Temporal and environmental influences on the early establishment and maintenance of coral-*Symbiodinium* symbioses

Thesis submitted by

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Publications resulting from the research in this thesis

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ABSTRACT

Understanding mechanisms underlying the formation and maintenance of coral-*Symbiodinium* symbioses as well as factors affecting the integrity of these symbioses is critical to predicting how coral holobionts might change in response to warming oceans predicted by climate change. Research reported in this thesis aims to enhance current knowledge of coral-*Symbiodinium* symbioses by: (1) examining temporal variation in *Symbiodinium* uptake by coral juveniles, (2) exploring the role of parental effects and ontogenetic stage in determining *Symbiodinium* associations, (3) assessing the impact of environmental parameters in the establishment of symbioses, and (4) evaluating whether some host-symbiont combinations are more resilient to environmental stress than others.

I found that newly settled juveniles of the corals *Acropora millepora* and *A. tenuis* do not necessarily take up the *Symbiodinium* type present in parental colonies, and that a potentially opportunist type D *Symbiodinium* quickly dominates symbioses in juveniles of both species at three sites in the central Great Barrier Reef. I also found that adult patterns of association may not become established for up to 2.5-3.5 years, suggesting a delay in the expression of symbiont specificity. In *A. tenuis,* continuing changes in *Symbiodinium* communities over the first 3.5 years are interpreted as fine-tuning of specificity mechanisms leading to establishment of the homologous algal symbiont characteristic of adult populations. Algal endosymbioses were much more stable over the same time period in juveniles of *A. millepora,* although further research is required to distinguish between absence of specificity and delayed expression of specificity. Changes in *Symbiodinium* communities in *A. tenuis* juveniles are not linked to the onset of reproductive maturity but may be linked to changes in micro-environmental conditions (possibly light intensity or access to nutrients) associated with growth of the colony.

Field studies investigating the role of environmental parameters in the establishment of symbioses revealed that light has little effect on the type of *Symbiodinium* initially acquired by both *A. millepora* and *A. tenuis*. This result was confirmed by experimental manipulations in aquaria where equal amounts of *Symbiodinium* types C1 and D were offered to newly settled juveniles maintained in two light levels by three temperature treatments. In contrast, I found that temperature has a significant effect on algal symbioses by affecting the type of *Symbiodinium* acquired by both coral species and by slowing and potentially stopping *Symbiodinium* uptake and the onset of symbioses at elevated temperatures. Type D *Symbiodinium* was found in larger proportions in juveniles at elevated temperatures (30 and 31°C), providing further evidence of the infective and potentially opportunistic nature of this *Symbiodinium* type. The benefits of type D to the host require further investigation as these juveniles had low levels of infection and it is unclear if their survival would depend on other mechanisms, such as a shift towards heterotrophy.

Comparisons of the resilience of corals hosting type C1 or D *Symbiodinium* to environmental stress indicate that *A. tenuis* juveniles have lower metabolic costs and enhanced physiological tolerance when hosting type C1 *Symbiodinium*. In other studies, the same D-type has been shown to confer higher thermal tolerance than both C2 in adults and C1 in juveniles of the closely related coral *A. millepora*. My results challenge speculations that associations with type D are universally most robust to thermal stress and highlight a potential role of host factors in determining the physiological performance of the holobiont. They also show that although the heat tolerance of corals may be contingent on the *Symbiodinium* strain *in hospite*, their response to heat and light stress is determined by species-specific interactions between both partners in the association.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1.0 Background and General Introduction

Reef-scape dominated by *Acropora* corals. Photo: Paul Costello

1.1 Background

Coral reefs are widely regarded as one of the most diverse ecosystems on the planet. The high structural complexity of these ecosystems provides habitat and refuge for thousands of species of marine animals and plants, contributing significantly to the high biodiversity and primary productivity characteristic of this ecosystem (Connell 1978). Scleractinian corals are the principal contributors to the structural framework of tropical reefs and their evolutionary success as reef builders is largely due to the symbiotic relationships they form with uni-cellular dinoflagellates of the genus *Symbiodinium* (Stanley & Fautin 2001). This relationship enables a highly efficient system of resource recycling in the clear oligotrophic waters in which coral reefs thrive (Muscatine 1977). The symbiont benefits from by-products of host respiration (e.g., nitrogen and phosphorous) in a safe and stable environment, while the host receives sugars and other molecular compounds derived from symbiont photosynthesis (Muscatine 1990; Gattuso *et al.* 1999; Douglas 2003). In addition, photosynthesis enhances calcification by removing CO_2 from host tissues, raising the pH and concentration of CO_3^{2-} which in turn raises the concentration of $CaCO_3$ and results in calcification (Barnes & Chalker 1990). While it is clear that the partnership provides benefits for both the algal symbiont and the coral host, many aspects of this symbiosis are poorly understood. Critical knowledge gaps include characterization of recognition and regulation mechanisms and the specific role each partner plays in the overall physiology of the holobiont.

In the last couple of decades, coral reefs have been devastated by multiple episodes of mass coral bleaching events (Hoegh-Guldberg 1999), increased prevalence of diseases (Harvell *et al.*; 2002 Bruno *et al.* 2007), over-harvesting of commercial species

(Pandolfi *et al.* 2003), and increased levels of pollution (McCulloch *et al.* 2003). All these impacts have raised substantial concerns regarding the future of these ecosystems. In addition to their considerable ecological value, coral reefs have enormous economic value arising from the many commercially important species they house that are consumed directly as food or are utilized by multiple industries (Moberg & Folke 1999), and from their role in reef tourism, which in 2005-2006 was estimated to be around 6 billion AUD on the Great Barrier Reef alone (Carr & Mendelsohn 2003). One of the greatest threats to corals is mass bleaching, which renders corals white by the loss of their symbionts or the pigments within them (Brown 1997). Due to the obligate nature of the coral-*Symbiodinium* symbiosis, the breakdown of the association as a result of increasingly more frequent and severe mass bleaching events has lead to high coral mortality and changes in community composition in coral reefs worldwide (Hoegh-Guldberg 1999; Hughes *et al.* 2003). Given the enormous value of coral reefs, understanding the underlying physiological basis of the establishment and regulation of coral-*Symbiodinium* symbioses is of paramount importance.

1.2 Patterns of *Symbiodinium* association, acquisition, and regulation mechanisms

Symbiodinium dinoflagellates are currently organized into eight major phylogenetic lineages or clades based on sequence differences in several molecular markers (reviewed in Coffroth & Santos 2005). At least five of these are known to occur in scleractinian corals. The genus is highly diverse, with several sub-clades or types existing within each clade. Most corals appear to associate with only one clade or a single type of *Symbiodinium* (Goulet 2006); but additional clades can be present at such low abundances that these have generally gone undetected (Mieog *et al.*, 2007). In

addition, low levels of replicate sampling may mask true diversity and prevent detection of corals with multiple symbionts (Baker & Romanski 2007). A few species host more than one symbiont either simultaneously (Rowan *et al.* 1997; Ulstrup & van Oppen 2003), at different depths (Rowan & Knowlton 1995; Toller *et al.* 2001b), in different reefs/bio-geographic areas (Loh *et al.* 2001; van Oppen *et al.* 2001), or in different seasons (Chen *et al.* 2005). Corals can acquire their symbionts in a vertical or a horizontal mode (Harrison & Wallace 1990). Corals with vertical mode of transmission obtain their symbionts from the parental colony via the eggs or larvae. In contrast, corals with a horizontal mode must acquire their symbionts directly from the environment. Each new generation is thus potentially exposed to a variety of symbiont types. This raises questions about the capacity of corals with horizontal transmission to take up new or different symbiont types from those in parental colonies, which has important implications for the fitness of the holobiont if the new or different symbionts have physiological attributes that differ from those in parental colonies.

To date, studies of coral species with horizontal transmission of *Symbiodinium* suggest that initial uptake is non-specific, based on corals exposed to multiple types of *Symbiodinium* in lab-based studies (Schwarz *et al.* 1999; Coffroth *et al.* 2001; Weis *et al.* 2001; Little *et al.* 2004) or in coral juveniles without symbionts (i.e. aposymbiotic) placed on the reef to acquire symbionts from the environment (Coffroth *et al.* 2001; Little *et al.* 2004; Gomez-Cabrera *et al.* 2008). In some cases the homologous symbiont is the best at establishing a lasting symbiosis, which implies some selectivity following initial uptake (Coffroth *et al.* 2001; Weis *et al.* 2001). In other studies, this is not the case and heterologous symbionts remain dominant, which may indicate active selection by the host to maximize symbiont effectiveness according to physiological demands of

the ontogenetic stage, or competitive dominance of the heterologous symbiont once inside the host (Little *et al.* 2004; Gomez-Cabrera *et al.* 2008). However, in studies monitoring uptake of symbionts on the reef (Coffroth *et al.* 2001; Little *et al.* 2004; Gomez-Cabrera *et al.* 2008), it has not been shown how availability of different types of symbionts may affect uptake patterns. Furthermore, the longest documented period of symbiont succession in juveniles is less that one year (Little *et al.* 2004), thus little is known about *Symbiodinium* dynamics within the coral host during early life history stages. Despite the short duration of these studies, the fact that most corals are dominated by only one type of *Symbiodinium* (Goulet 2006) argues for the existence of selective/regulating mechanisms underpinning stable associations observed in many adult corals (Goulet & Coffroth 2003; LaJeunesse *et al.* 2005; Thornhill *et al.* 2006). Longer monitoring periods of symbiont succession after initial uptake are required in order to explain how such seemingly stable associations are established in adults following apparently non-selective uptake. This will also help in discerning whether changes in symbiont communities are linked to ontogenetic changes in the host and thus, provide insights into factors regulating the symbiosis.

Virtually nothing is known about whether temperature and light affect the establishment of the symbiosis. Only one study has looked at the effect of light on the acquisition of symbionts by larvae of the coral *Fungia scutaria* but found no differences in the percentage of infected larvae in two light levels (Weis *et al.* 2001). However, temperature and light are the main triggers for the breakup of the association (bleaching). It is therefore critical to improve our understanding of how environmental conditions may affect the establishment of the symbiosis and the type of *Symbiodinium*

acquired in order to predict how these relationships may change in response to warming oceans.

1.3 Physiological diversity of *Symbiodinium* and implications for climate change

Several studies have shown that different symbiotic dinoflagellates vary in their physiological response to light and temperature. These responses include: 1) changing the size and number of photosynthetic units (Iglesias-Prieto & Trench 1994; Fitt & Cook 2001; Robison & Warner 2006); 2) synthesizing mycosporine-like amino acids (Banaszak & Trench 1995; Banaszak *et al.* 2000); 3) changing the concentration of chlorophyll-protein complexes (Iglesias-Prieto & Trench 1997a); 4) slowing down or stopping photosynthesis (Iglesias-Prieto *et al.* 1992); 5) modifying algal growth rates (Kinzie *et al.* 2001; Robison & Warner 2006); 6) disrupting CO_2 fixation and electron flow through the Calvin Cycle (Jones *et al.* 1998); and 7) modifying turnover rates of D1 proteins in reactions centers of photosystem II (Warner *et al.* 1999; Robison & Warner 2006). It is clear from these studies that there is physiological diversity amongst different *Symbiodinium* types but understanding how this diversity may impact holobiont physiology is critical in terms of assessing their response in the face of current threats to coral reefs.

One of the most intensely studied aspects of the coral-*Symbiodinium* relationship is the response of the holobiont to temperature and light stress as these are the main triggers for mass bleaching events (Brown 1997; Hoegh-Guldberg 1999). Changing symbiotic partners for new, heat-tolerant types has been proposed as a potential mechanism for corals to adapt to climate change (Buddemeier & Fautin 1993). Novel holobionts could

result from acquiring a new type from the exogenous environment (switching) or by increasing the relative abundance of types already present within the host (shuffling), but the extent to which either of these processes occurs is poorly understood.

Observations of increased prevalence of clade D symbionts in corals after bleaching events (Baker 2001; Glynn *et al.* 2001; Toller *et al.* 2001a; Baker *et al.* 2004; van Oppen *et al.* 2005; Jones *et al.* 2008a), as well as in corals living in reef lagoons exposed to higher temperature regimes than surrounding waters (Fabricius *et al.* 2004) has led to the characterization of symbionts within this clade as heat-tolerant (Baker 2003). However, direct comparisons of thermal tolerance of corals hosting clade D symbionts versus other types have only been experimentally tested in two studies (Rowan 2004; Berkelmans & van Oppen 2006). Although the results were consistent with field patterns, observations of thermally robust and thermally sensitive types within *Symbiodinium* clades (Tchernov *et al.* 2004) highlight the need for further comparative studies. Moreover, because clade D is relatively uncommon in Indo-Pacific corals (the epicenter of coral diversity and abundance), in contrast to the ubiquity of clade C (Baker & Rowan 1997; LaJeunesse 2001; Baker 2003; LaJeunesse *et al.* 2003), knowledge of the influence of symbiont clade (and types within a clade) on holobiont physiology has important implications for understanding the impact of warming oceans on coral communities.

1.4 Aims of thesis

The overarching aim of my research is to improve current understanding of the factors driving the uptake, establishment, and regulation of coral-*Symbiodinium* symbioses. My primary objectives were to investigate the role of environmental parameters in shaping

the establishment phases of the symbioses and to evaluate whether particular host-symbiont combinations are more resilient to environmental stress. The specific objectives for each chapter are as follows.

In Chapter 2, I examine temporal and geographic variations in symbiont uptake by juveniles of the horizontally transmitting scleractinian corals, *Acropora tenuis* and *A. millepora,* and briefly examine the effect of light on the uptake of symbionts in the field. This study provides important background on *in situ* patterns of *Symbiodinium* uptake, which enables evaluation of the importance of local availability and potential infective ability of different *Symbiodinium* types in the establishment of the symbiosis.

In Chapter 3, I evaluate experimentally the impact of light and temperature on the initial establishment of the symbiosis in *A. tenuis* and *A. millepora*. Knowledge of the role that environmental parameters might have in establishing algal endosymbiosis is important to enhance our understanding of how these symbioses might change with projected warming ocean scenarios.

In Chapter 4, I analyze temporal variations in the symbiont communities of both coral species and examine whether ontogenetic changes associated with reproductive maturity are linked to changes in symbiont communities. This chapter enhances our understanding of the impact of host development in the regulation of the symbioses under non-stress conditions.

In Chapter 5, I evaluate experimentally the physiological contributions of different symbiont types to thermal tolerance in corals. Analysis of the role each partner plays in

the bleaching response of the holobiont will enhance our understanding of the potential for different host-symbiont combinations to boost the physiological performance of the holobiont and hence, function as a potential mechanism for corals to persist through warming ocean predictions.

Chapter 6 synthesizes key findings from the preceding chapters into a cohesive discussion that highlights the significance of coral-*Symbiodinium* symbioses for the long-term resilience of coral reefs and comments on the potential of different host-symbiont combinations in coping with escalating threats linked to climate change. In addition to discussing implications of the research, I identify key areas for future research in coral-algal endosymbioses.

Chapter 2.0 Temporal and geographical variation in natural symbiont uptake by juvenile *Acropora tenuis* and *A. millepora*

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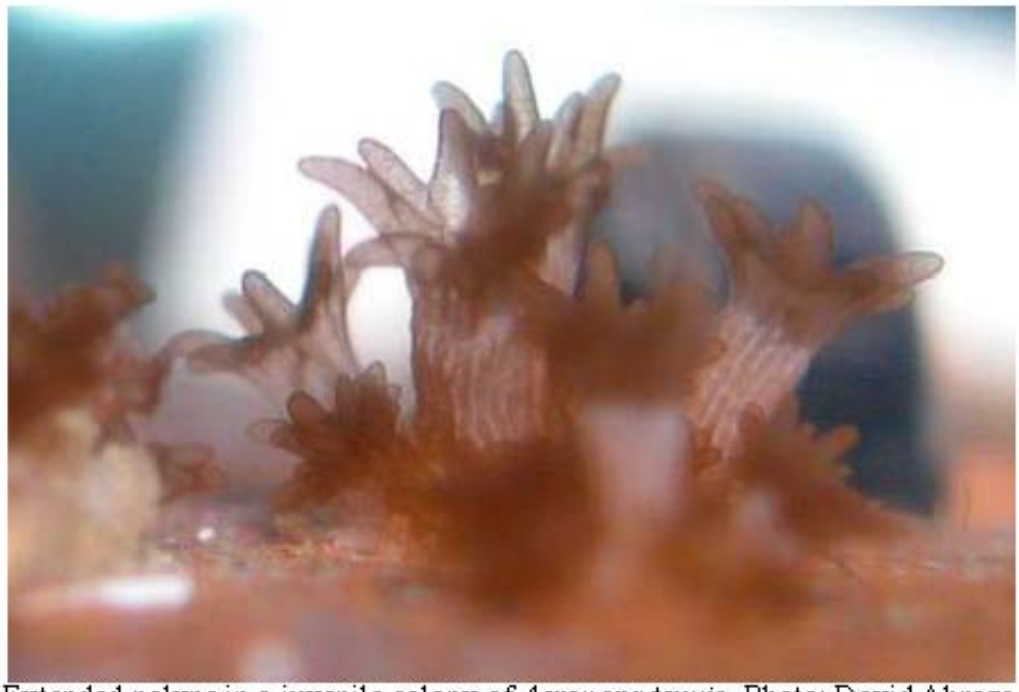
All the data was collected and analyzed by D. Abrego, who also wrote the chapter and manuscript after intellectual contributions by all co-authors.



Extended polyps in a juvenile colony of *Acropora tenuis*. Photo: David Abrego

2.1 Introduction

The success of corals as reef builders is intricately tied to their obligate symbiosis with dinoflagellates of the genus *Symbiodinium*. Nutrients and energy transferred between the two partners enable corals to build the carbonate structures that comprise the framework of modern reefs (Muscatine & Porter 1977). Based on a range of genetic markers, eight phylotypes or clades of *Symbiodinium* are known to associate with marine invertebrates, with at least five of these forming symbioses with reef-building corals (reviewed by Coffroth & Santos 2005). The genus is highly diverse, with several sub-clades or types existing within each clade (Baker 2003).

In most adult corals, the symbiosis is extremely stable over time and is usually dominated by one type of *Symbiodinium* (Goulet 2006; Thornhill *et al.* 2006), although background levels of different types (Mieog *et al.* 2007) present in some coral species may be shuffled in response to seasonal (Chen *et al.* 2005) or extreme environmental variations such as temperature-induced bleaching (Jones *et al.* 2008). It is clear that coral-*Symbiodinium* associations are not random and many species show specificity for their algal symbiont either at a local scale (within a reef or at a particular depth) or everywhere within the host range (strict specificity, reviewed in Baker 2003).

The mode of *Symbiodinium* acquisition has important implications for our understanding of how different holobionts (host-symbiont associations) are formed and maintained. Corals with a vertical mode of acquisition obtain their symbionts from parental colonies via the eggs or as brooded larvae. In contrast, in corals with a horizontal mode of acquisition, each new generation must acquire its symbionts from the environment. This is the dominant mode of *Symbiodinium* acquisition by corals

(Harrison & Wallace 1990). Corals that obtain their symbionts in this mode are potentially exposed to a variety of *Symbiodinium* types that are different from those found in the parental colonies. Thus, corals with horizontal symbiont acquisition offer unique systems to study the establishment and specificity mechanisms in the coral-algal symbiosis.

Although there is a large body of literature examining the fundamental nature of algal endosymbiosis in corals and documenting the large diversity of coral-*Symbiodinium* associations (reviewed by Baker 2003; Coffroth & Santos 2005), the processes underlying the recognition of the algal symbiont during initial uptake and establishment of the symbiosis remain poorly described. *Symbiodinium* may enter the host as early as the larval stage, when they are ingested as the larvae search for suitable surfaces upon which to metamorphose and settle. Upon ingestion, interactions between the cell surface glycoproteins of the symbiont and receptors in the host may result in the recognition of the symbiont and its incorporation into a host cell (Lin *et al.* 2000; Koike *et al.* 2004; Yuyama *et al.* 2005). However, it is not clear if recognition at this stage is specific enough to distinguish between different *Symbiodinium* types. There is some evidence that symbiont recognition may occur before incorporation into host cells in larvae of the solitary coral *Fungia scutaria* (Rodriguez-Lanetty *et al.* 2006). However, several studies have shown that aposymbiotic (without symbionts) corals have little specificity for algal symbionts at initial uptake (Schwarz *et al.* 1999; Coffroth *et al.* 2001; Weis *et al.* 2001; Little *et al.* 2004; Gomez-Cabrera *et al.* 2008). These studies involved exposure of juvenile corals to different types of *Symbiodinium* under laboratory conditions, or deployment of juveniles in the field at the parental reef to monitor symbiont uptake. The consensus from these studies is that juvenile corals are flexible in the type of

Symbiodinium initially taken up but in the case of field studies (Coffroth *et al.* 2001; Gomez-Cabrera *et al.* 2008; Little *et al.* 2004), the source and availability of symbionts different from those in the parental colonies was not thoroughly explored. Furthermore, it is not known how environmental conditions may shape the uptake of *Symbiodinium* during this initial stage.

The present study examines how environmental factors and local availability of *Symbiodinium* affect its initial uptake by coral juveniles. Specifically, temporal and geographic variations in uptake were examined by monitoring the *Symbiodinium* types acquired by coral juveniles reciprocally grown out between sites where parental colonies host different types of *Symbiodinium*.

2.2 Materials and methods

2.2.1 Study sites and experimental design

Three sites on the Great Barrier Reef (GBR) were chosen for this study based on differences in the dominant *Symbiodinium* community found in the corals *Acropora tenuis* and *A. millepora* (Fig. 2.1). To examine whether *Symbiodinium* uptake is affected by parental associations, aposymbiotic coral juveniles from each of these sites were reciprocally translocated and their *Symbiodinium* communities were determined after one month. At Magnetic Island, *A. tenuis* colonies are dominated by *Symbiodinium* type C1 (*sensu* van Oppen *et al.* 2001, GenBank Accession # AF380551), whereas at Orpheus Island and Davies Reef, this coral associates predominantly with type C2 (*sensu* van Oppen *et al.* 2001, GenBank Accession # AF380552). Similarly, *Acropora millepora* at Magnetic Island is dominated by type D (*sensu* van Oppen *et al.* 2001, GenBank Access # EU024793) but at Orpheus Island and Davies Reef it is dominated

by type C2. Magnetic Island is an inshore island surrounded by shallow and highly turbid waters facing a major shipping channel into the port of Townsville. Orpheus Island is approximately 40 nautical miles northwest of Magnetic Island. Like Magnetic Island, this is an inshore island with high turbidity levels (the Herbert River, a major river in Queensland discharges its waters approximately 10 nautical miles northwest of Orpheus Island). Davies Reef is located approximately 50 nautical miles northeast from Magnetic Island. It is a mid-shelf reef with clear and slightly cooler waters than Magnetic and Orpheus Islands (*in situ* data available from: http://www.aims.gov.au/docs/data-centre/seatemperatures.html).

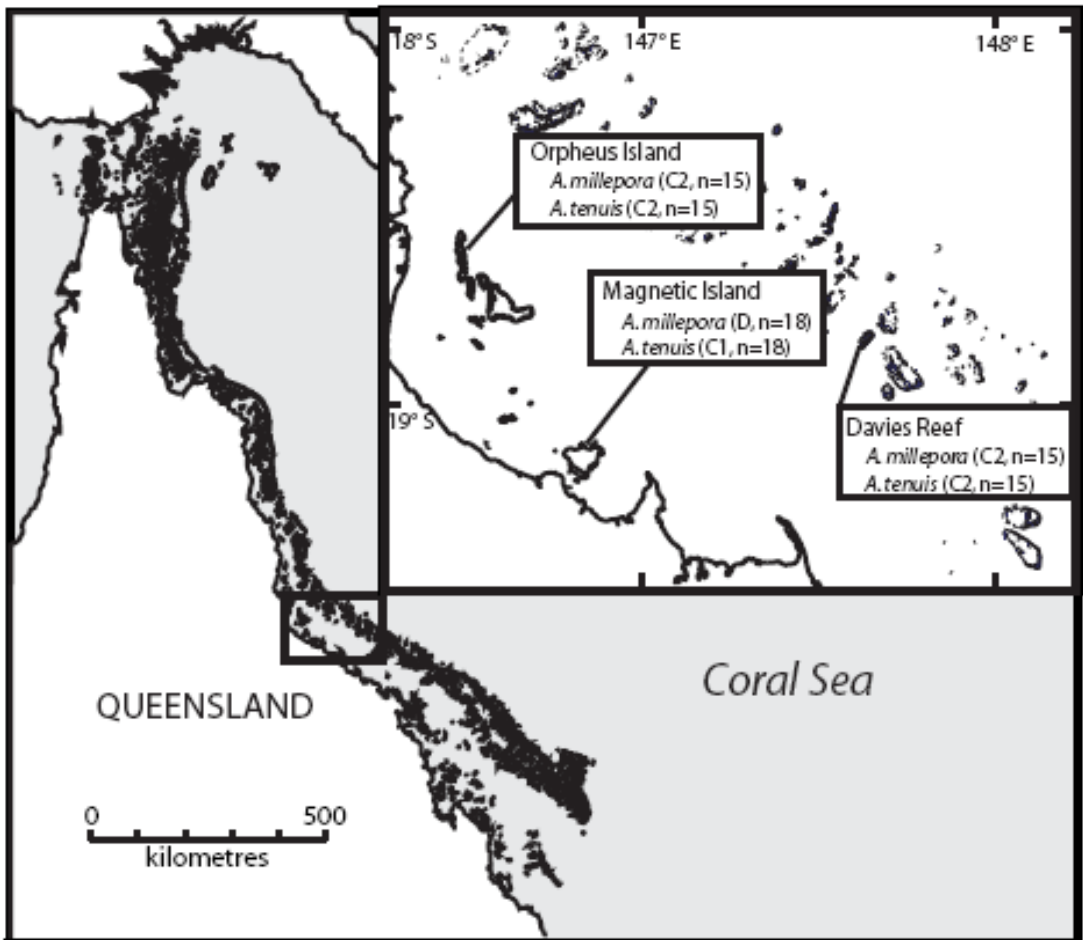


Fig. 2.1. Study sites. Inset shows the three sites on the Great Barrier Reef chosen for this study. For each site, the dominant *Symbiodinium* type in association with adult *Acropora tenuis* and *A. millepora* corals is shown in brackets along with the number of parental colonies used for each spawning event.

2.2.2 Collection of gametes, culture and settlement of juveniles, and reciprocal explants

Juveniles of *Acropora tenuis* and *A. millepora* were raised from fertilized gametes collected during the coral mass spawning events of 2003-2006 (see Little *et al.* 2004 for details on fertilization of gametes and culture conditions). *A. tenuis* juveniles were reciprocally explanted between Magnetic Island and Davies Reef in 2003 and between Magnetic Island and Orpheus Island from 2004-2006. *A. millepora* juveniles were reciprocally explanted between Magnetic Island and Orpheus Island from 2004-2006 (see Table 2.1 for summary of juveniles available from each site and for each cohort). Juveniles were raised in 1 μm filtered sea water and settled on pre-conditioned (in the field) terracotta tiles which had been previously autoclaved to ensure a *Symbiodinium*-free surface. Once settled (approximately 6-8 days after spawning), the tiles with juveniles were photographed to facilitate mapping and avoid sampling of juveniles that may have settled on the tiles after deployment on the reef. Settled juveniles were approximately 8 days old and were the size of a single polyp (~1mm). The number of juveniles per tile varied but most tiles had between 10-60 juveniles. The tiles were skewered onto a stainless steel rod and spaced by pieces of polyvinyl tubing to prevent them from coming into contact with one another. The rods were then transported to the grow-out location (half remained at the native reef and half were taken to a non-native reef, see Table 2.1) and suspended between star pickets driven into the reef substratum at the same depth as naturally occurring colonies of both species. The resulting vertical arrangement of the tiles prevented smothering of the juveniles by sediment while the spacers between tiles ensured that light reached every section of the tiles.

2.2.3 Determination of *Symbiodinium* genotype at initial uptake

Approximately one month after deployment on each reef, 30 juveniles were removed from the tiles to identify the *Symbiodinium* community acquired based on sequence differences in the nuclear rDNA ITS1 region using Single Stranded Conformation Polymorphism (SSCP). Samples were fixed in 100% EtOH for down-stream genotyping. Total (coral and algal) DNA was extracted using an adapted version of the protocol described by Wilson *et al*. (2002). Briefly, each sample (a few polyps) was ground in 250 µl of extraction buffer (100 mM Tris pH 9.0, 100 mM EDTA, 1% SDS, 100 mM NaCl) using a disposable paper clip. The sample was incubated at 65°C for 2-3 hrs. followed by addition of 62.5 µl KOAc. After vortexing for 10 seconds, the sample was incubated on ice for 20 minutes, followed by centrifugation (at room temperature) at maximum speed in bench top centrifuge for 15 minutes. The supernatant was decanted into a new 1.5 ml tube and 250 µl of isopropanol were added to precipitate the DNA. After 5 minutes of standing, the sample was centrifuged (maximum speed, room temperature) for 10 minutes and the supernatant decanted. The pellet was washed with 100 µl of 70% ethanol and centrifuged again for 5 minutes. The ethanol was decanted and after air drying the pellet (30 minutes), it was resuspended overnight in 200 µl of 0.01M Tris pH 7.5. The ITS1 region was amplified using fluorescently labeled Sym ITS1 PCR primers and SSCP was conducted (as described by Fabricius *et al.* 2004) to determine the symbiont types within each sample. Reference samples of known ITS1 sequences were run in each gel and SSCP profiles were scored manually from gel images. In most cases only a single band was detected in each sample. However, there were samples where more than one band was detected even though one band was usually dominant (highest intensity on the gel image). In these cases the sample was scored as having a mix of *Symbiodinium* types corresponding to each of the bands

present, but no inferences on relative abundances of each of these types were made. In addition to the juveniles, at least 15 adult colonies of each species, including every adult colony used at each spawning event were sampled (1 branch) to identify their *Symbiodinium* community.

Table 2.1. Summary of juveniles available for reciprocal grow-out experiments. For each cohort, the location from which the juveniles were obtained (Parental Reef) is listed along with the locations to which those juveniles were explanted (Grow-out Reef). Lack of sufficient gametes prevented reciprocal grow-out of both species every year. In some cases, mortality of the juveniles prevented their recovery for analysis. * indicates cohorts for which juveniles were recovered but in numbers too low to be included in analysis. + indicates juveniles that were pooled with 2006 cohort for analysis.

Species	**Cohort**	**Parental Reef**	**Grow-out Reef**	**Juveniles recovered for analysis?**
A. tenuis	2003	Magnetic Island	Magnetic Island	Y
			Davies Reef	N*
		Davies Reef	Magnetic Island	N*
	2004	Magnetic Island	Magnetic Island	Y
			Orpheus Island	N
		Orpheus Island	Magnetic Island	Y
			Orpheus Island	Y+
	2005	Orpheus Island	Magnetic Island	Y
			Orpheus Island	Y+
	2006	Magnetic Island	Magnetic Island	Y
			Orpheus Island	Y
		Orpheus Island	Magnetic Island	Y
			Orpheus Island	Y
A. millepora	2003	Magnetic Island	Magnetic Island	Y
	2004	Magnetic Island	Magnetic Island	Y
			Orpheus Island	N
		Orpheus Island	Magnetic Island	Y
			Orpheus Island	Y+
	2005	Magnetic Island	Magnetic Island	Y
			Orpheus Island	N
		Orpheus Island	Magnetic Island	Y
			Orpheus Island	Y+
	2006	Magnetic Island	Magnetic Island	Y
			Orpheus Island	Y
		Orpheus Island	Magnetic Island	Y
			Orpheus Island	Y

2.2.4 Effect of light environment on symbiont uptake

To determine if the light environment around newly settled juveniles affects the type of *Symbiodinium* acquired at initial uptake, tiles with settled juveniles were positioned in two orientations (Fig. 2.2) so that different light levels would reach the surface of the tiles. Juveniles of *A. tenuis* and *A. millepora* were raised from Magnetic Island colonies, settled on terracotta tiles, and then deployed on the reef at Magnetic Island as previously described. One set of tiles was positioned flat on a rack so that straight down-welling light would reach the top surface of the tiles with settled juveniles. A second set of tiles was skewered through rods so that the surface with settled juveniles would lie at an angle almost perpendicular to down-welling light. After approximately one month, 30 juveniles from each treatment were sampled for *Symbiodinium* genotyping following the procedures previously described. The light environment around these tiles was characterized by deploying submersible, cosine-corrected, Photosynthetically Active Radiation (PAR) sensors with data loggers (Odyssey, Dataflow Systems Pty. Ltd.) next to the tiles with the sensors pointed in the same orientation as the surface of the tiles. For the vertically deployed tiles, the sensor was positioned in the middle of the tile to account for potential variations in light levels on juveniles sitting on the top portion of the tile compared to those on the bottom portion of the same vertical tile (see Fig. 2.2). A measurement was recorded once every 10 minutes for one week. To prevent signal dimming due to fouling of the sensor, a SCUBA diver wiped the sensors once a day while deployed. The PAR sensors were calibrated with a LI-COR 1000 light meter.

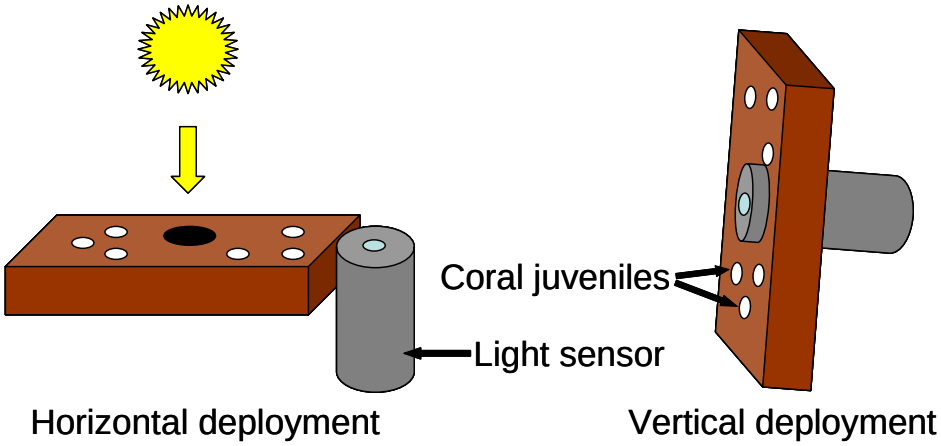


Fig. 2.2. Schematic representation of two tile arrangements deployed on the reef.

