thermal stress. First, bleaching events are relatively rare in the Red Sea (Cantin *et al.* 2010). Second, no low-level *Symbiodinium* were detected in any of the shallow water adult colonies sampled, even though thermal stress most strongly affects shallow water habitats (Glynn 1996). Conversely, some deep-water *S. pistillata* colonies hosted low-levels of clade A *Symbiodinium*. Clade A may be a highly infective, opportunistic symbiont (Baker 2001; Stat *et al.* 2008b), which may partially explain its presence in some deep-water colonies. It is also possible that real-time PCR detected *Symbiodinium* types that were not in symbiosis, but rather represent surface contaminants or ingested material (but see Silverstein *et al.* 2012). Lastly, the presence of clade A *Symbiodinium* in deep-water *S. pistillata* colonies may be a signature of the depth origin of these adult colonies.

All planulae processed, from both depths, hosted a single *Symbiodinium* clade with no detectable low-levels of a second clade. Even maternal colonies hosting both abundant and low-level *Symbiodinium* released planulae without low-level symbionts. Furthermore, based on the DGGE analysis, none of the planulae analyzed had a symbiont composition that differed from their parent. Therefore, planulae appear to only inherit the abundant *Symbiodinium* type, despite the presence of additional symbionts at low-levels in some adult colonies. In *S. pistillata*, *Symbiodinium* present in the maternal pharyngeal cavity are likely endocytosed by immature planulae (Benayahu & Schleyer 1998); therefore, if low-level symbionts are not proximate to egg cells, they will not be inherited by planulae. Fluorescent *in-situ* hybridization analysis may be

able to determine the spatial distribution of the low-level *Symbiodinium* in adult *S. pistillata* colonies (Loram *et al.* 2007).

Mixed Symbioses in Juvenile Colonies

Intriguingly, some juvenile *S. pistillata* colonies harbored a mixture of symbionts, in stark contrast to planulae, which only contained the abundant parental symbiont clade. Since one shallow and nine deep-water juveniles analyzed contained mixed symbioses, while none of the planulae from either depth hosted multiple symbionts, juveniles appear to be capable of horizontally acquiring *Symbiodinium* from the environment. Furthermore, given the dominance of clade C in deep-water adult colonies, it was unexpected that 11 of 25 deep-water juveniles analyzed solely contained clade A *Symbiodinium*. The incongruity between the abundant *Symbiodinium* clade found in deep-water adult *S. pistillata* and the majority of juveniles is likely explained by events occurring during the juvenile phase.

If planulae released from shallow water colonies, with their clade A *Symbiodinium* complement, settle and metamorphose in deep-water, the resulting juveniles will initially contain clade A *Symbiodinium* (Figure 11). Two possible, not mutually exclusive, scenarios may then follow, both leading to the observed *S. pistillata* symbiosis with abundant clade C *Symbiodinium* in deep-water. First, juveniles that continue to maintain only clade A *Symbiodinium* may not survive to adulthood (Figure 11). Alternatively, juveniles may be able to acquire symbionts from the environment. In *S. pistillata* the number of *Symbiodinium* is reduced two-fold in settled, as compared to swimming, planulae (Titlyanov *et al.* 1998). As *S. pistillata* planulae do not feed (Alamaru *et al.* 2009), this symbiont reduction may provide the opportunity for external *Symbiodinium* acquisition following metamorphosis. If clade A containing juveniles in deep-

water horizontally acquire clade C *Symbiodinium*, clade C may outcompete clade A and become the abundant symbiont present (Figure 11). I may have witnessed a snapshot of this process in the nine juveniles that contained predominantly clade C and low-levels of clade A. Similarly, planulae released from deep-water adults may settle in shallow water, as was potentially observed in one shallow water juvenile (Figure 11).

The above scenarios require that planulae are capable of settling in a non-natal depth. While laboratory studies have shown that in some species planulae preferentially settle in their natal habitat, occasional settlement outside the depth range of the parental colony can occur, especially in species with large depth distributions (Mundy & Babcock 1998; Baird *et al.* 2003). This has been supported by a number of population studies that have shown strong philopatric settlement with occasional long distance dispersal in several species, including *S. pistillata* in the Indo-Pacific (Nishikawa *et al.* 2003; Underwood *et al.* 2009). Preliminary analysis of the population structure of *S. pistillata* along a depth gradient in the Gulf of Eilat indicates that no population subdivision exists between colonies present in the 2-30m depth range (Daniel 2006). Horizontally acquiring *Symbiodinium* may allow juvenile *S. pistillata* colonies to survive at any depth, leading to a lack of coral population subdivision as a function of depth. Conversely, if population subdivision between depths occurred, it would imply post-settlement mortality of coral juveniles that settle outside of their natal depth. Therefore, a thorough population genetic analysis will determine the degree of coral host gene flow between depths, supporting either the hypothesis of horizontal symbiont acquisition in *S. pistillata* juveniles or the hypothesis of differential juvenile mortality.

Further evidence of at least occasional larval dispersal across large depth gradients was reported for the vertically transmitting coral *Seriatophora hystrix* in western Australia (van

Oppen *et al.* 2011). Similar to *S. pistillata*, *S. hystrix* displays symbiont zonation with depth, hosting *Symbiodinium* clade D in shallow and C in deep-water (Cooper *et al.* 2010; van Oppen *et al.* 2011). Population genetic analysis of the corals revealed that some shallow water adult *S. hystrix* colonies originated from the deep-water coral population. Interestingly, despite being of deep-water origin, these adult colonies possessed the shallow water symbiont (van Oppen *et al.* 2011). One potential explanation offered by the authors was that non-natal symbionts were horizontally acquired upon settlement, but as larval and juvenile colonies were not analyzed, their hypothesis remained untested. Therefore, the present study is the first to provide support for horizontal acquisition in conjunction with vertical symbiont inheritance in juvenile scleractinian corals.

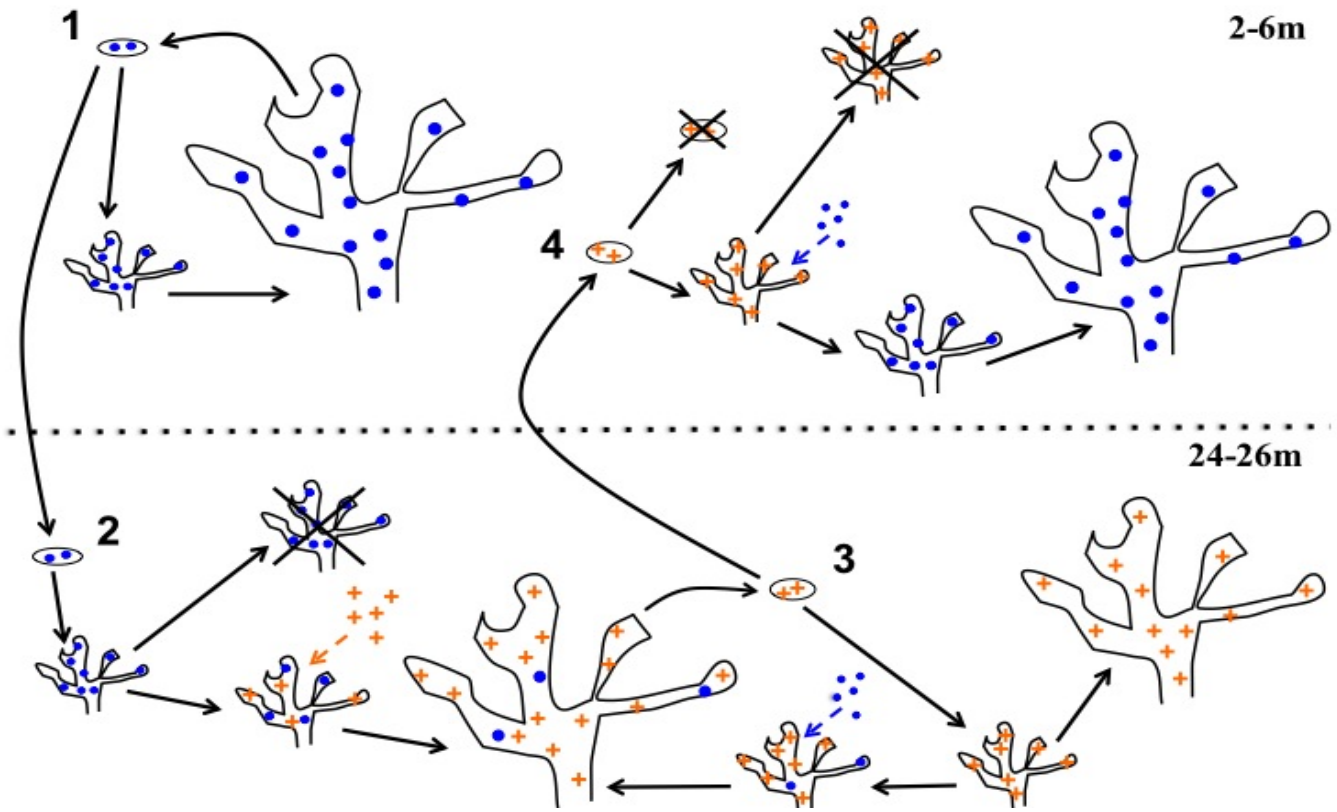

Figure 11: Schematic depicting symbiont inheritance and acquisition throughout *S. pistillata* ontogenesis. (1) Shallow water adults and planulae only host clade A *Symbiodinium* (blue circle). Planulae that settle in shallow water will grow to become adults hosting clade A. (2) Some planulae released from shallow water adults may settle in deep-water, as evidenced by juveniles with clade A found in deep-water. These juveniles may then horizontally acquire and maintain clade C *Symbiodinium* (orange plus sign) into adulthood. Conversely, juveniles in deep-water hosting only clade A may perish (black x). (3) All deep-water adult colonies abundantly hosted clade C *Symbiodinium* (with some hosting low-levels of clade A). As all planulae from deep-water adults inherit only clade C, upon settlement in deep-water, juveniles will either maintain clade C or horizontally acquire clade A *Symbiodinium* at low-levels. (4) Planulae released from deep-water adults may settle in shallow water. Indeed, one juvenile collected from shallow water contained clade A in addition to low-levels of clade C. Juveniles that only host clade C in shallow water may perish, but the horizontal acquisition of clade A symbionts may facilitate survival to adulthood.

