Flexibility of the coral-algal symbiosis in the face of climate change: investigating the adaptive bleaching hypothesis

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The research reported in this thesis was performed at the Department of Marine Benthic Ecology and Evolution (Center for Ecological and Evolutionary Studies, University of Groningen, PO Box 14, 9750 AA Haren, The Netherlands) in collaboration with the Australian Institute of Marine Science (PMB No. 3, Townsville MC, 4810 Townsville Qld, Australia). It was funded by the Foundation for the Advancement of Tropical Research within the Netherlands Organization for Scientific Research (NWO-WOTRO, Project Nr. W84-576) and the Australian Institute for Marine Science.

ISBN: 978-90-367-3871-2

Printed by Ipskamp Drukkers, Enschede, The Netherlands

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Cover by Abbi M. Mieog

RIJKSUNIVERSITEIT GRONINGEN

Flexibility of the coral-algal symbiosis in the face of climate change: investigating the adaptive bleaching hypothesis

Proefschrift

ter verkrijging van het doctoraat in de
Wiskunde en Natuurwetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. F. Zwarts,
in het openbaar te verdedigen op
vrijdag 4 september 2009
om 16.15 uur

door

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geboren op 29 december 1977
te Amersfoort

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Chapter 1

General introduction

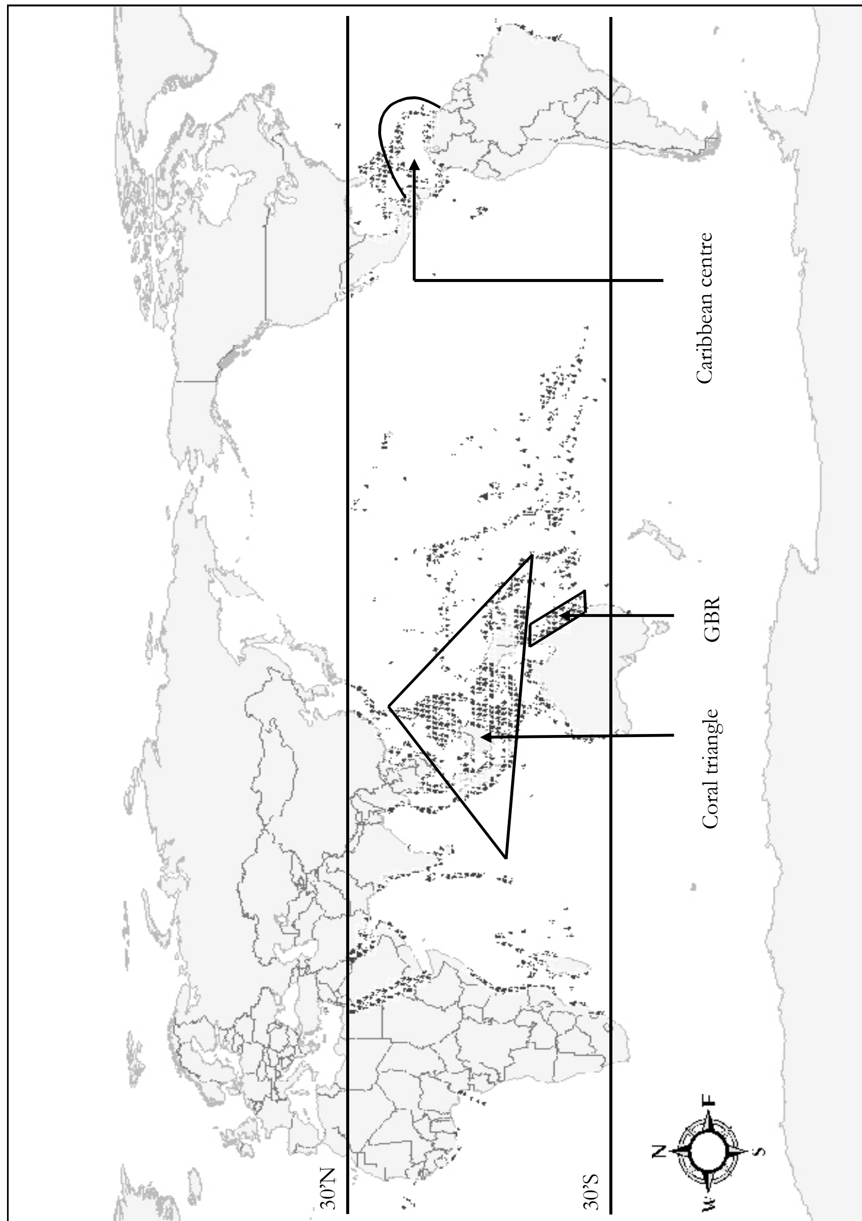

Fig. 1. World map showing the distribution of tropical coral reefs, the locations of the two main centers of reef biodiversity (coral triangle and Caribbean center) and the Great Barrier Reef (Map from ReefBase, http://reefgis.reefbase.org).

Tropical coral reefs are among the richest ecosystems on Earth, whether measured by biodiversity, productivity, biomass or structural complexity. Apart from their ecological value they also provide a vast number of goods and services to people, including food, tourism, coastal protection, aesthetic and cultural significance (Moberg & Folke 1999; Hoegh-Guldberg 2004; Wilkinson 2004). The vast majority of the coral reef systems are situated in the warm tropical seas between 30°N and 30°S (Fig. 1), where they fringe shorelines, form offshore barriers and atolls (Veron & Stafford-Smith 2000). Covering only about 0.1 % of the Earth's surface (Smith 1978; Spalding & Grenfell 1997), they accommodate almost 30% of the world's marine fish species and supply about 10% of the fish consumed by humans (Smith 1978). Annual revenues from tourism are estimated at US$105 billion for the Caribbean (Burke & Maidens 2004) and AU$7 billion for the Great Barrier Reef (Access Economics 2007).