2.2.5 *Symbiodinium* diversity and relative abundance on the reef

To estimate the diversity of the *Symbiodinium* pool potentially available to aposymbiotic coral juveniles from symbiotic hosts, a wide variety of symbiotic cnidarians were sampled on the reef in Nelly Bay at Magnetic Island and Pioneer Bay at Orpheus Island. This was done using SCUBA in the area surrounding the tiles by sampling different species of symbiotic cnidarians within an approximately 250 m radius from the tiles. A fragment was collected by hand or a small hammer and chisel from each different species identified and where possible, samples from 2-3 conspecific individuals were collected. Conspecific host samples were collected at least 10 meters apart to avoid sampling potential clone mates. In addition, each species was photographed underwater and one additional sample was collected for examination of skeletal structures and taxonomic verification following published descriptions (Veron 2000; Fabricius & Alderslade 2001). Samples for genotyping were immediately fixed in 100% EtOH. *Symbiodinium* genotypes were determined by ITS1 PCR and SSCP as described above. In most cases, SSCP profiles from all the cnidarians sampled were single bands identical to those used as references. However, some of the samples had multiple band profiles or were slightly different from the references used. All of these

samples along with a subset of those that were identical matches were re-amplified with non-fluorescent labeled primers and sequenced (Macrogen, Korea, ABI370XL sequencer) to verify the SSCP results. Prior to sequencing, PCR products with multiple bands were cloned (Invitrogen TOPO TA kit) and PCR products from bacterial colonies were run on an SSCP gel along with the original PCR product, so that all the cloned PCR products representing each band in the gel could be identified and sequenced. Sequences were proof-read and aligned using Sequencher 4.5 (Gene Codes Corporation) along with references from previously characterized *Symbiodinium* ITS1 rDNA sequences available from GenBank. Maximum-parsimony (heuristic search) analyses were conducted using PAUP 4.0b10 (Swofford 2000). Gaps were treated as fifth base. Bootstrap analysis was performed with 1000 replicates.

The relative abundance of *Symbiodinium* types potentially available for uptake by aposymbiotic corals was estimated by standardizing the results of the *Symbiodinium* diversity survey with data on the relative abundance of the hosts at both study sites. This approach allowed us to estimate the relative abundance of *Symbiodinium* based on the abundance of the host/s in which it is found *in hospite*. An important caveat of this approach is that it only estimates the diversity and abundance of *Symbiodinium in hospite* and may therefore miss other potential sources of *Symbiodinium* such as sediments and the water column. Data were collected by using four 20-meter line-intercept transects at six sites around the tiles at 2-6 m below LAT. For Magnetic Island, the relative abundances of the hosts were provided by Dr. A.M. Ailing (Sea Research, QLD) and were collected in September 2006 in the same area where tiles for the present study were deployed. The taxonomic resolution of these data was genus level. The relative abundance of each *Symbiodinium* clade was calculated as follows:

$$\text{Rel. abundance of clade x} = \left(\frac{\text{Relative abundance of host (by genus)}}{\text{Total \# of host spp. within genus surveyed}} \right) \text{\# of host spp. within genus harboring symbiont clade x}$$

This assumed that all the species within the genera of both data sets (host relative abundance and *Symbiodinium* survey) were the same, which was true in every case for which species-level resolution was available. For this calculation, the *Symbiodinium* types detected in the hosts were grouped within their respective clades (C1, C1-like and C• were all grouped into clade C). For host taxa where *Symbiodinium* types belonging to more than one clade were detected, the calculated relative abundance was proportionally assigned to each clade.

2.2.6 Statistical analysis

The distribution of *Symbiodinium* types in coral juveniles was analyzed by χ^2 tests. Comparisons were made between cohorts of the same parental reef (grouped by grow-out reef) to determine if there were significant differences in the distribution of symbiont types between years. As no inter-annual differences were detected, the data were pooled to compare symbiont distributions between juveniles from different parental reefs (grouped by grow-out reef), as well as between juveniles growing at different reefs (grouped by parental reef). Mortality of juveniles at Orpheus Island (possibly due to high sedimentation which smothered the corals) prevented the recovery of any juveniles from Magnetic Island parental colonies for the 2004 and 2005 cohorts. A few juveniles from these cohorts were recovered from Orpheus Island parental colonies but these were pooled with the 2006 cohort for the analysis. Data from juveniles reared from Davies Reef adults were excluded from the analysis because the number of survivors and samples for which the ITS1 region was successfully amplified was very low.

2.3 Results

2.3.1 Uptake in *A. tenuis* juveniles

Symbiodinium type D was the dominant (only band detected in SSCP profile) symbiont taken up by the majority of *A. tenuis* juveniles. This was particularly evident in juveniles growing at Magnetic Island, where the proportion of D-dominated juveniles consistently ranged from 81-100% regardless of cohort or parental reef (Fig. 2.3). *Symbiodinium* C1 was the only other type detected at Magnetic Island but was dominant in very few of the juveniles (up to 12% in a single cohort, 3% overall, Fig. 2.3). A small proportion of the juveniles (up to 19% in a single cohort, 12% overall) at Magnetic Island harboured types C1 and D simultaneously (Fig. 2.3). At Orpheus Island, type D *Symbiodinium* was dominant in 64-89% of the juveniles, regardless of parental reef (Fig. 2.3). Types C1 and A (GenBank Accession AF380513) were also detected in a few juveniles at Orpheus Island (Fig. 2.3). There were no significant differences in the distribution of *Symbiodinium* types between cohorts of the same parental reef or between juveniles from different parental reefs growing at Magnetic Island (Fig. 2.3 top row, see Table 2.2 for χ^2 test results). There were also no significant differences in the *Symbiodinium* types harbored by juveniles from Orpheus Island growing at different reefs (i.e., Orpheus Island juveniles growing at Magnetic Island or Orpheus Island), but this was not the case for juveniles from Magnetic Island, which harbored significantly different symbiont communities between grow-out reefs (Fig. 2.3, χ^2: 10.925, d.f. 4, $p = 0.019$, Table 2.2). This is illustrated by the higher proportion of juveniles with mixed symbiont communities, including combinations with clade A *Symbiodinium*, which were not found at Magnetic Island (Fig. 2.3).

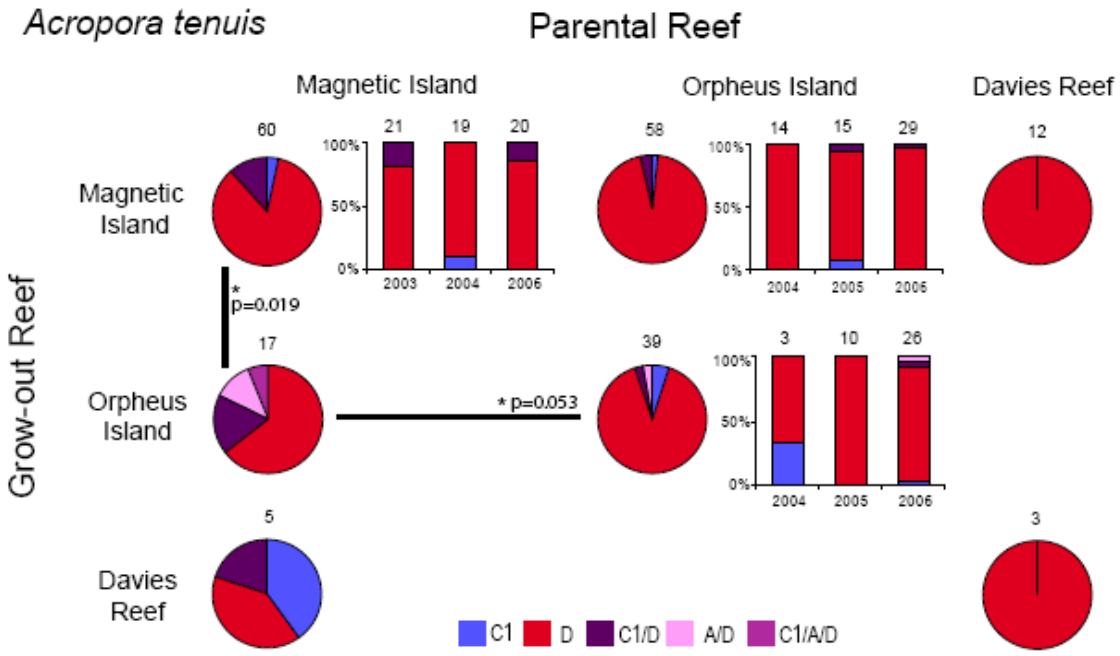


Fig. 2.3. Uptake of *Symbiodinium* in *Acropora tenuis* juveniles. Pie charts represent the proportion of juveniles dominated by different *Symbiodinium* types. Bar graphs next to some pie charts represent the uptake in juveniles where data for more than one cohort was obtained (year of cohort indicated on x-axis). Pie charts next to these graphs represent pooled data from the bar graphs. Numbers above all graphs/charts represent the number of juveniles for which the ITS1 region was successfully amplified. The symbiosis in adult *A. tenuis* at Magnetic Island, Orpheus Island and Davies Reef is dominated by *Symbiodinium* type C1, C2, and C2, respectively. Lines with * connecting pie charts indicate significant differences between corals at either end of the line (p value noted).

2.3.2 Uptake in *A. millepora* juveniles

Type D *Symbiodinium* was the dominant symbiont taken up by most of the *A. millepora* juveniles. Similar to the observations in *A. tenuis* juveniles, this was most evident at Magnetic Island, where 89-100% of all the juveniles were dominated by type D regardless of cohort or parental reef (Fig. 2.4). Type C1 *Symbiodinium* was detected in very few juveniles either as dominant (up to 5% in a single cohort, 1% overall) or in combination with type D (up to 11% in a single cohort, 1% overall, Fig. 2.4). Mortality of juveniles at Orpheus Island prevented the recovery of any juveniles from Magnetic Island parental colonies for the 2004-2005 cohorts. *Symbiodinium* types C1, C2 and A were also detected in juveniles at Orpheus Island but always in less than 15% of the

juveniles, except for 2004 juveniles, where 75% of the juveniles sampled had C1 and 25% had C2 (Fig. 2.4). Overall, type D was dominant in 70-76% of the juveniles growing at Orpheus Island, regardless of parental reef (Fig. 2.4). There were no significant differences in the *Symbiodinium* types harbored by different cohorts of the same parental reef or between juveniles from different parental reefs placed at the same grow-out reef (Fig. 2.4, Table 2.2). However, the *Symbiodinium* community in juveniles from the same parental reef but placed in different grow-out reefs was significantly different (Fig. 2.4, χ^2: 19.751, d.f. 3, $p < 0.001$ for juveniles from Magnetic Island parents and χ^2: 14.598, d.f. 4, $p = 0.005$ for juveniles from Orpheus Island parents, Table 2.2). This was illustrated by the higher proportion of juveniles with mixed symbiont communities found at Orpheus Island where up to five different associations were found, including combinations with clade A *Symbiodinium* which were not found at Magnetic Island (Fig. 2.4).

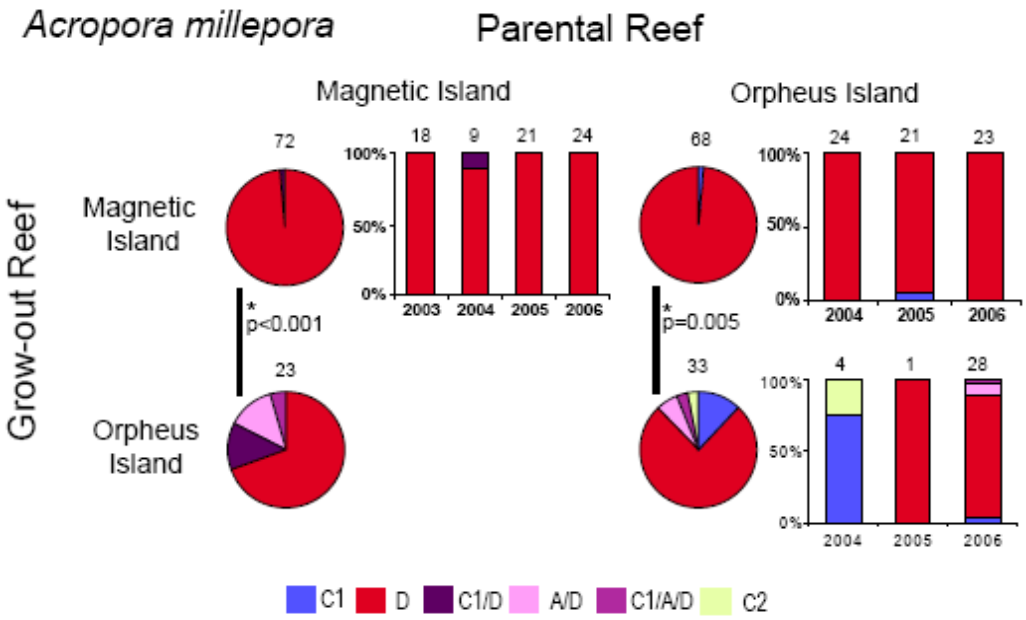


Fig. 2.4. Uptake of *Symbiodinium* in *Acropora millepora* juveniles. Pie charts represent the proportion of juveniles dominated by different *Symbiodinium* types. Bar graphs next to some pie charts represent the uptake in juveniles where data for more than one cohort was obtained (year of cohort indicated on x-axis). Pie charts next to these graphs represent pooled data from the bar graphs. Numbers above all graphs/charts represent the number of juveniles for which the ITS1 region was successfully amplified. The symbiosis in adult *A. millepora* at Magnetic Island and Orpheus Island is dominated by *Symbiodinium* type D and C2, respectively. Lines with * connecting pie charts indicate significant differences between corals at either end of the line (p value noted).

Table 2.2. Comparisons of *Symbiodinium* distributions in *A. tenuis* and *A. millepora* juveniles.

Comparisons between cohorts of the same parental reef (grouped by grow-out reef)

Spp.	Parental reef	Grow-out reef	Cohorts	χ^2	df	P
A. tenuis	Magnetic Island	Magnetic Island	2003, 2004, 2006	6.206	4	0.100
	Orpheus Island	Magnetic Island	2004 - 2006	4.468	4	0.197
A. millepora	Magnetic Island	Magnetic Island	2003 - 2006	7.099	3	0.069
	Orpheus Island	Magnetic Island	2004 -2006	2.272	2	0.321

Comparisons between juveniles from different parental reefs (grouped by grow-out reef, data pooled for groups with multiple cohorts)

Spp.	Grow-out reef	Parental reefs	χ^2	df	P
A. tenuis	Magnetic Island	Magnetic Island vs Orpheus Island	4.442	4	0.301
	Orpheus Island	Orpheus Island vs Magnetic Island	8.017	4	0.053
A. millepora	Magnetic Island	Magnetic Island vs Orpheus Island	2.004	2	0.367
	Orpheus Island	Orpheus Island vs Magnetic Island	8.666	5	0.123

Comparisons between juveniles growing at different reefs (grouped by parental reef, data pooled for groups with multiple cohorts)

Spp	Parental reef	Grow-out reefs	χ^2	df	P
A. tenuis	Magnetic Island	Magnetic Island vs Orpheus Island	10.925	4	0.019
	Orpheus Island	Orpheus Island vs Magnetic Island	2.485	3	0.478
A. millepora	Magnetic Island	Magnetic Island vs Orpheus Island	19.751	3	<0.001
	Orpheus Island	Orpheus Island vs Magnetic Island	14.598	4	0.005

2.3.3 Effect of light environment on *Symbiodinium* uptake

There was no difference in the type of *Symbiodinium* taken up by coral juveniles exposed to different irradiance levels. *A. tenuis* and *A. millepora* were both dominated by type D *Symbiodinium* even though juveniles on tiles receiving straight down-welling light (mean maximum irradiance of 540 μmol quanta m^{-2} s^{-1}) were exposed to almost

four times the amount of light as in the vertically-arranged tiles (mean maximum irradiance of 140 µmol quanta m^{-2} s^{-1}, Fig. 2.5).

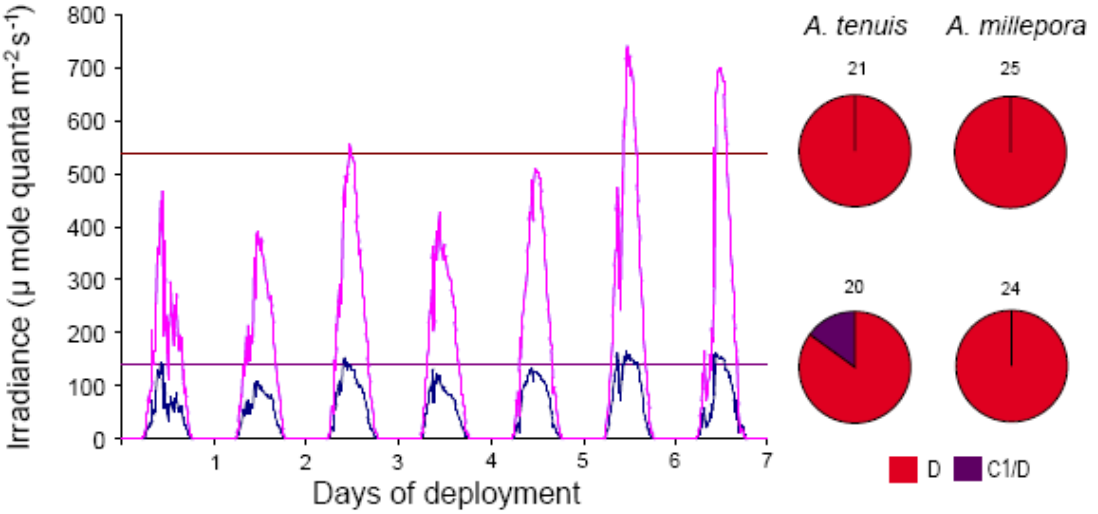


Fig. 2.5. Uptake of *Symbiodinium* in different light environments. Pie charts show the proportion of *A. tenuis* and *A. millepora* juveniles dominated by different *Symbiodinium* types. Top two represent juveniles on tiles horizontally deployed and bottom two represent juveniles on vertically deployed tiles. Graph on the left shows the typical light environment the juveniles were exposed to (horizontal tiles in pink, vertical tiles in blue). PAR sensors were deployed for one week next to the juveniles, which were sampled after approximately four weeks. Straight lines across this graph are the mean maximum irradiance in one week of monitoring.

2.3.4 *Symbiodinium* diversity and relative abundance on the reef

Fifty-two species of cnidarian hosts distributed across 23 scleractinian genera and 6 non-scleractinian genera at Magnetic Island (Nelly Bay) were all found to harbour *Symbiodinium* types belonging to clades C or D. Thirty-three species harboured *Symbiodinium* clade C, 15 had clade D and 4 harbored a mix of both clades (Fig. 2.6, Table 2.3). These proportions of *Symbiodinium* types in the adult coral community suggest that it would be unlikely that the proportions of juveniles with clade C, D, or C/D mix would fit a random distribution (33.3% for each, χ^2=24.986, p<0.001). Sequences from 20 of the 33 hosts harbouring clade C *Symbiodinium* were identical to type C1 *Symbiodinium*. Three were identical to *Symbiodinium* type C• (sensu van

Oppen 2004, GenBank Accession # AY327054). The remaining 10 sequences had 1-8 substitutions from sequences corresponding to types C1, C2 and C•. These sequences are referred to as C1-like or C2-like and were not named because determining whether these symbionts represent distinct strains based on ecological distributions or intragenomic variants was outside the scope of the present study. Fifteen taxa (23%) hosted *Symbiodinium* clade D (Fig. 2.6, Table 2.3). Fourteen of these were identical to the type D found in adult *A. millepora* as well as in the juveniles of both *A. millepora* and *A. tenuis*. The remaining host had *Symbiodinium* with a sequence that had one substitution from type D. This type is referred to as D-like. Four cnidarian hosts were found harbouring symbionts from both clades (Fig. 2.6). The estimated relative abundance of the two *Symbiodinium* clades found in cnidarians at Magnetic Island (Nelly Bay), based on the relative abundance of the same host genera was 66% for C, and 34% for D. Almost 70% of the estimated clade D abundance was found in *Acropora* spp. corals, where 12 out of 14 species sampled were found in association with clade D (Table 2.3).

Symbiodinium clades C or D were also the only ones found in fifty-four host species across 29 scleractinian genera and three genera of soft corals at Orpheus Island (Pioneer Bay). The vast majority (79.6%) of the hosts exclusively harboured *Symbiodinium* clade C (Fig. 2.6, Table 2.3) and only six scleractinian species (11%) were found exclusively hosting *Symbiodinium* clade D (Fig. 2.6, Table 2.3). Five species were found hosting a mix of clade C and D symbionts either simultaneously or in different replicate samples (Table 2.3). As with the Magnetic Island hosts, these proportions of *Symbiodinium* types in the adult coral community suggest that it would be unlikely that the proportions of juveniles with clade C, D, or C/D mix would fit a random distribution (χ^2=51.455,

p<0.001). Of the taxa hosting *Symbiodinium* clade C, 11 corresponded to type C1, 18 to type C2, 1 to type C•, and the remaining 13 had symbionts with sequences that had 1-4 substitutions from type C1 (C1-like, Table 2.3). For the taxa hosting *Symbiodinium* clade D, three of them corresponded to type D and the remaining three had sequences with two substitutions from the type D sequence (D-like, Table 2.3). The estimated relative abundance of the two *Symbiodinium* clades found in cnidarians at Orpheus Island (Pioneer Bay), based on the relative abundance of the hosts was 92% for C and only 8% for D. However, unlike Magnetic Island, there was no clear pattern in the distribution of host taxa harboring clade D. For example, only ten percent of the hosts in the family Acroporidae were found hosting clade D (Table 2.3).

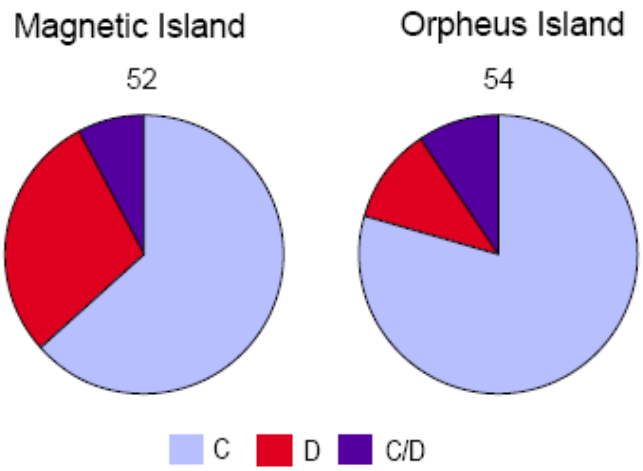


Fig. 2.6. *Symbiodinium* diversity and distribution in cnidarian hosts on the reefs at Magnetic and Orpheus Islands. Pie charts show the proportion of cnidarians species surveyed (n=52 for Magnetic Island and 54 for Orpheus Island) hosting *Symbiodinium* types within clade C, D, or a mix of both.

Table 2.3. *Symbiodinium* diversity in cnidarian hosts at Magnetic and Orpheus Islands (GBR). Number in brackets next to symbiont types indicates the number of colonies sampled. Not all hosts were found at both sites.

	***Symbiodinium* types in scleractinian hosts**		
Family	**Species**	**Magnetic Island**	**Orpheus Island**
Acroporidae	*Acropora caroliniana*		C2 (1)
	A. cytherea	D (6)	
	A. digitifera	C1-like/C2-like (1)	
	A. divaricata		C2 (3)
	A. florida		C2 (1)
	A. elseyi	D (3)	
	A. gemmifera		C2 (3)
	A. grandis		C2 (2)
	A. humilis	D (1)	
	A. hyacinthus	D (2)	C2 (2)
	A. latistella	D (2)	
	A. loripes		C2 (1)
	A. millepora	D (18)	C2 (15)
	A. muricata	D (3)	
	A. nasuta	D (1)	
	A. pulchra	D (3)	
	A. sarmentosa		C2 (1)
	A. secale		C2 (1)
	A. spathulata		C2 (2)
	A. subulata		C2 (1)
	A. tenuis	C1 (18)	C2 (15)
	A. torresiana	D (1)	
	A. valida	C1-like (2), D (4)	C1/D (1)
	A. vaughani		C2 (1)
	A. willisae	D (1)	
	A. Isopora cuneata		D (1)
	Astreopora myriophtalma		C1 (2)
	Montipora aequituberculata	C• (1)	
	M. crassituberculata	C• (3)	
	M. danae		C1 (1)
	M. peltiformis		C1 (1)
	M. turgescens		C2 (1)
Agariciidae	*Pachyseris rugosa*	C1 (1)	
	P. speciosa		C1-like (1)
	Pavona decussata		C1 (1)
	P. maldivensis		C1-like (1)
	P. varians		C1-like (1)
Caryophylllidae	*Euphyllia ancora*	C1 (1)	
Dendrophylliidae	*Turbinaria mesenterina*	C1 (3)	
	T. frondens	C1 (1), D (2)	
	T. peltata		C1 (2), C1-like

			(1)
Faviidae	*Caulastrea furcata*		C1-like (1)
	Cyphastrea decadia		C1 (1)
	C. serailia		C1 (2)
	Diploastrea heliopora		D (2)
	Echinopora horrida		C1-like (1)
	E. lamellosa	C1 (1), C1-like (1)	D-like (1)
	Favia maritima		C2 (1)
	F. matthaii	C1 (1)	
	Favites abdita	C1 (1)	
	F. complanata	C1 (1)	C2 (1)
	F. flexuosa	C1 (1)	
	F. halicora	C1-like (1)	
	F. russelli	C1 (1)	
	Leptoria phrygia		C1 (1)
	Montastrea valenciennesi		C1-like (1)
	Oulophyllia crispa		C1-like (1), D-like (1)
	Platygyra daedalea	C1 (2), D (1)	
	P. pini	C1-like (1)	
	P. verweyi	D (1)	C2 (1)
	Plesiastrea versipora	C1 (5)	
Fungidae	*Fungia fungites*	C1 (3)	
	Herpolitha limax	C1 (1), C1-like (1)	
	Podobacia crustacea		C1 (1), D-like (1)
	Polyphyllia talpina	C1 (1)	C1 (1)
Merulinidae	*Hydnophora exesa*	C1 (3)	C1-like (1), D-like (1)
	Merulina ampliata	D (1)	D-like (1)
Mussidae	*Acanthastrea hemprichii*		C1 (1)
	Lobophyllia hemprichii	C1 (1), C1-like (2)	C1-like (1), D-like (1)
Oculinidae	*Galaxea astreata*		C1-like (2)
	G. fascicularis	D (3)	
Pectinidae	*Echinophyllia aspera*	C1 (2)	
	E. orpheensis		D-like
	Mycedium elephantotus	C1 (2)	
	Pectinia paeonia		C1-like (1)
Pocilloporidae	*Pocillopora damicornis*	C1 (1)	
	P. verrucosa		C1 (1)
	Stylopora pistillata		D (1)
Poritidae	*Goniopora lobata*		C1 (1)
	G. tenuidens	C1 (2)	
	Porites australiensis	C• (3)	C• (2)
Siderastreidae	*Psammocora contigua*	C1 (2)	
	P. nierstraszi	C1 (2)	

***Symbiodinium* types in non-scleractinian hosts**

Order	Genus	*Symbiodinium* type	
Aclyonacea	*Briareum spp.*	C1 (3)	
	Capnella spp.	D/D-like (1)	
	Cladiella spp.	C1 (1), C1-like (1)	
	Lobophyton spp.	C1-like (1), D (1)	C1-like (1)
	Sarcophyton spp.		C1-like (1)
	Sinularia spp. 1	C1-like (1)	C1 (1), C1-like (1)
	Sinularia spp. 2	C1-like (1)	C1-like (1)
Hydrocorallina	*Millepora spp*	C1 (1)	

2.4 Discussion

2.4.1 Non-specific uptake of *Symbiodinium* in coral juveniles is dominated by highly infectious/opportunistic types

This study demonstrates that newly settled *A. millepora* and *A. tenuis* coral juveniles do not necessarily take up the *Symbiodinium* type present in the parental colonies, nor the one that is most dominant in the endosymbiotic communities of a variety of other hosts on the reef. Instead, juveniles of both species acquired mostly *Symbiodinium* type D regardless of parental association or the reef where they were placed to grow. With the exception of *A. millepora* colonies from Magnetic Island, type D *Symbiodinium* is not found in any of the parental colonies where the juveniles were sourced from (locally homologous, see Fig. 2.1). These results confirm findings of limited specificity in the type of symbiont initially acquired by corals with a horizontal mode of symbiont transmission. In larvae of the coral *Fungia scutaria,* multiple types of *Symbiodinium* are phagocytosed as soon as the mouth develops and short-term studies (less than one week) show that *Symbiodinium* from homologous as well as heterologous sources are able to enter the gastrodermal layer (Schwarz *et al.* 1999; Weis *et al.* 2001; Rodriguez-Lanetty *et al.* 2004). There are differences in infection rates, cell densities, and distribution of *Symbiodinium* in larvae with homologous symbionts when compared to

larvae with non-homologous symbionts (Rodriguez-Lanetty *et al*. 2006). Nevertheless, what these studies show is that, at least in the short term, non-homologous types are taken up and maintained. Similarly, studies with settled coral juveniles (Little *et al.* 2004; Gomez-Cabrera *et al.* 2008) or juvenile gorgonians (Coffroth *et al*. 2001) show non-specific uptake, even if the eventual symbiosis reflects homologous patterns (Coffroth *et al*. 2001). Hence, the evidence suggesting that there are few barriers to the uptake of non-homologous symbionts by corals during early ontogeny is compelling.

The overall dominance of type D in juveniles at all locations may be due to intrinsic properties of types within this clade that make it highly infectious and potentially opportunist (Toller *et al.* 2001b). This would allow it to quickly dominate the symbiosis in aposymbiotic juvenile corals. Clade D *Symbiodinium* was also found along with clade A *Symbiodinium* (another potentially opportunist clade, Toller *et al.* 2001a; Baker 2003) in 10 and 83 day old juveniles of *Acropora longicyathus* in the southern GBR (Gomez-Cabrera *et al.* 2008). Here again, none of the parental colonies from which the juveniles were sourced harboured clade D (Gomez-Cabrera *et al*. 2008). This ability to colonize vacant hosts may also explain why clade D *Symbiodinium* has been found in corals after episodes of bleaching or disease (Baker 2001; Toller *et al.* 2001a). Further support to the notion of a highly infectious type D *Symbiodinium* comes from the results of the surveys of *Symbiodinium* diversity at Magnetic and Orpheus Islands. Despite dominating the juvenile populations at both sites, it was only found in roughly one third of the host taxa surveyed at Magnetic Island, and less than 10% of the host taxa at Orpheus Island. It is also possible that juveniles may be actively taking up type D *Symbiodinium*, particularly at Magnetic Island, where the majority of *Acropora* corals surveyed were found hosting type D. However, *A. tenuis* was not one of these species,

thus it becomes more difficult to explain active selection, which would require development of specific recognition mechanisms for a *Symbiodinium* type that is not going to be kept for long. It is clear that type D is excellent at colonizing newly settled juveniles but, in the vast majority of corals, it does not remain as the dominant symbiont as shown by adult patterns of association at both sites in our study, as well as elsewhere in the GBR (van Oppen *et al.* 2001; LaJeunesse *et al.* 2003; van Oppen *et al.* 2005; Gomez-Cabrera *et al.* 2008). Several explanations are possible for these patterns including competitive exclusion by other symbionts after uptake, active selection by the host, mortality of juveniles with type D, or a combination of these. Further studies following the succession of symbionts in coral juveniles should help clarify this question.

There are three important caveats in the present study that require consideration. The first one involves the use of settled juveniles to examine uptake patterns. It has been shown that coral larvae are capable of acquiring symbionts well before settling and metamorphosing (Schwarz *et al.* 1999; Weis *et al.* 2001; Rodriguez-Lanetty *et al.* 2004). Coral larvae exhibit searching behaviour before settling (Harrison & Wallace 1990), which raises the possibility that they'll come into contact with sources of *Symbiodinium* before settling on the reef. Hence, the mobile nature of larvae may expose them to a potentially wider range of *Symbiodinium* than settled juveniles. In addition, using settled juveniles required a delay in symbiont acquisition while the larvae settled (when compared to larvae on the reef, which may potentially acquire symbionts as larvae). Although this was only a few days, it is possible that this delay may have compromised the health of the juveniles and resulted in the dominance of type D *Symbiodinium*. This would be consistent with literature showing that members of

clade D *Symbiodinium* are quick to colonize available space in health compromised corals (Baker 2001; Toller *et al*. 2001a). However, this does not change our conclusion that initial uptake is dominated by a highly infectious/opportunistic *Symbiodinium* type which is quick to colonize available spaces, regardless of whether they are in health-compromised corals or simply in corals which do not have any symbionts. Furthermore, in experiments with coral larvae exposed to sediments from the same sites as in the present study, type D *Symbiodinium* dominated the symbiosis initially (VR Cumbo, unpublished data). These larvae did not experience a delay in exposure to symbionts and had similar uptake patterns than the settled juveniles in our study.