***Differential Presence of Low-level* Symbiodinium**

Shallow and deep-water colonies differed in their abilities to host both *Symbiodinium* clades. While clade C *Symbiodinium* was only detected in one shallow water juvenile *S. pistillata* colony, clade A occurred in several deep-water adult and juvenile colonies. As clade C *Symbiodinium* routinely thrive at shallow depths around the world, including the Red Sea (Baker 2004; Barneah *et al.* 2004; LaJeunesse 2005), the symbiont depth zonation observed in *S. pistillata* in the Gulf of Eilat may be due to symbiont niche partitioning (Rowan & Knowlton 1995). Winters *et al.* (2009) reported differences in chlorophyll content and cell size between clade A and C *Symbiodinium* hosted by shallow and deep-water *S. pistillata* colonies, respectively. These physiological differences may explain why clade A is capable of surviving at both depths while clade C appears mostly limited to deeper waters. Clade A *Symbiodinium* hosted by shallow water *S. pistillata* are capable of altering chlorophyll density in response to seasonal changes in irradiance (Winters *et al.* 2009). Therefore, clade A symbionts hosted by deep-water colonies may be able to increase chlorophyll density in order to survive in deeper, low irradiance waters.

Clades A and C *Symbiodinium* also display differential responses to both elevated temperature and irradiance (Reynolds *et al.* 2008; Winters *et al.* 2009). When compared to clade A *Symbiodinium* cultures, clade C cultures are more sensitive to high irradiance and elevated temperature (Reynolds *et al.* 2008). In *S. pistillata* specifically, colonies hosting clade C were less resilient to thermal stress than colonies hosting clade A (Winters *et al.* 2009). Taken together, these studies suggest that not only is clade A *Symbiodinium* flexible enough to survive at both depths in *S. pistillata*, but that photoadaption to high irradiance and temperature may make clade A a superior competitor in shallow water. Alternatively, shallow and deep-water *S.*

pistillata colonies in the Gulf of Eilat may differ in their abilities to concurrently harbor multiple symbionts because they belong to two distinct coral populations or even coral morphospecies (Stefani *et al.* 2011).

Mixed Modes of Symbiont Transmission

Vertical symbiont transmission leads to highly stable, specific symbioses (Wilkinson & Sherratt 2001; Leigh *et al.* 2010; Sachs *et al.* 2011). Due to this fidelity, co-speciation often occurs, creating a strong congruence between host and symbiont phylogenies, as has been demonstrated in the *Buchnera*/aphid symbiosis for example (reviewed by Moran *et al.* 2008). In contrast, symbioses with horizontal acquisition typically show no signature of phylogenetic concordance or co-speciation (Bright & Bulgheresi 2010). Phylogenetic incongruence in vertically transmitted symbioses suggests that host switching or cheating occurs at least occasionally via horizontal symbiont acquisition (reviewed by Bright & Bulgheresi 2010). While these studies demonstrate predominant vertical and occasional horizontal transmission of prokaryotic symbionts, the frequency and life stage of acquisition could not be inferred. Here I demonstrate that in a coral species with vertically transmitted symbionts, horizontal acquisition of *Symbiodinium* may occur in the juvenile stage.

Several studies have shown a lack of congruence between *Symbiodinium* and host phylogenies (Rowan & Powers 1991; LaJeunesse *et al.* 2005). Distantly related hosts are known to harbor very closely related *Symbiodinium* types, leading some to conclude that the evolution of coral-algal symbioses are driven by frequent host switching events (Rowan & Powers 1991). Given that the majority of coral species utilize horizontal symbiont acquisition, phylogenetic incongruence is not unexpected. Additionally, many corals with vertical transmission host

specialized *Symbiodinium* types endemic to a particular coral species, unlike many horizontal transmitters that associate with cosmopolitan, host-generalist *Symbiodinium* types (LaJeunesse *et al.* 2004; LaJeunesse 2005; Thornhill *et al.* 2006a; Stat *et al.* 2008a). On the other hand, no phylogenetic congruence between host and symbiont was detected in an analysis of *Montipora spp.*, all of which maternally transmit *Symbiodinium*, suggesting that occasional host switching may occur even in species with vertical transmission (van Oppen 2004). Evidence of infrequent horizontal transmission was also documented in *Pocillopora spp.*, which exhibits maternal symbiont inheritance. Pettay *et al.* (2011) used microsatellite markers to investigate the genotypic diversity of *Symbiodinium glynni* (type D1) clones present within individual *Pocilloporid* colonies. The observed incongruence between host and *S. glynni* genotypes implies that switching occurs with some frequency (Pettay *et al.* 2011).

In laboratory studies of cnidarian hosts, horizontal acquisition in addition to vertical *Symbiodinium* transmission has been documented in both a scyphozoan (Montgomery & Kremer 1995) and an octocoral species (Zurel *et al.* 2008). The scyphozoan, *Linuche unguiculata* displays a very different life history as compared to scleractinian corals. Mature medusae release aposymbiotic eggs that are coated in mucus strands that contain maternally derived *Symbiodinium* (Montgomery & Kremer 1995). When developing embryos were exposed to fluorescently labeled *Symbiodinium* (of the same *Symbiodinium* type hosted by the maternal colony), embryos predominantly acquired the labeled *Symbiodinium* horizontally, but also acquired some symbionts from the maternal mucosal lining (Montgomery & Kremer 1995). Therefore, *L. unguiculata* embryos acquire *Symbiodinium* from the surrounding environment, which also contains maternally derived symbionts. This system differs from that observed in *S.*

pistillata planulae, which are released with internal maternal *Symbiodinium*. *S. pistillata* planulae appear to be capable of horizontal acquisition only after settlement and metamorphosis.

In a separate study, Zurel *et al.* (2008) documented horizontal acquisition of heterologous *Symbiodinium* in the vertically transmitting soft coral, *Litophyton crosslandi*. Young colonies were challenged with a fluorescently labeled *Symbiodinium* type not typically hosted by *L. crosslandi* colonies, and some fluorescently labeled *Symbiodinium* were acquired (Zurel *et al.* 2008). Many cnidarian hosts, including some scleractinian corals, can acquire herterologous *Symbiodinium* types in laboratory infection experiments, but acquisition is often transient (Rodriguez-Lanetty *et al.* 2004; Coffroth *et al.* 2010). Furthermore, in many studies the *Symbiodinium* types acquired in the laboratory have never been documented in natural populations (Thornhill *et al.* 2006c and references within). Therefore, laboratory experiments that challenge hosts with diverse *Symbiodinium* may not correlate to patterns of symbiont specificity in natural populations.

Vertical *Symbiodinium* transmission, coupled with occasional horizontal acquisition, has been proposed in various coral species (van Oppen *et al.* 2004; Stat *et al.* 2008a; Pettay *et al.* 2011; van Oppen *et al.* 2011), but has remained an untested hypothesis in scleractinian corals. In addition, while mixed symbiont transmission modes have been documented in a scyphozoan and octocoral, it has not been documented in a natural population. The *S. pistillata* juvenile colonies analyzed here therefore represent the first known documentation of vertical and horizontal *Symbiodinium* acquisition in a natural population of a scleractinian coral.

Vertical *Symbiodinium* transmission is generally associated with coral species that brood planulae, which only compose approximately 15% of coral species, implying that vertical *Symbiodinium* transmission may be relatively rare. This assumption, however, is not precise, as

approximately one quarter of all broadcast spawning coral species vertically transmit their *Symbiodinium* (Baird *et al.* 2009). Taken together, approximately 35% of coral species vertically transmit *Symbiodinium*. Furthermore, several widely distributed coral genera that exclusively vertically transmit *Symbiodinium*, such as *Porites*, *Montipora*, and *Pocillopora*, are among the most dominant genera on many reefs (Baker *et al.* 2008; Baird *et al.* 2009). Consequently, as observed in *S. pistillata*, numerous ecologically dominant coral species with vertical transmission may also be capable of horizontal acquisition in the juvenile phase.