Coral reefs have been in an unprecedented decline over the last century, as indicated by significant reductions in species abundances and degradation of reef communities (Hughes *et al.* 2003; Pandolfi *et al.* 2003). Reef losses are currently estimated at 20%; 24% of coral reefs are under imminent risk of collapse and a further 26% are threatened on the longer term (Wilkinson 2004). Numerous human-induced disturbances have been identified as causes of this decline, some of which started to affect reefs as early as a millennium ago (Jackson 2001; Pandolfi *et al.* 2003). The main threats to reef biodiversity include: (1) fishing and harvesting, (2) habitat destruction and fragmentation, (3) pollution, (4) disease, and (5) climate change. Fishing practices have long been unsustainable (Jackson 2001) and have resulted in the loss of key reef organisms such as herbivores that normally reduce the competition between corals and macroalgae (Hughes *et al.* 2007). Destructive practices such as dynamite fishing, coastal development and coral harvesting have directly impacted coral reef integrity. Pollution-based threats include sedimentation (which smothers coral and makes substrata unsuitable for juvenile settlement), eutrophication and herbicides/pesticides (Wilkinson 1999; Wilkinson 2004). The sources of pollution are often land-based (e.g. agriculture) and reach the reef through run-off as a result of poor land-management. Last but not least, climate change has been linked to a recent increase in coral diseases (Harvell *et al.* 2004), as well as to reduced coral calcification in conjunction with ocean acidification (Hoegh-Guldberg *et al.* 2007; Cooper *et al.* 2008). However, the main climate-induced threat to coral

reefs today is the heat-induced disruption of the delicate symbiotic relationship between corals and their algal endosymbionts (Coles & Brown 2003).

Stony corals (Order: Scleractinia) are the ecosystem engineers (Jones *et al.* 1994; Coleman & Williams 2002) of coral reefs. They produce hard calcium carbonate exoskeletons, which give rise to the characteristic structure of coral reefs. Stony corals form an obligate symbiosis with unicellular algae of the genus *Symbiodinium* (Division: Dinophyceae). The algal endosymbionts are photosynthetically active and transfer up to 95% of their photosynthate to the coral host (Muscatine *et al.* 1984); they also stimulate rates of calcification (Pearse & Muscatine 1971; Gattuso *et al.* 1999). In return, the coral host provides protection from predators and an environment optimal for photosynthesis, including the provision of several essential nutrients (Pearse & Muscatine 1971; Muscatine & Porter 1977). The evolution of tropical coral reefs in oligotrophic waters is heavily dependent on this successful partnership and anything that disrupts the partnership will, therefore, cause serious harm. Abnormally high seawater temperatures have been identified as the main cause for the periodic loss of the dark-pigmented algae from the host tissues (Glynn 1993; Berkelmans 2002) as witnessed by an increased frequency and severity of this phenomenon in the last 20 years. As the coral host tissue is mostly transparent, the calcium carbonate skeleton becomes visible making the coral appear white; hence, this response is called “coral bleaching”. Bleached corals may recover after weeks or even months but their growth and reproductive output will be significantly reduced (Szmant & Gassman 1990; Baird & Marshall 2002). If bleaching is too severe and/or prolonged, the corals will die.

In 1998, thermal bleaching of an unprecedented magnitude affected both the Pacific and the Caribbean, resulting in an estimated 16% loss of the world’s coral reefs (Wilkinson 2000). Since then, mass bleaching events have occurred (on a smaller scale) in 2002 (Indo-Pacific), 2005 (Caribbean) and 2006 (Indo-Pacific). Temperatures of as little as 1°C above the average summer maximum are already sufficient to cause bleaching (Glynn 1993; Berkelmans 2002). With expected increases of 1.5-4°C in the tropics by the end of this century (IPCC 2007; Fig. 2), coral reefs are projected to undergo major changes (Hughes *et al.* 2003)—to the extent that only remnant populations of corals may be left by the year 2050 (Hoegh-Guldberg 1999). However, a largely unknown variable in these “doomsday” predictions is the capacity of the coral holobiont (the coral plus the algal symbiont)

to increase its thermo-tolerance, either by adaptation or acclimatization (Coles & Brown 2003; Hoegh-Guldberg 2004; Box 1). Although very little is known about the capacity for thermal adaptation of the present-day coral-algal associations, their evolutionary response in a time frame of several decades is considered to be limited (Hoegh-Guldberg *et al.* 2007). Instead, it has been proposed that the flexibility of the coral-algal association provides a window of response to temperature increases that may allow it to cope with the at least some of the stress.

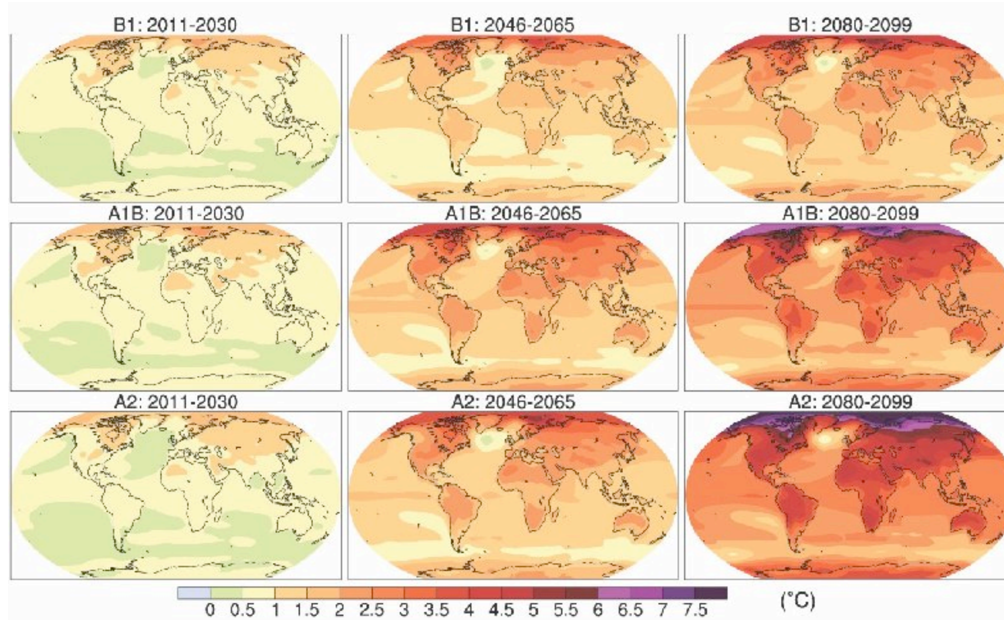

Fig. 2. Predictions of annual mean surface warming (°C) for three scenarios: low CO_2 emission (scenario B1 – top row), medium CO_2 emission (scenario A1B – middle row) and high CO_2 emission (scenario A2 – bottom row) and three time periods: 2011 to 2030 (left column), 2046 to 2065 (middle column) and 2080 to 2099 (right column). Anomalies are relative to the average of the period 1980 to 1999. Warming rates are fastest at the north pole but the tropical regions are expected to face temperature increases of 1.5°C (low CO_2 emissions scenario) to 4°C (high CO_2 emissions scenario) by the end of this century (Source: IPCC Fourth Assessment Report 2007).