The second caveat of the present study is that the estimate of diversity and abundance of *Symbiodinium* available for uptake was based on results from the survey of *Symbiodinium in hospite.* This ignores other potential and likely sources of *Symbiodinium* such as sediment on the reef and the water column itself and is illustrated by the observation of uptake of *Symbiodinium* clade A in combination with C1 and/or D in juveniles at Orpheus Island, despite the fact that *Symbiodinium* clade A was not detected in the survey of cnidarian hosts at that site. Invertebrate hosts with clade A *Symbiodinium* include giant clams (Baillie *et al*. 2000), which occur at Orpheus Island but were not sampled for this study. Furthermore, clade A *Symbiodinium* has been found in reef sediments and sand (Coffroth *et al.* 2006; Hirose *et al*. 2008; Adams *et al.* 2009). Using juvenile gorgonians as symbiont traps, Coffroth *et al*. (2006) detected *Symbiodinium* from clades A-C in the water column and cultured several isolates from reef sediments which had DNA sequences that fit within established *Symbiodinium* phylogenies, in some cases with identical or near identical sequences to established symbiotic types. However, many of these isolates were not culturable and/or were not

capable of establishing a symbiosis (Coffroth *et a*l. 2006). Another recent study examining the diversity of free living *Symbiodinium* found several types in the water column above the reef (Manning & Gates 2008). While this study did not specifically test whether the free living strains identified could infect cnidarian hosts, it did find several types with identical DNA sequences to named *Symbiodinium* types found *in hospite* on the same reefs. It was also noted that the diversity of *Symbiodinium* in the water column closely matched the diversity of *Symbiodinium in hospite* but some of the types found in the water column were new and not all the types which occur *in hospite* were found in the water column (Manning and Gates 2008). It is presently not known how abundant and how commonly the *Symbiodinium* types found in water and sediments are taken up by aposymbiotic corals, but this is clearly an area that requires further research. Finally, a third factor to consider is the possibility that not all *Symbiodinium* types were detected due to the low level of replication within host species, which may have masked intra-specific variation in symbiont assemblages (Ulstrup & van Oppen 2003; Sampayo *et al.* 2007) or due to their presence at background levels which the genetic technique employed in the present study failed to detect (Fabricius *et al.* 2004; Mieog *et al*. 2007). Due to these caveats it is likely that the diversity of *Symbiodinium* at Magnetic and Orpheus Islands estimated in the present study was an underestimation of the true level of diversity.

The reasons behind the apparent affinity of *Acropora* corals at Magnetic Island for *Symbiodinium* type D are not clear but may be related to the inherent characteristics of this site and possible physiological attributes of type D *Symbiodinium*. The slightly warmer waters surrounding Magnetic Island, relative to other reefs in the central GBR (*in situ* data available from: http://www.aims.gov.au/docs/data-

centre/seatemperatures.html), may be providing a haven for this *Symbiodinium* type, which appears more thermally tolerant than other types (Berkelmans & van Oppen 2006; but see Abrego *et al.* 2008). Fabricius *et al.* (2004) found a high proportion of corals hosting clade D *Symbiodinium* in a lagoon with a warmer temperature regime than surrounding waters in the Republic of Palau. In contrast to a nearby reef, the corals within this lagoon suffered little or no mortality following the global 1998 bleaching event, leading the authors to hypothesize that the thermal tolerance of these corals was largely attributable to the association with clade D *Symbiodinium* (Fabricius *et al.* 2004). Corals within the genus *Acropora* are amongst the most bleaching-sensitive taxa (Marshall & Baird 2000). Thus, it is possible that the high affinity of *Acropora* corals for type D *Symbiodinium* at Magnetic Island may be due to the potential thermal tolerance that may be gained from this association. It is worth noting that thermal tolerance may not always be gained by this association, as shown in *A. tenuis* juveniles (Abrego *et al.* 2008). This may also explain why this species does not host type D *Symbiodinium* at Magnetic Island.

2.4.2 No effect of light on *Symbiodinium* selection

The results of the tile orientation experiment showed that the amount of light reaching the juveniles did not affect the type of *Symbiodinium* taken up. This result was surprising since niche partitioning of different *Symbiodinium* types according to light environment in the reef has been documented for several corals (Rowan & Knowlton 1995; Iglesias-Prieto *et al.* 2004), including *Acropora tenuis* and *A. valida* (Ulstrup & van Oppen 2003;). Despite the almost four-fold difference in light levels between horizontal and vertical tiles, the juveniles of both species were overwhelmingly dominated by type D *Symbiodinium*, which suggests that factors other than light (at

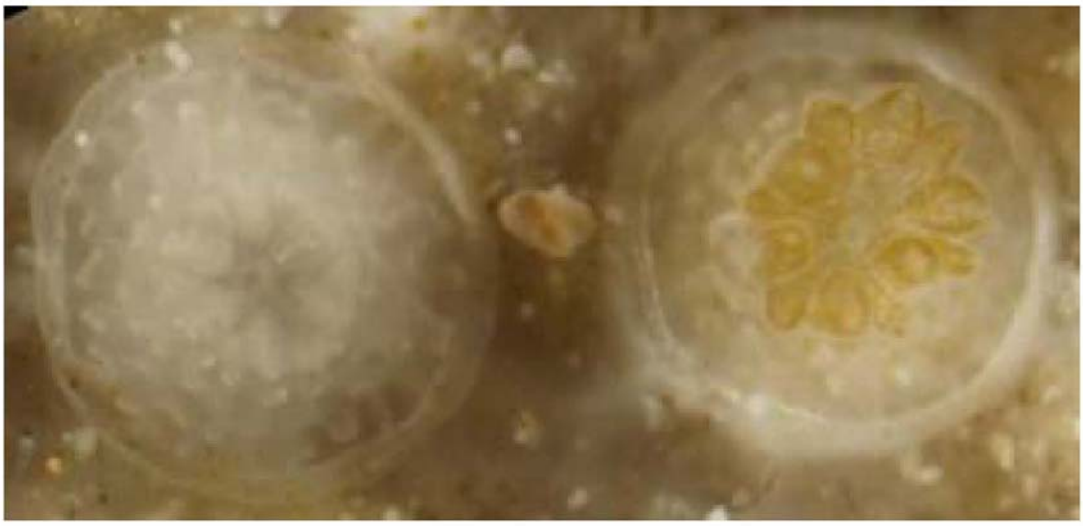
least at the levels detected in our study) determine the initial association in coral juveniles.

Although the average maximum irradiance reaching the low light treatment juveniles was only 140 μ moles quanta m^{-2} s^{-1} (Fig. 2.5), it is estimated that recruits at this site are exposed to very low light levels given that available spaces are often in partially shaded areas, such as crevices. Light levels in the high light treatment reflect the high turbidity characteristic of Magnetic Island, where underwater visibility rarely exceeds 4-6 m. In addition, the reef at Nelly Bay faces a major shipping channel into the Port of Townsville. Thus, the light levels that coral recruits are exposed to in this reef are likely to be quite low and the difference in light levels documented in this study corresponds well with in-situ measurements taken at Magnetic Island reefs in other published studies (Babcock & Mundy, 1996; Anthony *et al.* 2004; Sofonia & Anthony 2008).

It is also possible that the overall low light levels at Magnetic Island may favour a 'shade-loving' symbiont such as type D (Ulstrup & van Oppen 2003), and result in preferential uptake of type D by coral juveniles, even if this type does not remain as the dominant symbiont. This is consistent with the results of the *Symbiodinium* survey at the Magnetic Island site, which show that type D *Symbiodinium* is not the most common and abundant type *in hospite* on the reef, despite the fact that practically all the taxa surveyed were broadcast spawning species that acquire their symbionts horizontally (Babcock *et al.* 1986; Harrison & Wallace 1990; Richmond & Hunter 1990; Hayashibara *et al.* 1993). Nevertheless, the low light levels on the reef are not limiting the presence of other *Symbiodinium* types. Although the present study focused on uptake in only two species of coral; future studies examining uptake and succession of

Symbiodinium in other host taxa will clarify whether type D is also taken up initially by other species. The evidence on the flexibility of coral juveniles to acquire different symbiont types from those found in parental colonies is compelling. Future studies should also focus on the cellular mechanisms behind symbiont recognition to enhance our understanding of how these critically important symbioses are formed.

Chapter 3.0 Impact of light and temperature on the uptake of algal symbionts by juveniles of *Acropora tenuis* and *A. millepora*



Two juvenile *Acropora* corals in different stages of *Symbiodinium* uptake. Photo: David Abrego

3.1 Introduction

The horizontal mode of *Symbiodinium* acquisition of many symbiotic marine cnidarians, including the majority of reef-building corals, provides an ideal model to test questions relevant to the formation and maintenance of these symbioses, including specificity, timing of the processes leading to stable partnerships, and exploration and manipulation of factors that may affect these processes. The diversity of *Symbiodinium* (8 major lineages or clades and many sub-lineages, see reviews by Baker 2003, Coffroth and Santos 2005), has prompted many studies that have documented the uptake of multiple symbiont types by aposymbiotic cnidarian juveniles (Schwarz *et al.* 1999; Coffroth *et al.* 2001; Weis *et al.* 2001; Little *et al.* 2004; Gomez-Cabrera *et al.* 2008) or adult cnidarians (Fitt 1985; Davy *et al.* 1997; Kinzie *et al.* 2001; Berkelmans & van Oppen 2006; Jones *et al.* 2008a). These studies have shown that corals and other cnidarians can be infected by *Symbiodinium* types different from those previously detected in their tissues or different from those found in parental colonies. All of these studies have taken place at ambient, non-stressful temperatures either in the field or in controlled experiments. Although these works provide valuable insights into the formation of the symbioses, there is a gap in our understanding of whether such flexibility persists during stressful environmental conditions, such as those expected to become more common under climate change models (IPCC 2007), to provide options for survival of the coral holobiont.

Understanding how warming oceans are likely to affect the dynamics of *Symbiodinium* uptake will depend on knowledge of how different *Symbiodinium* types interact within the coral host under different temperature regimes. This is particularly relevant for corals which acquire their symbionts horizontally. Differences in the physiological

response of different *Symbiodinium* types to temperature and light are well documented (Iglesias-Prieto & Trench 1997a; Kinzie *et al.* 2001; Tchernov *et al.* 2004; Robison & Warner 2006). Because more than one type of *Symbiodinium*, including non-homologous types, may be taken up by these corals, there is the potential that intrinsic physiological differences between types may confer competitive advantages to some types under different thermal regimes and result in altered endosymbiont communities. Such competitive advantages under different environmental conditions may underlie the shuffling response documented in some adult corals hosting multiple *Symbiodinium* types (Rowan *et al.* 1997; Berkelmans & van Oppen 2006; Jones *et al.* 2008a). While this may be an important acclimatization mechanism in adult corals with established symbioses, it is not known whether similar competitive processes occur within coral juveniles during initial uptake of potentially different types of *Symbiodinium*.

Virtually nothing is known about whether temperature and light affect the establishment of the symbiosis. Only one study has examined the effect of light on the acquisition of symbionts by juvenile corals and it found no difference in the percentage of infected larvae of the coral *Fungia scutaria* when exposed to ambient levels of light or virtual darkness (Weis *et al.* 2001). The results of Chapter 2 also suggest that light, at least at relatively low levels, does not appear to play a significant role in symbiont selection on the reef. Given that temperature and light are the main triggers for the breakup of the association (bleaching), it is critical to improve our understanding of how they might affect the establishment of the symbiosis and the type of *Symbiodinium* acquired. Such knowledge will enable predictions about how these relationships might change in response to warming oceans. This study uses experimental manipulations to examine whether light and temperature influence the densities of *Symbiodinium* established in

juveniles of *Acropora tenuis* and *A. millepora*, as well as whether these factors affect the type of *Symbiodinium* acquired. *Symbiodinium* preference was tested by comparing relative uptake of type D, typically characterized as heat tolerant (Baker *et al.* 2004; Fabricius *et al.* 2004; Rowan 2004), versus type C1, typically characterized as light tolerant (Ulstrup & van Oppen 2003) under differing combinations of temperature and light. Specifically, I tested whether: (1) coral juveniles are equally able to acquire symbionts under different combinations of temperature and light and, (2) coral juveniles challenged with two different *Symbiodinium* types under these treatments establish symbioses with these types in equal ratios.

3.2 Materials and methods

3.2.1 Experimental corals, *Symbiodinium* inoculation, and genetic identification

Juvenile corals of *Acropora tenuis* and *A. millepora* were raised and settled on terracotta tiles after the coral spawning events of 2005 and 2007 at Magnetic Island (19°.10'S, 146°.50'E) in the Great Barrier Reef. Following the procedures described in Little *et al.* (2004), type C1 and D *Symbiodinium* (freshly isolated from adult *A. tenuis* and *A. millepora* colonies from Magnetic Island, respectively) were used to inoculate aposymbiotic juveniles of both species. These types were chosen because both have been detected in juveniles of both coral species at Magnetic Island (Little *et al.* 2004). Both types of *Symbiodinium* were offered simultaneously to the juveniles in equal concentrations and volumes.

3.2.2 Experimental design

Tiles with settled coral juveniles were placed at three temperatures (28°C, 30°C, 31°C) and two light treatments (Low light: 180 µmol photons m^{-2} s^{-1}; and High light: 390 µmol

photons m^{-2} s^{-1}) in temperature-controlled rooms at Orpheus Island Research Station. The photoperiod was 10 hrs light: 14 hrs dark. Within each of the six temperature by light treatments, tiles with corals were haphazardly assigned to four replicate containers with flow-through filtered seawater (1 μm). The number of settled juveniles on each tile varied but approximately the same number of juveniles was allocated to each treatment. The volume of water in the containers was 7 L and the flow rate into the containers was approximately 0.5 L $minute^{-1}$. Corals were maintained at these temperature by light treatments for 20 (*A. tenuis*) or 30 days (*A. millepora*).

To explore the differential uptake of *Symbiodinium* types in coral juveniles, types C1 and D were added simultaneously to the containers every day during the first half of each experiment. The density of symbionts added to the containers ranged between 1-5 x 10^4 cells mL^{-1}. Water flow through the tanks was stopped immediately before adding *Symbiodinium* and tanks were maintained as static cultures for 16-18 hours. After this incubation period, flow was restored into the containers to replace the water and flush them with new sea water for approximately 6 hours before inoculating again. Corals were maintained in filtered sea water (1 μm) and no *Symbiodinium* was added after the half-way point in each experiment in order to explore the dynamics of establishment of *Symbiodinium* after the initial inoculation phase.

3.2.3 Effects of temperature and light on *Symbiodinium* uptake

To assess the impact of temperature and light on the combined uptake of the two *Symbiodinium* types, the number of juveniles in each treatment was counted on the last day of *Symbiodinium* inoculation (day 10 for *A. tenuis* and day 15 for *A. millepora*) and the level of uptake was assessed visually. Each juvenile was assigned to one of two

categories (more categories would have introduced subjective bias): juveniles were scored as white when no pigmentation was visible under a dissecting microscope (see Fig. 3.1), or pigmented when either the main body of the juveniles or the area around the mouth/tentacles exhibited pigmentation (Fig. 3.1, see Table 3.1 for sample sizes). This procedure was repeated on the last day of the experiment (day 20 for *A. tenuis* and 30 for *A. millepora*). Given that almost every container had well over 100 juveniles, counts for white and pigmented juveniles were used to calculate a pigmentation ratio for all juveniles in each container (number of pigmented juveniles over the total number of juveniles in the container) as a measure of uptake efficiency. In addition, the relative survival of juveniles in each temperature by light treatment was calculated based on the number of surviving juveniles at the end of the experiment relative to the number of juveniles at the end of the inoculation phase (mid-point of the experiment). To account for juveniles sacrificed for analyses during the course of the experiment, the number of juveniles sub-sampled between the census at the mid-point of the experiment and that at the end of the experiment was subtracted from the calculation.

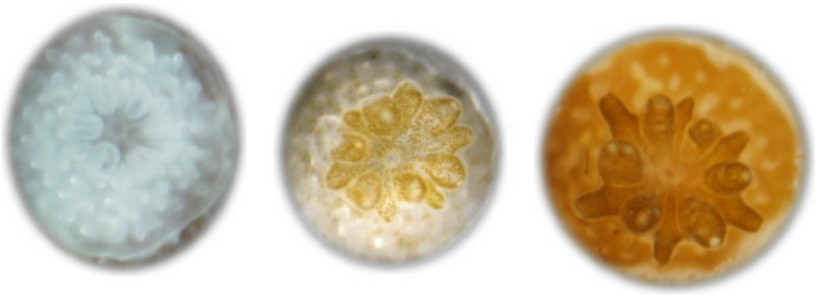


Fig. 3.1. Visual assessment of *Symbiodinium* uptake. Juveniles were scored in two categories (white or pigmented) according to pigmentation levels. The specimen on the left is a typical "white" juvenile while the two on the right represent a range of "pigmented" juveniles.

3.2.4 Effects of temperature and light on the type of *Symbiodinium* acquired and maintained by coral juveniles

To quantify whether the establishment of *Symbiodinium* types C1 and D occurred in equal ratios at each of the temperature by light treatments, coral juveniles were sub-sampled every other day during the experiment. At each treatment, 10 (*A. millepora*) or 20 (*A. tenuis*) juveniles were randomly sub-sampled and fixed in absolute ethanol for subsequent extraction of total (coral and algal) DNA following a cetyltrimethyl ammonium bromide (CTAB)-based protocol modified from Hoarau *et al.* (2007). Briefly, each sample was placed in 250 µl of extraction buffer (2% CTAB, 0.1M Tris (pH 9), 20 mM EDTA (pH 9), 1.4M NaCl) and macerated inside a 1.5 ml micro-centrifuge tube using a disposable paper clip. The sample was incubated overnight at 60°C followed by addition of 250 µl of chloroform/isoamyl-alchohol (24:1). After thorough mixing, the sample was centrifuged for 15 minutes at maximum speed in a bench top centrifuge. The aqueous phase was pipetted into a new tube and 250 µl of ice-cold 2-propanol was added followed by gentle mixing. The sample was incubated at -20°C for 20 minutes followed by centrifugation at maximum speed at room temperature for 20 minutes. The supernatant was discarded and the pellet washed with 150 µl of 70% ethanol followed by centrifugation for 5 minutes at maximum speed. The pellet was air-dried for 5 minutes and allowed to resuspend overnight in 400 µl of 0.01M Tris (pH 9).

To quantify the relative abundance of *Symbiodinium* C1 versus *Symbiodinium* D established under different temperature x light treatments, two µl of DNA template were used for a real-time PCR assay using C- and D-specific ITS1 primers developed by Ulstrup and van Oppen (2003). The real-time PCR reaction containing 10 µl Sybr-Green Super Mix (Invitrogen), 2 µl ITS1 universal forward primer (180nM), 2 µl ITS1

C or D reverse primer (180nM), 4 µl of milli-Q water, and 2 µl of DNA template, was run on a Rotor Gene 3000 (Corbett Research). The reaction profile (following an initial heating step to activate the *Taq* polymerase as per manufacturer recommendation) consisted of 40 two-step cycles of 15 s at 95°C and 30 s at 60°C. A melt curve was generated at the end of each run starting at 60°C and ramping to 95°C by increasing 0.5°C every five seconds (except for the first step, which was held for 45 seconds). Data acquisition took place during the 60°C step in each cycle as well as during the melt curve period. The cycle-threshold (C_T) was set to a fixed value for all runs to allow comparisons between runs. All reactions were run in duplicate. No-template controls and positive controls were included in every run. Data were collected using the Rotor Gene software (v 6.1). The relative abundance (cell ratios) of each *Symbiodinium* type was calculated by the 2-ΔΔ C_T method, taking into account a difference in copy number between clades C and D of three (3 copies of D for every C), as described in Mieog *et al.* (2007). Cell ratios (D:C ratios) were converted to proportions whereby 1 represented a *Symbiodinium* D only sample with no background of C and 0 represented a C sample with no background of D *Symbiodinium*.

3.2.5 Data analysis

Pigmentation ratio and relative survival data were arc-sin transformed and analyzed with a two-factor ANOVA with light (2 levels) and temperature (3 levels) as fixed factors. Homogeneity of variance and normality were verified by Levene's test and spread vs. residual plots. Where assumptions of ANOVA could not be met after arc-sin transformation of the data, the non parametric Kruskal-Wallis test was used to assess differences in pigmentation ratios or relative survival between temperature treatments. When this was the case, separate comparisons for each light level were carried out. The

effects of temperature and light on the D:C cell ratios were analyzed by a two factor repeated measures ANOVA. Time (day) was treated as the within subject factor and temperature (three levels, fixed) and light (two levels, fixed) were treated as between subject factors. The assumption of sphericity was checked by Mauchly's test. All analyses were performed using SPSS software v. 16.0.

3.3 Results

3.3.1 Effects of temperature and light on the onset of the symbiosis

Elevated temperature had a strong negative effect on the uptake and establishment of the symbiosis in juveniles of both coral species. At the end of the inoculation phase (day 10), the pigmentation ratios of *Acropora tenuis* juveniles differed significantly between the 28°C and 31°C treatment, being up to four times higher in juveniles at 28°C (Fig. 3.2a, ANOVA, $F= 1.233$, $p= 0.002$). The difference was much larger at the end of the experiment, when pigmentation ratios in 28°C juveniles were at least five times higher than in 31°C juveniles (Fig. 3.2b, ANOVA, $F= 1.304$, $p< 0.001$). Similarly, pigmentation ratios in *A. millepora* juveniles at the end of the inoculation phase (day 15) as well as by the end of the experiment were up to three times higher at 28°C and 30°C than at 31°C (Figs. 3.3a-b).

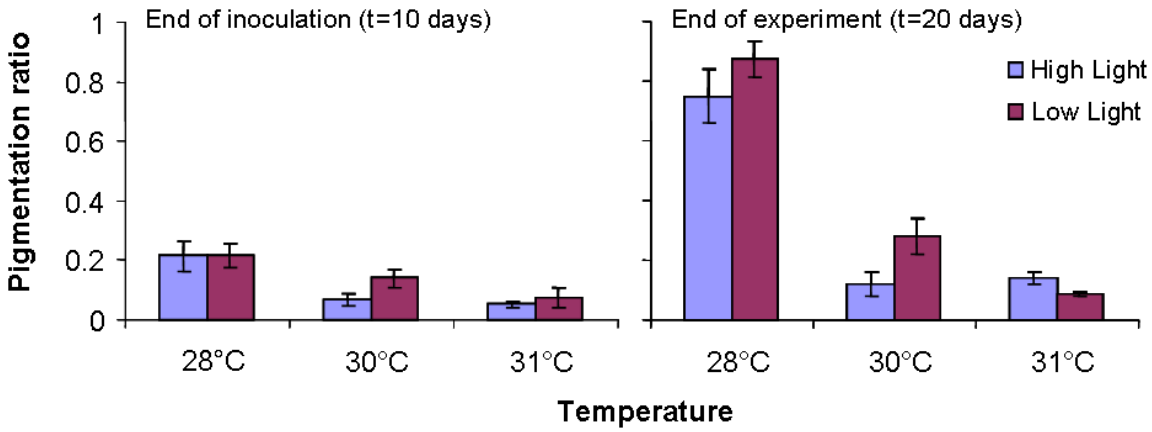


Fig. 3.2. Pigmentation ratios of *A. tenuis* juveniles kept at 28, 30, or 31°C and under high light (390 µmol photons m^{-2} s^{-1}) or low light (180 µmol photons m^{-2} s^{-1}) levels. Pigmentation ratios were calculated after 10 days of exposure to *Symbiodinium* and at the end of the experiment, after a further 10 days in filtered sea water (1 µm) without additional exposure to *Symbiodinium*. See Table 3.1 for sample sizes.

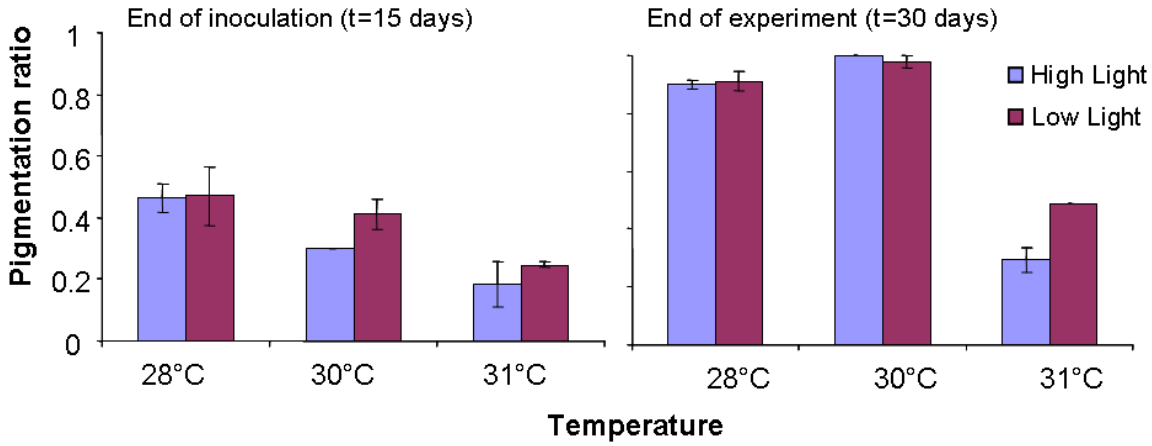


Fig. 3.3. Pigmentation ratio of *A. millepora* juveniles kept at 28, 30, or 31°C and under high light (390 µmol photons m^{-2} s^{-1}) or low light (180 µmol photons m^{-2} s^{-1}) levels. Pigmentation ratios were calculated after 15 days of exposure to *Symbiodinium* and after a further 15 days in filtered sea water (1 µm) without additional exposure to *Symbiodinium*. See Table 3.1 for sample sizes.

In contrast, light had little effect on the uptake and establishment of *Symbiodinium* as both coral species had very similar pigmentation ratios (within each temperature treatment) regardless of the light level they were exposed to (Figs. 3.2a-b, 3.3a-b). The only parameter affected by light levels was the relative survival of *A. millepora* juveniles, which was significantly higher in juveniles exposed to low light but did not

differ between temperatures for this light treatment (Fig. 3.4b. Kruskal-Wallis test, χ^2= 7.410, p= 0.006). The relative survival of *A. tenuis* juveniles in the low light treatment was up to four times higher at the control temperature than at the 31°C treatment, but comparative survival within a temperature treatment did not differ significantly for juveniles exposed to low versus high light treatments (ANOVA, F= 2.348, p= 0.132; Fig. 3.4a). Likewise, light levels had no effect on the relative survival of *A. tenuis* juveniles in the different temperature treatments (ANOVA, F= 0.285, p= 0.602; Fig 3.4a).

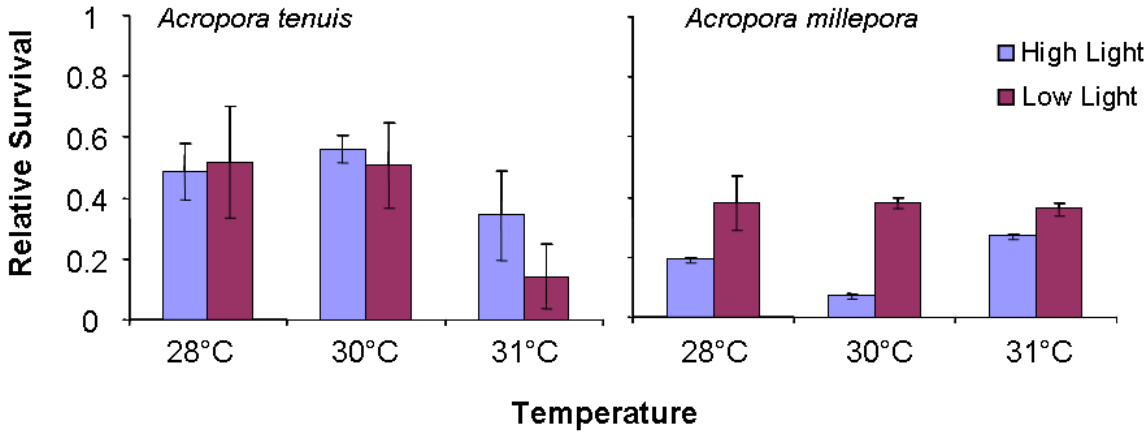


Fig. 3.4. Relative survival of juveniles in the 28, 30, or 31°C treatments and under high light (390 µmol photons m^{-2} s^{-1}) or low light (180 µmol photons m^{-2} s^{-1}) levels for *Acropora tenuis* after 20 days and *A. millepora* after 30 days. See Table 3.1 for sample sizes.

Table 3.1. Summary of total number of juveniles counted at each temperature by light treatment during the mid-experiment census (mid) and for the census at the end of the experiment (end).

	High light treatment		Low light treatment	
Temperature	n (mid)	n (end)	n (mid)	n (end)
Acropora tenuis				
28°C	2350	1316	2943	1844
29°C	2394	1279	2294	1050
31°C	2682	1064	2146	663
A. millepora				
28°C	354	59	319	121
30°C	147	7	235	73
31°C	180	40	271	84

3.3.2 Effects of temperature and light on the type of symbiont acquired and maintained

Elevated temperatures had the overall effect of significantly increasing the D:C cell ratio over time in juveniles of both coral species (Table 3.2a-b) regardless of the light level (Figs. 3.5a-b, 3.6a-b). In *A. tenuis* juveniles, *Symbiodinium* communities in all treatments started out dominated by type C1 (D:C cell ratios < 0.5, Figs. 3.5a-b). D:C cell ratios decreased during the inoculation phase (first 10 days) and were very similar across temperature or light treatments. However, after this point, when there was no additional exposure to *Symbiodinium*, there was an increase in the D:C cell ratios of corals at 30°C and 31°C but not at 28°C (Figs 3.5a-b). This change was very similar in both light levels until the end of the experiment, when the D:C ratio in corals at 30°C reached levels more than two-fold higher than those at 31°C (although they remained below 0.5, Figs. 3.5a-b).

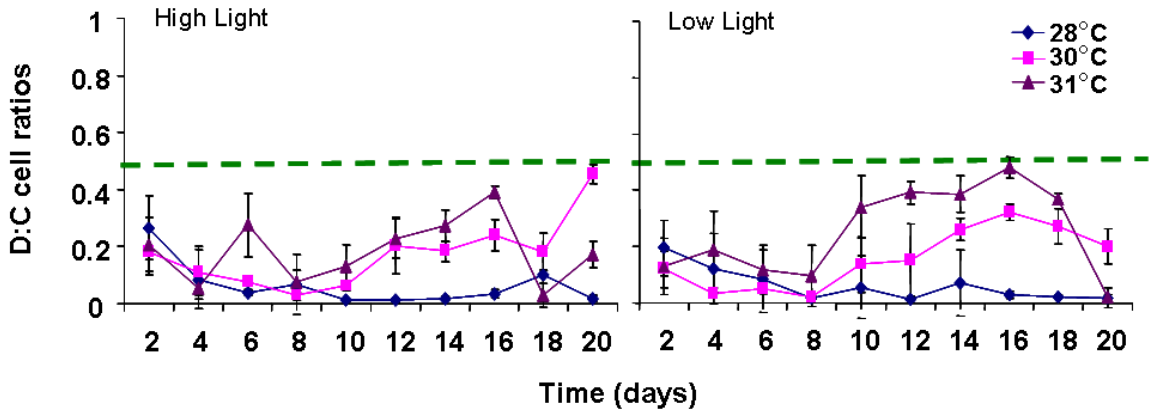


Fig. 3.5. Change in *Symbiodinium* D:C cell ratios over time in *Acropora tenuis* juveniles at 28, 30, or 31°C in high light (390 µmol photons m^{-2} s^{-1}) and low light levels (180 µmol photons m^{-2} s^{-1}). Dotted line represents equal proportions of *Symbiodinium* types D and C cells within the juveniles. Ratios closer to 1 are dominated by type D; ratios closer to 0 are dominated by type C. N = 20 per data point.

The *Symbiodinium* communities in *A. millepora* juveniles at high light in all temperatures also started out dominated by type C1 (Fig. 3.6a). However, unlike *A. tenuis* juveniles, D:C cell ratios increased during the inoculation phase, reaching

approximately equal concentrations by the end of this period (day 15, Fig 3.6a). D:C cell ratios in the higher temperature treatments continued to increase after exposure to *Symbiodinium* stopped but the D:C cell ratio in juveniles at 28°C remained close to 0.5 until the last sampling point, when it reached 0.8, indicating a D-dominated symbiosis (Fig. 3.6a). For juveniles at the low light level, the D:C cell ratios also increased over time in the 30°C and 31°C treatments but remained approximately at equal concentrations in the control treatment (28°C, Fig. 3.6b).

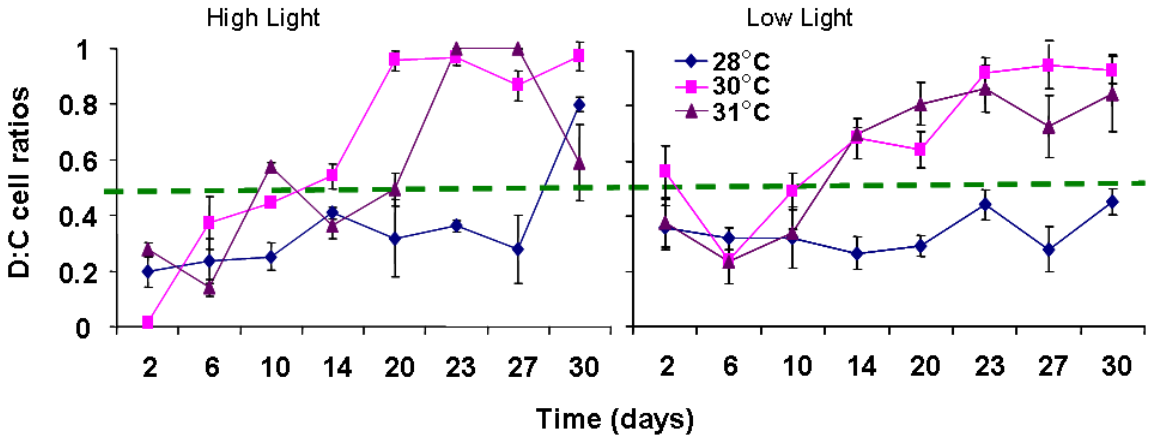


Fig. 3.6. Change in *Symbiodinium* D:C cell ratios over time in *Acropora millepora* juveniles at 28, 30, or 31°C in high light (390 µmol photons m^{-2} s^{-1}) and low light levels (180 µmol photons m^{-2} s^{-1}). Dotted line represents equal proportions of *Symbiodinium* types D and C cells within the juveniles. Ratios closer to 1 are dominated by type D; ratios closer to 0 are dominated by type C. N = 10 per data point.