Although the symbiont transmission strategy is unknown in a large number of coral species, in the majority of cases, all of the species within a given genus either vertically or horizontally transmit *Symbiodinium*. However, at least 7 coral genera feature both transmission strategies, as some species in the genera exhibit vertical transmission while others feature horizontal *Symbiodinium* transmission (Table 5). Additionally, three coral species display both vertical and horizontal transmission within the same species, as individual adult colonies can brood symbiotic larvae and spawn aposymbiotic gametes (Table 5; Sakai 1997; Lam 2000; Nozawa & Harrison 2005; Baird *et al.* 2009). It is unknown whether brooded symbiotic larvae can also horizontally acquire symbionts in these three species.

While some coral species are capable of releasing some progeny with, and other progeny without *Symbiodinium*, to my knowledge, this is the first report of the same individual progeny being capable of both symbiont transmission modes in scleractinian corals. Therefore, while other coral species may use both modes to increase their reproductive fitness, individual progeny are still limited to symbiont acquisition by a single mode. *S. pistillata* on the other hand, releases planulae with vertically inherited symbionts that are capable of subsequent environmental symbiont acquisition post-settlement, presenting a novel mechanism for symbiont acquisition.

Table 5. Coral genera in which symbiont transmission (vertical and horizontal) modes vary among species. The number of species in each genera that exclusively vertically or horizontally transmit *Symbiodinium*, or those species that are capable of doing both in the same colony (but has not been demonstrated in the same progeny). The total number of species in each genus illustrates that more data are needed to characterize the remaining species in most of these genera. Table is based on data compiled by Baird *et al.* 2009 (supplementary material).

Genera	Vertical Transmission	Horizontal Transmission	Both	Total No. of Species/Genera
Galaxea	1	1	-	6
Siderastrea	2	1	-	5
Cyphastrea	1	3	-	8
Goniastrea	-	4	1	12
Heliofungia	-	-	1	1
Oulastrea	-	-	1	1
Madracis	3	1	-	15

The Perpetuation of Novel Symbionts

Environmental acquisition of new symbionts may be a means by which corals can acclimate and/or adapt to environmental changes (Buddemeier *et al.* 2004), but symbiont acquisition in adult corals may either not occur or may be transient (Goulet & Coffroth 2003; Goulet 2006; Thornhill *et al.* 2006b; LaJeunesse *et al.* 2009; Coffroth *et al.* 2010). In coral species with horizontal symbiont acquisition, the juvenile stage appears to be more flexible in acquiring symbionts not present in the adult population (Coffroth *et al.* 2001; Weis *et al.* 2001; Little *et al.* 2004; Rodriguez-Lanetty *et al.* 2006; Gomez-Cabrera *et al.* 2008; Abrego *et al.* 2009a,b). However, even if a juvenile colony were able to horizontally acquire and maintain a novel *Symbiodinium* type into adulthood, these novel symbionts would not be transmitted to the next generation, since in corals with horizontal symbiont transmission, progeny must acquire symbionts anew. Horizontal transmission may provide an opportunity for symbiont switching, and enable short-term acclimation on an individual level, but it does not provide a mechanism for the perpetuation of novel *Symbiodinium* across generations.

In contrast, if juvenile corals with vertically transmitted symbionts are capable of acquiring novel, advantageous *Symbiodinium* that are maintained into adulthood, the subsequent vertical symbiont inheritance to their progeny would facilitate the maintenance of novel *Symbiodinium* over generations. If progeny with the advantageous novel symbiont have a higher survival rate and/or fecundity, the novel, advantageous symbiosis will be perpetuated through natural selection. Consequently, unlike horizontal symbiont transmission, vertical symbiont transmission provides an evolutionary mechanism for rapid adaptation to environmental change.

Conclusions

S. pistillata may benefit from both modes of symbiont transmission. On the one hand, the progeny are equipped with *Symbiodinium*, eliminating the risk of not obtaining their obligate symbionts. On the other hand, under certain conditions, juveniles may acquire symbionts from the environment, potentially increasing their chances of survival in a new environment. Employing both horizontal and vertical symbiont acquisition modes provides a mechanism for the acquisition and maintenance of novel *Symbiodinium*, which may prove adaptive during changing environmental conditions, such as those occurring due to global climate change.

II. RECIPROCAL DEPTH TRANSPLANTATION OF *STYLOPHORA PISTILLATA* COLONIES IN EARLY ONTOGENY

Introduction

Reef building (scleractinian) corals rely on the photosynthate provided by obligate algal symbionts (*Symbiodinium*) for growth and calcification (Muscatine 1990; Allemand *et al.* 2011). Consequently, scleractinian corals are restricted to the photic zone of tropical and subtropical waters, where light levels are sufficient to sustain photosynthesis (Bongaerts *et al.* 2010a). Some coral species inhabit broad depth distributions, ranging from 1m to over 30m in depth. In contrast, the majority of coral species are distributed within narrow depth ranges due to differential physiological tolerances to abiotic factors such as irradiance or temperature (Mundy & Babcock 1998; Bongaerts *et al.* 2010a; Baird *et al.* 2003 and references therein). As scleractinian corals host *Symbiodinium*, this niche partitioning may occur at the host, symbiont, or holobiont (host and symbiont) level. Indeed, differences in host morphology (Rowan & Knowlton 1995; Bruno & Edmunds 1997; Smith *et al.* 2007; Einbinder *et al.* 2009) and *Symbiodinium* fitness (Iglesias-Prieto *et al.* 2004; Warner *et al.* 2006; Reynolds *et al.* 2008; Winters *et al.* 2009) have been recorded as a function of differences in depth or irradiance.

Many species with wide depth distributions host distinct *Symbiodinium* as a function of depth. For instance, Bongaerts *et al.* (2010a) determined that of the ten species with broad depth

distributions and known *Symbiodinium* composition, nine exhibit clade-level depth zonation. In western Australia, shifts from clade D to clade C dominated colonies have been observed in *Seriatophora hystrix* as a function of depth (Cooper *et al.* 2010) and in the Gulf of Eilat, Red Sea, *Stylophora pistillata* associates with clade A in shallow water but clade C in deeper water (Daniel 2006; Winters *et al.* 2009). Shifts in *Symbiodinium* with depth have also been reported at the subcladal, or type, level. In the Great Barrier Reef, *Stylophora pistillata* and *Pocillopora damicornis* both associate with different clade C subtypes as a function of depth (Sampayo *et al.* 2007). Similarly, in the Caribbean, *Madracis pharensis* associates with type B7 exclusively between 5-10m, but at depths greater than 25m, also harbors type B15 *Symbiodinium* (Frade *et al.* 2008).

Given the pattern of symbiont zonation in species with broad depth distributions, several studies have attempted to determine if *Symbiodinium* composition changes in response to reciprocal transplant experiments. Most studies did not demonstrate changes in *Symbiodinium* even after depth transplantations (Toller *et al.* 2001; Goulet & Coffroth 2003; Iglesias-Prieto *et al.* 2004; LaJeunesse *et al.* 2004). Changes in the abundant *Symbiodinium* clade present was observed following transplantation in five Caribbean coral species, all of which have broad depth distributions and host specific symbionts with depth (Baker 2001). Following transplantation from deep (20-23m) to shallow (2-4m) water, all five species hosted the *Symbiodinium* clade characteristic of shallow water colonies of each species. Interestingly, Baker (2001) did not document any *Symbiodinium* changes over 12 months in colonies of the same species that were transplanted from shallow to deep depth. Similarly, in reciprocal depth transplantation of *Stylophora pistillata* in the Gulf of Eilat, shallow water (5m) coral fragments transplanted to 50m showed no change in the dominant *Symbiodinium* clade present after 6 months, but when deep-

water fragments were transplanted to shallow water, some colonies transitioned from one clade C type to another, but in most cases, the change was transient and did not persist (Daniel 2006).

Most reciprocal transplant experiments have focused on adult corals, and within these studies, few have shown changes in *Symbiodinium* after either transplantation to geographically distinct reefs or following transplantation to different depths (change: Baker 2001; Daniel 2006; Berkelmans & van Oppen 2006; no change: Toller *et al.* 2001; Goulet & Coffroth 2003; Iglesias-Prieto *et al.* 2004; LaJeunesse *et al.* 2004; Smith *et al.* 2007). This is not necessarily surprising given the strict fidelity most adult coral hosts have to a single *Symbiodinium* type (Goulet 2006). However, juvenile corals are known to host *Symbiodinium* with less specificity than adults (present study- Chapter 1; Coffroth *et al.* 2001; Weis *et al.* 2001; Little *et al.* 2004; Rodriguez-Lanetty *et al.* 2006; Gomez-Cabrera *et al.* 2008; Abrego *et al.* 2009a,b). If corals are able to acquire unique *Symbiodinium* from the environment, it is more likely that this will occur at the juvenile phase than the adult phase. Few studies have attempted reciprocal transplants of juvenile corals and none, to my knowledge, have considered both *Symbiodinium* composition and reciprocal depth transplantation in the same experiment. Mundy and Babcock (2000) conducted reciprocal depth transplants (between 0, 5, and 10m) of 10 day-old juvenile corals from three different species, one species with a shallow water distribution, one species restricted to deep-water only, and one species with a broad depth distribution. Surprisingly, 12 months after transplantation, juveniles from all three species survived equally well over all three depths (Mundy & Babcock 2000). The authors explained the lack of post-settlement morality in habitats outside the parental distribution as evidence for selective settlement behavior in larvae (planulae).