The fact that many different *Symbiodinium* types form symbiotic relationships with corals has only been established in the last two decades (Blank & Trench 1985; Rowan & Powers 1992; Rowan 1998; Baker 2003) and it is this observation that has led to the formulation of the “adaptive bleaching hypothesis” (ABH) (Buddemeier &

Fautin 1993; Buddemeier *et al.* 2004; Fautin & Buddemeier 2004). The ABH hypothesizes that bleaching provides an opportunity for corals to re-establish a symbiosis with a different, more stress-tolerant (e.g. heat-tolerant) *Symbiodinium* type, resulting in a coral holobiont better suited to the altered environmental circumstances. Initially, it was thought that uptake of new symbiont types could only take place from the environment and that this adaptive response involved a genetic change in the coral holobiont (Buddemeier & Fautin 1993). Later, it was recognized that change could also take place by an increase in the low abundance, pre-existing *in hospite* populations of symbionts (Fautin & Buddemeier 2004). In order to capture both modes of symbiont change in the ABH, the term "adaptation" was reverted to its meaning in common usage (Box 1) causing considerable confusion in the literature (Hoegh-Guldberg *et al.* 2002; Hughes *et al.* 2003). In this thesis, any changes predicted by the ABH are considered "acclimatizations" (or acclimations when in an experimental setting), as they happen within the life-time of a coral colony and no new genes need to evolve.

Box 1: Definitions of adaptation/acclimatization

The adaptive bleaching hypothesis (ABH) employs the common usage definition of the term "adaptation" : …. modification of an organism or its part in a way that makes it more fit for existence under the conditions of its environment….. (Fautin & Buddemeier 2004).

This definition is more relaxed than those applied in evolutionary biology:

Adaptation: physiological, biochemical, or anatomical modifications within a species, directed by selection, that happen over generations. Adaptations involve a genetic change and are heritable.

Acclimatization: physiological, biochemical, or anatomical modifications in an individual's lifetime due to phenotypic plasticity. Acclimatizations do not involve a genetic change and are not heritable. For example, changing to a more thermo-tolerant symbiont type provides a wider envelop of plasticity to increased temperature for the coral holobiont.

Acclimation: similar to acclimatization, but in an experimental, human-manipulated setting.

The central aim of this thesis was to assess the potential of the mechanisms described by the ABH to: (1) induce changes in the symbiotic communities of coral, and (2) mitigate the effects of global warming on coral reefs.

General questions included:

1. Where do heat-tolerant *Symbiodinium* types come from — from already present, low-abundance-algal cells in the coral host before bleaching, or taken-up anew from the environment — and how can we detect and quantify them?

2. To what extent does the symbiont type shape coral fitness — in terms of heat-tolerance, growth and mortality — and what are the relative contributions of host-factors and the local environmental conditions?

3. What are the *Symbiodinium*-related trade-offs between thermo-tolerance and growth/survival, and how are they shaped by coral host and environmental factors?

4. What is the field evidence for the ABH?

To address these questions, a combination of lab and field experiments were designed to increase the sensitivity of *Symbiodinium* detection and ease of quantification, and to experimentally partition and quantify the coral-algal and holobiont-environmental interactions.

BACKGROUND

Scleractinian corals

The fossil record shows that scleractinian corals have been around since the early Triassic, about 237 my BP (Stanley Jr & Fautin 2001). These early corals did not build reefs and the formation of reefs in the late-Triassic is thought to have co-incided with the evolution of the coral-dinoflagellate symbiosis (Stanley 2006).

Scleractinian corals have survived massive climatic perturbations, including the mass-extinction events at the end of the Triassic (200 my BP) and Cretaceous (145 my BP).

Corals belong to the metazoan phylum Cnidaria, Class Anthozoa to which hydroids, jelly fish and sea anemones also belong. Their body-plan is simple: radially symmetrical polyps, containing a sac-like body cavity (the stomach) with one opening that serves as both mouth and anus (Fig. 3A). The tentacles surrounding the opening contain stinging cells (nematocysts), which they use to filter particulate matter or small organisms (immobilized by the nematocyst stings) from the water column. The "zooxanthellae" (the symbiotic algal cells) live in specialized vacuoles inside the host's endodermis cells (Fitt & Trench 1983). Most corals form modular colonies consisting of thousands to even millions of polyps (Veron & Stafford-Smith 2000).

Reef-building corals are highly diverse: 25 living families are currently recognized, containing 246 genera and ~1000 species (Veron 2000). The genus *Acropora* is the most species-rich, containing over a hundred species with a large variety of growth forms (Fig. 3B). Branching forms are the most common in the genus *Acropora* and these dominate Indo-Pacific reefs. Their relatively fast growth rate allows them to outcompete other corals, especially in clear waters. However, the branching habit of many *Acropora* species makes them particularly sensitive to environmental disturbances and breakage. They are among the first taxa to exhibit a bleaching response (McClanahan *et al.* 2004; Carpenter *et al.* 2008). For these reasons, *Acropora* species are often used as model organisms in physiological and ecological studies of reef coral responses.