Table 3.2. Repeated measures ANOVA results comparing changes in D:C cell ratios in (a) *Acropora tenuis* and (b) *A. millepora* juveniles kept at three temperatures (28, 30, or 31°C) and two light levels (390 µmol photons m^{-2} s^{-1} or 180 µmol photons m^{-2} s^{-1}). Within-subjects factor Day was 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 for *A. tenuis* and 2, 6, 10, 14, 20, 23, 27, and 30 for *A. millepora*.

Source of variation	*Sum of squares*	*Degrees of freedom*	*Mean square*	*F*	*Sig.*	*Tukey's*
a*) Acropora tenuis*						
Within Subjects analysis						
Day	0.497	9	0.055	3.241	0.001	
Day x Light	0.380	9	0.042	2.478	0.012	
Day x Temp	0.987	18	0.055	3.216	<0.001	
Day x Light x Temp	0.317	18	0.018	1.034	0.427	
Between Subject analysis						
Light	0.024	1	0.024	1.229	0.286	
Temp	0.758	2	0.379	19.020	<0.001	28<30, 31
Light x Temp	0.084	2	0.042	2.118	0.157	
b) Acropora millepora						
Within Subject analysis						
Day	4.874	7	0.696	33.621	<0.001	
Day x Light	0.400	7	0.057	2.757	0.013	
Day x Temp	1.613	14	0.115	5.565	<0.001	
Day x Light x Temp	1.149	14	0.082	3.964	<0.001	
Between Subject analysis						
Light	0.020	1	0.020	1.458	0.253	
Temp	2.412	2	1.206	85.858	<0.001	28<30, 31
Light x Temp	0.041	2	0.020	1.456	0.275	

3.4 Discussion

This study highlights the importance of temperature and light for the uptake and establishment of *Symbiodinium* during the initial stages of the coral-*Symbiodinium* symbiosis. While the combined effects of high temperature and light stress on the break-down of the association have been well documented (Brown 1997; Hoegh-

Guldberg 1999), this is one of the first studies to explore the impact of these stressors on the formation of the symbiosis. Elevated temperatures slowed down or impeded the uptake and establishment of *Symbiodinium* in coral juveniles of both coral species. This has important implications for coral persistence given projected increases of sea surface temperatures associated with climate change (IPCC 2007). First, acquisition of *Symbiodinium* by newly settled juveniles is most likely to occur during late spring following coral spawning events (Babcock *et al.* 1986), when sea surface temperatures are typically increasing. Low rates of infection due to elevated temperatures may result in diminished survival of recruits and/or slow growth of juveniles. Although the relative survival of juveniles over the time span covered here was not significantly different across all temperatures, it is unlikely that uninfected juveniles would have survived for an extended period of time given the fundamental role of algal endosymbiosis in coral survival (Muscatine 1977). Second, elevated temperatures may favour uptake or early establishment of thermally tolerant or opportunistic *Symbiodinium* types, even if such types are not homologous to the juveniles. In the present study the increase over time of D:C cell ratios in juveniles of both species provides evidence that type D is better at infecting and/or growing in corals at high temperatures. This result is also consistent with field observations of *Symbiodinium* uptake by coral juveniles a few weeks after the annual spawning events, when water temperatures are at their highest levels (Chapter 2). However, the tradeoffs (to the host) of this initial association with a potentially thermally tolerant or opportunistic symbiont remain to be investigated. This is particularly important given that: (1) juveniles at high temperatures had low pigmentation ratios and may therefore be photosynthate-limited and more prone to suffer mortality and, (2) type D *Symbiodinium* may not provide enough photosynthate for rapid growth of the juvenile colony (Little *et al.* 2004). Nevertheless, these corals

may be able to compensate for possible low photosynthate output through heterotrophy (Grottoli *et al.* 2006) or other mechanisms. Such a strategy would 'buy some time' for the corals until higher densities are reached by the proliferation of existing symbionts or newly acquired ones. Long term studies monitoring the succession of *Symbiodinium* in coral juveniles through several seasons will provide more insights into whether these corals (initially D-dominated in summer months) persist through time (see Chapter 4).

The limited effect of light on both the establishment of *Symbiodinium* and type of symbiont acquired was surprising given that types C1 and D *Symbiodinium* have been characterized as light-loving and shade-loving, respectively (Ulstrup & van Oppen 2003). However, this result is consistent with field observations, where the light level reaching newly settled juveniles had no effect on the type of *Symbiodinium* acquired (Chapter 2). Weis *et al.* (2001) found that densities of algal symbionts did not change over a four day sampling period in coral larvae maintained in either ambient light levels or virtual darkness. The short duration of their experiment, in combination with a possible lag phase in *Symbiodinium* population growth immediately following inoculation (Davy *et al.* 1997), may have prevented the detection of light effects in the infection rates of the larvae. However, the lack of relationship between light and pigmentation in the present study, which was run for 20 or 30 days, suggests that temperature may play a bigger role than light in driving the early establishment phase of the symbioses. Alternatively, it is possible that the difference between light levels used in this study was not large enough to elicit a response, but these levels are in agreement with documented *in-situ* measurements taken at Magnetic Island reefs (Anthony *et al.* 2004; Sofonia & Anthony 2008), thus making these levels ecologically relevant.

The visual assessment of *Symbiodinium* uptake in the present study may have underestimated the actual proportion of juveniles that acquired symbionts. However, given the constraint of working with small, single-polyp juveniles with a calcium carbonate skeleton and thus a lack of material for calculating meaningful *Symbiodinium* densities, the visual assessment method provided a means of estimating symbiont uptake across a range of pigmentation levels (Fig. 3.1). Furthermore, the use of only two pigmentation categories minimized observer bias. Other studies have assessed uptake by squashing either larvae or soft coral polyps and counting algal cells under a compound microscope (Schwarz *et al.* 1999; Coffroth *et al.* 2001; Weis *et al.* 2001). However, this method was not a reliable way of counting algal cells in this study because the skeleton of the juveniles prevented uniform squashing and the skeletal debris blocked the field of view. Subsequent to this study, a molecular method of quantifying algal vs. host cell ratios has become available (Mieog *et al.* 2009) and will enable a more quantitative approach in future studies.

The results of this study also revealed species-specific differences in the uptake of *Symbiodinium*, highlighting the potential role of host factors in the early stages of the symbiosis. These differences were particularly evident at the control temperatures, where high pigmentation levels indicated successfully established symbioses. In *A. tenuis* juveniles, the association was almost entirely C-dominated by the end of the experiment (D:C ratio < 0.02). This association mirrored the homologous symbiosis in *A. tenuis* adults at Magnetic Island (type C1) and suggests specificity for the algal symbiont. In contrast, in *A. millepora* juveniles at the control temperature, the symbiosis was approximately equal in proportion (D:C ratio of 0.45 at low light), or dominated by type D *Symbiodinium* (D:C ratio of 0.8 at high light). Furthermore, juveniles at 30°C,

which also had high pigmentation ratios were almost completely dominated by type D *Symbiodinium* (D:C ratio > 0.93). This is also the type found in *A. millepora* adults at Magnetic Island. Thus, the dominance of the homologous *Symbiodinium* type in both coral species at the control temperature shows that the host plays a factor in the early establishment of the symbiosis. However, elevated temperatures may disrupt this interaction. *A. tenuis* juveniles at high temperatures had a higher proportion of type D *Symbiodinium* than conspecifics at 28°C. On the reef, uptake of *Symbiodinium* by *A. tenuis* juveniles is dominated by type D (Chapter 2). Initial uptake on the reef occurs in early summer, when sea-surface temperatures are rising towards maximum levels. Hence, high temperatures in the field may inhibit mechanisms by which host factors play a role in the uptake of homologous symbionts and result in non-specific uptake.

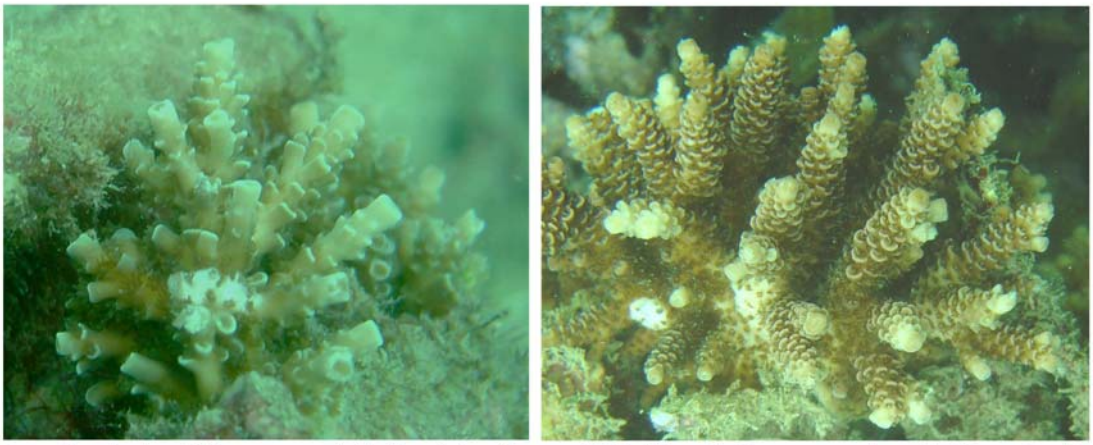
It is clear that inherent physiological differences between the two *Symbiodinium* types provide a competitive edge to type D at elevated temperatures. The increase in D:C ratios at higher temperatures after the inoculation phase suggests that type D can outgrow or out-compete *Symbiodinium* type C1 under high temperature stress conditions. However, as mentioned above, it is important to investigate whether this competitive advantage has a net positive effect on the holobiont at this early stage, or if it comes at the expense of the host (Stat and Gates 2008). Ultimately, it is important to establish if juveniles that form associations with potentially opportunistic symbionts under conditions of high temperature stress are able to establish lasting symbioses which allow them to survive extended periods of heat stress. It is also important to point out that differences in competitive abilities alone can not explain the host-specific patterns in uptake and establishment detected in this study (at the control temperatures). Whether these differences are due to host factors selecting for a specific symbiont, or

differences in intracellular environments that favor one type and allow it to outcompete other types (without specifically selecting for it) remains to be investigated. Characterization of any differences in intracellular environments along with possible host factors involved in the uptake and regulation of *Symbiodinium* will enhance understanding of mechanisms likely to be important for the persistence of coral-*Symbiodinium* associations in warming oceans.

Chapter 4.0 Long term patterns in succession of *Symbiodinium* types in juveniles of *Acropora tenuis* and *A. millepora*

This chapter is inserted without abstract as published in the journal *Molecular Ecology*: Abrego, D, van Oppen, MJH, Willis, BL. **2009** Algal endosymbiont specificity varies among closely related species of *Acropora* corals. *Molecular Ecology*. (accepted).

All the data was collected and analyzed by D. Abrego, who also wrote the chapter and manuscript after intellectual contributions by all co-authors.



Juvenile colonies of *Acropora tenuis* (left) and *A. millepora* (right). The colonies are approximately 18 months old. Photos: David Abrego.

4.1 Introduction

Dinoflagellates of the genus *Symbiodinium* form obligate mutualistic symbioses with reef building corals. Discovery of high genetic diversity within the genus (eight sub-generic lineages or clades that are further divided into several sub-clades or types; Baker 2003; Coffroth & Santos 2005), has fueled substantial efforts to determine whether this genetic diversity might be correlated with physiological diversity and enhance the fitness of the partnership (the holobiont). Several studies have demonstrated that the *Symbiodinium* type in the partnership can vary spatially according to reef location and even microhabitat within colonies of some coral species (Rowan & Knowlton 1995; Ulstrup & van Oppen 2003; Iglesias-Prieto *et al.* 2004; Ulstrup *et al*. 2007), but little is known about temporal patterns in the association (Thornhill *et al.* 2006a; Jones *et al.* 2008), especially during the first few years following the establishment of the partnership.

In the vast majority of corals, each new generation must acquire its *Symbiodinium* partners from the environment (horizontal uptake; Harrison & Wallace 1990). This has important implications for the initial development of the association, as newly-recruited, aposymbiotic (i.e., symbiont-free) corals can potentially be exposed to multiple types of symbionts during the uptake phase. This includes the possibility that heterologous symbionts may enter the symbiosis; that is, *Symbiodinium* types different from those found in the parent colony. The ability to associate with a diverse pool of *Symbiodinium* types may confer significant fitness advantages to the holobiont if such assemblages include a variety of types with physiologically different attributes. Such advantages could be further enhanced if processes involved in acquiring, maintaining and regulating *Symbiodinium* types varied with changing conditions to optimize physiological

performance, for example with changing environmental regimes or life history requirements. In the present study, as well as in the literature cited within, the term aposymbiotic is used to describe cnidarians lacking dinoflagellate symbionts but capable of associating with them (i.e. coral juveniles with horizontal symbiont transmission). This is different from the term asymbiotic, which is used to describe cnidarians which do not associate nor have the ability to associate with dinoflagellate symbionts (e.g. *Tubastrea* species of corals).

A number of studies demonstrate that flexibility exists in the types of *Symbiodinium* acquired by a range of cnidarians, including scleractinian coral juveniles (Schwarz *et al.* 1999; Weis *et al.* 2001; Little *et al.* 2004; Rodriguez-Lanetty *et al.* 2004, Gomez-Cabrera *et al.* 2008; Dunn & Weis 2009), gorgonians (Coffroth *et al.* 2001), sea anemones (Davy *et al.* 1997; Kinzie *et al.* 2001; Belda-Baillie *et al.* 2002; Schwarz *et al.* 2002), and jellyfish (Thornhill *et al.* 2006b). These studies show that uptake of *Symbiodinium* is a relatively non-selective process and that aposymbiotic cnidarians may be infected with a range of symbiont types from different sources. Some of these same studies show that establishment of the homologous or typical adult association involves a "winnowing process" (*sensu* Nyholm & McFall-Ngai 2004) (Weis *et al.* 2001; Coffroth *et al.* 2001, Belda-Baillie *et al.* 2002, Rodriguez-Lanetty *et al.* 2004, Dunn & Weis 2009), although factors influencing the timing of winnowing process/es are not well understood. In juvenile acroporid corals, Little *et al*. (2004) and Gomez-Cabrera *et al.* (2008) demonstrated that corals can acquire and maintain *Symbiodinium* types different from those dominant in parental colonies for several months. These authors speculated that non-selective uptake may reflect either a more infectious heterologous *Symbiodinium* type that quickly enters the juveniles but is eventually out

competed by the homologous symbiont (Gomez-Cabrera et al. 2008), or active selection by the host to maximize symbiont effectiveness according to ontogenetic changes in physiological demands (Little *et al.* 2004). However, the longest documented period of succession of *Symbiodinium* types in coral juveniles is less than one year (Little *et al*. 2004). Longer term monitoring is necessary to determine the stage at which the *Symbiodinium* type characteristic of the adult coral population eventually becomes dominant in juveniles and to determine if there are links between the identity of *Symbiodinium* communities and changing physiological needs associated with the ontogenetic stage of the host. Amongst the most important of these changes are those associated with the onset of reproductive maturity, when energetic requirements for gamete development start to place new demands on coral energy budgets.

The objectives of the present study are: (1) to document the succession of *Symbiodinium* in coral juveniles sourced from a number of reef populations that harbour different *Symbiodinium* types, and (2) to evaluate whether temporal changes in dominant *Symbiodinium* populations are linked to ontogenetic changes associated with the onset of reproductive maturity of the coral host.

4.2 Materials and methods

Study sites and species

Three sites on the Great Barrier Reef (GBR) were chosen based on differences in *Symbiodinium* association patterns in the corals *Acropora tenuis* and *Acropora millepora* (see Fig. 2.1 in Chapter 2). We define the *Symbiodinium* type found in adult populations at a given reef as the locally homologous type. At Magnetic Island, *A. tenuis* adults host type C1 *Symbiodinium* (sensu van Oppen *et al.* 2001, GenBank

Access # AF380551), but at Orpheus Island and Davies Reef they host type C2 (sensu van Oppen *et al.* 2001, GenBank Accession # AF380552). Similarly *A. millepora* adults at Orpheus Island and Davies Reef host type C2 but at Magnetic Island they host type D (sensu van Oppen *et al.* 2001, GenBank Access # EU024793). Magnetic and Orpheus Islands are inshore islands with highly turbid waters. Davies Reef is a mid-shelf reef located approximately 50 nautical miles northeast from Magnetic Island. This reef is surrounded by comparatively clear and slightly cooler waters than either Magnetic or Orpheus Islands (*in situ* data available from: http://www.aims.gov.au/docs/data-centre/seatemperatures.html).

4.2.1 Specificity and succession of *Symbiodinium* types in coral juveniles

To determine whether differential specificity in coral-dinoflagellate symbioses among different populations of *Acropora tenuis* and *A. millepora* is genetically or environmentally determined, juveniles from reefs where the locally homologous *Symbiodinium* type differed from the Magnetic Island type were reciprocally translocated and their symbiont communities monitored through time (see Fig. 2.1 in Chapter 2). Juvenile corals from both species were raised from fertilized gametes obtained after the spawning events of 2004-2006 (*A. millepora* only in 2005), settled on terracotta tiles under standardized sterile conditions as described in Little *et al*. (2004), and then reciprocally translocated between Magnetic and Orpheus Islands prior to symbiont uptake. In addition, *A. tenuis* corals from Davies Reef, where adult populations are dominated by type C2, were reciprocally translocated to Magnetic Island after the spawning event of 2003. High mortality of juveniles at Orpheus Island and Davies Reef within the first three months prevented long-term monitoring of their symbiont populations. However, Orpheus Island and Davies Reef juveniles raised at

Magnetic Island had excellent survivorship, thus their *Symbiodinium* populations, as well as those associated with Magnetic Island juveniles maintained in the local habitat, were monitored for up to 3.5 years. No bleaching events were recorded at any of the sites for the duration of the study.

4.2.2 Monitoring of algal types over time

Juveniles were sampled approximately one month after deployment on the reef. Each cohort was subsequently sampled 3 months later, then again 6 months after initial deployment and every six months thereafter until May 2007. In total, juvenile cohorts from 4 years (2003-2006) were followed for up to 3.5 years (see Figure 4.1). At each sampling time, 30 juvenile colonies were sampled from the tiles to identify their symbiont communities based on nuclear rDNA sequence differences in the ITS1 region using Single Stranded Conformation Polymorphism (SSCP). In addition, approximately one week before the spawning event of each year, one branch was sampled from at least 15 adult colonies (approximately 35-50 cm in diameter) for each species, including every adult colony from which gametes were collected at each spawning event, to identify its symbiont community. Samples were stored in 100% EtOH prior to genotyping.

Total (coral and algal) DNA was extracted from each juvenile coral sample using an adapted version of the protocol described by Wilson *et al.* (2002) (see Chapter 2 for description of method modification). The ITS1 region was amplified using the Sym ITS1 PCR primers, and Single Stranded Conformation Polymorphism (SSCP, which has a limit of detection of ~10% for background populations, as described by Fabricius *et al.* 2004) was used to determine the symbiont types within each sample. Reference

samples of known ITS1 sequence were run on each gel and SSCP profiles were scored manually from gel images. In most cases, only a single band was detected for each sample. However, there were samples where more than one band corresponding to different *Symbiodinium* clades was detected even though one band was usually dominant (highest intensity on the gel image). In these cases, the sample was scored as having a mix of *Symbiodinium* types corresponding to each of the bands present.

4.2.3 Onset of reproductive maturity

To investigate whether the onset of reproductive maturity correlated with changes in *Symbiodinium* populations harboured by corals, samples of *A. tenuis* at Nelly Bay, Magnetic Island were collected from five size classes ranging from early recruits through to reproductively mature adults (mean diameters: 1-5cm, 6-10cm, 11-15cm, 16-20cm, and 21-25cm). Healthy colonies of *Acropora* larger than 25 cm in diameter are generally reproductively mature (Harrison, Wallace, 1990), although the specific size of *A. tenuis* at the onset of reproductive maturity was unknown for this population. Ten to fifteen naturally-recruited colonies in each of these five size classes were examined for the presence of mature egg bundles a few days prior to the predicted spawning event of 2004 (Willis *et al.* 1985; Babcock *et al.* 1986;). Two branchlets from the center of each colony were broken off and the presence or absence of mature eggs, as denoted by their typically pink to red colouration at this late pre-spawning stage, was recorded. To ensure that sampled colonies were not fragments of larger colonies, only attached, regularly-shaped, corymbose colonies at least 5 meters away from another colony of *A. tenuis* were sampled. Colonies were temporarily tagged to avoid duplicate sampling. One of the branches sampled was fixed in 10% formalin (in seawater) for further examination of mature egg-sperm bundles under a dissecting microscope. The other

branch was fixed in ethanol for identification of the *Symbiodinium* community by ITS1 PCR and SSCP as described above.

4.2.4 Statistical analysis

The distributions of *Symbiodinium* types associated with coral juveniles were compared over time using χ^2 homogeneity tests of contingency tables, which use observed versus expected values (generated according to standard formulae) to calculate χ^2 statistics (Sokal & Rohlf 1996; Quinn & Keough 2002). Separate comparisons were made for cohorts originating from each of the three source reefs to determine if significant changes in the frequencies of *Symbiodinium* types occurred over time (i.e. 1 month old vs. 1.5 yr old juveniles, 1.5 yr old vs. 3.5 yrs). χ^2 tests were also used to determine if frequencies of *Symbiodinium* types were similar at a given age in different cohorts originating from the same parent reef (i.e. 2.5 yr old corals from 2004 vs. 2.5 yr old corals from 2003 originating from the same source reef). Where no differences between cohorts of the same age and source reef were found, data were pooled to compare differences in the frequencies of *Symbiodinium* types between local juveniles originating from Magnetic Is vs. explanted juveniles of the same age originating from Orpheus Is and Davies Reef.

4.3 Results

4.3.1 Symbiont succession in *Acropora tenuis* juveniles

The only *Symbiodinium* types detected in juvenile colonies of *Acropora tenuis* during this study were C1 and D. Symbiont communities were initially dominated by type D *Symbiodinium* in all cohorts raised at Magnetic Island, regardless of their natal reef (Figs. 4.1a-d). Hence, the distribution of symbiont types in one-month old juveniles was

independent of reef of origin (χ^2: 4.5382, d.f. 2, p = 0.103, Table 4.1)**.** This pattern continued for the first 12 months following settlement, with type D or mixed C1-D *Symbiodinium* communities detected in 75% - 100% of juveniles at each sampling time (Figs. 4.1a-c). By 1.5 years, the proportion of juveniles dominated by type D *Symbiodinium* had decreased in every cohort, thus all symbiont communities at 1.5 years differed significantly from initial (1 month old) communities (Figs. 4.1a-c, see Table 4.1 for χ^2 test results). Temporal differences in *Symbiodinium* types were particularly marked in juveniles originating from Magnetic Island. Symbiont communities in this group shifted significantly throughout the first three years, from *Symbiodinium* D, which was the only type detected in more than 80% of juveniles at one month, to a mixed C1-D community in approximately half of all juveniles at 1.5 years (Figs. 4.1a-b), to exclusively *Symbiodinium* C1 in greater than 90% of juveniles after 3.5 years (Fig. 4.1a, Table 4.1). Furthermore, *Symbiodinium* communities in 3.5 yr old juveniles originating from Magnetic Island were indistinguishable from those of local adult colonies of *A. tenuis* (χ^2: 1.7225, d.f. 2, p = 0.423, Fig. 4.1a).

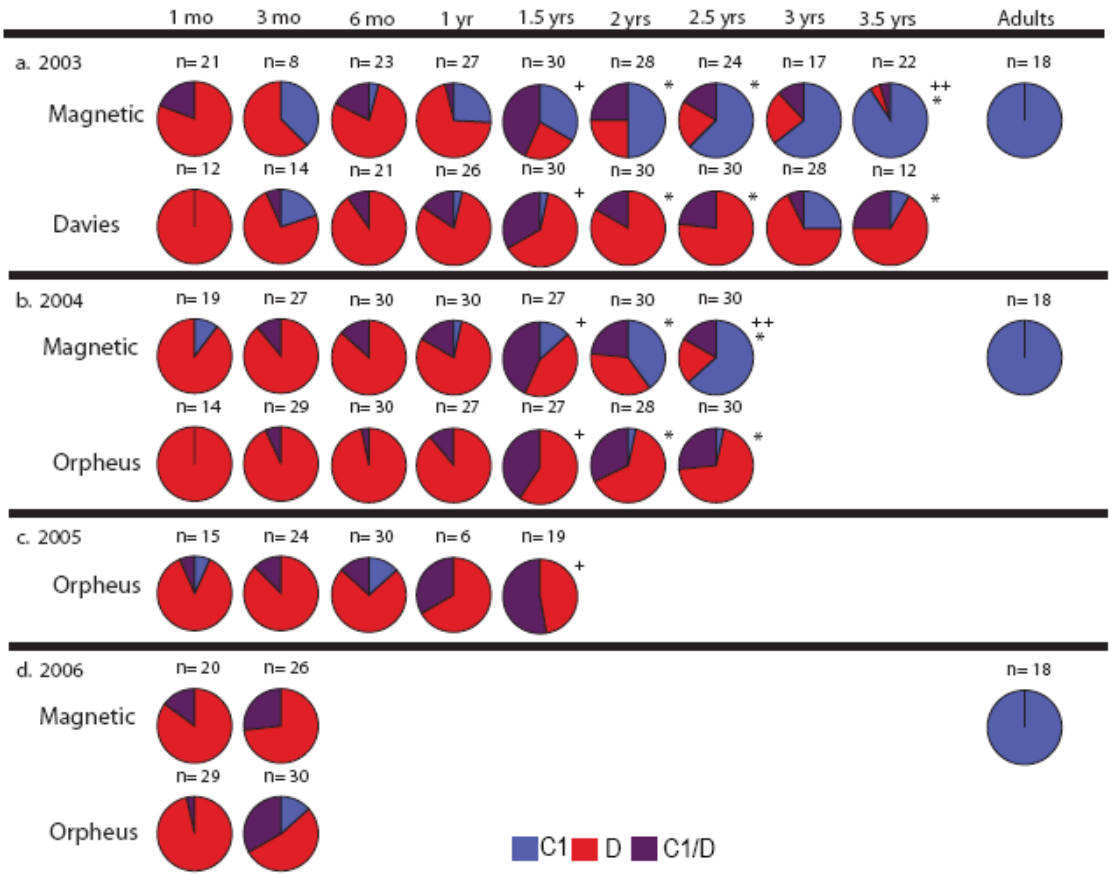


Fig. 4.1. Succession of *Symbiodinium* types in *Acropora tenuis* juveniles raised at Magnetic Island after four spawning events (2003- 2006). Reef of origin is listed for each cohort. Pie charts show the proportion of juveniles hosting type C1 (blue), D (red) or a combination (purple) of both *Symbiodinium* types. In all cases, the number of samples for which the ITS-1 region was successfully amplified is shown above each pie chart. This number was low in initial samples, possibly because of low symbiont densities in the juveniles. + denotes significant differences between juveniles at 1month and the same cohort at 1.5yrs. ++ denote significant differences between corals at 1.5 years and the same cohort at 2.5 or 3.5 yrs. * denotes significant differences between corals of the same age originating from different parental reefs. The column labeled 'Adults' denotes *Symbiodinium* communities in adult colonies of *A. tenuis* at Magnetic Island, including all those from which gametes were obtained. *A. tenuis* adults at Orpheus Island and Davies Reef remained C2-dominated throughout the study.

Table 4.1. Comparisons of *Symbiodinium* type distributions in four cohorts (2003-2006) of *A. tenuis* juveniles raised at Magnetic Island. The reef of origin for each cohort is denoted as: MTM (juveniles from Magnetic Island); DTM (juveniles from Davies Reef); or OTM (juveniles from Orpheus Island). Age indicates the time point of sampling.

Comparison within cohorts of different ages (grouped by origin)

	Cohort	*Age*	χ^2	*Df*	*p*
Local Juveniles	2003 MTM	1 month vs 1.5 yrs	22.8825	2	<0.001
	2003 MTM	1.5 yrs vs 3.5 yrs	17.2977	2	<0.001
	2004 MTM	1 month vs 1.5 yrs	12.3532	2	0.002
	2004 MTM	1.5 yrs vs 2.5 yrs	15.9171	2	<0.001
	2006 MTM	1 month vs 3 months	0.9446	1	0.331
Transplanted Juveniles	2003 DTM	1 month vs 1.5 yrs	5.9612	1	0.015
	2003 DTM	1.5 yrs vs 3.5 yrs	0.0415	1	0.839
	2004 OTM	1 month vs 1.5 yrs	7.7951	1	0.005
	2004 OTM	1.5 yrs vs 2.5 yrs	1.9970	2	0.368
	2005 OTM	1 month vs 1.5 yrs	5.6685	1	0.017
	2006 OTM	1 month vs 3 months	14.6236	2	<0.001

Comparisons between different cohorts of the same age (grouped by origin)

	Age	*Cohort*	χ^2	*Df*	*P*
Local Juveniles	2.5 yrs	2003 MTM vs 2004 MTM	0.0060	2	0.997
	2 yrs	2003 MTM vs 2004 MTM	0.9749	2	0.614
	1.5 yrs	2003 MTM vs 2004 MTM	8.9807	2	0.011
	1 yr	2003 MTM vs 2004 MTM	7.6113	2	0.022
	1 month	2003 MTM vs 2004 MTM vs 2006 MTM	7.7888	4	0.100
Transplanted Juveniles	2.5 yrs	2003 DTM vs 2004 OTM	1.1576	2	0.691
	2 yrs	2003 DTM vs 2004 OTM	3.2173	2	0.200
	1.5 yrs	2003 DTM vs 2004 OTM vs 2005 OTM	3.1210	4	0.537
	1 yr	2003 DTM vs 2004 OTM	1.3245	2	0.516
	1 month	2003 DTM vs 2004 OTM vs 2005 OTM vs 2006 OTM	4.2071	3	0.240

Comparisons of same-age cohorts by reef of origin

Age	*Reef of Origin*	χ^2	*Df*	*P*
3.5 yrs	MTM (Local) vs DTM (Transplanted)	15.3950	1	<0.001
2.5 yrs	MTM (Local, pooled) vs DTM/OTM (Transplanted, pooled)	52.2432	2	<0.001
2 yrs	MTM (Local, pooled) vs DTM/OTM (Transplanted, pooled)	33.3941	2	<0.001
1 month	MTM (Local, pooled) vs DTM/OTM (Transplanted, pooled)	4.5382	2	0.103

In contrast, *A. tenuis* juveniles originating from other reefs did not have the same shift from D-dominated to C1-dominated *Symbiodinium* communities through time. There

were clear and consistent patterns of symbiont succession within explanted juveniles that were significantly different from patterns found for Magnetic Island juveniles after the first 6-12 months (Figs. 4.1a-c, see Table 4.1 for χ^2 test results of local vs. explanted comparisons). Juveniles originating from Davies Reef initially hosted only type D *Symbiodinium* and, although the proportion of juveniles dominated by type D *Symbiodinium* declined significantly to approximately two-thirds by 1.5 yrs (χ^2: 5.9612, d.f. 1, p = 0.015, Fig. 4.1a), it had increased back to 80% by 2 years and remained relatively invariant thereafter (χ^2: 0.0415, d.f. 1, p = 0.839, Fig. 4.1a). The succession of symbionts in juveniles reared from Orpheus Island parents was very similar. Type D *Symbiodinium* was detected in more than 90% of juveniles in the first month (Fig. 4.1b) but the proportion of juveniles dominated by type D declined significantly between one month and 1.5 yrs (Figs. 4.1b-c, Table 4.1). The proportion of juveniles with mixed symbiont communities was again relatively small up to one year after settlement but increased over time. In all non-native juveniles, the highest proportion of mixed symbiont communities was detected at approximately 1.5 years after settlement. This was as high as 53% in the case of the 2005 cohort from Orpheus Island (Fig. 4.1c). Nevertheless, subsequent sampling of the two non-native cohorts that were monitored for longer than 1.5 years consistently demonstrated that type D was the dominant symbiont and no further significant changes in symbiont communities were detected after this point (Figs. 4.1a-b, Table 1). Adult colonies of *A. tenuis* at Magnetic Island maintained a stable C1-dominated symbiont community for the duration of the study.

4.3.2 Symbiont succession in *A. millepora* juveniles

As was found for *A. tenuis*, *Symbiodinium* C1 and D were the only types detected in *A. millepora* juveniles and, at one month, all *Symbiodinium* communities were dominated

by type D, i.e. all local and explanted juveniles of *A. millepora* that were raised at Magnetic Island from all four cohorts (2003-2006) were dominated by *Symbiodinium* D (Fig. 4.2a-d). However, unlike *A. tenuis*, *Symbiodinium* communities in the majority of *A. millepora* juveniles remained dominated by type D for the duration of the study, regardless of reef of origin (Fig. 4.2a-d). With just two exceptions, C1 or mixed-symbiont communities were not detected in more than 15% of *A. millepora* juveniles. The two exceptions were the 2003 and 2004 cohorts from Magnetic Island at approximately 3 months after settlement, but in both cases, sampling over the following two to three years revealed that *Symbiodinium* type D remained the dominant symbiont in more than 85% of juveniles (Fig. 4.2a-b). *Symbiodinium* communities in adult colonies of *A. millepora* at Magnetic Island remained dominated by type D for the entire 3.5 years of the study.