For most species, it is not clear whether adult distributions are dictated by differential settlement behavior in planulae or differential post-settlement survival. Studies have demonstrated that coral planulae are not simply at the whim of ocean currents, but rather exhibit various preferential settlement behaviors in response to varying substrates, light intensity, and light spectrum (Mundy & Babcock 1998; Baird *et al.* 2003; Harrington *et al.* 2004; Golbuu & Richmond 2007; Vermeij *et al.* 2009). Mundy and Babcock (2000) did not consider the *Symbiodinium* type present in the transplanted colonies. It is possible that juveniles acquired *Symbiodinium* from the environment that allowed at least short-term survival in the non-parental habitat. If this is the case, the possibility of differential mortality at older juvenile stages cannot be eliminated. Abrego *et al.* (2009a) did consider changes in *Symbiodinium* but did not transplant colonies to different depths. Eight-day old juveniles representing two different *Acropora* species were reciprocally transplanted between geographically separate reefs. When transplanted outside of their parental habitat, most juveniles, from both species, environmentally acquired *Symbiodinium* types and/or clades not present in parental colonies (Abrego *et al.* 2009a). Therefore, in order to fully understand the depth distributions of coral species, it is important to consider the holobiont with focus towards the host juvenile phase. Neither Mundy and Babcock (2000) nor Abrego *et al.* (2009a) included in their analyses coral species that vertically transmit their *Symbiodinium* from parent to offspring.

In coral species with vertical transmission, the *Symbiodinium* inherited by planulae may influence larval settlement behavior. Additionally, if juvenile corals with vertically transmitted symbionts are restricted to the inherited symbiont type, post-settlement mortality in non-parental habitats may occur. Here, I report evidence of environmental acquisition of non-parental *Symbiodinium* post-settlement in *Stylophora pistillata*, a coral species with vertical symbiont

transmission (Chapter 1). Consequently, the acquisition of non-parental symbionts may support post-settlement survival at any depth, allowing species to inhabit wide bathymetric distributions. *S. pistillata* in the Gulf of Eilat, Red Sea, has a wide depth distribution and clade level *Symbiodinium* zonation. Adult colonies present from 2-17m exclusively host clade A *Symbiodinium*, while colonies inhabiting depths of 20-30m dominantly host clade C *Symbiodinium* (Chapter 1; Daniel 2006; Winters *et al.* 2009).

Adult *S. pistillata* in the Gulf of Eilat survive reciprocal transplant experiments, although mortality rates are often high (Falkowski & Dubinsky 1981; Daniel 2006). However, no studies of this population have investigated the survivability of young recruits when settled in an environment that differs from their maternal colony. Therefore, several manipulative studies were attempted to establish how host/symbiont combinations affect post-settlement survival over depth. Reciprocal depth transplant experiments were run in order to gauge survival as well as any changes in symbiont associations. Using various experimental designs, I was interested in addressing the null hypothesis that survival will not be affected by placing settled recruits in a depth that differs from their maternal colony. I additionally addressed the null hypothesis that the *Symbiodinium* types hosted by newly settled recruits will not change following reciprocal depth transplantation. While I was unable to adequately address these hypotheses, here I will explain each attempted experiment, outline why they were ineffective, and describe what can be done in future studies to investigate the proposed hypothesis with success.

Methods

Transplantation of Recruits Reared from Field-collected Planulae

All experiments were performed at the Interuniversity Institute for Marine Sciences in Eilat (IUI), Eilat, Israel, Red Sea in May through July of 2010. Planulae were collected from reproductively active adult *S. pistillata* colonies with planula collection nets as described previously (Chapter 1-Methods). In order to identify colonies that consistently released planulae each night, 67 different shallow water (2-8m) adult colonies were sampled. In an effort to repeatedly sample only the most fecund colonies, if, after one night of collection, a colony released less than seven planulae, that colony was no longer used. Ten shallow water adult colonies were identified that consistently released planulae. In total, 970 planulae were collected from shallow water adults over 10 days of collection. SCUBA diving restrictions prevented the extensive sampling of deep-water colonies (24-26m), but planulae were collected from deep-water colonies over two nights, collecting 119 planulae in total from 12 adult colonies.

Following collection, each net was emptied into a bucket and planulae were individually collected with a transfer pipette. The isolated planulae were placed in petri dishes, which had lids with several small holes to allow for water circulation. Individual petri dishes contained planulae collected over one night from a single adult, and housed no more than 24 planulae per petri dish. Each petri dish also contained a piece of pre-conditioned settlement paper (Figure 12A,B). Settlement paper (underwater paper) was pre-conditioned in an indoor flow through seawater table (no coral was in the tank), supplied with unfiltered seawater pumped directly from the Gulf of Eilat. Pre-conditioning is important because it allows the paper to develop a bacterial biofilm that induces planula settlement (Vermeij *et al.* 2009).

To determine if settlement success rate was affected by the substrate provided, a subset of shallow water planulae were exposed to alternative pre-conditioned settlement surfaces. Each petri dish had at least one or a combination of all three different settlement surfaces: 1) small pieces of underwater paper that had been pre-conditioned in the ocean for approximately three weeks (the paper had substantial amounts of macroalgal growth), 2) small pieces of plastic baskets that were preconditioned for at least three weeks in a flow through seawater table that contained multiple adult corals, and 3) pebbles collected from the sea floor of the shallow reef. In total, 230 shallow water planulae were exposed to various alternative per-conditioned surfaces that were added to the petri dishes. All of the petri dishes, regardless of settlement surface, were then placed in an outdoor flow through seawater table with shading nets that provided similar irradiance levels to those of the planulae's maternal colonies (Figure 12C,D).

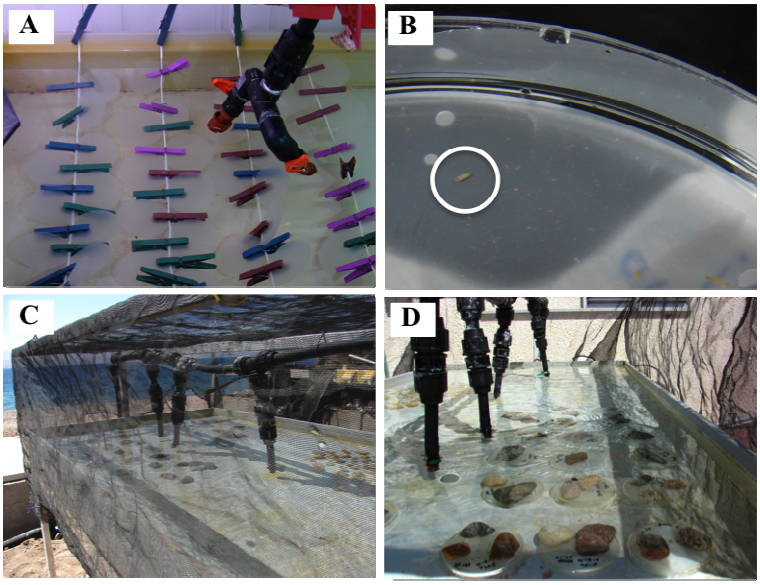

Figure 12. Experimental setup to rear field-collected planulae in a flow through seawater table. (A) Underwater paper that was used as a settlement surface is held in place with clothespins and preconditioned in an indoor flow through seawater table. (B) Each petri dish was lined with settlement paper. A free-swimming planula that has not yet settled is circled. (C) Flowing seawater table with shading nets to control irradiance levels. (D) Petri dishes containing planulae, weighed down with rocks, in the flow through seawater table. Each petri dish contained planulae from a single adult colony.

After six to 18 days in the petri dishes, all successfully settled, healthy recruits were prepared for transplantation. I use the term recruit to refer to newly settled colonies, approximately 3-5mm in diameter with minimal three-dimensional structure, to avoid confusion with older and larger juvenile colonies that featured three-dimensional structure. Each settled recruit was gently removed from its settlement surface and, using commercial super glue, was glued to an individual glass microscope slide and photographed. Recruits were out of seawater for approximately one minute during processing. All microscope slides were placed in a shaded flow through seawater table for several days to allow the recruits to recover from processing and handling prior to transplantation. The slides were then placed in Plexiglas microscope slide holders that were secured to one of two large crates placed at 7m and 30m on the reef (Figure 13).

Forty-six recruits reared from planulae released by shallow water adults were successfully transplanted to the reef. Due to the reduced sampling effort applied to deep-water planula collection, only five recruits reared from deep-water were transplanted. Half (23) of the shallow water recruits were transplanted to 30m while the remaining half were placed at 7m as a control (Figure 13). All five of the recruits reared from deep-water planulae were placed at 7m. While the intention was to collect seven to eight recruits reared from shallow water planulae from each depth once a week for three weeks, high mortality rates only allowed six shallow water recruits from each depth to be collected two weeks following transplantation. One deep-water recruit was collected after two weeks at 7m. Once collected, recruits were photographed to record growth before they were preserved in 100% ethanol.