New coral colonies can be formed asexually through simple mitosis and eventual colony fragmentation or by one of two modes of sexual reproduction. Broadcast spawning corals (~75% of the reef-building corals including all *Acropora* spp.) release massive numbers of gametes (eggs plus sperm) into the water column in a process called mass spawning. Many species can spawn synchronously within a period of several days, once a year (Harrison *et al.* 1984; Babcock *et al.* 1986; Guest *et al.* 2005), although this multi-species reproductive synchrony is not a characteristic of all coral communities (Richmond & Hunter 1990). To accomplish synchronized spawning, broadcasters rely on seasonality in light (solar radiance,

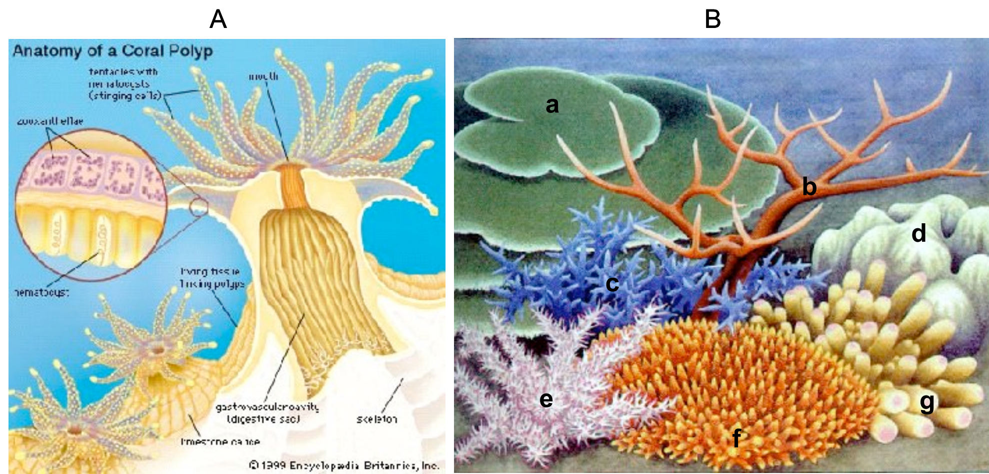

Fig. 3. A: schematic representation of a coral polyp (Courtesy of Encyclopaedia Britannica, Inc., copyright 1999, used with permission), B: examples of *Acropora* growth forms: a) tables and plates, b) staghorn, c) bushy, d) massive, e) bottlebrush, f) corymbose, g) digitate (Figure from Geoff Kelley).

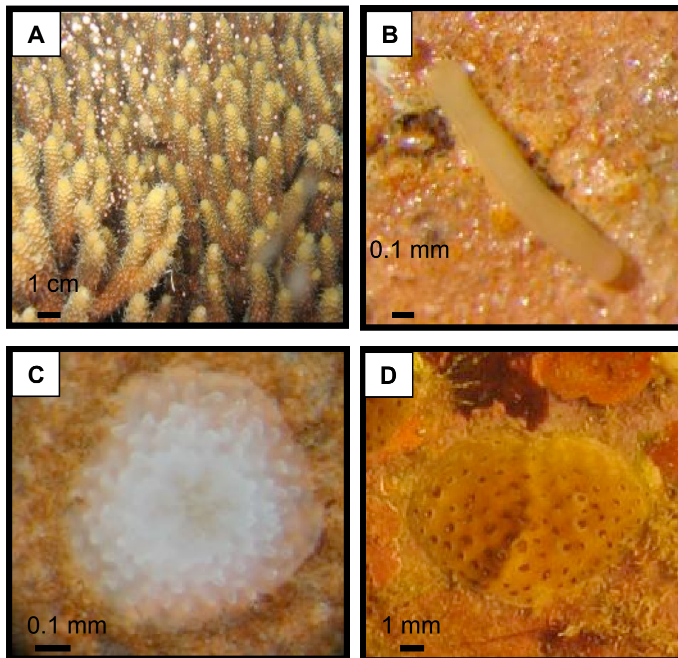

Fig. 4. Sexual reproduction in *Acropora millepora.* A: adult colony releasing egg-sperm bundles, B: planula larva searching for a suitable settlement site, C: newly metamorphosed coral polyp, D: juvenile coral colony ~6 months old (Photos by Jos Mieog).

night/day cycles and lunar radiance) and temperature (Fadlallah 1983; Babcock *et al.* 1986; van Woesik *et al.* 2006). Eggs are fertilized at the water surface where they develop into planula larvae over the course of a few days. Once at the planula stage, the larvae become negatively buoyant, sink and attach themselves via chemical cues (Heyward & Negri 1999) to the substratum. There, they undergo metamorphosis into the recognizable coral polyp. Subsequently, the coral grows into a colony by asexual budding and clonal growth (Fig. 4).

Brooding corals fertilize their eggs internally. Sperm is received from neighboring colonies and fertilization occurs inside the coral polyps. Mature planulae are released, which are mostly capable of immediate settlement. Brooding corals generally produce much smaller numbers of eggs at any one time, but the planula larvae are larger and have higher survival rates. Brooding corals tend to release larvae over longer period (several months) (Fadlallah 1983).

The genus *Symbiodinium*

The unicellular, symbiotic algae of corals belong to the Dinophyceae — an ancient class of protist algae—and are commonly referred to as zooxanthellae. The algal life history involves a vegetative and a motile phase (Schoenberg & Trench 1980; Fig. 5). The vegetative phase ranges in size from 5-15 μm and is the dominant form when in symbiosis with corals. The flagellate stage (zoospore) is known from cultures and is particularly active under illumination. Multiplication of *Symbiodinium—in vitro* and *in hospite*) occurs mainly through mitotic divisions. Sexual reproduction has, so far, not been observed in *Symbiodinium*, although it has been inferred from molecular data (Stat *et al.* 2006). Until recently, only a few studies attempted to address the free-living phase of *Symbiodinium* (Carlos *et al.* 1999; LaJeunesse 2001; LaJeunesse 2002), because data collection was hampered by technical difficulties associated with detection in the natural environment.

Encouragingly, significant progress has recently been made by using azooxanthellate polyps as "environmental samplers" (Coffroth *et al.* 2006), sensitive automated particle counters (Littman *et al.* 2008) and better water filtering techniques (Manning & Gates 2008), as well as culturing *Symbiodinium* from water samples taken from macroalgal beds and fish feces (Porto *et al.* 2008). These studies show that the free-living forms are genetically diverse, mainly reside in the benthos

and that corallivorous fish may aid the dispersal through their feces. Future studies are urgently needed to increase our limited understanding of the distribution and dispersal of free-living *Symbiodinium* spp.