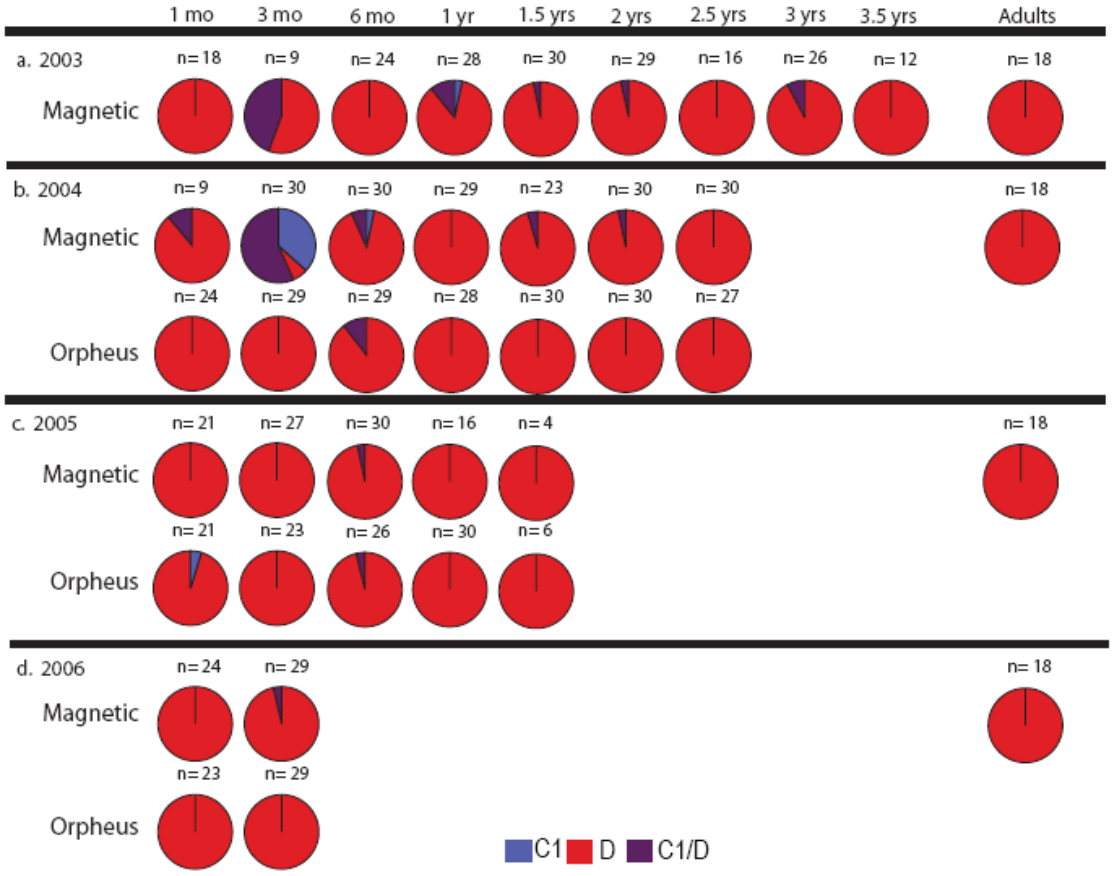


Fig. 4.2. Succession of *Symbiodinium* types in *Acropora millepora* juveniles raised at Magnetic Island after four spawning events (2003-2006). Reef of origin is listed for each cohort. Pie charts show the proportion of juveniles hosting type C1 (blue), D (red), or a combination (purple) of both *Symbiodinium* types. In all cases, the number of samples for which the ITS-1 region was successfully amplified is shown above each pie chart. This number was low in initial samples possibly because of low symbiont densities in the juveniles. The column labeled 'Adults' denotes *Symbiodinium* communities in adult colonies of *A. millepora* at Magnetic Island, including all those from which gametes were obtained. *A. millepora* adults at Orpheus Island remained C2-dominated throughout this study.

4.3.3 Onset of reproductive maturity and *Symbiodinium* community *in hospite*

Naturally recruited colonies of *A. tenuis* smaller than 10 cm in mean diameter were not reproductively mature. Mature egg bundles were detected in only 18% of colonies in the 11-15 cm size class; whereas 75% of colonies in the 16-20 cm and 100% of those in the 21-25 cm size class had mature bundles (Fig. 4.3a). There was no indication that changes in sexual maturity are linked to the identity of symbionts *in hospite*. Symbiont communities across all size classes were dominated by type C1 (Fig. 4.3b). Three small colonies were dominated by type D symbionts; two in the 1-5cm. size class and the

third in the 11-15 cm size class (Fig. 4.3b). Thus, changes in sexual maturity were not linked to any detectable changes in the identity of symbionts *in hospite*.

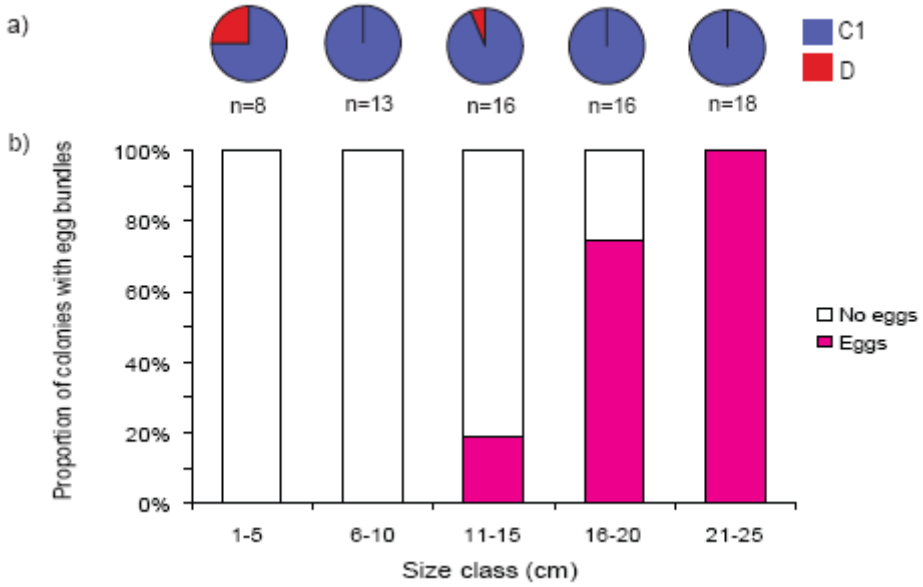


Fig. 4.3. a) Symbiont communities in naturally recruited juveniles of *A. tenuis* at Magnetic Island. Pie charts show the proportion of juveniles hosting: *Symbiodinium* C1 (blue), or D (red). b) Percentage of the same colonies with mature eggs (pink) or with no mature eggs (white).

4.4 Discussion

Analyses of *Symbiodinium* communities during early ontogeny in two closely related species of *Acropora* indicate that adult patterns of coral-algal endosymbioses may not become established for up to 2.5 – 3.5 years. During these early life history stages, *Symbiodinium* communities in *Acropora tenuis* and *A. millepora* from 4 of 5 study populations and 10 of 14 cohorts were dominated by types that were non-homologous in their parental populations. These results extend the duration of the period over which algal endosymbioses remain flexible reported in previous studies of juvenile corals (Little *et al.* 2004, Gomez-Cabrera *et al.* 2008).

4.4.1 Delayed onset of specificity in *Acropora tenuis* juveniles

The present study shows that succession of *Symbiodinium* types in the early life history stages of *A. tenuis* is a dynamic process but, providing that juveniles have access to *Symbiodinium* types characteristic of the natal adult population (i.e. locally homologous *Symbiodinium* types), the stable adult partnership becomes established by 3.5 years. Given that it took several years to establish the stable adult partnership, we conclude that the onset of specificity for the locally homologous *Symbiodinium* type is delayed in at least some populations of *A. tenuis*. Previous studies have shown that multiple types of symbionts may be acquired initially by cnidarians, but the homologous symbiont characteristic of the adult/parental association generally dominates within days to months (Coffroth *et al.* 2001; Weis *et al.* 2001; Belda-Baillie *et al.* 2002; Rodriguez-Lanetty *et al.* 2004). On the other hand, Little et al. (2004) and Gomez-Cabrera et al. (2008) found a high degree of flexibility for the algal symbionts acquired by juvenile acroporid corals, which took up and maintained *Symbiodinium* types different from those found in their parental colonies. However, neither of these two studies nor any other study of juvenile cnidarians have monitored the symbioses for more than seven months, thus knowledge of the dynamics of cnidarian-endosymbiont associations between the very early juvenile and adult stages has been based on short-term studies.

The rapid dominance of *A. tenuis* juveniles from all three reef populations by type D *Symbiodinium* when raised at Magnetic Island, even though none of the adult populations host this type, demonstrates flexibility in the early establishment phases of the symbiosis. Moreover, if the locally homologous type is not available for uptake, as was the case for Orpheus Island and Davies reef juveniles raised at Magnetic Island (*Symbiodinium* type C2 has not been found at Magnetic Island, see Chapter 2), *A. tenuis*

juvenile corals are able to persist for at least 3.5 years with heterologous *Symbiodinium* types. Survival of these juveniles for 3.5 years provides evidence of extended flexibility in coral-endosymbiont partnerships in early life history stages.

The gradual shift towards C1-dominated symbioses in both cohorts of Magnetic Island juveniles monitored for at least 2.5 years indicates that, although the symbiosis is initially flexible, one or more mechanisms operate to establish the association characteristic of the adult population (type C1) over 3.5 years. This period is much longer than any previously documented study and explains why Little *et al.* (2004) and Gomez-Cabrera *et al.* (2008) did not find evidence for specificity in juvenile acroporid corals. There are several possible explanations for the lag in establishing the homologous association in Magnetic Island juveniles of *A. tenuis*. First, differences in relative abundance and/or ability to initially infect juveniles may result in type D dominating the symbiosis at this early stage. Indeed, types within clade D have been characterized as opportunistic (Toller *et al.* 2001a; Baker 2003) and in a recent survey of symbionts in cnidarian taxa at the Magnetic Island site, type D was found in more than 85% of *Acropora* spp. (Chapter 2) and was thus the most abundant type in acroporid corals at this location. In such conditions, type D may be quick to initiate a symbiosis but may not persist through time in *A. tenuis*. A similar pattern has been detected in *A. longicyathus* elsewhere in the Great Barrier Reef, where a potentially more infectious *Symbiodinium* type A was the dominant symbiont taken up by 10-day old juveniles even though it was rare in the local adult population (Gomez-Cabrera *et al*. 2008).

Once inside the host, patterns of symbiont succession in coral juveniles may be explained by differences in competitive abilities among *Symbiodinium* types (Fitt 1985). The proportion of juveniles dominated by type C1 in our study started to increase at approximately 18 months, corresponding to the age at which juveniles started to grow into the water column as small, multi-branched colonies occupying a much more three-dimensional space than younger, two-dimensional juveniles (Fig. 4.4). It is possible that changing environmental conditions associated with vertical growth of juvenile colonies favored type C1 *Symbiodinium* over type D. According to this hypothesis, both types may be acquired simultaneously, but one type remains at background levels (Mieog *et al.* 2007) until more favorable conditions enable it to out-compete the other symbiont. Such conditions might include increased exposure to light and/or nutrients in the water column, both of which have been shown to differentially affect the physiology of different *Symbiodinium* types (Rodriguez-Roman & Iglesias-Prieto 2005; Robison & Warner 2006; Ulstrup *et al.* 2007). However, the corollary to this hypothesis, that symbiont succession would follow the same pattern regardless of reef of origin, was not fulfilled. Hence differences in competitive abilities alone cannot explain endosymbiont succession patterns in this coral species. Furthermore, the results from Chapters 2 and 3 suggest that the light environment, at least at the levels measured on the site of this study, does not appear to have an impact on the type of *Symbiodinium* initially acquired (Chapter 2) or how these symbionts may change inside the host in the first few weeks after initial uptake (Chapter 3).

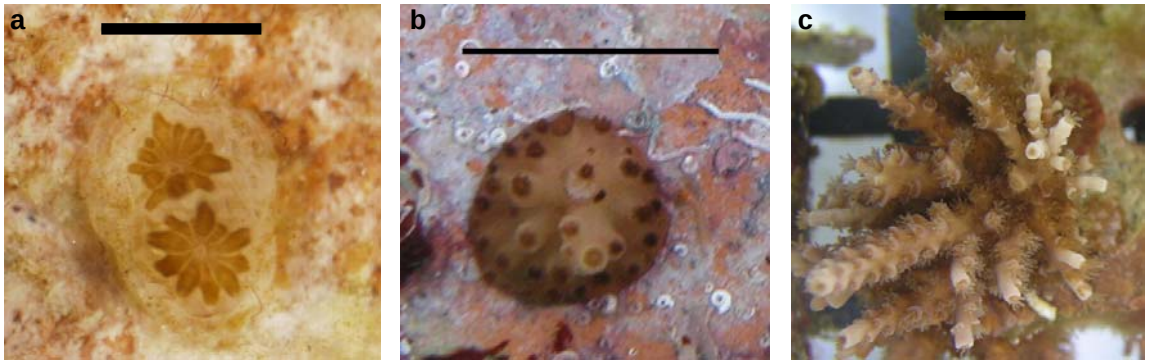


Fig. 4.4. Changes in growth form of *Acropora tenuis* juveniles over time. Juveniles typically exhibited: a) a two dimensional growth form in the first few months as they grew from single polyps; b) small dome-shaped colonies at six months; and c) three-dimensional branching morphologies between 1.5 and 2.5 yrs. Scale bar is 1.0 mm (a) or 1.0 cm (b and c).

Differential mortality of juveniles dominated by type D *Symbiodinium* is a potential but unlikely explanation for the progressively declining proportion of *A. tenuis* juveniles dominated by type D in the Magnetic Island cohorts. In a recent study of a *Stylophora pistillata* population following bleaching stress, repeated sampling of the same colonies established that differential mortality of colonies hosting symbionts differing in their bleaching susceptibility explained changing patterns in *Symbiodinium* communities, rather than internal shuffling of *Symbiodinium* types within surviving colonies (Sampayo *et al.* 2008). Although the small size of juveniles in our study precluded repeated sampling, a number of lines of evidence suggest that differential mortality was not driving the observed patterns in *Symbiodinium* communities for Magnetic Island cohorts. First, given that *Symbiodinium* D was the only type detected in 80% of *A. tenuis* juveniles at one month, mortality rates of D juveniles would have had to have been in the order of 80% to cause the observed shift to *Symbiodinium* C1 communities through time. Although mortality was not quantified formally, it would not have been possible to follow the cohorts for up to 3.5 years if mortality rates had been this high. Secondly, the ongoing dominance of Orpheus Island and Davies Reef cohorts by

Symbiodinium D throughout the study argues against this association being particularly fatal during the study period. Finally, the acquisition of multiple types of symbionts simultaneously in early ontogeny in corals with horizontal symbiont transmission (as shown for *A. tenuis* in this study) provides greater opportunities for shuffling than vertical transmission in species like *S. pistillata,* which acquire a fixed symbiont population from the parental colony and are therefore less likely to display shuffling (unless a mixed population is acquired from the parental colony). In support of this argument, shuffling has been observed in colonies of the closely related coral *A. millepora* (Berkelmans & van Oppen 2006; Jones *et al.* 2008). We conclude that it is more likely that shuffling, rather than differential mortality, accounted for the changing patterns in *Symbiodinium* associations in *A. tenuis* juveniles.

Alternatively, the change from a D-dominated to a C1-dominated symbiosis in Magnetic Island juveniles of *A. tenuis* could have been driven by a maturation and gradual fine-tuning of host factors or cell recognition mechanisms that favor the locally homologous symbiont. This hypothesis assumes that there are population level differences in host factors that have led to the establishment of *Symbiodinium* C2 as the locally homologous type in *A. tenuis* populations at Orpheus Island and Davies reef (van Oppen *et al.* 2001; Berkelmans & van Oppen, 2006). The fact that *Symbiodinium* C2 is rare at Magnetic Island and is unlikely to have been available for these explanted juveniles to take up, would have enabled a more opportunistic or highly infectious type D symbiont to become dominant. The increased proportion of explanted juveniles dominated by mixed or C1 symbionts after 1.5 years may indicate that the fine-tuning process was impeded in the absence of the native *Symbiodinium* C2 type and is consistent with our conclusion that *A. tenuis* exhibits delayed specificity. Although a

mechanistic explanation of the processes and time scales involved in the cellular recognition and regulation of symbionts in cnidarians remains to be shown, recent studies have described differences in host gene expression linked to the symbiotic state of the host (Rodriguez-Lanetty *et al.* 2006) and the type of symbiont hosted (Reynolds *et al.* 2000). Furthermore, increasing up-regulation of host-derived proteins (or the mRNAs coding for the host proteins) up to 31 days after symbiont inoculation (Yuyama *et al.* 2005) suggests that the regulation of host proteins potentially involved in symbiont-recognition processes continues after initial uptake. Likewise, cell surface glycoproteins on the symbiont have been identified as important factors during the onset of symbiosis (Lin *et al.* 2000; Wood-Charlson *et al.* 2006). Finally, there is the possibility that the intracellular environment of the juveniles may favor a particular symbiont type without actually selecting or recognizing it (Huss *et al*. 1993). This alternative hypothesis combines the potential for host factors, such as intracellular environment in host cells (which may have an impact on the symbiont), with differences in competitive abilities inherent to the different *Symbiodinium* types. An important aspect of this hypothesis is that the host factors do not necessarily recognize and select for a specific symbiont type, they simply facilitate dominance of that type. This hypothesis also allows for changes in such factors to favor different types of symbionts over time. However, as with specific host factors, further research is required to describe whether differences in intracellular environments and changes to these over time may influence the *Symbiodinium* types hosted by corals.

4.4.2 Unresolved specificity in *A. millepora* juveniles

In contrast to the delayed specificity documented for *A. tenuis* juveniles, it is unclear whether specificity for *Symbiodinium* types occurs in *A. millepora* juveniles. All *A.*

millepora juveniles acquired and maintained a remarkably stable D-dominated symbiosis, regardless of reef of origin. For juveniles from Magnetic Island, this could be interpreted as immediate onset of specificity, given that adult populations at this site host type D *Symbiodinium*. However, adult populations of *A. millepora* at Orpheus Island host type C2 *Symbiodinium,* thus the persistence of D *Symbiodinium* in explanted juveniles could reflect either unavailability of the native homologous *Symbiodinium* type or lack of specificity. Given the lack of change in symbiont communities in all native and explanted juveniles, it is not possible to distinguish whether the absence of specificity allowed these juveniles to simply take up and maintain the more infectious type D *Symbiodinium* or whether delayed specificity enabled Orpheus Island juveniles to persist for 2.5 years with the D (heterologous) type in the absence of the C2 (native homologous) type. It is also possible that there is little specificity for the algal symbiont initially in these juveniles (as in *A. tenuis*), but that there is a delayed onset of specificity that allows for the maintenance of type D *Symbiodinium*. Furthermore, type D *Symbiodinium* may be the best type for this environment, which may explain why it remained as the dominant type in these corals. Finally, as mentioned for *A. tenuis*, there is the possibility that the intracellular environment of the host cells during the time span of this study may have been more benign towards type D *Symbiodinium*. Further studies monitoring juveniles sourced from parental colonies dominated by C2 *Symbiodinium* and raised at sites where type C2 is known to occur is required to distinguish between these two possible explanations for the persistence of *Symbiodinium* D in explanted *A. millepora* juveniles.

4.4.3 No link between onset of sexual maturity and symbiont composition

Studies of both experimentally settled and naturally recruited juveniles also show that the onset of reproductive maturity is not linked to changes in *Symbiodinium* communities in *A. tenuis*. In experimentally settled juveniles, there were clear changes in *Symbiodinium* communities that occurred in the absence of changes in reproductive state. In the vast majority (96%) of naturally recruited *A. tenuis*, *Symbiodinium* communities were stable and dominated by type C1, despite colonies spanning the full range of reproductive states, from immature small colonies to reproductively mature larger colonies. For the first time at this site, a few naturally recruited colonies of *A. tenuis* were found to be dominated by type D. However, this was in three small, reproductively immature colonies. Two of these colonies were in the smallest size class (1-5 cm.), accounting for 25% of the colonies in this group (Fig. 4.3). This was consistent with the proportion of colonies dominated by type D in experimentally settled colonies at 18 months (23%, Fig. 4.1a), which were comparable in size (approximately 5-7 cm. in diameter). Taken together, the results from naturally recruited and experimentally settled corals show that changes in symbiont composition leading to the locally homologous association take place much earlier than sexual maturity.

4.4.4 Conclusion

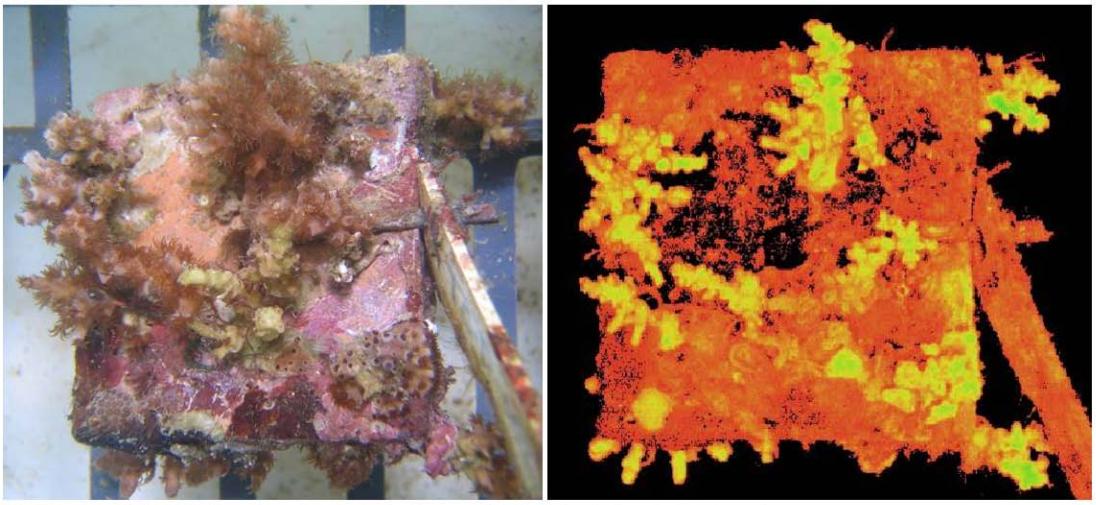
This study shows that closely related corals can differ in the onset of specificities for their algal symbionts and that changes in endosymbiotic communities leading to the locally homologous association can take several years. For *A. tenuis* juveniles, dynamic changes in *Symbiodinium* communities in the first 3.5 years are in stark contrast to the stable C1-dominated symbiosis documented in adults at Magnetic Island (van Oppen *et al.*, 2001; Ulstrup, van Oppen, 2003; van Oppen *et al.*, 2005) and may be driven by

maturation and fine tuning of host factors regulating endosymbiont populations. In contrast, algal endosymbioses were much more stable throughout the same 3.5 year period in *A. millepora* juveniles raised at Magnetic Island, although whether this is due to early onset of specificity, delayed onset of specificity, or lack of specificity requires further study. It is also clear from the present study that changes in algal endosymbiont communities associated with *A. tenuis* juveniles are not linked with the onset of reproductive maturity but may be linked with changes in micro-environmental conditions associated with three-dimensional growth of juvenile. Future studies to characterize host factors and their interactions with *Symbiodinium* types are required to enhance our understanding of the cellular processes involved in the recognition of these algal endosymbionts. Our findings suggest that multiple mechanisms are at play in the establishment of homologous associations, and unraveling those acting at a cellular level as well as those acting at ecological levels will provide much needed insights into the regulation of coral-algal endosymbioses.

Chapter 5.0 Physiological contributions of different *Symbiodinium* types to thermal tolerance of *Acropora tenuis* juveniles

This chapter is inserted without abstract as published in the journal *Proceedings of the Royal Society B*: Abrego, D., Ulstrup, K.E., Willis, B.L., van Oppen, M.J.H. 2008 Species-specific interactions between algal endosymbionts and coral hosts define their bleaching response to heat and light stress. *Proc. R. Soc. B.* **275**, 2273-2282. DOI 10.1098/rspb.2008.0180. The Electronic Supplementary Material published with this paper has been incorporated into the present chapter for ease of reading.

All the data was collected and analyzed by D. Abrego, except for that involving oxygen microelectrode characterization of photosynthesis and respiration, which was collected and analyzed by K.E. Ulstrup. D. Abrego wrote the chapter and resulting paper after editorial and intellectual contributions by all co-authors.



Left image: tile with *Acropora tenuis* juveniles used during heat stress experiment.
Right image: same tile as observed through an Imaging PAM, showing chlorophyll a fluorescence of symbionts as bright yellow/green areas. Images: David Abrego

5.1 Introduction

The obligate symbiosis between reef-building corals and dinoflagellates of the genus *Symbiodinium* has been fundamental to the evolution of reef corals. However, over the last few decades, this relationship has been disrupted on global scales by mass bleaching events, which render corals white through the loss of symbionts or pigments within them. The main triggers for these events are elevated sea surface temperatures acting synergistically with high irradiance levels (Brown 1997; Fitt *et al.* 2001; Lesser & Farrell 2004). Predicted increases in the frequency and severity of anomalously warm summers present a significant threat to coral reefs worldwide and to the goods and services they provide (Hoegh-Guldberg 1999; Hughes *et al.* 2003).

Recent studies demonstrating high genetic diversity within the genus *Symbiodinium* raise new possibilities regarding their potential role in the resilience of reef corals to climate stress. The genus consists of eight lineages or clades (A-H), each of which comprises multiple types (Baker 2003; Coffroth & Santos 2005). Although some coral colonies appear to harbour only a single symbiont type (Goulet 2006), others harbour two or more types simultaneously (Rowan & Knowlton 1995; Ulstrup & van Oppen 2003), which may include a dominant type and background levels of other types (Mieog *et al.* 2007).

It has been proposed that corals may adapt to warmer oceans by changing their symbiotic partners for new, heat tolerant types (Buddemeier & Fautin 1993; Baker 2001), and form novel host-symbiont combinations, either by acquiring a new symbiont type (switching) or by increasing the relative abundance of a symbiont type already present within the host (shuffling *sensu* Baker 2003). Among coral endosymbionts,

clade D *Symbiodinium* has been characterized as heat or stress tolerant based on increased prevalence of types within this clade in Caribbean and Indo-Pacific corals after bleaching events (Glynn *et al.* 2001; Toller *et al.* 2001a; Baker *et al.* 2004; van Oppen *et al.* 2005, Jones *et al.* 2008a), or in corals living in reef lagoons exposed to higher temperature regimes than surrounding waters (Fabricius *et al.* 2004). However, only a few published studies have tested and compared the physiological response to heat stress among corals hosting D types versus types in other *Symbiodinium* clades. In one study (Rowan 2004), adult corals hosting clade D *Symbiodinium* had higher rates of photochemical efficiency of Photosystem II (PSII) and higher ratios of maximum net photosynthesis to respiration than corals hosting clade C. In a second study (Berkelmans & van Oppen 2006), adult corals that had shuffled their dominant endosymbiont from C2 to D (ITS1 defined types) following bleaching had higher photochemical efficiency and higher symbiont densities than C2-dominated colonies when subsequently tested in a heat stress experiment. These studies are consistent with field observations and support the notion that the upper thermal tolerance of corals is enhanced when hosting clade D *Symbiodinium*. Nevertheless, observations of both thermally robust and thermally sensitive types within *Symbiodinium* clades caution against making clade-wide generalizations (Tchernov *et al.* 2004) and highlight the need for comparative physiological studies of types within *Symbiodinium* clades. Moreover, because clade D is relatively uncommon in Indo-Pacific corals, in contrast to the ubiquity of clade C (Baker & Rowan 1997; LaJeunesse 2001; Baker 2003; LaJeunesse *et al.* 2003), knowledge of the influence of symbiont type on holobiont physiology has important implications for understanding the impact that warming oceans may have on coral communities.

Recent studies showing that symbiont stress responses differ between freshly isolated and *in hospite* cells suggest that the host may play a significant role in regulating the response of the holobiont (host-symbiont combination) to heat/light stress (Bhagooli & Hidaka 2003; Goulet *et al.* 2005). Host-driven protective mechanisms that could contribute to regulation of the holobiont's bleaching response include production of anti-oxidant enzymes (Lesser *et al.* 1990), mycosporine-like amino acids (MAA's) (Dunlap & Shick 1998), and fluorescent pigments (Salih *et al.* 2000). Greater understanding of the host-symbiont interactions that govern holobiont physiology in intact coral-algal endosymbioses would provide fresh insights into the resilience of reef building corals.

Here we use physiological indicators to compare bleaching tolerance between corals hosting *Symbiodinium* type C1 or D to test the hypothesis that Indo-Pacific corals achieve optimal bleaching tolerance when dominated by *Symbiodinium* clade D. Hereafter we use the term "clade" to denote the sub-generic level of *Symbiodinium* classification; the term "type" to denote genetic types within a clade; and "C1" and "D" to denote specific ITS1 types (*sensu* van Oppen *et al.* 2001) when discussing our study species. Contrary to expectations, we show that C1-corals have higher thermal/light tolerance than D-corals in juveniles of the common Indo-Pacific coral *Acropora tenuis*. Our results challenge the view that clade D is universally associated with thermal robustness and provide evidence that the heat/light tolerance of *Symbiodinium* types differs with host species.

5.2 Materials and methods

5.2.1 Experimental corals, *Symbiodinium* inoculation and genetic identification

Three independent heat stress experiments were carried out using *Acropora tenuis* juveniles raised after the spawning events of 2003-2005 at Magnetic Island (19°.10'S, 146°.50'E) in the central section of the Great Barrier Reef. The pilot study and first experiment used six-month old juveniles, while those in the second experiment were 16 months old. Type C1 or D symbionts were isolated from adult *A. tenuis* and *A. millepora* colonies, respectively, and used to inoculate aposymbiotic coral juveniles, which had been settled on terra-cotta tiles and maintained in 1μm filtered seawater as described in Little *et al.* (2004). Following uptake of *Symbiodinium,* tiles with attached juveniles were suspended vertically on rods, which were then hung between star pickets to grow on the reef. Complete ITS1 sequences of D symbionts isolated from *A. millepora* (GenBank accession number EU024793) were identical to those found in *A. tenuis* juveniles naturally infected at this location (Little *et al.* 2004). Requirements for large quantities of D symbionts during inoculations precluded the use of naturally occurring *A. tenuis* juveniles as a source of symbiont cells, however, D symbionts from adult *A. millepora* colonies were readily taken up by the juveniles in this study. Furthermore, a recent survey of 52 taxa at the same study site including hard and soft corals revealed that at least 21 of these taxa had symbionts with identical sequences to both the D symbionts used to inoculate juveniles in this study and the D symbionts taken up naturally by recruits in the reef (Chapter 2).

Genetic verification of *Symbiodinium* type hosted by the juvenile corals took place after initial inoculation and again immediately before the start of each experiment. This was performed using Single Stranded Conformation Polymorphism (SSCP) of the ITS1

region of the nuclear rDNA. For the pilot study, 6 colonies per association were randomly selected for verification of symbiont identity and all had only the *Symbiodinium* type initially offered, within the limit of detection of the SSCP technique (5-10% of relative abundance; Fabricius *et al.* 2004). Additionally, 8 colonies per clade were randomly selected at the end of this study to verify that surviving colonies retained only the *Symbiodinium* type offered. For the first experiment, 20 colonies were randomly selected per association and in all but two juveniles, the presence of only the symbiont initially offered was confirmed. The two exceptions were from D-juveniles, where type C1 was detected in approximately equal proportions. Because the coral colonies in the second experiment were larger compared to those in the previous experiments, a small piece of every experimental colony used was checked prior to experimentation and confirmed to harbor only the *Symbiodinium* type initially offered.

5.2.2 Experimental design

For the pilot study and the first full scale experiment, four temperature treatments (28°C, 30°C, 31°C, 32°C) were selected ranging from ambient, non-stress conditions to the temperature at which bleaching occurs on local reefs. For the second full scale experiment, only three temperatures were selected (26°C, 29°C, 32°C) due to the reduced number of colonies available for replication (n=45 C1-corals; n=26 D-corals). In the latter experiment, a control temperature of 26°C was selected to match ambient, winter water temperatures at the field site where corals were kept. For each experiment, C1 and D-corals were divided among three replicate tanks per temperature treatment. The number of replicate colonies per temperature treatment ranged from 26-370 depending on the experiment (See Table 5.1 for a summary of assays, experimental conditions, and sample size for each experiment).

Irradiance levels were selected based on the range of intensities (120-320 μmol photons m^{-2} s^{-1}) experienced by juveniles *in situ* at the grow-out site. Intensities were at the low end of the range in the pilot study to avoid light stress (130 μmol photons m^{-2} s^{-1}). To test the impact of light dose, the photoperiod was initially 7.5 hours per day and then raised to 10 hours per day for the second half of the experiment. The increased number of bleached juveniles after the photoperiod was lengthened provided evidence for the importance of light in determining the bleaching response of corals. Hence, two light treatments were used in the first experiment, corresponding to the low (160 μmol photons m^{-2} s^{-1}) and high (360 μmol photons m^{-2} s^{-1}) ends of the *in situ* range. For the second experiment, irradiance intensity for the single light treatment was selected to approximate the middle of the *in situ* range (250 umol photons m^{-2} s^{-1}). Throughout both the first and second experiments, the photo-period was maintained at 10hrs light: 14hrs dark.

5.2.3 Experimental setup

In preparation for all three experiments, tiles with attached juveniles were retrieved from the reef and macro-algae and other organisms were removed before being assigned haphazardly to experimental tanks. Due to differences in initial rates of settlement and survival of corals whilst on the reef, the number of colonies per tile varied but there was approximately the same number of C1 and D colonies per temperature treatment (Table 5.1). To ensure accurate temperature control in experimental treatments, filtered seawater (0.5 μm) was pumped into 300-500 l reservoirs in a temperature-controlled room. Each reservoir was fitted with a single 1 or 2 kW titanium heater with digital controllers that switched on/off if the water temperature varied by more than ± 0.1°C

from target temperatures. From each reservoir, the water was pumped at 3 l/min into three replicate tanks (approx. 60 l each) fitted with a small power head pump to maintain water movement, and an air stone that provided a small stream of bubbles. Water temperature in the tanks was monitored by thermistors connected to data loggers (Innotech Genesis II) that recorded temperatures every 15 seconds. Target temperatures never varied by more than ± 0.3°C throughout each experiment (see Table 5.1 for summary of experimental setups for all three experiments). Metal halide lights provided the appropriate spectral output for photosynthesis (250W, 20K, Sylvania). In every experiment coral juveniles were allowed to acclimate to experimental light levels for at least five days before the start of heating. This period was sufficient for maximum quantum yield values to stabilize. Temperatures were increased (0.3°C/4hrs) from a staggered start so that target temperatures in each treatment were reached at the same time.