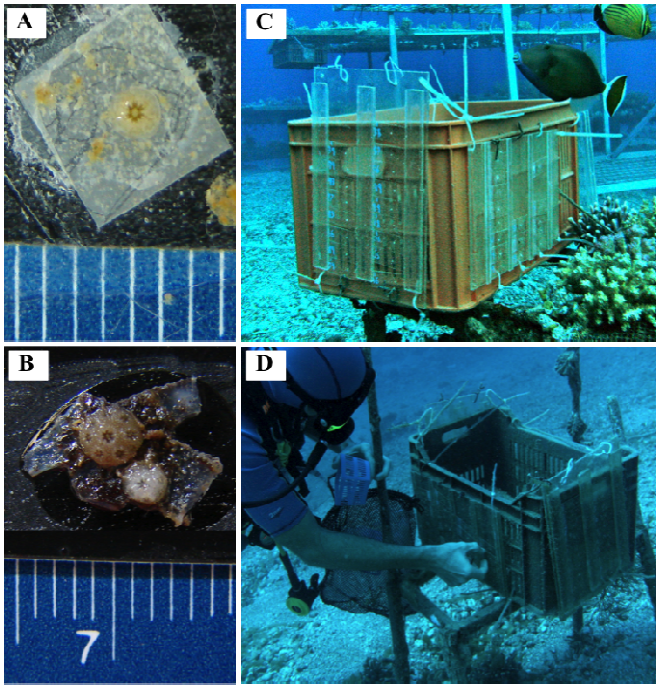

Figure 13. Experimental setup of the reciprocal transplant using newly settled recruits several weeks of age. Settled recruits were glued to individual microscope slides and transplanted onto the reef. (A) A newly settled, healthy recruit that metamorphosed from a shallow water planula and settled on the preconditioned settlement paper. (B) Two newly settled recruits collected from a flow through seawater table containing reproductively active *S. pistillata* colonies. The larger recruit on the left is healthy, with a complement of *Symbiodinium*, as indicated by the brown pigmentation. The recruit on the right lacks *Symbiodinium*, as indicated by the white coloration. These recruits settled on a plastic basket conditioned in a flow through seawater table that contained live coral. Microscope slides were placed in Plexiglas holders and tied to crates. One crate was placed at 7m (C) and the other at 30m (D).

Transplantation of Flow Through Seawater Table Recruits

Due to the low sample sizes obtained from rearing recruits from field-collected planulae, I attempted to transplant settled recruits collected from experimental flow through seawater tables. There were several seawater tables at IUI that were used for various unrelated studies and contained gravid adult *S. pistillata* colonies, as well as other scleractinian coral species. These adult *S. pistillata* colonies were initially collected from the coral nursery on the IUI reef (10m). The colonies released planulae that successfully settled on various plastic surfaces present in the flow through seawater table.

A total of 206 settled recruits (3-5mm; hence forth referred to as watertable recruits) were removed from the various plastic surfaces, glued to microscope slides, and photographed as described above (Figure 13C). Twenty-three watertable recruits were preserved in 100% ethanol to determine the *Symbiodinium* present at time zero of the transplantation experiment. Additionally, a 2cm branch piece was collected from all adult colonies (irrespective of species) present in the watertable in order to establish which *Symbiodinium* types were present in the seawater table and more specifically, which symbionts the adult *S. pistillata* colonies hosted. One hundred three of the watertable recruits were transplanted to each of the crates secured at 7m and 30m (these watertable recruits were placed on the same crates used to hold the recruits that we reared from planulae in the experiment described above; Figure 13). Approximately five to 11 recruits were subsequently collected from each depth four, 10, 24, and 30 days after transplantation. An additional eight colonies were collected from the 30m-transplant depth one year after transplantation. Those watertable recruits that survived one year were preserved in 100% ethanol prior to DNA extraction and analyzed with DGGE and real-time PCR described previously (Chapter 1- Methods).

Transplantation of Juvenile Colonies

To monitor the effects of transplantation on older colonies, juvenile *S. pistillata* colonies, ranging in size from approximately 5-30.5mm diameter were collected from three different sources: the shallow (2-6m; n=50) and deep reef (24-26m; n=42) at IUI, and from an outdoor aquarium from the local commercial underwater observatory (hence forth referred to as observatory juveniles; n=28). Entire juvenile colonies were removed by hammer and chisel from both rock and artificial substrates present on the reef or from the concrete walls of the outdoor

aquarium. The juveniles were then transferred to an outdoor flow through seawater table under shading equivalent to the irradiance levels present at the depth from which they were collected. The observatory juveniles were placed under the same level of shading as the juveniles collected form the shallow water reef. All juveniles were acclimated in the seawater table for several days following collection. All colonies appeared healthy with no signs of bleaching prior to further handling.

To prepare the juveniles for transplantation, six juveniles were glued to each of 20, 30.48cm by 30.48cm ceramic tiles. To insure appropriate controls on each tile, when possible, at least one juvenile each from deep-water, shallow water, and the observatory were glued to each tile. Six juveniles were randomly selected at a time and carried into the laboratory where they were measured for height, width, and length, photographed, and glued to the ceramic tile (Figure 14A,B, and C). Additionally, a small piece of coral (approximately 4mm by 4mm) was clipped from each juvenile colony and preserved in 100% ethanol in order to establish which symbiont(s) were present at time zero of the experiment. The juveniles were out of seawater for approximately one-two minutes during processing. The completed tile was carried back to the outdoor flow through seawater table and placed under irradiance levels equivalent to a 30m depth in order to avoid exposing the deep juveniles on the tile to high irradiance.

Ten tiles, with 60 juveniles, were transplanted to each depth of either 7m or 30m. In total, 23 shallow, 22 deep, and 15 observatory juveniles were transplanted to 7m, while 27 shallow, 20 deep, and 13 observatory juveniles were transplanted to 30m. The 10 tiles that were transplanted to 30m were left in the flowing seawater table for only one day before they were transferred to the reef (Figure 14C). The remaining 10 tiles that were to be transplanted to 7m were left in the flowing seawater table under heavy shading. Over a three-day period, the

shading was slowly decreased in order to acclimate the juveniles to the irradiance level they would experience at 7m. After three days of acclimation, the tiles were secured to a platform on the reef set at 7m (Figure 14D). All juveniles appeared healthy immediately prior to and following transplantation with no visible bleaching. While complete mortality prevented additional sampling, I intended to have collaborators collect small branch pieces from every juvenile colony at each depth after one, three, and six months post transplantation.

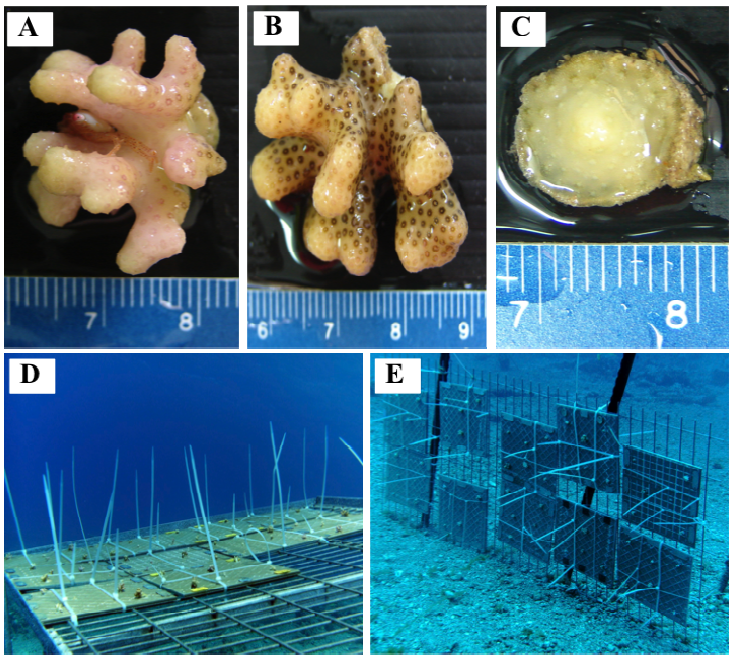

Figure 14. Experimental setup of the reciprocal transplant using juveniles several months of age. (A) Juvenile collected from the shallow reef (3-8m). B) Juvenile collected from the observatory outdoor aquarium. (C) Juvenile collected from the deep reef (20-30m). Centimeter ruler shown for scale. Ten tiles with six juvenile colonies on each were placed on a rack at 7m (D) and 30m (E).

Results

Transplantation of Recruits Reared from Field-collected Planulae

The most fecund shallow water adult colony identified (ID number 226) released 216 planulae over five nights, releasing as many as 99 planulae in a single night and averaging 43

planulae per night. This colony was exceptional however, as on average colonies released only 14.3 planulae per night (Table 6). Deep-water *S. pistillata* colonies are known to be less fecund (Rinkevich & Loya 1987) and released on average 6.3 planulae per night (Table 6).

Table 6. Settlement and survival rates of field-collected planulae reared *ex situ* in a flow through seawater table. As not all recruits that successfully settled survived, settlement rate was calculated in two ways. Settlement success rate is the percent of planulae that settled successfully independent of whether they survived or subsequently died in the flow through seawater table ([no. of alive recruits + no. of dead recruits]/total number of planulae collected). Survival rate is the percent of planulae that successfully settled and survived until transplantation onto the reef (no. of alive recruits/total number of planulae collected).