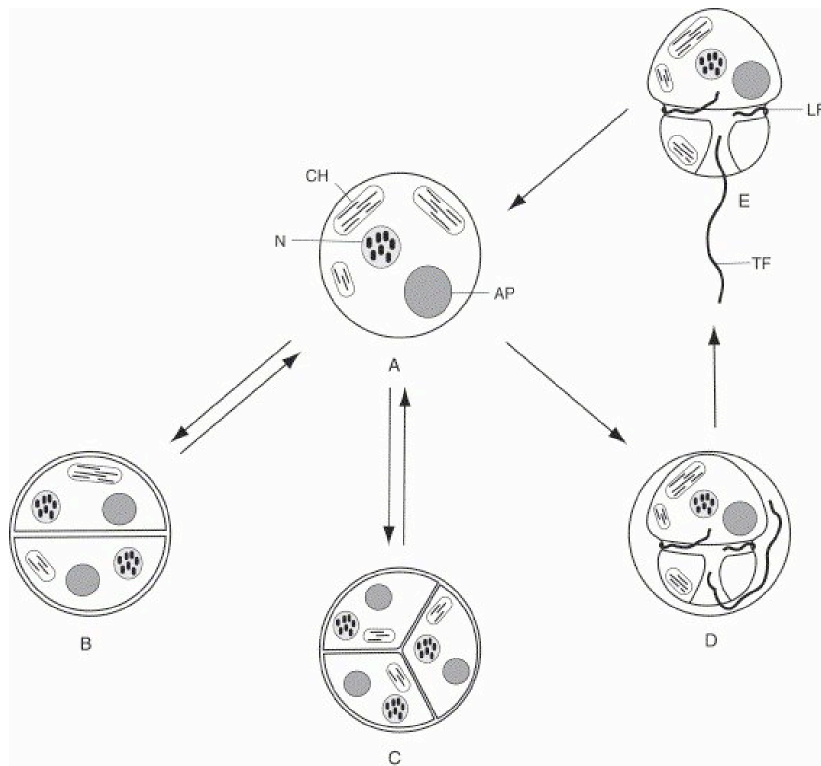

Fig. 5. *Symbiodinium* life cycle. A: vegetative cell, B: dividing vegetative cell producing two daughter cells, C: dividing vegetative cell producing three daughter cells, D: developing zoospore and E: zoospore. CH: chloroplast; N: nucleus; AP: accumulation product; LF: longitudinal flagella; TF: transverse flagella (Reprinted from Stat *et al.* 2006, with permission from Elsevier).

CORAL BLEACHING

The whitening of corals by the loss of zooxanthellae was first described in detail by Yonge and Nicholls (1931). Bleaching is considered a general stress response, as it can be induced by a variety of stimuli including abnormal temperatures, salinities, irradiance levels and various diseases (reviewed by Douglas

2003). Causes for bleaching are likely to be complex and vary per stressor. In the case of thermal bleaching, it is known to be associated with a disruption in the photomachinery that operates in the zooxanthellae (Jones 1998; Smith *et al.* 2005). The typical rise in the excitation pressure over photosystem II is thought to be caused by increased damage to the D1 protein of the photosystem II reaction centers (Warner *et al.* 1999) and/or disruption of the Calvin cycle (Jones *et al.* 1998). Large quantities of reactive oxygen species (ROS) are produced (Smith *et al.* 2005) that swamp the protective mechanisms available and induce, through poorly understood interactions between the coral host and the symbiont, the loss of up to >90% of the symbiotic cells from the coral host tissues (Fitt *et al.* 2000; Weis 2008).

Corals are extremely sensitive to increases in temperature because their upper temperature tolerances are locally adapted to only 1-3°C above the long-term average summer seawater temperature (Hoegh-Guldberg 1999; Berkelmans 2002). Worldwide, temperatures which induce coral bleaching range over 8°C, from 27°C at cool, high latitudes, to 35-36°C in the very warm Arabian gulf (Coles & Brown 2003). Living close to the temperature threshold probably conveys advantages in the absence of large temperature fluctuations—as seen in the last 8,000 years—but is detrimental in an era of rapid warming.

The variability in bleaching thresholds suggests a capacity for reef corals to adapt to higher temperatures, although the time period needed for this adaptation is unknown (Coles & Brown 2003). It is feared that the increases in global temperature are happening so rapidly that there will be insufficient time available for thermal adaptation (Hoegh-Guldberg *et al.* 2007). Corals are believed to evolve slowly, as they have long life spans, overlapping generations and high incidences of asexual reproduction (Hughes *et al.* 2003). In contrast, *Symbiodinium* may be able to provide the needed adaptive potential as a suite of thermo-tolerant *Symbiodinium* genotypes already exist which corals may be able to take advantage of.

THE ADAPTIVE BLEACHING HYPOTHESIS

The Adaptive Bleaching Hypothesis (ABH) poses that the loss of photosymbionts allows some coral species to re-establish a symbiosis with a different dominant *Symbiodinium* type, resulting in a new coral holobiont that is better suited to the altered environmental circumstances (Buddemeier & Fautin

1993; Ware *et al.* 1996; Buddemeier *et al.* 2004; Fautin & Buddemeier 2004). Such a change has the potential to enhance long-term survival of the hosts and may explain how coral reefs have survived repeated climatic fluctuations over geological time (Buddemeier & Smith 1999). There are five fundamental assumptions that underpin the ABH: (1) multiple types of both coral host and *Symbiodinium* species commonly co-exist, (2) both symbiont and coral host have a degree of flexibility in their associations, (3) symbiont types are physiologically different and influence important aspects of the coral's physiology (especially stress-responses), (4) coral bleaching provides an opportunity for repopulation of a host with a different dominant algal symbiont, and (5) stress-sensitive holobionts have competitive advantages in the absence of stress, which implies a reversion to stress-prone combinations under non-stressful conditions. In the next paragraphs these assumptions are discussed in detail.

(1) Diversity and co-existence

There are two areas of exceptional coral reef biodiversity in the world. The primary center, containing the highest coral diversity in the world, lies within the "coral triangle" in the Indo-Pacific (Fig. 1) with an outward attenuation in all directions. A secondary center lies in the southern Caribbean (Fig. 1). Only a few genera are shared between the two areas illustrating the extent of the geographic and temporal isolation between the two oceans (Briggs 2005). Such deep differences were not appreciated until fairly recently because of the notoriously difficult taxonomic identification of many coral species and lack of molecular-based phylogenies, especially those which exhibit high levels of morphological plasticity. The complexities and uncertainties in identifying the coral hosts has also hampered the correct identification of symbiotic combinations (e.g. Santos *et al.* 2001).