Table 5.1. Summary of physiological assays, experimental setup and number of colonies for each heat stress experiment. HL: High light, LL: Low light.

Physiological assay for each experiment					
Experiment	Assay				
Pilot	Bleaching condition, photochemical efficiency				
1	Bleaching condition, photochemical efficiency				
2	Photochemical efficiency, pressure over PSII, O_2 production and consumption, pigment quantification and absorption efficiency				
Experimental conditions for each heat stress experiment					
Experiment	Temperature treatments (ºC)		Light intensity (µmol photons m^{-2} s^{-1})		Length of experiment (days)
Pilot	28, 30, 31, 32		128 ± 20		31
1	28, 30, 31, 32		HL: 362 ± 9 LL: 158 ± 8		17
2	26, 29, 32		240 ± 15		18
Sample size (n) used in each experiment					
Exp	n per type		Range in sample size (n) per *Symbiodinium* type for each temp.		n for fluorescence measurements
	C1	D	C1	D	
Pilot	370	324	77-105	71-86	24
1 HL	98	121	18-33	16-52	10-17
1 LL	137	97	20-46	13-34	10-17
2	45	26	13-16	7-10	7-13

5.2.4 Bleaching condition of corals – Pilot study and Experiment 1

Bleaching was quantified every other day by visual scoring of all experimental colonies. Colonies were scored as normal (normally pigmented), pale (including moderately bleached colonies), bleached (completely translucent tissue), or dead (bare skeleton in various stages of overgrowth by other organisms).

5.2.5 Photochemistry of heat stressed corals

The maximum quantum yield of PSII (F_v/F_m), a proxy for photochemical efficiency of C1- vs. D-corals, was measured using a Pulse Amplitude Modulated Fluorometer

(PAM). For the Pilot Study and Experiment 1, we used a Mini PAM (Walz, Germany) fitted with a 2 mm diameter fiber optic probe. For Experiment 2, we used an Imaging-PAM (I-PAM, Walz, Germany) that allowed us to haphazardly select three 'areas of interest' (AOIs) within each replicate colony using the Imaging-PAM software (ImagingWin v2.12a). Dark-adapted colonies were measured every morning before the lights went on. In addition to measuring F_v/F_m as in the pilot study and experiment 1, we calculated the maximum excitation pressure over PSII (Q_m) in experiment 2 to better characterize the physiological performance of the symbiont (Iglesias-Prieto *et al.* 2004). Pressure was calculated using the equation:

$$Q_m = 1 - [(\Delta F/Fm')/(F_v/F_m)] \quad (1)$$

where $\Delta F/Fm'$ is the effective quantum yield of fluorescence in light-saturated conditions and F_v/F_m is the maximum quantum yield in a dark-adapted state. Excitation pressure was calculated based on a $\Delta F/Fm'$ measurement after one hour of exposure to lights, following observations that Q_m did not change significantly after 1, 4, and 7 hours of exposure to light in a pilot study preceding this experiment. Fluorescence measurements were taken every 3rd day.

5.2.6 Oxygen microelectrode characterization of photosynthesis and respiration

To further characterize the physiological impact of heat and light stress on the juvenile holobiont, rates of gross and net photosynthesis as well as respiration were measured for 4 colonies per *Symbiodinium* type per temperature during the second experiment. Measurements were performed on days 1, 8, and 15 of heating using an oxygen microelectrode (~50 μm in diameter) connected to a pico-amperemeter (PA2000, Unisense A/S, Denmark) which recorded measuring signals on a strip chart recorder (Kipp & Zonen, The Netherlands). Each colony was placed in a custom-built flow

chamber (25 × 10 × 10 cm) through which seawater at experimental temperatures ± 0.5ºC was circulated at a flow velocity of ~1 cm s^{-1}. An adjustable fibre-optic light source (Schott KL-2500) with a 250 W halogen lamp, fitted with a collimating lens and calibrated against a quantum irradiance meter (LiCor 192),was used for homogeneous, vertical illumination of the coral samples. The maximum irradiance level matched the level in the heat experiment (250 µmol photons m^{-2} s^{-1}). To minimize heterogeneity in the light climate caused by complexity in the skeletal structures of corals, a fully light exposed coral surface was selected for positioning of the microelectrode. A manual micromanipulator (MM33, Märtzhäuser, Germany) enabled us to place the microelectrode in direct contact with the coral tissue whilst observing the samples with a dissecting microscope. Linear calibration of the electrode was performed at experimental temperatures by recording signals in air-saturated seawater and O_2-free seawater, respectively. The O_2 concentration of air-saturated seawater at experimental temperatures and salinity (35 ppm) was obtained from tabulated values (www.unisense.com).

Gross photosynthesis rate (Pg) was determined using the light-dark shift technique in units of nmol O_2 cm^{-3} s^{-1}(Revsbech & Jorgensen 1983). The light-dark shift technique is based on the assumption that the immediate (<1 second) oxygen depletion following the eclipse of the light source is equal to the photosynthetic oxygen production during the previous light period and thus requires physiological steady-state (obtained in this case after 10 minutes at 250 µmol photons m^{-2} s^{-1}) (Revsbech & Jorgensen 1983; Kühl *et al.* 1996). In order to obtain accurate Pg measurements, the oxygen microelectrode must be small (< 100 µm) and exhibit low stirring sensitivity and fast response time (Revsbech 1989). After attaining physiological steady-state, oxygen microprofiles were measured

through the diffusion boundary layer (DBL) in darkness and at 50, 150 and 250 μmol photons m^{-2} s^{-1}. Oxygen flux measurements were then calculated from the oxygen concentration profiles (Kühl *et al.* 1995) as:

$J = -D_0 \times dC/dz$ (2)

where D_0 is the molecular diffusion coefficient of oxygen which is temperature and salinity specific (tabulated values available from www.unisense.com), and dC/dz is the slope of the oxygen gradient in the DBL (which was measured in 10 μm vertical steps from the surface of the tissue). This calculation assumes a 1-dimensional diffusion geometry (Kühl *et al.* 1995). The ratio ($Pn:R_D$) of the resulting flux in the dark (R_D) and at 250 μmol photons m^{-2} s^{-1} (Pn) was calculated as a measure of metabolic cost incurred during stress. In order to estimate the irradiance above which the tissue exhibited net oxygen production, known as the compensation irradiance (E_c), oxygen flux estimates obtained at 0, 50, 150 and 250 μmol photons m^{-2} s^{-1} were linearly integrated and the E_c was estimated as the irradiance where the oxygen flux was 0.

5.2.7 Chorophyll *a* content and xanthophyll pigments

The concentrations of chlorophyll *a* (chl *a*) and xanthophyll pigments in each coral juvenile were determined by reverse-phase High Performance Liquid Chromatography (HPLC) using an integrated PC-interfaced Waters HPLC system in the second experiment. The HPLC system comprised: In-Line vacuum degasser, controller and quaternary pump (Waters™, 600), autosampler (Waters™, 717plus) and a photodiode array detector (PDA, Waters™, 996). System control, data collection and integration were performed using the software Empower Pro (Waters Corporation, 2002). The method was adapted from van Heukelem and Thomas (2001). Small branch fragments (~0.5 cm long) were sub-sampled on days 1, 8, and 15 of heating (n=3 per

Symbiodinium type in each temperature treatment). After measurement of their reflectance spectra (see following section), fragments were snap-frozen in liquid nitrogen and stored at -80ºC. Following pigment extraction, the surface area of each fragment was calculated using photogrammetry and digital model construction as described in Jones *et al.* (2008b).

Pigments were extracted in 1 ml of methanol for two hours in the dark at -20ºC. Coral fragments were disrupted with an ultrasonic probe for 1 minute before and after extraction. The extract was then centrifuged at 15,000 rpm for 2 minutes and the clarified supernatant extract was mixed 1:1 (v/v) in solvent A (see below), before injection (10 µl) into a Gemini C18 column (3 µm particle size, 110Å pore, 50 x 4.6 mm, Phenomenex®) at room temperature. The coral skeletons were kept for surface area calculations. Pigments were separated using a binary mobile phase system (see Table 5.2 for Analytical Gradient Protocol). All the organic solvents used were HPLC-grade. Solvent A was prepared by diluting 2.8 ml 1.0M tetra butyl ammonium acetate (TBAA, Sigma-Aldrich) into 90 ml Milli-Q water. The pH was adjusted to 6.5 and the mixture was diluted to 100 ml to give 28 mM TBAA. Ninety ml of this solution was then diluted with 210 ml of methanol and filtered under vacuum through Millipore organic solvent filters. Solvent B was prepared by mixing 1:1 (v/v) methanol:acetone and filtered under vacuum as above.

Chl *a* and xanthophylls (diadinoxanthin and diatoxanthin) were detected by PDA spectroscopy (350-750 nm) and by fluorescence (excitation: 440nm, emission: 650nm). Absorbance chromatograms were extracted at 440nm. Pigment identity was confirmed by co-chromatography with authentic standards (Sigma Aldrich, and DHI, Denmark).

The area of the peaks corresponding to chl *a* (retention time 11.74 ±0.02 min), diadinoxanthin (retention time 9.26 ±0.02 min), and diatoxanthin (retention time 9.70 ±0.02 min) were obtained using the Empower Pro software. Manual correction of peak baselines was performed if necessary to make sure that no adjacent peaks/shoulders were used in the calculation of peak areas. Pigment concentrations were calculated using relative response factors for each pigment standard. Pigment amounts were standardized to the surface area of the fragment from which they were extracted.

Table 5.2. HPLC analytical gradient protocol. Flow rate was mainained at 1 ml min^{-1} for the duration of the analysis.

Time (min)	Solvent A % [70:30(v/v) methanol:28mM aqueous tetrabutyl ammonium acetate (TBAA)]	Solvent B % [50:50(v/v) acetone:MeOH]	Condition
0	75	25	Injection
5	0	100	Linear gradient
10	0	100	Linear gradient
11	75	25	Linear gradient
18	75	25	Equilibration

The xanthophyll ratio was calculated as the ratio of diatoxanthin to the total xanthophyll pool [diadinoxanthin plus diatoxanthin] (Ambarsari *et al.* 1997). Due to the small size of fragments (38-66 mm^2) and the limited number of colonies available for sub-sampling, it was not possible to collect samples for quantification of algal cells. While this restricted the interpretation of pigment data (i.e. whether differences were due to changes in the presence of pigment, or the number of algal cells), standardizing pigment concentrations to coral surface area allowed for comparison of general patterns among treatments.

5.2.8 Reflectance spectra of corals and calculation of chlorophyll *a* specific absorption coefficient ($a^*_{Chl\ a}$)

To quantify changes in the light absorption efficiency of chlorophyll *a* in the symbionts, reflectance spectra of the corals and skeletons used in the second experiment were measured between 400 and 750 nm with 0.3 nm resolution using a USB2000 Fiber Optic Spectrometer (25 μm optical slit with grating #3 installed, Ocean Optics™). The method was adapted from Enriquez *et al.* (2005). Coral colonies were sub-sampled by taking a small branch fragment and placing it on a black, non-reflecting surface in a small container filled with seawater. The fragment was positioned so that the side that received more down-welling light while attached to the colony was facing up. Illumination was provided by a metal halide lamp approximately 40 cm above the sample. Reflected light was collected with a 200 μm diameter waveguide attached to the spectrometer. The waveguide was placed underwater 0.5 cm away from the sample at a 45º angle. To avoid complications due to morphological variance, the waveguide was always pointed to the coenosarc (tissue that joins adjacent polyps). The field of view of the waveguide was approximately 0.1 cm^2. Reflectance was calculated as the ratio of the radiance measured from the coral surface relative to the radiance obtained from a reference white diffusing surface. The specific absorption coefficient of chlorophyll *a* ($a^*_{Chl\ a}$) was calculated as described in Enriquez *et al.* (2005) using the equation:

$$a^*_{Chl\ a} = -(D/\rho)\ \ln 10 \quad (3)$$

where D is the absorbance calculated from reflectance (R) measurements as the log (1/R), and ρ is the content of chl *a* per projected surface area (in mg m^{-2}). The absorption coefficient was only calculated for the absorbance values at 675 nm.

5.2.9 Statistical analysis

Physiological parameters measured for C1- and D-corals, including F_v/F_m, Q_m, Pg, $Pn:R_D$, E_c, chl *a*, $a^*_{Chl\ a}$, and Dt/(Dt+Dn), were compared among temperature treatments at the end of the three experimental exposures. In addition, values for each parameter were compared between the first and last day of each experiment for each *Symbiodinium* type to examine changes within each association over the period of heat stress. Non-parametric tests (Mann-Whitney U) were used in all cases as transformation of the data did not satisfy the assumption of homogeneous variances required by ANOVA.

5.3 Results

5.3.1 Bleaching condition of corals

Elevated temperatures had a much greater impact on juvenile corals of *Acropora tenuis* when they hosted ITS1 type D compared to type C1 *Symbiodinium,* both in terms of bleaching intensity and mortality. In the pilot study, all D-corals exposed to 32ºC bleached or died after 29 days, whereas the proportion of C1-corals that bleached under the same conditions was only 5% (Fig. 5.1). Results from the two light level treatments in experiment 1 emphasize the importance of light dose and intensity on the bleaching response of corals. Corals exposed to elevated light levels bleached more rapidly than those exposed to lower light levels. The proportion of colonies that bleached or died in the 32ºC treatment was 3-4 times greater for D-corals compared to C1-corals (70% at low light and 94% at high light for D-corals, compared to 13% and 33% for C1-corals. Fig. 5.1). In both the pilot study and experiment 1, the response was diminished at lower temperatures but the pattern of bleaching by type association was consistent (Fig. 5.2).

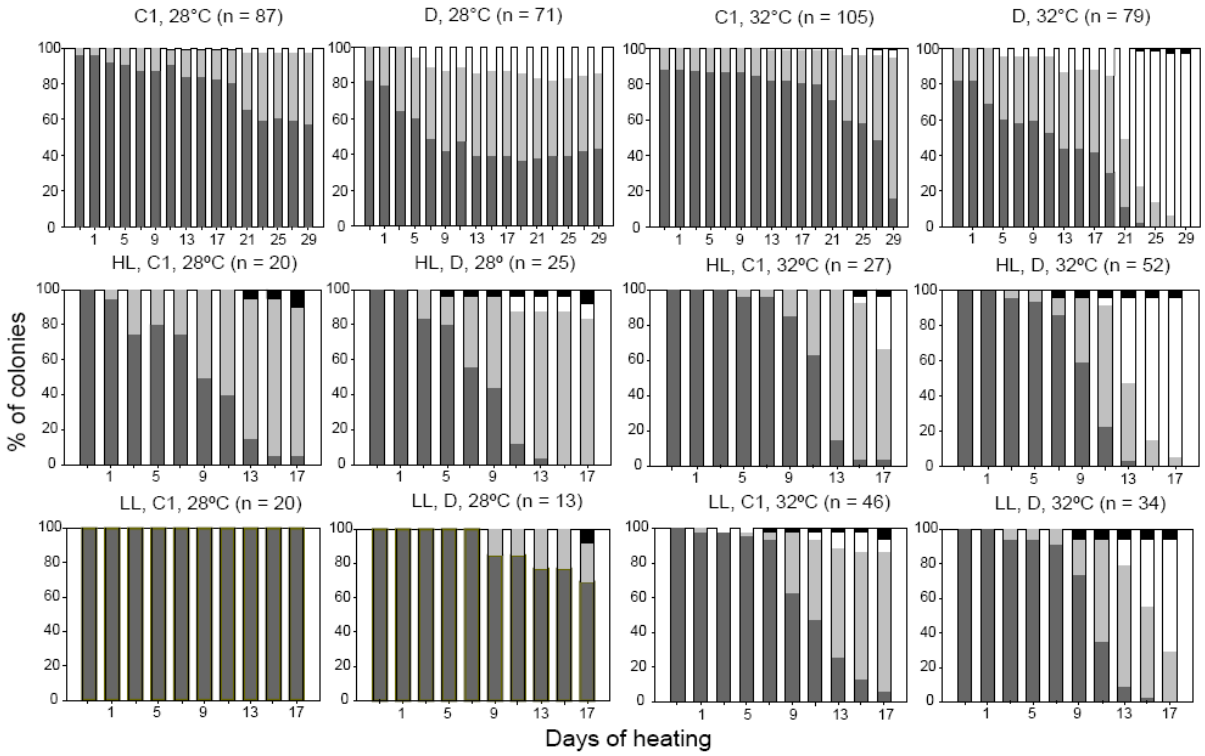


Fig. 5.1. Visual scoring of colonies hosting *Symbiodinium* type C1 or D. Top row shows results for the pilot study and bottom rows are from experiment 1. HL denotes high light treatment (362 µmol photons m^{-2} s^{-1}), LL denotes low light treatment (158 µmol photons m^{-2} s^{-1}). Four categories were used to quantify bleaching condition and mortality (normal, pale, bleached, dead). *Symbiodinium* type, temperature treatment (28ºC or 32ºC) and sample size (n) are shown for each graph.

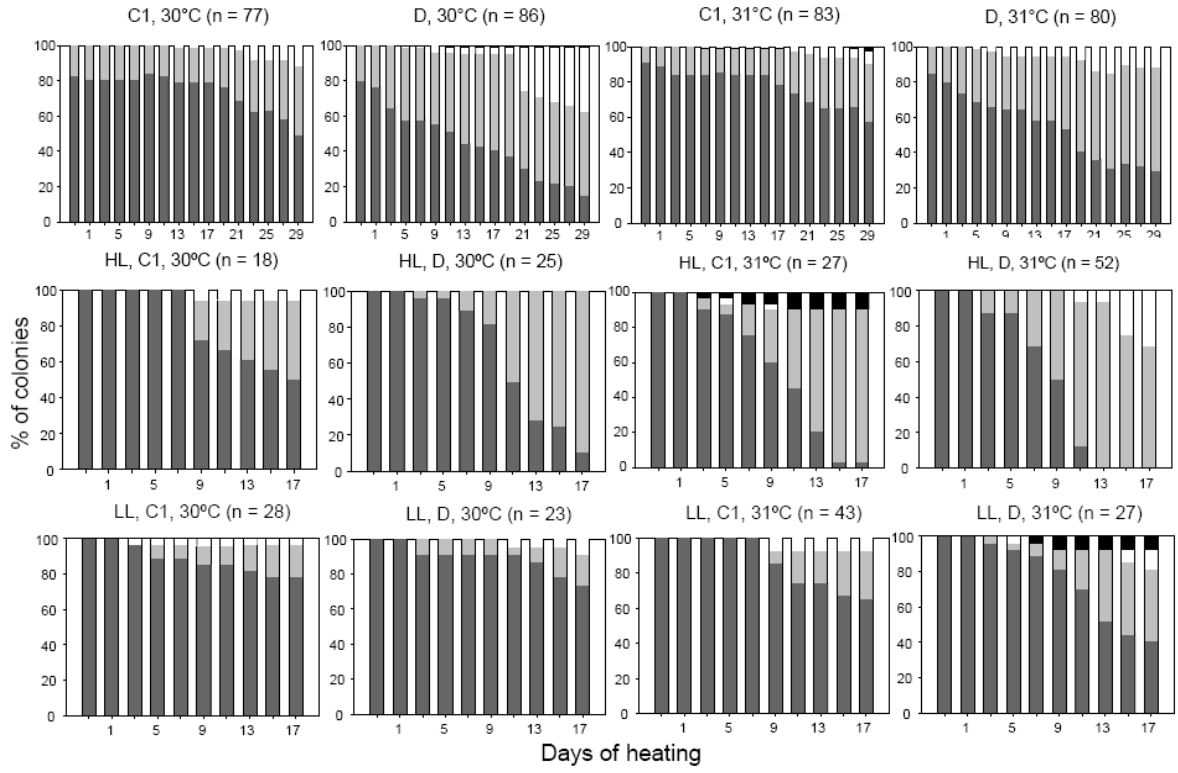


Fig. 5.2. Visual scoring of colonies hosting *Symbiodinium* type C1 or D. Top row shows results for pilot study and bottom rows are from experiment 1. HL denotes high light treatment (362 µmol photons m^{-2} s^{-1}), LL denotes low light treatment (158 µmol photons m^{-2} s^{-1}). Four categories were used to quantify bleaching condition and mortality (normal, pale, bleached, dead). *Symbiodinium* type, temperature treatment (30ºC or 31ºC) and sample size (n) are shown for each graph.

5.3.2 Photochemistry of heat-stressed coral juveniles

C1-corals had consistently greater maximum quantum yields (F_v/F_m) than D-corals in our three independent experiments (Figs. 5.3-5.6). At the end of the pilot study (33 days after heating began), F_v/F_m in C1-corals at 32˚C was only 5% lower than the same association at 28˚C (control temperature). In contrast, F_v/F_m for D-corals was 65% lower at 32˚C than at the control temperature and almost three-fold lower than in C1-corals ($p< 0.001$, Mann-Whitney U, Fig. 5.3a-b). Both associations showed a significant decline in F_v/F_m over time at 32˚C ($p<0.001$, Mann-Whitney U). However, the decline for D-corals was over 70% from initial values, compared to only 20.4% for C1-corals (Fig. 5.3b). At 31˚C, the extent of the response was smaller but F_v/F_m was still

significantly higher for C1- than for D-corals ($p<0.001$, Mann-Whitney U, Fig. 5.4b). Declines in F_v/F_m of both associations after day 17 (Fig. 5.3a-b, Fig. 5.4a-b) coincided with an increase in photoperiod (from 7.5 to 10hrs) that was initiated on this day. The much more rapid decline of F_v/F_m for D-corals in the 32˚C treatment after this point (Fig 5.3b) emphasizes the additive and potentially synergistic interaction of light and temperature to substantially increase the sensitivity of this clade to heat stress.

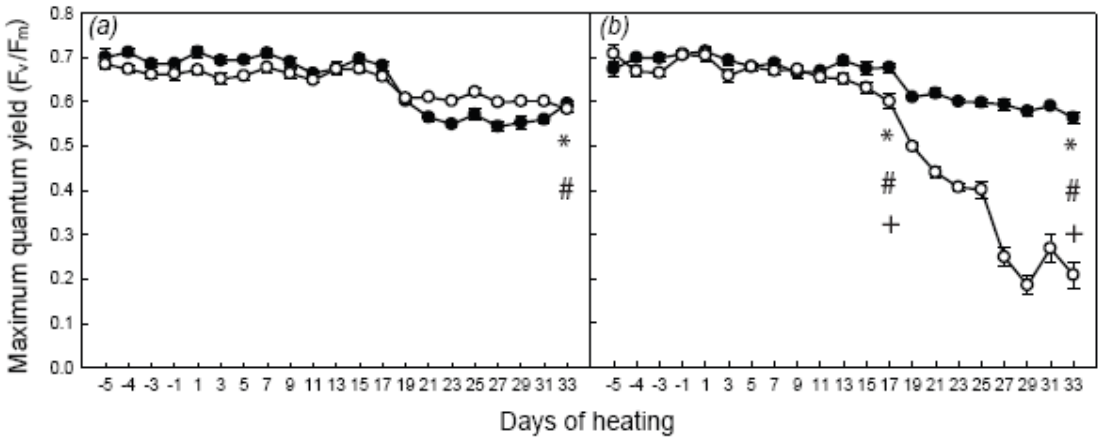


Fig. 5.3. Maximum quantum yield (F_v/F_m) of corals hosting either *Symbiodinium* C1 (●) or D (○) at 28°C (a) or 32°C (b) during the Pilot Study. Values are means ± SE for each *Symbiodinium* type (n=24). HL denotes high light treatment (360 µmol photons m^{-2} s^{-1}), LL denotes low light treatment (160 µmol photons m^{-2} s^{-1}). * and # notations refer to significant differences over time within C1 and D-corals respectively. + denotes a difference between C1 and D-corals. Comparisons are Mann-Whitney U test.

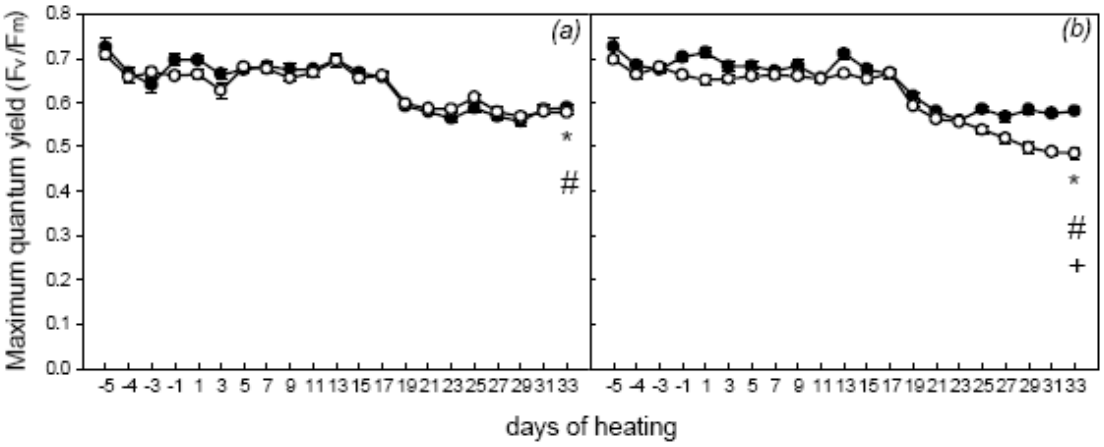


Fig. 5.4. Maximum quantum yield (F_v/F_m) of corals hosting either *Symbiodinium* C1 (●) or D (○) at 30°C (a) or 31°C (b) during the Pilot Study. Values are means ± SE for each *Symbiodinium* type (n=24). HL denotes high light treatment (360 µmol photons m^{-2} s^{-1}), LL denotes low light treatment (160 µmol photons m^{-2} s^{-1}). * and # notations refer to significant differences over time within C1 and D-corals respectively. + denotes a difference between C1 and D-corals. Comparisons are Mann-Whitney U test.

In the first full-scale experiment, which incorporated two light levels, we again found that F_v/F_m of heat-stressed C1-corals was higher compared to D-corals. F_v/F_m declined significantly in all corals under high light in both the 31˚ and 32˚C treatments ($p<0.05$, Mann-Whitney U, Fig. 5.5c-d). Changes in the photochemistry of corals were smaller in the low light treatments and significant differences in maximum quantum yields between C1 and D-corals were only detected at 32˚C (Fig. 5.5h).

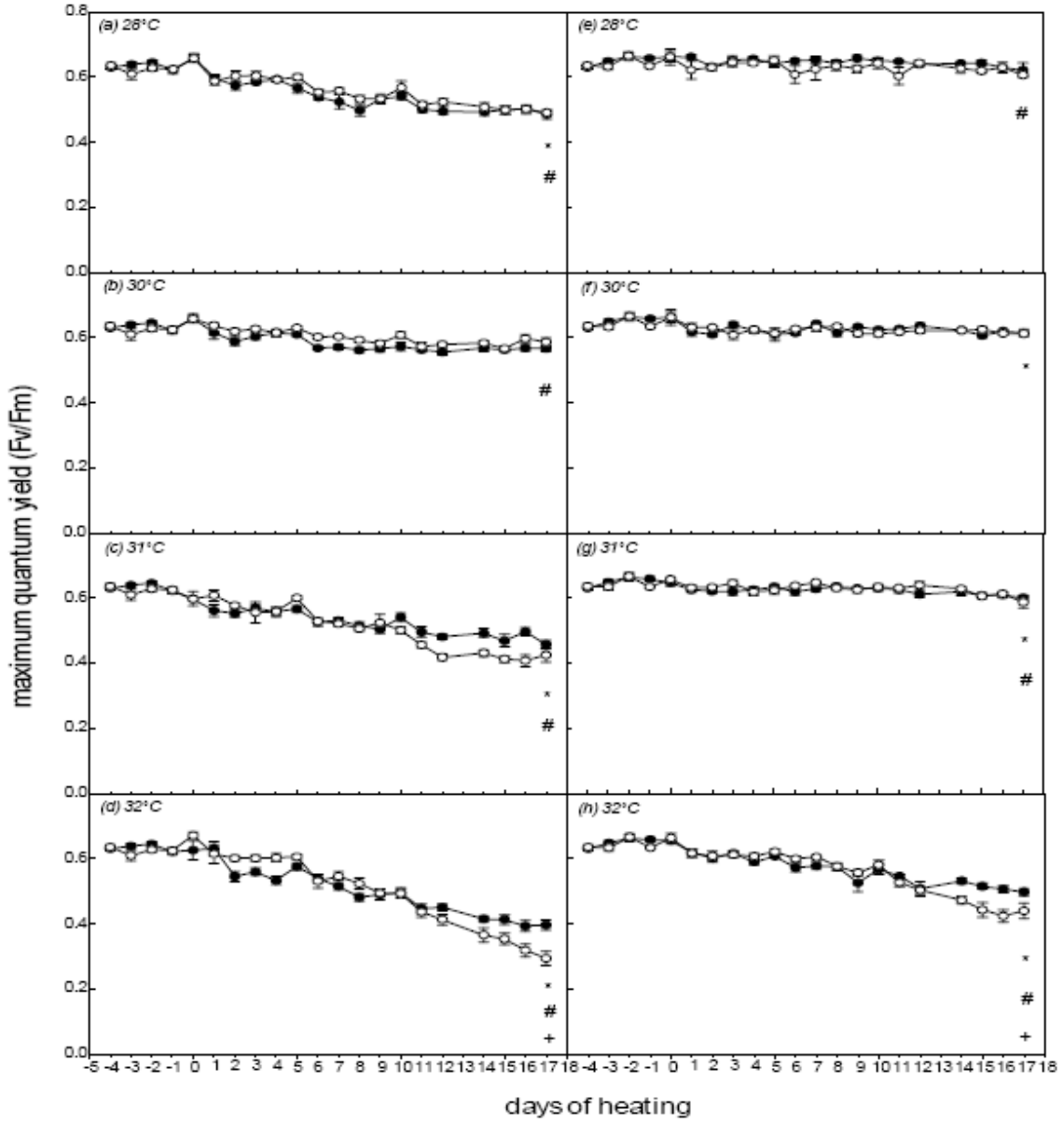


Fig. 5.5. Maximum quantum yield (F_v/F_m) of corals hosting either *Symbiodinium* C1 (●) or D (○). Graphs on the left side (a-d) are for corals in the high light treatment (362 µmol photons m^{-2} s^{-1}) at 28 (a), 30 (b), 31 (c) and 32°C (d). Graphs on the right side (e-h) are for corals in the low light treatment (158 µmol photons m^{-2} s^{-1}) at 28 (e), 30 (f), 31 (g) and 32°C (h).Values are means ± SE for each *Symbiodinium* clade (n=10-17). * and # notations refer to significant differences over time within C1 and D-corals respectively. + denotes a difference between C1 and D-corals. Comparisons are by Mann-Whitney U test.

In the second full-scale experiment, we monitored the maximum excitation pressure of PSII (Q_m) in addition to F_v/F_m to further analyze the response of these coral-algal associations to heat stress. At 32˚C, Q_m was always significantly higher in D-corals than in C1-corals ($p<0.001$, Mann-Whitney U, Fig. 5.6a). In general, there was a

significant increase in Q_m by the end of heat exposure (Day 17) for D-corals but not for C1-corals ($p<0.001$, Mann-Whitney U, Fig. 5.6a). At the intermediate temperature, Q_m was always around 25% higher in D-corals but levels did not differ significantly between the start and end of the experiment for either association (Fig. 5.6a). Colonies at the control temperature showed a small but significant ($p<0.05$, Mann-Whitney U) decline in Q_m for both associations throughout the experiment but by the end there was no significant difference between them (Fig. 5.6a). As in the previous experiments, corals hosting C1-symbionts at 32˚C had significantly higher F_v/F_m compared to D-corals ($p<0.001$, Mann-Whitney U, Fig. 5.6b). This was in spite of lower initial F_v/F_m in the C1-corals than in the D-corals. At the intermediate temperature (29˚C) there was a small but non-significant drop in F_v/F_m for both associations (Fig 5.6b). At the control temperature, F_v/F_m in C1-corals increased initially but levels did not differ significantly between the start and end of the experiment (Fig. 5.6b). In contrast, D-corals showed a sustained and significant decline in F_v/F_m in the control temperature treatment ($p<0.05$, Mann-Whitney U, Fig. 5.6b), but had slightly higher F_v/F_m than C1-corals by the end of the experiment.