Colony ID No.	No. of Planulae Collected	Avg No. of Planulae Collected per Day	No. of Days	No. of Alive Settled Recruits	No. of Dead Settled Recruits	Settlement Success Rate (%)	Survival Rate (%)
Shallow Water Colonies							
212	91	13	7	8	2	10.99	8.79
214	228	23	10	17	15	14.04	7.46
210	54	5	3	0	0	0.00	0.00
207	54	9	6	3	1	7.41	5.56
211	129	13	10	3	2	3.88	2.33
204	52	9	6	1	0	1.92	1.92
209	37	7	5	1	2	8.11	2.70
208	55	7	8	5	2	12.73	9.09
217	54	13.5	4	11	5	29.63	20.37
226	216	43	5	23	3	12.04	10.65
Total	**970**	**14.25**	**-**	**72**	**32**	**10.72**	**7.42**
Deep-Water Colonies							
229	12	6	2	0	0	-	0.00
230	10	5	2	1	0	-	10.00
235	9	4.5	2	0	0	-	0.00
236	12	6	2	1	0	-	8.33
238	14	7	2	0	0	-	0.00
240	29	14.5	2	1	0	-	3.45
241	3	-	1	0	0	-	0.00
234	9	-	1	1	0	-	11.11
249	11	-	1	1	0	-	9.09
244	7	-	1	0	0	-	0.00
247	2	-	1	0	0	-	0.00
239	1	-	1	0	0	-	0.00
Total	**119**	**6.33**	**-**	**5**	**0**	**-**	**4.20**

While some planulae metamorphosed and settled in the petri dishes supplied, settlement success and survival rates were extremely low (Table 6). When petri dishes were checked to monitor settlement success, some planulae had successfully settled, but many had already died soon after settling. Therefore, the settlement success rate is expressed as the percent of planulae that settled successfully regardless of whether they survived in the petri dishes after settlement. Alternatively, survival rate was calculated as the number of planulae that successfully settled and survived in the flow through seawater table until transplantation to the reef. Although using settlement paper had been successful in the past (M. Fine pers. comm.), in the current experiment, the planulae avoided the settlement paper. Actively swimming planulae were observed after as many as 23 days in the petri dishes indicating that the planulae did not find a favorable substrate, which likely caused the high mortality. Only 9.32% of the planulae exposed to the settlement paper successfully settled, and only 5.56% survived post-settlement (Table 7).

The survival rate of successfully settled recruits was significantly higher when the planulae were exposed to the alternative substrates as compared to the settlement paper pre-conditioned in the empty flow through seawater table (test of two proportions, z-test, $P<0.001$). However, this statistical difference was primarily due to the large effects of colony 226 ($P<0.001$) and colony 214 ($P=0.031$; Figure 15).

Table 7. Settlement and survival rates of field-collected planulae exposed to alternative settlement substrates. Planulae collected from seven different shallow water adult colonies were presented with either settlement paper that was pre-conditioned in a flow through seawater table with no live coral or various alternative substrates pre-conditioned in the presence of live coral. Alternative substrates included: small pieces of underwater paper that had been pre-conditioned in the ocean for approximately three weeks, small pieces of plastic baskets that were preconditioned for at least three weeks in a flow through seawater table that contained multiple adult corals, and 3) pebbles collected from the sea floor of the shallow reef.

Colony ID No.	No. of Planulae Collected	No. of Alive Settled Recruits	No. of Dead Settled Recruits	Settlement Success Rate (%)	Survival Rate (%)
Settlement Paper					
214	206	12	12	11.65	5.83
207	46	3	1	8.70	6.52
211	95	2	0	2.11	2.11
204	46	1	0	2.17	2.17
208	34	4	2	17.65	11.76
217	42	7	3	23.81	16.67
226	89	2	3	5.62	2.25
Total	**558**	**31**	**21**	**9.32**	**5.56**
Alternative Substrates					
214	22	5	3	36.36	22.73
207	8	0	0	0.00	0.00
211	34	1	2	8.82	2.94
204	6	0	0	0.00	0.00
208	21	1	0	4.76	4.76
217	12	4	2	50.00	33.33
226	127	21	0	16.54	16.54
Total	**230**	**32**	**7**	**16.96**	**13.91**

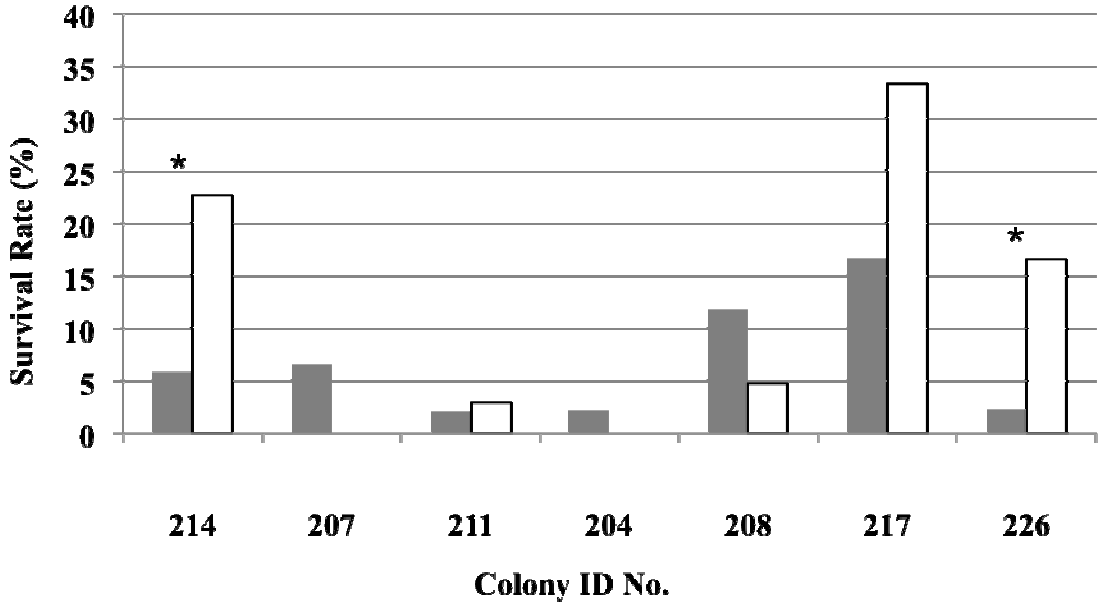

Figure 15. Percent survival of field-collected planulae exposed to alternative settlement substrates. Planulae were collected from seven different shallow water adult colonies. Gray bars represent the survival rate when planulae were presented with settlement paper that was pre-conditioned in a flow through seawater table with no live coral. White bars represent survival rate when planulae were presented with alternative substrates. Survival rate was calculated as the number of planulae that successfully settled and survived. Asterisks represent significant differences ($p<0.05$) between substrates offered.

In total, from the 970 planulae collected from 10 different shallow water adult colonies, 46 settled recruits, representing 10 unique parent colonies, were transplanted to the reef. Although Table 6 indicates that 72 recruits successfully settled and survived, I was unable to transfer all of the recruits to the microscope slides for various reasons. First, although extreme care was taken in the process of removing recruits from their substrate, several samples were crushed. Second, many planulae appeared to preferentially settle on the small pebbles that were added to petri dishes as an alternative settlement substrate, but recruits could not be removed from the pebbles without being crushed. A total of 119 planulae were collected from 12 deep-water colonies, which yielded five successfully settled recruits.

Six recruits reared from shallow water planulae were collected from the 7m-transplant depth, while only five were collected from the 30m-transplant depth after two weeks on the reef. Of the six recruits collected from the 7m-transplant depth, only three appeared healthy while the other three were at least bleached if not dead. Additionally, two of the bleached colonies actually decreased in size over the two-week period. Similarly, three of the five recruits collected from the 30m-transplant depth appeared healthy, while the other two were bleached and had decreased in size. Only one recruit that was reared from a deep-water planula was collected after two weeks at 7m and the colony was severely bleached if not dead. No additional recruits were collected as all the colonies later died.

Transplantation of Flow Through Seawater Table Recruits

Transplanted watertable recruits were sampled five times over the course of one year. At time one (four days post transplantation), of the eight recruits collected from 7m, seven appeared healthy but several recruits were less pigmented compared to time zero, indicating the recruits were likely stressed; 10 recruits were collected from 30m, three of which were severely bleached or dead. At time two (10 days post transplantation), only two of 11 recruits collected from 7m were completely healthy, five were dead, and the remaining four were extremely pale; four of 11 recruits collected from 30m appeared healthy. At time three (24 days post transplantation), most of the recruits had grown in size. However, only four of 11 recruits collected from 7m and two of nine recruits collected from 30m appeared healthy. After 32 days on the reef (time four), only five recruits were collected from 30m, of which one appeared healthy. Since the health of the recruits appeared to be declining, no more recruits were sampled. One year later, nine recruits transplanted to 30m had survived and were therefore collected.

Genomic DNA was only extracted from the nine recruits collected after one year on the reef, with extractions failing to obtain usable DNA from one sample. Therefore, eight watertable recruits were run on DGGE and real-time PCR (according to the methods described in Chapter 1). All eight sampled displayed a DGGE fingerprint characteristic of *Symbiodinium* type A1 (Figure 16). Real-time PCR did not detect any low-levels of clade C *Symbiodinium*.