All symbiotic dinoflagellates that form symbiotic relationships with corals were once considered to be a single cosmopolitan species, *Symbiodinium microadriaticum* (Freudenthal 1962). This misconception was mainly due to the lack of distinguishing morphological features of both the motile and vegetative stages of *Symbiodinium*. However, subsequent morphological, biochemical, physiological and karyotypic (e.g. Schoenberg & Trench 1980; Blank & Trench 1985), and especially comparative DNA sequencing (e.g. Rowan & Powers 1991a,b) have since shown

that the genus *Symbiodinium* is extraordinarily diverse, exhibiting much higher sequence diversity than observed between genera of non-symbiotic dinoflagellates.

Studies of *Symbiodinium* diversity have mainly utilized the nuclear ribosomal DNA (nrDNA) cistron and associated spacers which provide several regions with different evolutionary rates (Fig. 6). Based on analyses of the relatively slowly evolving 18S, 5.8S and 28S units, *Symbiodinium* has been divided into eight (A-H) phylogenetic clades (Fig. 7) (Rowan & Powers 1991a; LaJeunesse 2001; Pochon *et al.* 2006). The evolutionary position of each clade has received further support from analyses based on mitochondrial (Takabayashi *et al.* 2004) and chloroplast DNA (Santos *et al.* 2002; Pochon *et al.* 2006).

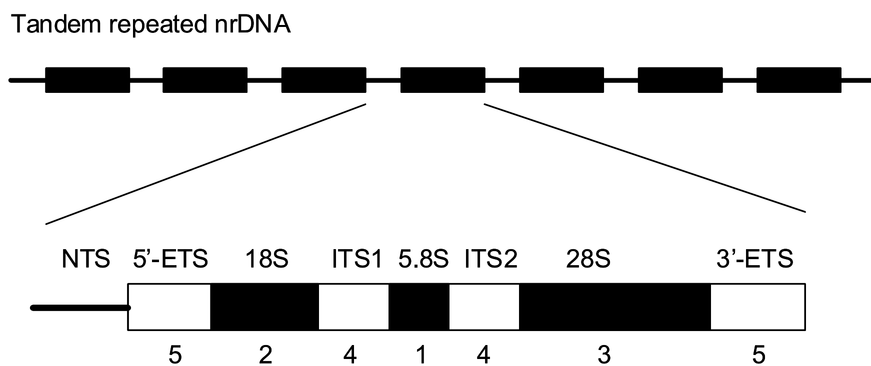

Fig. 6. Organization of the nuclear ribosomal genes. Each consists of three coding units (18S, 5.8S and 28S), which are separated by the internal transcribed spacers (ITS) 1 and 2 and the external transcribed spacers (ETS) on both ends. The repeats are separated from one another by the non-transcribed spacer (NTS). The regions differ in their evolutionary rates, as indicated below each region, with 1 representing the slowest and 5 the fastest rate. The number of repeats per cell is variable both within and between taxa, ranging from 1 to several thousands (Long & Dawid 1980; Rogers & Bendich 1987; Loram *et al.* 2007).

Scleractinian corals are mainly associated with *Symbiodinium* from the clades A, B, C, D (Baker 2003); associations with clades F and G are rare (LaJeunesse 2001; van Oppen *et al.* 2005b; Pochon *et al.* 2006; Goulet 2007). Clade E may represent a free-living subgenus of *Symbiodinium* and H is a lineage specific to the Foraminifera (Pochon *et al.* 2006). Biogeographic and habitat surveys of *Symbiodinium* in scleractinian corals have established two main patterns. First,

clades A, B and F are more common at higher latitudes and clade C is more abundant at tropical latitudes. Second, clades A and B are much more common in the Caribbean than in the tropical Indo-Pacific (LaJeunesse *et al.* 2003; LaJeunesse 2005). The first pattern is thought to be the result of adaptations to the environment (Savage *et al.* 2002; Baker 2003; LaJeunesse *et al.* 2003), whereas the second may be the result of different vicariant histories following the closure of the Central American Isthmus 3.1-3.5 million years ago (LaJeunesse *et al.* 2003; Pochon *et al.* 2004; LaJeunesse 2005). In the Caribbean, an intercladal habitat pattern is present in relation to depth and, thus, irradiance. In general, clades A and B have been found in shallower waters (0-3 m) than clade C (3-14 m) (Rowan & Knowlton 1995; Rowan *et al.* 1997; Toller 2001; LaJeunesse 2002). However, these patterns are dependent on the species and the location. For instance, Diekmann *et al.* (2002) surveyed the symbiont community of five morphospecies of *Madracis* at Curaçao over a depth gradient from 2 to 45 meter and found no differences correlated to habitat.

Clade D has been given special attention, as it is mostly found in marginal habitats where other clades struggle, indicating an opportunistic character and a high stress-tolerance (Baker 2003). Importantly, clade D has increased in abundance in scleractinian corals after bleaching events (Glynn *et al.* 2001; Toller *et al.* 2001; Baker *et al.* 2004; van Oppen *et al.* 2005a) and is favored at reefs exposed to unusually high temperature regimes (Fabricius *et al.* 2004), indicating that symbionts from this clade may play an important role on future reefs.

Below the cladal level, subclades, strains and types have been distinguished. Molecular markers that have been used to resolve within-clade diversity include microsatellites and flanking regions (Santos *et al.* 2004), DNA fingerprinting (Goulet & Coffroth 2003) and, especially, the nrDNA ITS regions (LaJeunesse 2001; van Oppen *et al.* 2001b). In this thesis, I will refer to “types” as the taxonomic unit below the cladal level. Which taxonomic rank (clade, subclade, strain, type) should be considered “species” is open to discussion. Importantly, the many types turn out to have the most ecological relevance and these have not always been identified in ecological studies. For example, in the Indo-Pacific, different types within clade C show zonation with depth/light (van Oppen *et al.* 2001b; LaJeunesse *et al.* 2003).