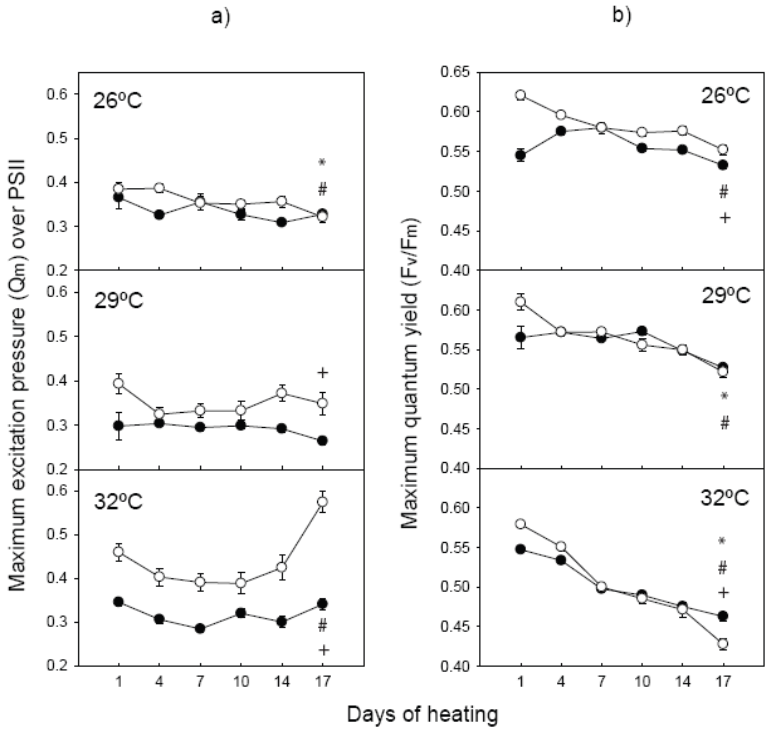


Fig. 5.6. **a)** Maximum excitation pressure over PSII (Q_m) of C1 (●) or D-corals (○) at 26ºC, 29ºC, and 32 ºC. **b)** Maximum quantum yield (F_v/F_m) of the same corals. Values are means ± SE for each *Symbiodinium* type (n=7-13). * and # notations refer to significant differences over time within C1 and D-corals respectively. + denotes a difference between C1 and D-corals. Comparisons are Mann-Whitney U test.

5.3.3 Oxygen microelectrode characterization of photosynthesis and respiration

Comparisons of gross photosynthesis rate (Pg) confirm results of reduced photochemical efficiency of D-corals at elevated temperatures obtained using chlorophyll *a* fluorescence. In the highest temperature treatment (32˚C), Pg in C1-corals was significantly higher ($p<0.05$, Mann-Whitney U, Fig. 5.7a) after 15 days of heating than in D-corals, which exhibited significant declines in Pg over time ($p<0.05$, Mann-Whitney U, Fig. 5.7a). Rates of gross photosynthesis of corals in the intermediate temperature treatment (29˚C) were not significantly different from rates of corals in the control (26˚C) treatment. Pg was similar for both coral-algal associations throughout the duration of the experiment in both the control and intermediate temperature treatments (Fig. 5.7a).

The ratio between rates of net photosynthesis and dark respiration ($Pn:R_D$) decreased significantly during heating in D-corals at 32˚C, but not in C1-corals ($p<0.05$, Mann-Whitney U, Fig. 5.7b). There was no significant difference in $Pn:R_D$ between C1 and D-corals at the start of heating (Day 1, Fig 5.7b). However, at day 15, $Pn:R_D$ of C1-corals was significantly higher at 32 ˚C ($p<0.05$, Mann-Whitney U, Fig. 5.7b). At day 15, E_c in D-corals was significantly higher than in C1-corals in the 32˚C treatment ($p<0.05$, Mann-Whitney U, Fig. 5.7c).

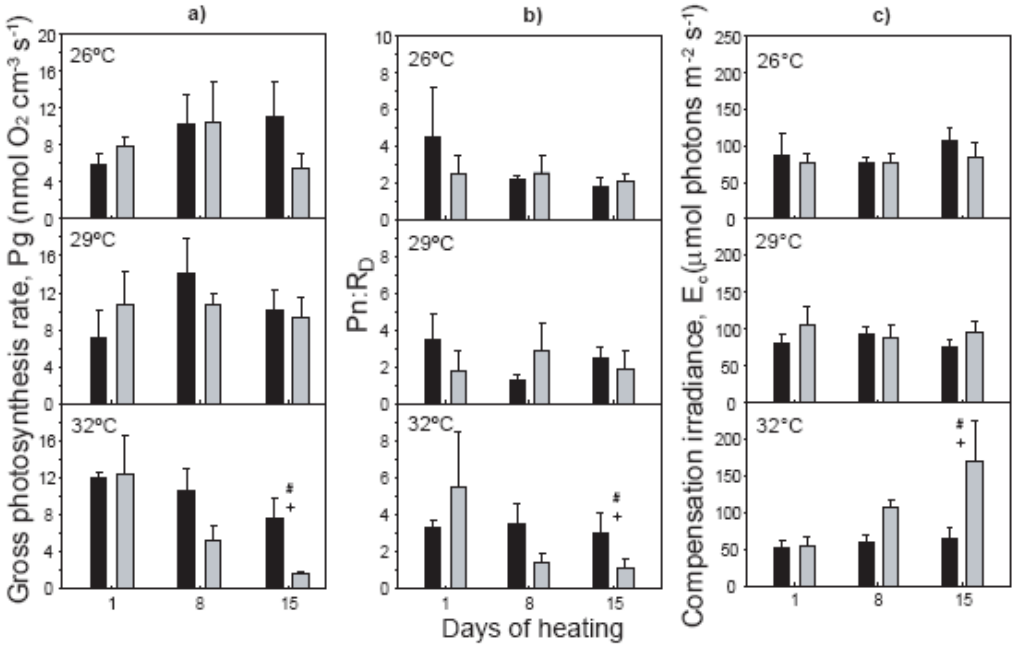


Fig. 5.7. O_2 microelectrode measurement of photosynthesis in C1 (black columns) or D-corals (grey columns). **a)** Gross photosynthesis rate, Pg (nmol O_2 cm^{-3} coral surface s^{-1}), **b)** net photosynthesis rate vs dark respiration rate ($Pn:R_D$), **c)** compensation irradiance, E_c, at 26ºC, 29ºC, and 32ºC. Values are means ± SE (n=4 for each *Symbiodinium* type). * and # notations refer to significant differences over time within C1 and D-corals respectively. + denotes a difference between C1 and D-corals. Comparisons are Mann-Whitney U test.

5.3.4 Chlorophyll *a* content, absorption coefficient ($a^*_{Chl\ a}$), and xanthophyll pigments

After 15 days of heating at 32ºC, the chl *a* content in both associations was significantly lower relative to initial levels ($p<0.05$, Mann-Whitney U). However, the chl *a* content of C1-corals was 1.8 times higher ($p<0.05$, Mann-Whitney U) compared to that of D-corals, even though the latter had slightly higher initial chl *a* concentrations (Fig. 5.8a). No significant differences were found within or between associations at the lower temperatures (26ºC and 29ºC).

The absorption coefficient of chl *a* in D-corals was 2.4 times higher compared to C1-corals ($p<0.05$, Mann-Whitney U, Fig. 5.8b) after 15 days of heating at 32°C. Despite having similar initial levels, $a^*_{Chl\ a}$ increased significantly over time in both associations but in D-corals this increase was more than four-fold by day 15 ($p<0.05$, Mann-Whitney U, Fig. 5.8b). No significant differences were found within or between associations at the lower temperatures.

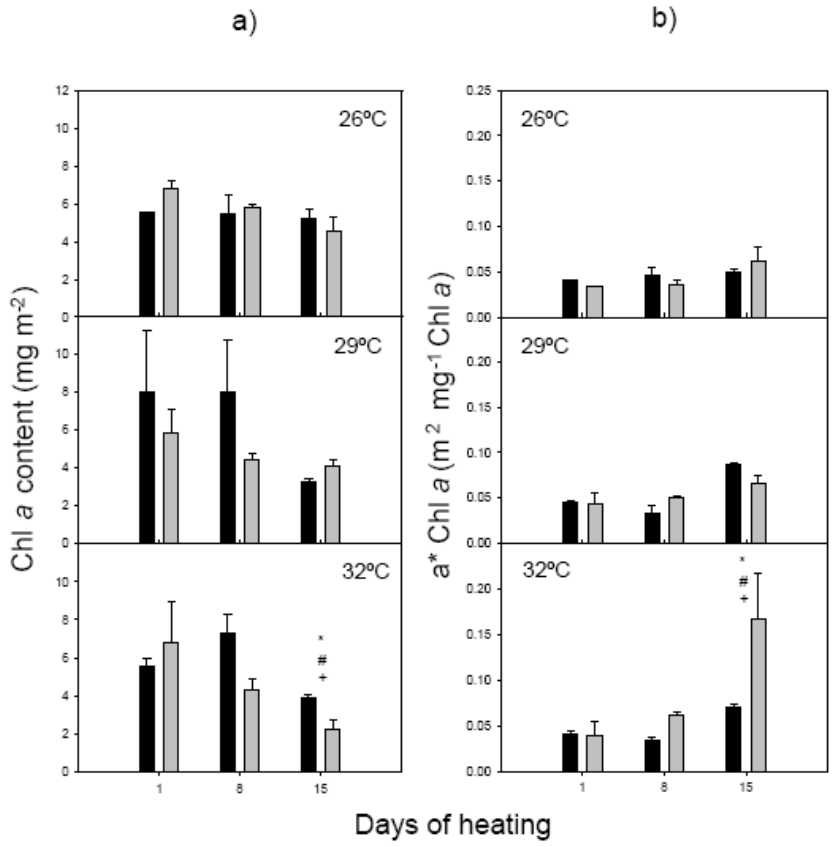


Fig. 5.8. **a)** Chl *a* content in sub samples of C1 (black bars) or D-corals (grey bars). **b)** Specific absorption coefficient of Chl *a* ($a^*_{chl\ a}$) in the same samples as in **a)**. Values are means ± SE (n=3 for each *Symbiodinium* type). Where error bars are not visible, they are small and hidden by the columns. * and # notations refer to significant differences over time within C1 and D-corals respectively. + denotes a difference between C1 and D-corals. Comparisons are Mann-Whitney U test.

There was a significant decline in the total pool of xanthophylls in both associations at 29ºC and 32ºC ($p<0.05$, Mann-Whitney U; data not shown). The change in the xanthophyll pool was driven by a significant decline in diadinoxanthin ($p<0.05$, Mann-Whitney U). However, when normalized to the amount of chl *a,* the differences in xanthophyll pigments over time or between D and C1 types were not significant. The xanthophyll ratio of both associations at 32ºC increased during the experiment but by day 15, it was significantly higher in D-corals than in C1-corals ($p<0.05$, Mann-Whitney U, Fig. 5.9). Although changes in the ratio of xanthophyll pigments at 32°C suggested the activation of xanthophyll cycling as a photo-protective mechanism, there

was no correlation between this elevated ratio and the bleaching response of the corals. The xanthophyll ratio of corals at 29ºC was not significantly different between associations but it showed a small but significant decline in C1 corals relative to initial levels ($p<0.05$, Mann-Whitney U, Fig. 5.9). At 26°C, the xanthophyll pool in C1-corals did not change significantly during the course of the experiment, whereas there was a significant drop in both pigments during the experiment for D-corals ($p<0.05$, Mann-Whitney U; data not shown). For both associations at this temperature, the xanthophyll ratio changed significantly from initial values and was significantly different between associations by the end of the experiment ($p<0.05$, Mann-Whitney U, Fig. 5.9).

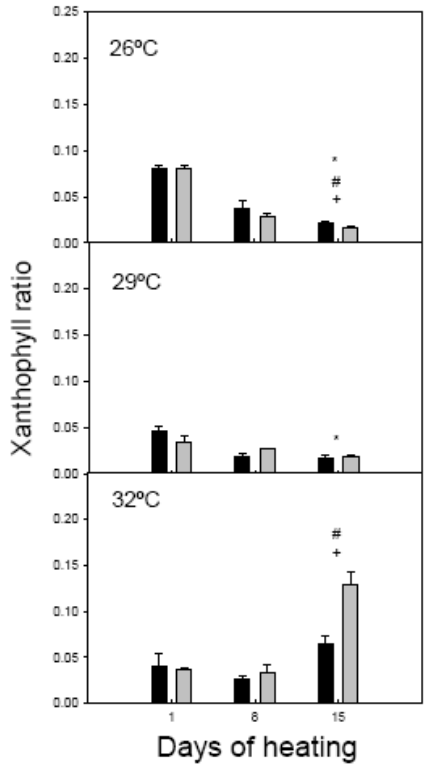


Fig. 5.9. Changes in xanthophyll ratio (ratio of diatoxanthin to the sum of diatoxanthin and diadinoxanthin) of C1 (black bars) or D-corals (grey bars). Values are means ± SE (n=3 for each *Symbiodinium* type). Where error bars are not visible, they are small and hidden by the columns. * and # notations refer to significant differences over time within C1 and D-corals respectively. + denotes a difference between C1 and D-corals. Comparisons are Mann-Whitney U test.

5.4 Discussion

Our results demonstrate that the bleaching response of corals can vary dramatically depending on the *Symbiodinium* type with which they associate. Juveniles of the common coral *Acropora tenuis,* which naturally establish symbioses with both type C1 and D *Symbiodinium* in field uptake studies (Little *et al.* 2004), were found to have much greater thermal tolerance when associated with type C1. The greater robustness of C1-juveniles to temperature and light stress was supported by all physiological parameters measured in each of three independent heat/light stress experiments. The only exception was the increased xanthophyll ratio in D-juveniles, which may have been induced to counter stress. However, it is possible that the xanthophyll pigments of these juveniles were still overwhelmed and therefore no improvement in the overall physiological state was observed. Moreover, the proportion of D-corals that bleached and/or died at 31°C and 32°C was higher than C1-corals in all three experiments. Although a number of studies have suggested that corals associated with clade D have greater thermal tolerance (Glynn *et al.* 2001; Toller *et al.* 2001a; Baker *et al.* 2004; Fabricius *et al.* 2004; Rowan 2004; Berkelmans & van Oppen 2006), our results demonstrate that enhanced bleaching tolerance of corals is not universally associated with *Symbiodinium* types within clade D. Moreover, our conclusion that type C1 is thermally robust is consistent with recent field observations showing a dramatic shift in the symbiont community of *A. millepora* from type C2 to type C1 or a mix of C1/D after bleaching (Jones *et al.* 2008a). Although it is possible that clade D includes algal types that differ in thermal tolerance (see Tchernov *et al.* 2004), the specific D-type associated with lower bleaching tolerance in juveniles of *A. tenuis* in our study was shown to confer higher thermal tolerance in adults of *A. millepora* (Berkelmans and vanOppen 2006). Thus, caution must be exercised in making generalizations about the

performance of *Symbiodinium* clades *in hospite*, and there is need for further studies to explore host-symbiont interactions and their impact on the physiology of the coral holobiont.

5.4.1 Photochemical confirmation of enhanced thermal tolerance of C1-juveniles

In addition to macroscopic indicators of holobiont health, photochemical measures clearly demonstrate enhanced thermal tolerance of coral juveniles when associated with *Symbiodinium* type C1. The steady and up to three-fold greater decline in photochemical efficiency of PSII in D-corals well into the period when they started to bleach suggests that these corals were experiencing chronic photo-inhibition (Brown *et al.* 1999; Gorbunov *et al.* 2001). In contrast, the smaller decline of F_v/F_m and lack of substantial bleaching in C1-corals is consistent with photo-acclimation (Robison & Warner 2006). Moreover, measurements of maximum excitation pressure over PSII (Q_m) corroborated the reduced photochemical efficiency of clade D *Symbiodinium* when associated with *A. tenuis*. Q_m takes into account the induction of photo-chemical and non-photo-chemical processes competing within the reaction centers of PSII for de-activation of chlorophyll *a* excited states (Maxwell *et al.* 1995; Iglesias-Prieto *et al.* 2004) and hence enables the distinction between photo-acclimation and photo-inhibition. Values close to one indicate photo-inhibition whereas values close to zero indicate light-limitation. Our conclusion that the smaller decline of F_v/F_m and lack of bleaching in C1-corals in the high temperature treatment represented photo-acclimation is supported by the lack of change in excitation pressures of PSII (Q_m) at 32°C for C1-corals, in contrast to the significant increase found for D-corals. Under normal conditions, photosynthetic marine organisms can regulate Q_m by changing the concentration of chlorophyll *a*, thereby modifying the light absorption efficiency of this

pigment ($a^*_{Chl\ a}$) (Enriquez *et al.* 2005; Robison & Warner 2006), and thus decrease the probability of damage to PSII by chronic photo-inhibition. However, under thermal stress conditions, when the chain of degradation events leading to coral bleaching is activated by damage to PSII (Iglesias-Prieto & Trench 1994; Warner *et al.* 1996; Iglesias-Prieto & Trench 1997b; Warner *et al.* 1999; Takahashi *et al.* 2004) or downstream from PSII (Jones *et al.* 1998), this photo-acclimation mechanism can break down. The higher values of Q_m at 32°C in D-corals (Fig. 5.6), combined with lower amounts of chlorophyll *a* and higher absorption efficiency of this pigment (Fig. 5.8) provide strong evidence of decreased physiological performance of *A. tenuis* at high temperatures when associated with *Symbiodinium* D.

5.4.2 The role of light in the bleaching response of heat-stressed corals

The rapid decline in photochemical efficiency in the pilot study when the photoperiod was increased highlights the importance of light in the bleaching response of corals. In D-corals in particular, rapid declines in photochemical efficiency correlated with rapid increases in the proportion of bleached colonies. Such inverse correlations may be explained by feedback loops that magnify photoinhibition as coral tissues become more translucent. The highly reflective nature of coral skeletons (Kühl *et al.* 1995), particularly when the number of symbionts and/or amount of pigment is reduced during bleaching, results in a magnified light field within the host tissue and further exacerbates damage to remaining cells (Enriquez *et al.* 2005). Ironically, the rise in absorption efficiency of chl *a* ($a^*_{Chl\ a}$) in the high temperature treatment during the second experiment would imply that the efficiency of light capture increases with temperature. However, due to the loss of reaction center integrity, this light becomes a liability and contributes to further degradation of PSII. In combination with the more

rapid onset of bleaching in the first experiment in the high light treatment, our results underscore the enormous role that light pre-history and dose can have on the bleaching response of heat-stressed corals (Brown *et al.* 2002).

5.4.3 Contribution of symbionts to metabolic costs incurred during heat stress

Our oxygen microelectrode measurements add important insights into the photosynthetic performance of *Symbiodinium* types when associated with *Acropora tenuis* juveniles and further corroborate our conclusion that *A. tenuis* juveniles are more tolerant to combined heat and light stress when associated with *Symbiodinium* C1. The reduced rate of photosynthesis (Pg) found for D-juveniles indicates a reduced capacity for carbon fixation (Li *et al.* 1984; Jones *et al.* 1998) that is consistent with photo-inhibition. Moreover, the decreased ratio of net photosynthesis to dark respiration ($Pn:R_D$) for D-corals, but not C1-corals (Fig. 5.7), suggests that when associated with D *Symbiodinium,* the holobiont invests more heavily in maintenance and repair processes associated with metabolic costs incurred during heat stress (Warner *et al.* 1996; Takahashi *et al.* 2004). Such energetic costs are likely to impact other important parameters such as growth and reproduction of the holobiont (Michalek-Wagner & Willis 2001; Baird & Marshall 2002).

In addition, the lower efficiency of light utilization in photosynthesis found for heat and light stressed D-juveniles, as indicated by their increased compensation irradiance (E_c) (Epping & Kühl 2000), suggests that greater energetic costs contributed to their poorer performance. The linear integration by which E_c was calculated may skew the irradiance intensity at which net energy acquisition occurs due to the normal shape of the photosynthesis-irradiance curve (Platt *et al.* 1980). However, the significant increase in

E_c observed in D-corals at 32°C after 15 days (Fig. 5.7c) corresponds well with our other estimates of photosynthetic activity. Furthermore, due to the highly reflective nature of the coral skeleton, as the experiment progressed and corals bleached, the light field around the symbionts is amplified (Enriquez *et al.* 2005) and therefore the effects of underestimating E_c can be considered negligible. While every method has its limitations, the use of microsensors permits minimally invasive and accurate mapping of oxygen and photosynthesis activity at high spatial resolution. It has not been shown whether reactive oxygen species could influence these measurements or by how much, however, the cleavage of ROS molecules to form free oxygen would be required to bias our results.

5.4.4 Potential role of host factors in the heat stress response

Differences in the tolerances of C1- versus D-corals to heat and light stress between ours and previous studies may be explained partially by host factors, or interactions between hosts and symbionts that may modify the physiological response of the holobiont. For example, each partner in the symbioses is capable of producing protective enzymes involved in protein re-generation and/or anti-oxidant defense pathways (Shick *et al.* 1995; Downs *et al.* 2000; Brown *et al.* 2002). Synthesis of one or more of these enzymes in one partner may elicit a response in the other that differs according to its identity. Previous studies, which have shown that corals associated with type D are more thermally tolerant, have involved species in which type D is homologous, highlighting a potential role for host factors. Berkelmans and van Oppen (2006) showed that adult corals of *Acropora millepora* that had shuffled their dominant symbiont population after bleaching, from type C2 to D, were more thermally tolerant in a subsequent heat-stress experiment. Similarly, juvenile *A. millepora* achieved

superior thermal tolerance when associated with type D (Mieog *et al.* pers. comm.), the type normally hosted by adults of this species at Magnetic Island. In both of these studies the D-type was the same as those used in our study (GenBank accession number EU024793). Although juveniles of *A. tenuis* host *Symbiodinium* type D at this location (Little *et al.* 2004), adult colonies do not (van Oppen *et al.* 2001), thus host factors required to maintain this association past an initial flexible stage may not have evolved.

Interestingly, *A. tenuis* juveniles initially establish a symbioses with a mix of type D and C1 at this location, and although they rapidly become dominated by type D during early ontogeny (less than 1 year old), they grow much faster when hosting *Symbiodinium* type C1 (Little *et al.* 2004). Why juveniles of *A. tenuis* should establish and maintain a symbiosis with a *Symbiodinium* type not found in adults remains to be investigated. Possible explanations for the change from D to C1 dominance include: 1) onset of as yet undescribed host factors in early ontogeny that may regulate the symbiosis and favor type C1 symbionts (Rodriguez-Lanetty *et al.* 2004); 2) accumulation of deleterious impacts arising from associating with type D *Symbiodinium* that increases mortality of D-juveniles through time (Little *et al.* 2004); 3) superior competitive ability of type C1 symbionts within host cells (Fitt 1985); 4) changing physiological needs associated with life history stage; and/or 5) changing micro-environmental conditions associated with the growth of the host that differentially favor one type over the other through time.

In summary, juvenile *A. tenuis* achieved superior thermal tolerance when associated with *Symbiodinium* C1, the type normally hosted by adults at the study location. Type C1 is a very common and widespread symbiont in *A. tenuis* on the GBR, but the most common type found associated with this coral throughout the GBR is C2 (van Oppen *et*

al. 2005). This and the fact that C is the most common and diverse clade in Indo-Pacific corals (LaJeunesse 2005), call for further exploration of how genetic diversity within clade C correlates to physiological diversity. Along with this, continued efforts to understand the cellular mechanisms underlying host-symbiont interactions will provide insights into how corals and the reefs they build may respond to environmental change.

Chapter 6.0 General discussion, major findings, and future research

Reef-scape dominated by *Acropora* corals. Photo: Yui Sato

6.1 General Discussion

The grim outlook for coral reefs in the face of predicted climate change demands comprehensive research into potential mechanisms for corals to adapt to warming oceans. Given the fundamental nature of the coral-*Symbiodinium* symbiosis (Muscatine 1977), one of the most relevant areas of research is the establishment and maintenance of this relationship. The major aim of this thesis has been to enhance current understanding of environmental factors driving the uptake, establishment, and regulation of coral-*Symbiodinium* symbioses in the context of climate change. A logical first step towards this aim was to examine symbiont specificity by analysis of temporal and geographical variation in *Symbiodinium* uptake. The extraordinary genetic diversity within the genus *Symbiodinium* (Baker 2003; Coffroth & Santos 2005) has prompted many researchers to examine how open or flexible these relationships are in corals or other invertebrates that exhibit a horizontal mode of *Symbiodinium* acquisition (Belda-Baillie *et al.* 1999; Coffroth *et al.* 2001; Weis *et al.* 2001; Little *et al.* 2004; Rodriguez-Lanetty *et al.* 2004; Gomez-Cabrera *et al.* 2008). The consensus from these studies is that corals are initially flexible in the type(s) of *Symbiodinium* taken up, but there is mixed evidence in terms of the fidelity of the symbiont type eventually established in the mature holobiont. For example, juveniles of the gorgonian *Plexaura kuna* can take up multiple types of *Symbiodinium*, including non-homologous types, but the homologous type is the best in establishing a stable association (Coffroth *et al.* 2001). On the other hand, initial uptake in juveniles of the coral genus *Acropora* appears more successful with non-homologous symbionts (Gomez-Cabrera *et al.* 2008) or at least equally successful with non-homologous or mixed symbionts (Little *et al.* 2004).

An important issue that emerges from these studies is how specificity for the algal symbiont is defined. The notion of strict specificity for the algal symbiont, that is, the same *Symbiodinium* type is always found in a host throughout its distribution range, cannot be supported in light of evidence that the same host species may harbour different symbionts in different photic zones on the reef (Rowan & Knowlton 1995) or in different reefs across its biogeographical range (Loh *et al.* 2001; van Oppen *et al.* 2001). However, fidelity for a specific *Symbiodinium* type appears to occur at a local level (van Oppen *et al.* 2001; LaJeunesse *et al.* 2004), raising the possibility of local specificity for an algal symbiont, an issue that has received little attention in the literature. This raises the question of whether uptake patterns of *Symbiodinium* in juveniles from coral species that vary in association across localities follow the parental association or that found in conspecific corals at the new location. Both species of coral used in the present study associate with different *Symbiodinium* types on reefs that are less than 100 km apart. Analysis of *Symbiodinium* communities initially acquired by 24 cohorts of newly settled coral juveniles from 11 study populations of *Acropora tenuis* and *A. millepora* overwhelmingly confirmed that uptake of *Symbiodinium* is non-specific (Chapter 2). However, in 5 out of 7 populations that were monitored for up to 3.5 years, I found differences in *Symbiodinium* succession that suggested local specificity for the algal symbiont in *A. tenuis,* although I was unable to draw definitive conclusions for *A. millepora* (Chapter 4). These results are by far the longest, most comprehensive data set on symbioses in the very early life stages of corals and show that: (1) it can take several years to establish the homologous association characteristic of parental colonies and, (2) closely related corals exhibit different specificities for their algal symbionts during the establishment phase. These results may help reconcile the conflicting evidence from a variety of studies that indicate specificity in some cases

(Coffroth *et al.* 2001; Weis *et al.* 2001; Belda-Baillie *et al.* 2002; Rodriguez-Lanetty *et al.* 2004) but lack of specificity in others (Little *et al.* 2004; Gomez-Cabrera *et al.* 2008). The very long lag period in the acquisition of the homologous symbiont characteristic of adult populations by *A. tenuis* juveniles prevented Little *et al.* (2004) from detecting the patterns shown in the present study.

The surprisingly high proportion of newly settled juveniles that acquired type D *Symbiodinium* on the reef (Chapters 2 & 4) provides strong evidence that this type is highly infectious and potentially opportunistic. This has important implications for the survival of the holobiont. Given the obligate nature of algal endosymbioses, flexibility in the type of symbionts that can be taken up would ensure the survival of corals recruiting to a reef where the parental or homologous type is absent or rare (as in the case of the Orpheus Island juveniles growing at Magnetic Island, Chapter 4).

A second step towards the major aim of this thesis was to determine the impact of environmental factors in the establishment of the symbiosis. Impeded uptake of *Symbiodinium* as a consequence of high temperature stress (Chapter 3), compounds the already gloomy outlook for corals in the context of warming oceans. This is particularly worrying as *Symbiodinium* uptake in corals with horizontal acquisition (~ 85% of all corals, Harrison & Wallace 1990; Richmond & Hunter 1990) occurs in the weeks following the late-spring spawning events (Babcock *et al.* 1986) when sea-surface temperatures are approaching annual peaks. This is also when corals are at the highest risk of bleaching as a consequence of high temperature stress (Brown 1997; Hoegh-Guldberg 1999). Considering predicted increases in sea surface temperatures over the coming decades (IPCC 2007), these results suggest potentially devastating summer

scenarios where coral recruits fail to establish a symbiosis while adult corals fail to maintain it.

The results from this thesis provide some encouraging signs for the future persistence of coral-algal endosymbioses. The non-specific uptake of *Symbiodinium* provides a mechanism by which a variety of symbiont types may be acquired, including potentially heat tolerant types. *Symbiodinium* types within clade D have been characterized as heat tolerant and opportunistic by many studies (Baker 2001; Glynn *et al.* 2001; Toller *et al.* 2001a; Baker *et al.* 2004; Fabricius *et al.* 2004; Rowan 2004; Berkelmans & van Oppen 2006). In the present study, type D *Symbiodinium* dominated initial symbioses in the field (Chapters 2 & 4) and increased in proportion in juveniles kept at high temperatures in laboratory experiments (Chapter 3). Taken together, these results show a promising capacity for flexible associations early on that may, at the very least, "buy time" for juveniles until summer sea temperatures decrease. It is important to point out that although opportunistic *Symbiodinium* types may be able to initiate a symbiosis at high temperatures, further research is required to ascertain whether these symbioses can persists through extended periods at high temperatures and to investigate potential tradeoff to the host.

An emerging theme from the results of this thesis is that species-specific interactions between the algal endosymbiont and the coral host play a large part in (1) the uptake of *Symbiodinium* under non-stress temperatures, (2) the attainment of a steady symbiosis, and (3) the response of the holobiont to thermal stress. First, *A. tenuis* juveniles quickly acquired their homologous *Symbiodinium* type at 28°C while *A. millepora* acquired roughly equal proportions of different types or slightly higher concentrations of a non-

homologous type (with the exception of the last data point at high light, Chapter 3). Second, the steady increase in the proportion of type C1 *Symbiodinium* in *A. tenuis* juveniles, which had been outplanted to the reef for *in situ* acquisition of *Symbiodinium* types, suggests the existence of specificity for the algal symbiont, whereas the remarkably stable symbiosis with type D *Symbiodinium* in *A. millepora* prevented any conclusions to be drawn regarding specificity (Chapter 4). Finally, the bleaching response of *A. tenuis* juveniles hosting type D *Symbiodinium* was significantly higher than that in juveniles hosting type C1 (Chapter 5). This result challenges the notion that associations with type D *Symbiodinium* are universally more thermally robust (Baker 2001; Baker *et al.* 2004; Rowan 2004; Fabricius *et al.* 2004; Berkelmans & van Oppen 2006). It is important to point out that the results of Chapter 3 show that *Symbiodinium* type D was better at initiating a symbiosis in both species at high temperatures, which appears to contradict the results of Chapter 5. However, as mentioned above, further research is required to ascertain whether this association could persist and actually benefit the host, since the juveniles at high temperatures were significantly less pigmented and had lower relative survival compared to those in the control temperature (which for *A. tenuis* juveniles was dominated by *Symbiodinium* type C1). Another key difference between these chapters is that Chapter 3 examined uptake at elevated temperatures while Chapter 5 examined the response of well-established symbioses to thermal stress (i.e. the corals did not need to acquire any symbionts as they had already been engaged in stable associations with either type C1 or D *Symbiodinium*). However, in both chapters the juveniles with the best established association (control temperature, Chapter 3) and the highest thermal tolerance (Chapter 5) was with type C1 for *A. tenuis*. This may not be the case with other corals, as shown for *A. millepora* adults subjected to heat stress (Berkelmans & van Oppen 2006). Hence, in line with the emerging theme

of this thesis, the results from chapter 5 show that generalizations about the physiological attributes of particular *Symbiodinium* types cannot be extended to the holobiont.

6.2 Major findings of this thesis

1. Uptake of symbionts by newly settled coral juveniles is non-specific (Ch. 2). Highly infectious symbionts are able to quickly dominate the symbiosis in juveniles early on but may not be the only ones retained (Ch. 2).
2. Light intensity has little or no influence on the type of symbiont initially acquired by juvenile corals (Ch. 2 and Ch. 3).
3. Elevated temperatures substantially delay or stop the initial establishment of the symbiosis in coral juveniles (Ch. 3).
4. Elevated temperatures affect the type of symbiont initially acquired by closely related coral species (Ch. 3).
5. Closely related corals express different levels of specificity for algal symbionts over time and it may take several years for coral juveniles to establish a homologous association (Ch. 4).
6. Metabolic or physiological changes associated with the onset of reproductive maturity are not related to the type of *Symbiodinium in hospite* (Ch. 4).
7. Species-specific interactions between corals and their algal symbionts define their bleaching response to heat and light stress (Ch. 5).

6.3 The future

Research on coral-*Symbiodinium* symbioses remains a critical priority to understand how corals and the reefs they build might adapt to climate change. The present study has demonstrated that uptake in the reef is non-specific. This highlights the importance of identifying the potential pools of symbionts (outside of symbiotic hosts) which are available to coral recruits. Recent studies have found *Symbiodinium* belonging to

several clades in the water column above the reef (Manning & Gates 2008) as well as in sediments on the reef (Coffroth *et a*l. 2006, Hirose *et al*. 2008, Adams *et al*. 2009) and in algal beds on the reefs (Porto *et al*. 2008). Although it has been shown that some of these can initiate symbioses with cnidarians (Coffroth *et al*. 2006, Adams *et al.* 2009), this is a wide open area of research as it is currently not known how abundant these potential reservoirs of *Symbiodinium* are and how many of these *Symbiodinium* types can establish symbioses with reef corals.

The survival of coral juveniles with non-homologous symbionts also demands closer inspection to evaluate if and how such symbionts might meet the metabolic demands of the host and to understand the trade-offs that enable these symbioses to persist. For example, juveniles associating with type D *Symbiodinium* may be able to cope with higher summer temperatures but colony growth may be stunted (Little *et al.* 2004, but only a few species have been examined this way) or they may be forced to shift towards heterotrophy in order to meet metabolic demands. Comprehensive characterization of physiological interactions in these symbioses (relative contribution of *Symbiodinium* types to energy budgets, stress resilience, and growth of the holobiont, to name a few) will enhance our understanding of how they are maintained, with the potential to revise our definition of what constitutes a homologous symbiont. Not only may the homologous symbiont type of adult populations vary with local conditions, it is also possible that one *Symbiodinium* type may be homologous during early ontogeny but another becomes the characteristically homologous type in adulthood.

Our understanding of how these symbioses are formed and maintained will be significantly advanced by unraveling the cellular mechanisms behind these processes.