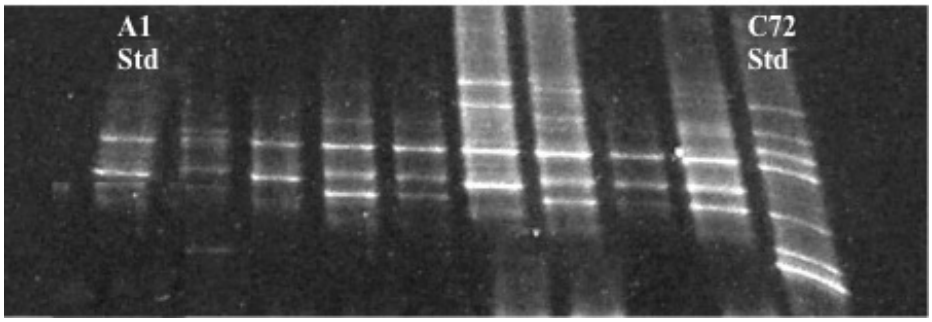

Figure 16. Denaturing-gradient gel electrophoresis image of the *Symbiodinium* hosted by watertable recruits. Nine watertable recruits were transplanted to 30m and collected after one year. Since the maternal colonies of the watertable recruits were most likely of shallow water origin, they probably contained *Symbiodinium* type A1. After one year at 30m, all eight watertable recruits analyzed displayed a DGGE fingerprint characteristic of *Symbiodinium* type A1. The profiles on the extreme left and right of the image represent DGGE standards of A1 and C72 *Symbiodinium*, respectively.

Transplantation of Juvenile Colonies

One month following transplantation, collaborators reported that all juvenile colonies at both transplant depths suffered 100% mortality.

Discussion

Transplantation of Recruits Reared from Field-collected Planulae

Low Fecundity in* Stylophora pistillata *Colonies

The success of this experiment hinged on an ability to collect large numbers of planulae from reproductively active adults. For unknown reasons, reproductive output from the shallow

water adult colonies in 2010 was extremely low. *S. pistillata* has a long reproductive season from December through July with peak planulation occurring between March and June (Rinkevich & Loya 1979b; Zakai *et al.* 2006). In the Gulf of Eilat, *S. pistillata* colonies begin to develop mature ova when they reach a geometric mean radius of approximately 4.5-5cm (Loya 1976b; Rinkevich & Loya 1979b). The smallest adult colonies sampled had geometric mean radii of approximately 6-7cm, indicating that all adult colonies sampled were of a reproductive age class and potentially capable of releasing planulae. Further, *S. pistillata* are known to release as many as 400 planulae in a single night (Loya 1976b; Rinkevich & Loya 1979b; Zakai *et al.* 2006). In 2009, an adequate number of planulae were collected from shallow water colonies in a single night in mid-July, well past the peak planulation period. It is not clear then why shallow water adult colonies in May 2010 did not release similarly large numbers of planulae. The most fecund colony identified only released 99 planulae in a single night, while most colonies released less than 10-20 planulae per night.

In the winter of 2010, very strong storms in the northern Gulf of Eilat caused a great deal of heavy wave action on the IUI reef. A large amount of physical damage was observed in many shallow water branching coral species including *S. pistillata*. It is possible that the winter storms caused physical damage that negatively affected the fecundity of the shallow water colonies in the 2010 reproductive season.

Low Settlement Success Rate

Despite difficulties in collecting an adequate number of planulae, 970 planulae were ultimately collected from 10 different shallow water adult colonies. However, an extremely low settlement success rate prevented the experiment from being executed with sufficient statistical

power to address the original hypothesis. It is most likely that an unfavorable substrate prevented most planulae from successfully settling in the petri dishes. The majority of planulae collected (740 shallow water) were exposed to settlement paper that was pre-conditioned in an indoor flow through seawater table, with no live coral present, for approximately two weeks. When exposed to this settlement surface in the petri dishes, only 8.78% successfully settled. The settlement success rate is extremely low in comparison to the 60% settlement success rate for shallow water *S. pistillata* planulae reported by Linden and Rinkevich (2011). Among other methodological differences described below, Linden and Rinkevich (2011) exposed field-collected planulae to petri dishes lined with polyester underwater paper that had been preconditioned in a flow through seawater table with live corals for at least 2 months. Linden and Rinkevich (2011) used a slightly different type of underwater paper than used here, and importantly, exposed their settlement paper to a longer preconditioning period in a flow through seawater table with live coral. The use of an unfavorable settlement surface is further supported by the significantly different settlement success rates reported in the present study when planulae were exposed to alternative substrates preconditioned in the presence of live coral. Therefore, it is clear that a long preconditioning period is necessary for the establishment of a biofilm and the growth of crustose coralline algae that are known to induce settlement (Harrington *et al.* 2004; Golbuu & Richmond 2007).

Settlement success rate in symbiotic planulae has also been linked to light exposure (Isomura & Nishihira 2001; Harii *et al.* 2010). The symbiotic planulae of *Pocillopora damicornis* became inactive, white, and irregularly shaped after 30 days of exposure to no light in contrast to active, pigmented, and uniformly shaped planulae exposed to the light treatment (Harii *et al.* 2010). This difference translated to a significant increase in settlement success rate

of *P. damicornis* planulae exposed to the light treatment (Harii *et al.* 2010). Harii *et al.* (2010) determined that under dark conditions, *P. damicornis* planulae used approximately two times more of their lipid energy reserves, indicating that the *Symbiodinium* provide a substantial amount of energy to the planulae. In the present study, *S. pistillata* planulae that survived 23 days in the petri dishes appeared pale in color and were less active compared to freshly released planulae. Therefore, it is possible that in the present study *S. pistillata* planulae were not exposed to light conditions that favored photosynthesis. With the lack of a favorable substrate, the planulae would have exhausted their energy reserves quickly if the *Symbiodinium* were unable to supplement the energy requirements of the planulae (Harii *et al.* 2010). Using nets that provide less shading may increase the settlement success rate.

Recent research conducted by Linden and Rinkevich (2011) provides a detailed method for rearing field-collected *S. pistillata* from planulae for conservation applications. Following the successful rearing of planulae to young recruits *ex situ*, 89% of recruits survived four months after transplantation to the shallow reef (Linden & Rinkevich 2011). There were several differences between the methods applied here and those developed by Linden and Rinkevich (2011). For instance, while I only placed settlement paper on the bottom of the petri dish and not on the lid, Linden and Rinkevich (2011) placed paper on both sides of the dish. To allow for water circulation I added holes to the petri dish lids, while no efforts were made to facilitate water circulation by Linden and Rinkevich (2011). Additionally, Linden and Rinkevich (2011) reported reduced settlement success if new settlement paper and water was not added to the dishes every few days; I did not change the water or settlement surfaces. Following successful settlement and metamorphosis, Linden and Rinkevich (2011) allowed recruits to grow outside of the petri dishes for one month *ex situ* prior to further handling. One-month-old recruits were

then individually transferred to plastic pegs before transplantation to an *in situ* nursery. Allowing newly settled recruits time to grow and develop prior to transferring them to a new surface is likely critical for successful transplantation to the reef. Others have successfully transplanted laboratory reared recruits of various coral species by exposing planulae to preconditioned tiles that were then transferred to the reef, with no efforts made to remove the recruits from their substrate (although high mortality rates were documented; Mundy & Babcock 2000; Abrego *et al.* 2009a). In an effort to transplant the youngest colonies possible, survivorship was likely reduced in the present study, but following the protocol outlined by Linden and Rinkevich (2011) should increase the success of future experiments.

Transplantation of Flow Through Seawater Table Recruits

The nearly complete mortality observed in transplanted watertable recruits is likely an indication of the difficulty in transplanting extremely young recruits. Attempts would have likely been more successful had recruits been given the time to grow to a larger size class before transplantation. As documented in *Pocillopora damicornis*, recruits reared in the lab and transplanted to the reef at a size of 10.1-29mm featured 47.5% survivorship after one year (Raymundo & Maypa 2004). This is in comparison to 0% and 2.5% one-year survivorship for juveniles transplanted at ≤3mm and 3.1-6mm in diameter, respectfully (Raymundo & Maypa 2004). Others have also demonstrated that juvenile survivorship increases with increased colony size (Loya 1976a,b; Glassom & Chadwick 2006; Vermeij & Sandin 2008). To prevent predation, caging may also increase survivorship following transplantation to the reef (Baria *et al.* 2010; Linden & Rinkevich 2011). Finally, Oren and Benayahu (1997) reported that *S. pistillata* juvenile survivorship increased with depth when transplanted to artificial reefs ranging

in depth from 6-15m. At the shallow depths, macroalgae more easily out-compete juvenile corals thereby decreasing survivorship (Oren & Benayahu 1997). Therefore, moving the shallow transplant depth from 7m to approximately 10m may increase survivorship.