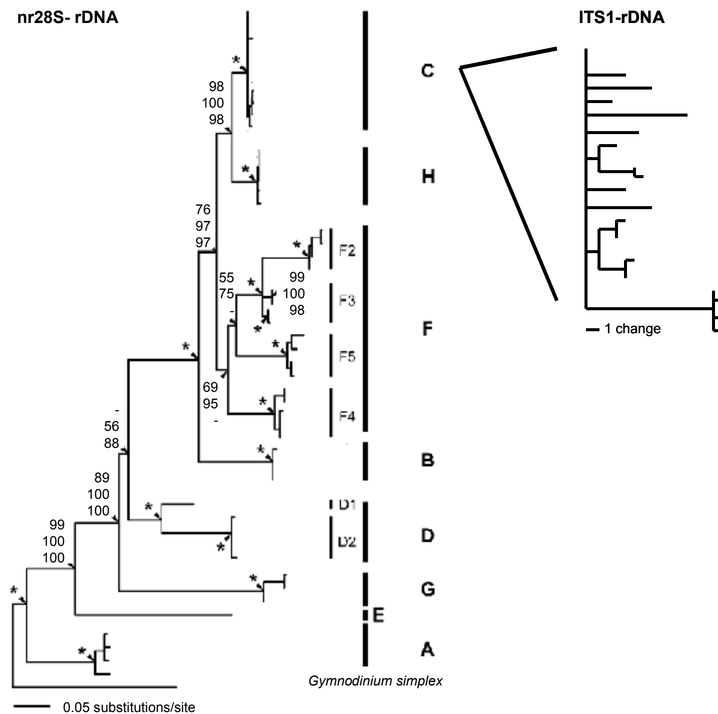

Fig. 7. Maximum likelihood (ML) phylogram of *Symbiodinium*, based on 28S nrDNA. The principle eight clades are A-H. Numbers at nodes are, from top, the bootstrap values obtained with ML, Bayesian posterior probabilities (in percentage) and the bootstrap values obtained with maximum parsimony; * = 100% for all three. Clade C (right) remains unresolved even with ITS1, one of the most variable markers currently available (adapted from Pochon *et al.* 2006).

The use of several different molecular markers at the within-clade level has resulted in considerable confusion about type designations (C1, C2, C2*, C2a, etc.). Lajeunesse (2001) identifies each unique, dominant ITS2 sequence as a type with >100 ITS2 types having been distinguished within clade C alone (Fig. 7). These types do not always correspond to the ITS1 designations used by van Oppen *et al.* (2001b). In this thesis I will use the types *sensu* van Oppen and link these to the types *sensu* Lajeunesse where relevant.

(2) Specificity and flexibility

The specificity of the coral-*Symbiodinium* interactions varies considerably (reviewed in Baker 2003) (Fig. 8). Some symbiont types are widely distributed and found associated with many hosts (called generalists), whereas others are only found in one or a few hosts and/or locations (called specialists). Similarly, some coral hosts can associate with many different symbiont types, whereas others are limited to only one or a few. Hence, a symbiosis can be both flexible (defined here as the ability to associate with multiple partners) and specific, depending on the partner. The mode of symbiont transmission from one generation to the next also plays an important role in shaping specificity/flexibility, as described below.

In horizontal transmission (Richmond 1997) the eggs do not contain algal symbionts and each generation must acquire their own symbionts anew from the environment. This mode of transmission is generally found in broadcasting corals and, hence, is the main mode of transmission in scleractinian corals. Horizontal transmission is likely to involve a certain degree of flexibility of the coral host, as symbiont uptake is dependent on what is available in the environment. Indeed, several studies have described the initial uptake of symbionts by azooxanthellate coral juveniles as relatively non-specific (Coffroth *et al.* 2001; Goulet and Coffroth 2003; Little *et al.* 2004; Gomez-Cabrera *et al.* 2008).

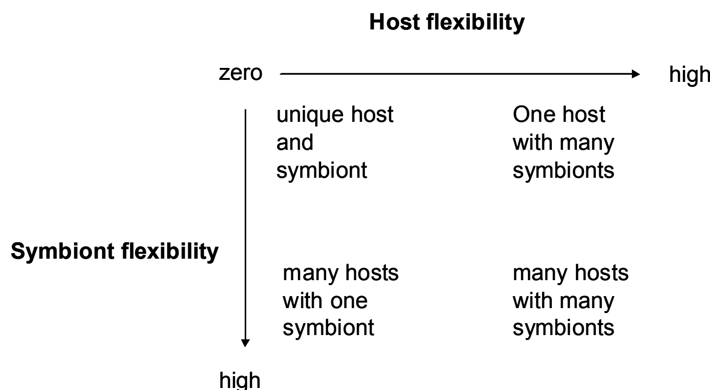

Fig. 8. Conceptual framework for symbiosis specificity (adapted from Baker 2003).

In vertical transmission, the eggs already contain symbiont cells from the parent colony and this mode of transmission is generally found in brooding corals (Fadlallah 1983). Vertical transmission reduces the risk of not finding a suitable symbiotic partner and favors the evolution of specialist symbiont lines (Douglas 1998). Specialist symbiont types are often found associated with vertical transmitters (LaJeunesse *et al.* 2004).

In general, one or a few generalist types and many specialist types of *Symbiodinium* dominate the population at a given site (LaJeunesse 2001, 2002, 2005; Diekmann *et al.* 2002). Both specialists and generalists are spread over the different clades. For clade C, two main generalists have been identified, designated C1 and C2 (C1 and C3, respectively, based on ITS2 *sensu* LaJeunesse 2001). C1 and C2 are common to both the Indo-Pacific and the Caribbean, but other than these two, each ocean has a unique assemblage of locally evolved specialist types. It has been proposed that this pattern may be the result of a worldwide selective sweep of C1 and C2, or their progenitor, before the closure of the Central American Isthmus (3.1-3.5 million years ago), followed by bursts of diversification (LaJeunesse 2005).

Coral species may host different symbionts depending on the depth (e.g. Rowan & Knowlton 1995), geographic location (e.g. van Oppen *et al.* 2005a) and sampling time (e.g. Thornhill *et al.* 2006). Variation within a single colony is also possible (e.g. Ulstrup & van Oppen 2003). Importantly, several studies have documented bleaching-related changes in the symbiont community composition of reef-building corals (Baker 2001; Toller *et al.* 2001; Berkelmans & van Oppen 2006; Thornhill *et al.* 2006). A recent meta-analysis (Goulet 2006) suggested that 29% of the scleractinian coral species associate with more than one symbiont type. This notion was challenged by Baker and Romanski (2007), who re-analyzed Goulet's (2006) data from a different perspective. They concluded that the majority (>>50%) of scleractinian coral species, including virtually all of the dominant reef-building taxa, may be able to host multiple symbiont types — even at the clade level — pointing to the fact that: (1) almost two-thirds of the coral species investigated were represented by a very low number of samples (N≤5), (2) within-clade flexibility was not taken into account, and (3) the standard molecular typing techniques used were only able to detect the dominant symbiont type within a coral colony, with types with abundances below 5-10% being overlooked (Diekmann *et al.* 2002; LaJeunesse 2002; Goulet & Coffroth 2003; Fabricius *et al.* 2004).