To this end, several studies are starting to examine gene expression patterns in coral hosts (Reynolds *et al.* 2000; Yuyama *et al.* 2005; Rodriguez-Lanetty *et al.* 2006a; Desalvo *et al.* 2008; Schwarz *et al.* 2008) and symbionts (Lin *et al.* 2000) in order to identify the proteins involved in cellular recognition processes, but how these patterns change in response to different types of *Symbiodinium* remains to be shown.

This thesis has examined how different coral-*Symbiodinium* associations change through time and how temperature and light shape the establishment and regulation of these symbioses in order to enhance our understanding of potential impacts of warming oceans on future reefs. The results presented here highlight the complexity of the interactions that shape the association between the two partners and the physiological performance of the holobiont. Future studies should be directed at teasing apart the cellular mechanisms underlying healthy symbioses in order to estimate the scope for adaptation to increasingly warmer and acidic oceans.

References

Abrego, D., Ulstrup, K. E., Willis, B. L., van Oppen, M. J. H. 2008 Species-specific interactions between algal endosymbionts and coral hosts define their bleaching response to heat and light stress. *Proceedings of the Royal Society B* **275**, 2273-2282.

Adams L. M., Cumbo V. R., Takabayashi M. 2009 Exposure to sediment enhances primary acquisition of *Symbiodinium* by asymbiotic coral larvae. *Marine Ecology Progress Series* **377**, 149-156.

Ambarsari, I., Brown, B. E., Barlow, R. G., Britton, G. & Cummings, D. G. 1997 Fluctuations in algal chlorophyll and carotenoid pigments during solar bleaching in the coral *Goniastrea aspera* at Phuket, Thailand. *Marine Ecology Progress Series* **159**, 303-307.

Anthony, K., Ridd, P., Orpin, A., Larcombe, P., Lough, J. 2004 Temporal variation of light availability in coastal benthic habitats: Effects of clouds, turbidity, and tides. *Limnology and Oceanography* **49**, 2201-2211.

Babcock, R., Mundy, C. 1996 Coral recruitment: Consequences of settlement choice for early growth and survivorship in two scleractinians. *Journal of Experimental Marine Biology and Ecology* **206**, 179-201.

Babcock, R. C., Bull, G. D., Harrison, P. L., Heyward, A., Oliver, J. K., Wallace, C. C., Willis, B. L. 1986 Synchronous spawnings of 105 scleractinian coral species on the Great Barrier Reef. *Marine Biology* **90**, 379-394.

Baillie B. K., Belda-Baillie C. A., Maruyama T. 2000 Conspecificity and Indo-Pacific distribution of *Symbiodinium* genotypes (Dinophyceae) from giant clams. *Journal of Phycology* **36**, 1153-1161.

Baird, A. H., Marshall, P. A. 2002 Mortality, growth and reproduction in scleractinian corals following bleaching on the Great Barrier Reef. *Marine Ecology Progress Series* **237**, 133-141.

Baker, A. C. 2001 Reef corals bleach to survive change. *Nature* **411**, 765-766.

Baker, A. C. 2003 Flexibility and specificity in coral-algal symbiosis: Diversity, ecology, and biogeography of *Symbiodinium*. *Annual Review of Ecology, Evolution & Systematics* **34**, 661-689.

Baker, A. C., Rowan, R. 1997 Diversity of symbiotic dinoflagellates (zooxanthellae) in scleractinian corals of the Caribbean and Eastern Pacific. In *Proceedings of the 8th International Coral Reef Symposium*, vol. 2 (ed. H. A. Lessios & I. G. Macintyre), pp. 1301-1306. Panama: Smithsonian Tropical Reseach Institute.

Baker, A. C., Starger, C., McClanahan, T., Glynn, P. W. 2004 Coral's adaptive response to climate change. *Nature* **430**, 741.

Baker, A. C., Romanski, A.M. 2007 Multiple symbiotic partnerships are common in scleractinian corals, but not in octocorals: Comment on Goulet (2006). *Marine Ecology Progress Series* **335**, 237-242.

Banaszak, A. T., Trench, R. K. 1995 Effects of ultraviolet (UV) radiation on marine microalgal-invertebrate symbioses. I. Response of the algal symbionts in culture and *in hospite*. *Journal of Experimental Marine Biology and Ecology* **194**, 213-232.

Banaszak, T., Anastazia, LaJeunesse, T. C., Trench, R. K. 2000 The synthesis of mycosporine-like amino acids (MAAs) by cultured, symbiotic dinoflagellates. *Journal of Experimental Marine Biology and Ecology* **249**, 219-233.

Barnes, D. J., Chalker, B. E. 1990 Calcification and photosynthesis in reef-building corals and algae. In *Coral Reefs*, vol. 25 (ed. Z. Dubinsky), pp. 109-131. Amsterdam: Elsevier.

Belda-Baillie, C. A., Baillie, B. A., Maruyama, T. 2002 Specificity of a model cnidarian-dinoflagellate symbiosis. *The Biological Bulletin* **202**, 74.

Belda-Baillie, C. A., Sison, M., Silvestre, V., Villamor, K., Monje, V., Gomez, E. D., Baillie, B. K. 1999 Evidence for changing symbiotic algae in juvenile tridacnids. *Journal of Experimental Marine Biology and Ecology* **241**, 207-221.

Berkelmans, R., van Oppen, M. J. H. 2006 The role of zooxanthellae in the thermal tolerance of corals: a 'nugget of hope' for coral reefs in an era of climate change. *Proceedings of the Royal Society of London, Series B: Biological Sciences* **273**, 2305-2312.

Bhagooli, R., Hidaka, M. 2003 Comparison of stress susceptibility of *in hospite* and isolated zooxanthellae among five coral species. *Journal of Experimental Marine Biology and Ecology* **291**, 181-197.

Brown, B. E. 1997 Coral bleaching: causes and consequences. *Coral Reefs* **16**, 129-138.

Brown, B. E., Ambarsari, I., Warner, M. E., Fitt, W. K., Dunne, R. P., Gibb, S. W., Cummings, D. G. 1999 Diurnal changes in photochemical efficiency and xanthopyll concentrations in shallow water reef corals: evidence for photoinhibition and photoprotection. *Coral Reefs* **18**, 99-105.

Brown, B. E., Downs, C. A., Dunne, R. P., Gibb, S. W. 2002 Exploring the basis of thermotolerance in the reef coral *Goniastrea aspera*. *Marine Ecology Progress Series* **242**, 119-129.

Bruno, J. F., Selig, E. R., Casey, K. S., Page, C. A., Willis, B. L., Harvell, C. D., Sweatman, H., Melendy, A. M. 2007 Thermal stress and coral cover as drivers of coral disease outbreaks. *PloS Biology* **5**, 1220-1227.

Buddemeier, R. W., Fautin, D. G. 1993 Coral bleaching as an adaptive mechanism. *Bioscience* **43**, 320-326.

Carr, L., Mendelsohn, R. 2003 Valuing coral reefs: A travel cost analysis of the Great Barrier Reef. *Ambio* **32**, 353-357.

Chen, C. A., Wang, J.-T., Fang, L.-S., Yang, Y.-W. 2005 Fluctuating algal symbiont communities in *Acropora palifera* (Scleractinia: Acroporidae) from Taiwan. *Marine Ecology Progress Series* **295**, 113-121.

Coffroth, M. A., Santos, S. R. 2005 Genetic Diversity of symbiotic dinoflagellates in the genus *Symbiodinium*. *Protist* **156**, 19-34.

Coffroth, M. A., Santos, S. R., Goulet, T. L. 2001 Early ontogenetic expression of specificity in a cnidarian-algal symbiosis. *Marine Ecology Progress Series* **222**, 85-96.

Coffroth M. A., Lewis C. F., Santos S. R., Weaver J. L. 2006 Environmental populations of symbiotic dinoflagellates in the genus *Symbiodinium* can initiate symbioses with reef cnidarians. *Current Biology* **16**, R985-R987.

Connell, J. H. 1978 Diversity in tropical rain forests and coral reefs. *Science* **199**, 1302-1310.

Davy, S. K., Lucas, I. A. N., Turner, J. R. 1997 Uptake and persistence of homologous and heterologous zooxanthellae in the temperate sea anemone *Cereus pedunculatus* (Pennant). *The Biological Bulletin* **192**, 208.

Desalvo, M., Voolstra, C., Sunagawa, S., Schwarz, J., Stillman, J., Coffroth, M., Szmant, A., Medina, M. 2008 Differential gene expression during thermal stress and bleaching in the Caribbean coral *Montastrea faveolata*. *Molecular Ecology* **17**, 3952-3971.

Douglas, A. E. 1998 Host benefit and the evolution of specialization in symbiosis. *Heredity* **81**, 599-603.

Douglas, A. E. 2003 Coral bleaching--how and why? *Marine Pollution Bulletin* **46**, 385-392.

Downs, C. A., Mueller, E., Phillips, S., Fauth, J., Woodley, C. 2000 A molecular biomarker system for assessing the health of coral (*Montastrea faveolata*) during heat stress. *Marine Biotechnology* **2**, 533-544.

Dunlap, W. C., Shick, J. M. 1998 Ultraviolet radiation-absorbing mycosporine-like amino acids in coral reef organisms: a biochemical and environmental perspective. *Coral Reefs* **20**, 201-210.

Dunn, S. R., Weis, V. M. 2009 Apoptosis as a post-phagocytic winnowing mechanism in a coral-dinoflagellate mutualism. *Environmental Microbiology* **11**, 269-276.

Enriquez, S., Mendez, E. R., Iglesias-Prieto, R. 2005 Multiple scattering on coral skeletons enhances light absorption by symbiotic algae. *Limnology and Oceanography* **50**, 1025-1032.

Epping, E., Kühl, M. 2000 The responses of photosynthesis and oxygen consumption to short-term changes in temperature and irradiance in a cyanobacterial mat (Ebro Delta, Spain). *Environmental Microbiology* **2**, 465-474.

Fabricius, K., Alderslade, P. 2001 *Soft Corals and Sea Fans: A comprehensive guide to the tropical shallow-water genera of the Central-West Pacific, the Indian Ocean and the Red Sea*. AIMS Townsville (Australia). 264p.

Fabricius, K. E., Mieog, J. C., Colin, P. L., Idip, D., vanOppen, M. J. H. 2004 Identity and diversity of coral endosymbionts (zooxanthellae) from three Palauan reefs with contrasting bleaching, temperature and shading histories. *Molecular Ecology* **13**, 2445-2458.

Fitt, W. K. 1985 Effect of different strains of the zooxanthella *Symbiodinium microadriaticum* on growth and survival of their coelenterate and molluscan hosts. In *Proceedings of the Fifth International Coral Reef Congress*, vol. 6, pp. 131-136. Tahiti, French Polynesia.

Fitt, W. K., Brown, B. E., Warner, M. E., Dunne, R. P. 2001 Coral bleaching: interpretation of thermal tolerance limits and thermal thresholds in tropical corals. *Coral Reefs* **20**, 51-65.

Fitt, W. K., Cook, C. B. 2001 Photoacclimation and the effect of the symbiotic environment on the photosynthetic response of symbiotic dinoflagellates in the tropical marine hydroid *Myrionema amboinense*. *Journal of Experimental Marine Biology and Ecology* **256**, 15-31.

Gattuso, J.-P., Allemand, D., Frankignoulle, M. 1999 Photosynthesis and calcification at cellular, organismal and community levels in coral reefs: A review on interactions and control by carbonate chemistry. *American Zoologist* **39**, 160-183.

Glynn, P. W., Mate, J. L., Baker, A., Calderon, M. O. 2001 Coral bleaching and mortality in Panama and Ecuador during the 1997-1998 EL NINO-Southern Oscillation event: Spatial/ temporal patterns and comparisons with the 1982-1983 event. *Bulletin of Marine Science* **69**, 79-109.

Gomez-Cabrera, M., Ortiz, J., Loh, W., Ward, S., Hoegh-Guldberg, O. 2008 Acquisition of symbiotic dinoflagellates (*Symbiodinium*) by juveniles of the coral *Acropora longicyathus*. *Coral Reefs* **27**, 219-226.

Gorbunov, M. Y., Kolber, Z., Lesser, M. P., Falkowski, P. G. 2001 Photosynthesis and photoprotection in symbiotic corals. *Limnology and Oceanography* **46**, 75-85.

Goulet, T. L. 2006 Most corals may not change their symbionts. *Marine Ecology Progress Series* **321**, 1-7.

Goulet, T. L., Coffroth, M. A. 2003 Stability of an octocoral-algal symbiosis over time and space. *Marine Ecology Progress Series* **250**, 114-124.

Goulet, T. L., Cook, C. B., Goulet, D. 2005 Effect of short-term exposure to elevated temperatures and light levels on photosynthesis of different host-symbiont combinations in the *Aiptasia pallida Symbiodinium* symbiosis. *Limnology and Oceanography* **50**, 1490-1498.

Grottoli, A. G., Rodrigues, L. J., Palardy, J. E. 2006 Heterotrophic plasticity and resilience in bleached corals. *Nature* **440**, 1186-1189.

Harrison, P. L., Wallace, C. C. 1990 Reproduction, Dispersal and recruitment of scleractinian corals. In *Coral Reefs*, vol. 25 (ed. Z. Dubinsky), pp. 133-208. Amsterdam: Elsevier.

Harvell, C. D., Mitchell, C. E., Ward, J. R., Altizer, S., Dobson, A. P., Ostfeld, R. S., Samuel, M. D. 2002 Climate warming and disease risks for terrestrial and marine biota. *Science* **296**, 2158-2162.

Hayashibara, T., Shimoike, K., Kimura, T., Hosaka, A., Heyward, A., Harrison, P., Kudo, K., Omori, M. 1993 Patterns of coral spawning at Akajima Island, Okinawa, Japan. *Marine Ecology Progress Series* **101**, 253-262.

Hirose M., Reimer J. D., Hidaka M., Suda S. 2008 Phylogenetic analyses of potentially free-living *Symbiodinium* spp. isolated from coral reef sand in Okinawa, Japan. *Marine Biology* **155**, 105-112.

Hoarau, G., Coyer, J., Stam, W., Olsen, J. 2007 A fast and inexpensive DNA extraction/purification protocol for brown macroalgae. *Molecular Ecology Notes* **7**, 191-193.

Hoegh-Guldberg, O. 1999 Climate change, coral bleaching and the future of the world's coral reefs. *Marine & Freshwater Research*, 839-866.

Hughes, T. P., Baird, A. H., Bellwood, D. R., Card, M., *et al*. 2003 Climate change, human impacts, and the resilience of coral reefs. *Science* **301**, 929.

Huss, V. A. R., Holweg, C., Seidel, B., Reich, V., Rahat, M., Kessler, E. 1993 There is an ecological basis for host/symbiont specificity in *Chlorella*/*Hydra* symbioses. *Encocytobiosis & Cell Research* **10**, 35-46.

Iglesias-Prieto, R., Beltran, V., LaJeunesse, T. C., Reyes-Bonilla, H., Thome, P. E. 2004 Different algal symbionts explain the vertical distribution of dominant reef corals in the eastern Pacific. *Proceedings of the Royal Society of London, Series B: Biological Sciences* **271**, 1757-1763.

Iglesias-Prieto, R., Matta, J., Robins, W., Trench, R. 1992 Photosynthetic response to elevated temperature in the symbiotic dinoflagellate *Symbiodinium microadriaticum* in culture. *Proceedings of the National Academy of Sciences USA* **89**, 10302-10305.

Iglesias-Prieto, R., Trench, R. K. 1994 Acclimation and adaptation to irradiance in symbiotic dinoflagellates. 1. Responses of the photosynthetic unit to changes in photon flux density. *Marine Ecology Progress Series* **113**, 163-175.

Iglesias-Prieto, R., Trench, R. K. 1997a Acclimation and adaptation to irradiance in symbiotic dinoflagellates. 2. Response of chlorophyll-protein complexes to different photon-flux densities. *Marine Biology* **130**, 23-33.

Iglesias-Prieto, R., Trench, R. K. 1997b Photoadaptation, photoacclimation and niche diversification in invertebrate-dinoflagellate symbioses. In *Proceedings of the 8th International Coral Reef Symposium*, vol. 2 (ed. H. A. Lessios & I. G. Macintyre), pp. 1319-1324. Panama: Smithsonian Tropical Research Institute.

IPCC. 2007 *Climate Change 2007: Synthesis Report. Contribution of Working Groups I, II, and III to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change*. Geneva. 104p.

Jones, A. M., Berkelmans, R., van Oppen, M. J. H., Mieog, J. C., Sinclair, W. 2008a A community shift in the symbionts of a scleractinian coral following a natural bleaching event: field evidence of acclimatization. *Proceedings of the Royal Society B* **275**, 1359-1365.

Jones, A. M., Cantin, N., Berkelmans, R., Sinclair, B., Negri, A. P. 2008b A 3D modelling method to calculate the surface areas of coral branches. *Coral Reefs*.

Jones, R. J., Hoegh-Guldberg, O., Larkum, A. W. D., Schreiber, U. 1998 Temperature-induced bleaching of corals begins with impairment of the CO2 fixation mechanism in zooxanthellae. *Plant Cell Environment* **21**, 1219-1230.

Kinzie, R. A., III, Takayama, M., Santos, S. R., Coffroth, M. A. 2001 The adaptive bleaching hypothesis: experimental tests of critical assumptions. *The Biological Bulletin* **200**, 51-58.

Koike, K., Jimbo, M., Sakai, R., Kaeriyama, M., Muramoto, K., Ogata, T., Maruyama, T., Kamiya, H. 2004 Octocoral chemical signaling selects and controls dinoflagellate symbionts. *The Biological Bulletin* **207**, 80-86.

Kühl, M., Cohen, Y., Dalsgaard, T., Jorgensen, B., Revsbech, N. P. 1995 Microenvironment and photosynthesis of zooxanthellae in scleractinian corals studied with microsensors for O2, pH and light. *Marine Ecology Progress Series* **117**, 159-172.

Kühl, M., Glud, R., Ploug, H., Ramsing, N. 1996 Microenvironmental control of photosynthesis and photosynthesis-coupled respiration in an epilithic cyanobacterial biofilm. *Journal of Phycology* **32**, 799-812.

LaJeunesse, T. C. 2001 Investigating the biodiversity, ecology, and phylogeny of endosymbiotic dinoflagellates in the genus *Symbiodinium* using the ITS region: In search of a "species" level marker. *Journal of Phycology* **37**, 866-880.

LaJeunesse, T. C. 2005 "Species" radiations of symbiotic dinoflagellates in the Atlantic and Indo-Pacific since the Miocene-Pliocene transition. *Molecular Biology & Evolution* **22**, 570-581.

LaJeunesse, T. C., Bhagooli, R., Hidaka, M., de Vantier, L., Done, T., Schmidt, G. W., Fitt, W. K., Hoegh-Guldberg, O. 2004 Closely related *Symbiodinium* spp. differ in relative dominance in coral reef host communities across environmental, latitudinal and biogeographic gradients. *Marine Ecology Progress Series* **284**, 147-161.

LaJeunesse, T. C., Lee, S., Bush, S., Bruno, J. F. 2005 Persistence of non-Caribbean algal symbionts in Indo-Pacific mushroom corals released to Jamaica 35 years ago. *Coral Reefs* **24**, 157-159.

LaJeunesse, T. C., Loh, W. K., van Woesik, R., Hoegh-Guldberg, O., Schmidt, G. W., Fitt, W. K. 2003 Low symbiont diversity in southern Great Barrier Reef corals, relative to those of the Caribbean. *Limnology and Oceanography* **48**, 2046-2054.

Lesser, M. P., Farrell, J. H. 2004 Exposure to solar radiation increases damage to both host tissues and algal symbionts of corals during thermal stress. *Coral Reefs* **23**, 367-377.

Lesser, M. P., Stochaj, W. R., Tapley, D. W., Shick, J. M. 1990 Bleaching in coral-reef anthozoans - effects of irradiance, ultraviolet-radiation, and temperature on the activities of protective enzymes against active oxygen. *Coral Reefs* **8**, 225-232.

Li, W. K. W., Smith, J. C., Platt, T. 1984 Temperature response of photosynthetic capacity and carboxylase activity in Artic marine phytoplankton. *Marine Ecology Progress Series* **17**, 237-243.

Lin, K.-L., Wang, J.-T., Fang, L.-S. 2000 Participation of glycoproteins on zooxanthellal cell walls in the establishment of a symbiotic relationship with the sea anemone, *Aiptasia pulchella*. *Zoological Studies* **39**, 172-178.

Little, A. F., van Oppen, M. J. H., Willis, B. L. 2004 Flexibility in algal endosymbioses shapes growth in reef corals. *Science* **304**, 1492-1494.

Loh, W. K. W., Loi, T., Carter, D., Hoegh-Guldberg, O. 2001 Genetic variability of the symbiotic dinoflagellates from the wide ranging coral species *Seriatopora hystrix* and *Acropora longicyathus* in the Indo-West Pacific. *Marine Ecology Progress Series* **222**, 97-107.

Manning M. M., Gates R. D. 2008 Diversity in populations of free-living *Symbiodinium* from a Caribbean and Pacific reef. *Limnology and Oceanography* **53**, 1853-1861.

Marshall, P., Baird, A. 2000 Bleaching of corals on the Great Barrier Reef: differential susceptibilities among taxa. *Coral Reefs* **19**, 155-163.

Maxwell, D. P., Falk, S., Huner, N. 1995 Photosystem II excitation pressure and development of resistance to photoinhibition (I. light-harvesting complex II abundance and zeaxanthin content in *Chlorella vulgaris*). *Plant Physiol.* **107**, 687-694.

McCulloch, M., Fallon, S., Wyndham, T., Hendy, E., Lough, J., Barnes, D. 2003 Coral record of increased sediment flux to the inner Great Barrier Reef since European settlement. *Nature* **421**, 727-730.

Michalek-Wagner, K., Willis, B. L. 2001 Impacts of bleaching on the soft coral *Lobophytum compactum*. I. Fecundity, fertilization and offspring viability. *Coral Reefs* **19**, 231-239.

Mieog, J., van Oppen, M., Berkelmans, R., Stam, W., Olsen, J. 2009 Quantification of algal endosymbionts (*Symbiodinium*) in coral tissues using real-time PCR. *Molecular Ecology Resources* **9**, 74-82.

Mieog, J., VanOppen, M. J. H., Cantin, N., Stam, W. T., Olsen, J. L. 2007 Real-time PCR reveals a high incidence of *Symbiodinium* clade D at low levels in four scleractinian corals across the Great Barrier Reef: implications for symbiont shuffling. *Coral Reefs* **26**, 449-457.

Moberg, F., Folke, C. 1999 Ecological goods and services of coral reef ecosystems. *Ecological Economics* **29**, 215-233.

Muscatine, L. 1990 The role of symbiotic algae in carbon and energy flux in reef corals. In *Coral Reefs*, vol. 25 (ed. Z. Dubinsky), pp. 75-87. Amsterdam: Elsevier.

Muscatine, L., Porter, J. W. 1977 Reef corals: mutualistic symbioses adapted to nutrient-poor environments. *Bioscience* **27**, 454-460.

Nyholm, S. V., McFall-Ngai, M. J. 2004 The winnowing: establishing the squid-*Vibrio* symbiosis. *Nature Reviews Microbiology* **2**, 632-642.

Pandolfi, J. M., Bradbury, R. H., Sala, E., Hughes, T. P., Bjorndal, K. A., Cooke, R. G., McArdle, D., McClenachan, L., Newman, M. J. H., Paredes, G., Warner, R. R., Jackson, J. B. C. 2003 Global trajectories of the long-term decline of coral reef ecosystems. *Science* **301**, 955-958.

Platt, T., Gallegos, C. L., Harrison, W. G. 1980 Photoinhibition of photosynthesis in natural assemblages of marine phytoplankton. *Journal of Marine Research* **38**, 687-701.

Porto, I. C., Granados, C., Restrepo, J. C., Sanchez, J. A. 2008 Macroalgal-associated dinoflagellates belonging to the genus *Symbiodinium* in Caribbean Reefs. *PLoS ONE* **3**, e2160-e2164.

Revsbech, N. P. 1989 An oxygen microelectrode with a guard cathode. *Limnology and Oceanography* **34**, 474-478.

Revsbech, N. P., Jorgensen, B. 1983 Photosynthesis of benthic microflora measured with high spatial resolution by the oxygen microprofile method: Capabilities and limitations of the method. *Limnology and Oceanography* **28**, 749-756.

Reynolds, W. S., Schwarz, J. A., Weis, V. M. 2000 Symbiosis-enhanced gene expression in cnidarian-algal associations: cloning and characterization of a cDNA, sym32, encoding a possible cell adhesion protein. *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology* **126**, 33-44.

Richmond, R., Hunter, C. 1990 Reproduction and recruitment of corals: comparisons among the Caribbean, the Tropical Pacific and the Red Sea*. *Marine Ecology Progress Series* **60**, 185-203.

Robison, J. D., Warner, M. E. 2006 Differential impacts of photoacclimation and thermal stress on the photobiology of four different phylotypes of *Symbiodinium* (Pyrrhophyta). *Journal of Phycology* **42**, 568-579.

Rodriguez-Lanetty, M., Krupp, D. A., Weis, V. M. 2004 Distinct ITS types of *Symbiodinium* in clade C correlate with cnidarian/dinoflagellate specificity during onset of symbiosis. *Marine Ecology Progress Series* **275**, 97-102.

Rodriguez-Lanetty, M., Phillips, W. S., Weis, V. M. 2006a Transcriptome analysis of a cnidarian-dinoflagellate mutualism reveals complex modulation of host gene expression. *BMC Genomics* **7**, 23.

Rodriguez-Lanetty, M., Wood-Charlson, E. M., Hollingsworth, L. L., Krupp, D. A., Weis, V. M. 2006b Temporal and spatial infection dynamics indicate recognition events in the early hours of a dinoflagellate/coral symbiosis. *Marine Biology* **149**, 713-719.

Rodriguez-Roman, A., Iglesias-Prieto, R. 2005 Regulation of photochemical activity in cultured symbiotic dinoflagellates under nitrate limitation and deprivation. *Marine Biology* **146**, 1063-1073.

Rowan, R. 2004 Thermal adaptations in reef coral symbionts. *Nature* **430**, 742.

Rowan, R., Knowlton, N. 1995 Intraspecific diversity and ecological zonation in coral-algal symbiosis. *Proceedings of the National Academy of Sciences USA* **92**, 2850-2853.

Rowan, R., Knowlton, N., Baker, A., Jara, J. 1997 Landscape ecology of algal symbionts creates variation in episodes of coral bleaching. *Nature* **388**, 265-269.

Salih, A., Larkum, A., Cox, G., Kühl, M., Hoegh-Guldberg, O. 2000 Fluorescent pigments in corals are photoprotective. *Nature* **408**, 850-853.

Sampayo, E. M., Franceschinis L., Hoeugh-Guldberg O., Dove S. 2007 Niche partitioning of closely related symbiotic dinoflagellates. *Molecular Ecology* **16**, 3721-3733.

Smpayo, E. M., Ridgway, T., Bongaerts, P., Hoegh-Guldberg, O. 2008 Bleaching susceptibility and mortality of corals are determined by fine-scale differences in symbiont type. *Proceedings of the National Academy of Sciences USA* **105**, 10444-10449.

Schwarz, J., Brokstein, P., Voolstra, C., Terry, A., Miller, D., Szmant, A., Coffroth, M., Medina, M. 2008 Coral life history and symbiosis: Functional genomic resources for two reef building Caribbean corals, *Acropora palmata* and *Montastrea faveolata*. *BMC Genomics* **9**.

Schwarz, J. A., Krupp, D. A., Weis, V. M. 1999 Late larval development and onset of symbiosis in the scleractinian coral *Fungia scutaria*. *The Biological Bulletin* **196**, 70.

Schwarz, J. A., Weis, V. M., Potts, D. C. 2002 Feeding behavior and acquisition of zooxanthellae by planula larvae of the sea anemone *Anthopleura elegantissima*. *Marine Biology* **140**, 471-478.

Shick, J. M., Lesser, M. P., Dunlap, W. C., Stochaj, W. R., Chalker, B. E., Won, J. W. 1995 Depth-dependent responses to solar ultraviolet-radiation and oxidative stress in the zooxanthellate coral *Acropora-Microphthalma*. *Marine Biology* **122**, 41-51.

Sofonia, J. J., Anthony, K. R. N. 2008 High-sediment tolerance in the reef coral *Turbinaria mesenterina* from the inner Great Barrier Reef lagoon (Australia). *Estuarine, Coastal and Shelf Science* **78**, 748-752.

Stat, M. E., Morris, E., Gates, R. D. 2008 Functional diversity in coral-dinoflagellate symbiosis. *Proceedings of the National Academy of Sciences USA* **105**, 9256-9261.

Stanley, G. D., Jr., Fautin, D. G. 2001 The origins of modern corals. *Science* **291**, 1913-1914.

Swofford, D. 2000 PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). ver. 4. (ed. S. Associates).

Takahashi, S., Takahashi, N., Sakamizu, M., van Woesik, R., Yamasaki, H. 2004 Repair machinery of symbiotic photosynthesis as the primary target of heat stress for reef-building corals. *Plant Cell Environment* **45**, 251-255.

Tchernov, D., Gorbunov, M. Y., de Vargas, C., Narayan Yadav, S., Milligan, A. J., Haggblom, M., Falkowski, P. G. 2004 Membrane lipids of symbiotic algae are diagnostic of sensitivity to thermal bleaching in corals. *Proceedings of the National Academy of Sciences USA* **101**, 13531-13535.

Thornhill, D. J., LaJeunesse, T., Kemp, D., Fitt, W., Schmidt, G. 2006a Multi-year, seasonal genotypic surveys of coral-algal symbioses reveal prevalent stability or post-bleaching reversion. *Marine Biology* **148**, 711-722.

Thornhill, D. J., Daniel, M. W., LaJeunesse, T. C., Schmidt, G. W., Fitt, W. K. 2006b Natural infections of aposymbiotic *Cassiopea xamachana* scyphistomae from environmental pools of *Symbiodinium*. *Journal of Experimental Marine Biology & Ecology* **338**, 50-56.

Toller, W. W., Rowan, R., Knowlton, N. 2001a Repopulation of zooxanthellae in the Caribbean corals *Montastraea annularis* and *M. faveolata* following experimental and disease-associated bleaching. *The Biological Bulletin* **201**, 360.

Toller, W. W., Rowan, R., Knowlton, N. 2001b Zooxanthellae of the *Montastraea annularis* species complex: Patterns of distribution of four taxa of *Symbiodinium* on different reefs and across depths. *The Biological Bulletin* **201**, 348.

Ulstrup, K. E., van Oppen, M. J. H. 2003 Geographic and habitat partitioning of genetically distinct zooxanthellae (*Symbiodinium*) in *Acropora* corals on the Great Barrier Reef. *Molecular Ecology* **12**, 3477-3484.

Ulstrup, K. E., van Oppen, M. J. H., Kuhl, M., Ralph, P. J. 2007 Inter-polyp genetic and physiological characterisation of *Symbiodinium* in an *Acropora valida* colony. *Marine Biology* **153**, 225-234.

Van Heukelem, L., Thomas, C. S. 2001 Computer-assisted high-performance liquid chromatography method development with applications to the isolation and analysis of phytoplankton pigments. *Journal of Chromatography* **910**, 31-49.

van Oppen, M. 2004 Mode of zooxanthella transmission does not affect zooxanthella diversity in acroporid corals. *Marine Biology* **144**, 1-7.

van Oppen, M., Mahiny, A. & Done, T. 2005 Geographic distribution of zooxanthella types in three coral species on the Great Barrier Reef sampled after the 2002 bleaching event. *Coral Reefs* **24**, 482-487.

van Oppen, M. J. H., Palstra, F. P., Piquet, A.-T., Miller, D. J. 2001 Patterns of coral-dinoflagellate associations in *Acropora*: significance of local availability and physiology

of *Symbiodinium* strains and host-symbiont selectivity. *Proceedings of the Royal Society of London, Series B: Biological Sciences* **268**, 1759-1767.

Veron, J. 2000 *Corals of the World*. Townsville: AIMS.

Warner, M. E., Fitt, W. K., Schmidt, G. W. 1996 The effects of elevated temperature on the photosynthetic efficiency of zooxanthellae *in hospite* from four different species of reef coral: A novel approach. *Plant Cell Environment* **19**, 291-299.

Warner, M. E., Fitt, W. K., Schmidt, G. W. 1999 Damage to photosystem II in symbiotic dinoflagellates: A determinant of coral bleaching. *Proceedings of the National Academy of Sciences USA* **96**, 8007-8012.

Weis, V. M., Reynolds, W. S., deBoer, M. D., Krupp, D. A. 2001 Host-symbiont specificity during onset of symbiosis between the dinoflagellates *Symbiodinium* spp. and planula larvae of the scleractinian coral *Fungia scutaria*. *Coral Reefs* **20**, 301-308.

Willis, B. L., Babcock, R. C., Harrison, P. L., Oliver, J. K. 1985 Patterns in the mass spawning of corals on the Great Barrier Reef from 1981 to 1984. In *Proceedings of the Fifth International Coral Reef Cogress*, vol. 4, pp. 343-348. Tahiti.

Wilson, K., Li, Y., Whan, V., Lehnert, S., Byrne, K., Moore, S., Pongsomboon, S., Tassanakajon, A., Rosenberg, G., Ballment, E., Fayazi, Z., Swan, J., Kenway, M., Benzie, J. 2002 Genetic mapping of the black tiger shrimp *Penaeus monodon* with amplified fragment length polymorphism. *Aquaculture* **204**, 297-309.

Wood-Charlson, E. M., Hollingsworth, L. H., Krupp, D. A., Weis, V. M. 2006 Lectin/glycan interactions play a role in recognition in a coral/dinoflagellate symbiosis. *Cellular Microbiology* **8**, 1985-1994.

Yuyama, I., Hayakawa, H., Endo, H., Iwao, K., Takeyama, H., Maruyama, T., Watanabe, T. 2005 Identification of symbiotically expressed coral mRNAs using a model infection system. *Biochemical and Biophysical Research Communications* **336**, 793-798.