Despite these numerous difficulties, eight watertable recruits survived one year after transplantation to 30m. Interestingly, even after one year in the deep-water environment, where clade C is the dominant *Symbiodinium* clade hosted by adults, all eight watertable recruits only contained clade A *Symbiodinium*. These results indicate that watertable recruits, presumably reared from maternal colonies of shallow water origin, are capable of surviving at least one year in the deep-water habitat with clade A *Symbiodinium*. This is in accordance with the results reported in Chapter 1. Eleven of the 25 juveniles collected from deep-water solely contained clade A *Symbiodinium*, with no detectable low-levels of clade C. Therefore, planulae released from shallow water adults may successfully settle and grow in deep-water. No low-levels of clade C *Symbiodinium* were detected in the one-year old watertable recruits placed in 30m, which may indicate one of two things. Either, the watertable recruits would have died before reaching adulthood, or they had not yet acquired clade C *Symbiodinium* from the environment. Juvenile *Acropora tenuis* colonies can associate with non-parental *Symbiodinium* for up to 3.5 years (Abrego *et al.* 2009b). Consequently, in order to detect potential changes in *Symbiodinium* composition following reciprocal transplant, juvenile colonies should be monitored for multiple years.

Transplantation of Juvenile Colonies

The main goal in conducting reciprocal transplant experiments was to monitor possible changes in survival and in the *Symbiodinium* type(s) hosted. Juvenile colonies three to 16

months old can associate with both clade A and C *Symbiodinium* simultaneously (Chapter 1). Assuming that several month-old juveniles can still acquire exogenous symbionts, in future experiments, one to two-month old juvenile colonies can be used, as these colonies should demonstrate increased survival (Linden & Rinkevich 2011; Raymundo & Maypa 2004). To ensure the identity of the maternal colony, juveniles could be reared from planulae and allowed to grow in flowing seawater tables for several months before attempting transplantation, as described by Linden and Rinkevich (2011). Beyond increased survival rate, using older individuals allows for multiple collections from the same individual over time, which provides more accurate monitoring of changes in *Symbiodinium*.

However, when older juveniles were transplanted they still suffered 100% mortality. Even control colonies, collected from shallow or deep-water and transplanted back to shallow or deep-water died after only one month on the reef. The complete mortality observed at both transplant depths is an indication that the processing and handling of the juvenile colonies was likely too stressful. Unfortunately, due to time constraints, juvenile samples were processed very quickly and likely did not have an adequate amount of time to recover from handling. Survivorship will likely increase if recruits are allowed a slow acclimation period, over the course of several weeks, to the irradiance levels they will experience when eventually transplanted. Once the appropriate irradiance level has been reached, the juveniles should be left in flow through seawater tables for several days in order to closely monitor health prior to transplantation. Finally, gluing each individual to its own ceramic tile or plastic peg, as opposed to gluing multiple colonies from various depths on the same tile, would provide the capability of varying exposure for shallow versus deep juveniles during the recovery and acclimation periods (Linden & Rinkevich 2011).

Conclusion

By employing various techniques in an effort to execute the reciprocal transplantation of *S. pistillata* colonies in early ontogeny, valuable methodological insight was gained that will benefit future experimentation. By following recently available protocols (Linden & Rinkevich 2011) settlement success and initial survival rates may improve. This, in turn, will allow sufficient sample sizes for the continued monitoring of transplanted young (several week-old) recruits on the reef. High mortality rates, however, may make this approach challenging. Transplanting older (several month-old) juveniles may still allow for the documentation of horizontal *Symbiodinium* acquisition and may also increase survivorship. Consequently, future experiments may benefit from transplanting colonies of various ages. Regardless of the initial age class used, in order to adequately address the possibility of horizontal acquisition and *Symbiodinium* switching, juvenile colonies should be monitored for several years. While the data collected in Chapter 1 strongly suggests horizontal *Symbiodinium* acquisition in a vertically transmitting species, it would be beneficial to empirically document this in *S. pistillata* juveniles. Monitoring potential *Symbiodinium* changes in juveniles of a coral species with a large depth distribution will lend insight into understanding whether symbiont zonation patterns observed in that species are mediated by larval settlement behavior or post-settlement survival.

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VITA

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EDUCATION

2004-2008	B.S. Biology Concentration in Environmental and Conservation Biology Minor in Ocean and Estuarine Science Graduated with biology honors and Magna Cum Laude George Mason University, Fairfax, VA

PROFESSIONAL EXPERIENCE

2010-2012	Graduate Research Assistant (June 2010-May 2012) The University of Mississippi, University, MS Funded by National Science Foundation (Grant DUE-0942290) to TL Goulet
2009-2010	Graduate Teaching Assistant (August 2009-May 2010) The University of Mississippi, University, MS Inquiry into Life Laboratory I (BISC 103)
2009	Lab Assistant (Full-time; February-June 2009) Novozymes Biologicals, Inc., Salem, VA Quality Control Department
2008	Internship (May-July 2009) Humane Society International, Gaithersburg, MD Principal writer of U.S. Endangered Species Act petition proposal
2008	Lab Assistant (February-May and August-December 2008) George Mason University Histology Lab, Fairfax, VA Histology and coral disease research with Dr. Esther Peters
2007	Nature Guide (Part-time; May-June 2007) Apple Ridge Farms, Floyd, VA Led groups of urban children (K-7) on guided nature hikes

2006 Internship (Full-time; May-July 2006)
Hopewell Culture National Historical Park, Chillicothe, OH
Student Conservation Association
Invasive plant control and assisted with school groups and educational displays

2005 Volunteer (July 2005)
International Student Volunteers, Inc., Queensland, Australia
In association with the Bush Heritage Fund

GRANTS AND AWARDS

2011 2nd Place (Basic Sciences) at The University of Mississippi (UM) Graduate School Council Research Poster Symposium
2011 UM Graduate School Council Research Grant, $500
2011 UM Graduate School Travel Grant, $200
2010 Lerner Gray Memorial Fund of the American Museum of Natural History, $1,000
2010 Sigma Xi Grant-in-Aid of Research, $400
2010 UM Graduate School Summer Research Assistantship, $2,000
2010 UM Graduate School Council Research Grant, $500
2009 George Mason University (GMU) Department of Environmental Science and Policy Outstanding Graduate in Conservation Biology Award
2008 GMU John Souza III Endowed Scholarship for the Outstanding Undergraduate Student in Environmental Science and Policy
2008 GMU College of Science Merit Award Scholarship
2008 GMU Writing Across the Curriculum Award for Excellence in Undergraduate Writing in Biology

TEACHING EXPERIENCE

2010-2012 Guest lecturer for non-biology majors course Inquiry into Life Human Biology (BISC 102, two sections of approximately 100 students; The University of Mississippi); lecture topic: cellular transcription and translation
2009-2010 Instructor for Inquiry into Life Laboratory I (BISC 103; The University of Mississippi); human biology and physiology for non-biology majors
2007 Taught Virginia Department of Education Standards of Learning curriculum in science to elementary and middle school students (K-7) as a nature guide at Apple Ridge Farms, Floyd, VA

CERTIFICATIONS AND MEMBERSHIPS

2011-present Society for Conservation Biology Member
2010-present SCUBA Diving International Advanced Adventure Diver (advanced open-water)
2010-present Technical Diving International Nitrox Diver
2008-present SCUBA Schools International Open-water Diver

CONFERENCES AND POSTERS

2012 Byler K.A., Fine M., Goulet T.L. Do corals with maternal (vertical) symbiont transmission acquire symbionts? Abstract accepted. Oral presentation to be presented by T.L. Goulet at the 12th International Coral Reef Symposium, Cairns, Queensland, Australia, July 9-13.

2012 Byler K.A., Fine M., Carmi-Veal M., Goulet T.L. Symbiont identity and acquisition through ontogenesis in the coral *Stylophora pistillata* in the Red Sea. Poster presented by K.A. Byler at the 2012 University of Mississippi Graduate School Council Poster Symposium, April 5.

2011 Byler K.A., Fine M., Carmi-Veal M., Goulet T.L. Identification of symbiotic dinoflagellates (*Symbiodinium*) in the larvae of the coral *Stylophora pistillata* in the Red Sea. Poster presented by K.A. Byler at the 2011 University of Mississippi Graduate School Council Poster Symposium, April 8.

2011 Byler K.A., Fine M., Carmi-Veal M., Goulet T.L. Identification of symbiotic dinoflagellates (*Symbiodinium*) in the larvae of the brooding coral *Stylophora pistillata* in the Red Sea. Oral presentation, presented by K.A. Byler at the 2011 Benthic Ecology Meeting, Mobile, Alabama, March 16-20.

2011 Goulet T.L., Day L.B., Byler K.A., Sullivan K. Class generated community clicker cases: Testing a novel pedagogic approach connecting science and students in non-major biology classes. Poster presented by T.L. Goulet at the 2011 CCLI/TUES Principal Investigators (PIs) Conference- Transforming Undergraduate Education in STEM: Making and Measuring Impacts, Washington D.C., January 26-28, 2011

2010 Goulet T.L., Day L.B., Byler K.A., Sullivan K. Class generated community clicker cases: Testing a novel pedagogic approach connecting science and students in non-major biology classes. Poster presented by T.L. Goulet at the National Association of Biology Teachers Conference, Minneapolis, MN, November 3-6

2009 Symbiofest hosted by the University of Georgia; attendee, April 30

SCIENTIFIC SERVICE

2012 Mississippi Public Schools 2012 Region VII Science Fair Judge (Grades 7-12)
2011 Mississippi Public Schools 2011 Region VII Science Fair Judge (Grades 1-6 & 7-12)
2010 Mississippi Public Schools 2010 Region VII Science Fair Judge (Grades 2-6)
2008 National Ocean Sciences Bowl- Chesapeake Bay Bowl volunteer