In this thesis I elaborate on the above discussion, particularly in relation to the last point. I hypothesize that many multi-clade associations have been overlooked because of the insufficient sensitivity of the molecular typing techniques used. To test this hypothesis, I develop a new, highly sensitive real-time PCR assay to re-screen samples already typed by commonly used molecular methods (Chapter 2).

(3) Symbiont type affects coral performance

Patterns in the distribution of *Symbiodinium* types — across and especially within coral species — are indicators of holobiont acclimitization. For example, zonation with depth indicates that corals can acclimatize to irradiance levels by associating with certain *Symbiodinium* types (Iglesias-Prieto *et al.* 2004), which can be classified as "high-light" and "low light" types (Baker 2003). Geographic zonation (Rodriguez-Lanetty *et al.* 2001; Fabricius *et al.* 2004; van Oppen *et al.* 2005a; Ulstrup *et al.* 2006) may reflect acclimatization to local environmental conditions such as water quality and temperature regime. Importantly, patterns in the natural bleaching response are strongly correlated with symbiont type (Rowan *et al.* 1997; Rowan 2004; Berkelmans & van Oppen 2006; Sampayo *et al.* 2008). The notion that holobiont physiology is affected by the symbiont identity is supported by differences in the physiological performance of symbiont types. Differences in photo-acclimatory responses to light (Iglesias-Prieto & Trench 1997), growth (Kinzie *et al.* 2001; Robinson & Warner 2006) and heat-tolerance (Bhagooli & Hidaka 2003) have been found between types isolated from different coral hosts. However, extrapolating these findings to the physiology of the holobiont is questionable (Bhagooli & Hidaka 2003), as the symbiont culture conditions are very different from the *in hospite* situation and the holobiont physiology is shaped by the coral host as well. Recently, however, the genetic identity of the symbionts *in hospite* has been unambiguously linked to a 2-3 fold difference in growth rates (Little *et al.* 2004) and in a 1-1.5°C difference in heat-tolerance (Rowan 2004; Berkelmans & van Oppen 2006) in a few coral species. This highlights the influence of the symbiont on important proxies of fitness.

The significance of the symbiont type for holobiont fitness is further examined in this thesis. I hypothesize that within a coral species, harboring different symbiont types leads to stronger differences in coral fitness than can be induced by differences

between coral host populations or their local environmental conditions during development. To test this hypothesis, I construct custom holobionts to partition symbiont type, host population and environmental effects (Chapter 5).

As a spin-off of Chapter 5, the connection between symbiont type and coral growth is further examined in a collaborative study with fellow PhD student Neal Cantin (Chapter 7). By using two custom coral groups and several different techniques (PAM fluorometry, radio-labelling, DCMU exposure, HPLC analyses), we compare the photosynthetic efficiency of the algal symbiont to the photosynthate incorporation by the coral host.

(4 & 5) Bleaching as a trigger for changes and post-bleaching reversal

Annual fluctuations in algal densities, correlated with seasonal changes in light and temperature, appear to be a common feature of scleractinian corals (Fitt *et al.* 2000). This seasonal fluctuation can be coupled with a shuffling of different *Symbiodinium* types (Chen *et al.* 2005). Bleaching may, therefore, not always be required for symbiont changes. With increasing temperature, flexibility of this degree can result in a smooth change towards more heat-tolerant symbionts with little to no coral mortality. However, most scleractinian corals (including those which are known to be flexible) are dominated by a single *Symbiodinium* type (Diekmann *et al.* 2002; Baker 2003; Goulet 2006). Symbiont types that are heat-tolerant (e.g. clade D symbionts) may be weak competitors (e.g. low growth rates) compared to heat-sensitive types in the absence of heat-stress (Little *et al.* 2004), explaining why they generally do not dominate the coral hosts. Bleaching, therefore, provides an opportunity for a symbiont change, as heat-tolerant types can take advantage of the space made available by the loss of the dominant symbiont (Baker 2001; Fautin & Buddemeier 2004). However, when conditions return to normal for prolonged periods of time, the heat-susceptible type may return to dominance as it is competitively superior under those conditions (Thornhill *et al.* 2006). Such a post-bleaching reversal, predicted by the ABH, would leave the corals susceptible to the next bleaching event.

In a second collaborative study with fellow PhD student Alison Jones, we follow the *Symbiodinium* community of an *Acropora millepora* population through a natural mass bleaching event (Chapter 6). We investigate whether bleaching can

induce a temporal change in coral-algal associations resulting in a higher coral thermo-tolerance on the scale of a reef and, if a change occurs, whether post-bleaching reversal is evident within six months after the bleaching event.

IMPLEMENTING CHANGE: SWITCH OR SHUFFLE?

There are two proposed ways by which changes in the symbiont population can occur (Baker 2003):

(1) Symbiont switching involves the uptake of a new algal type from the environment, which then becomes the dominant symbiont. It allows the coral to sample the present pool of *Symbiodinium* that may contain different symbiont types compared to what the coral initially established in symbiosis. Uptake of exogenous zooxanthellae by adult individuals has been shown experimentally in anemones (Kinzie *et al.* 2001), in a soft coral (Lewis & Coffroth 2004) and in the scleractinian coral *Porites divaricata* (Coffroth *et al.* 2008). Coffroth *et al.* (2008) exposed bleached *P. divaricata* from the Caribbean to four atypical types and recovered one atypical type after several weeks, as well as the original type. They suggested that secondary infections are restricted by the limited flexibility of the symbiosis. There is also evidence to suggest that the temporal window for uptake of zooxanthellae in *Acropora* spp. is narrow and restricted to the juvenile stage (Little *et al.* 2004).

In this thesis, I investigate whether adult, experimentally bleached *Acropora millepora* were able to take up atypical symbionts, that were provided in large dosage in their environment, to establish a new symbiosis (Chapter 4).

(2) Symbiont shuffling requires the new dominant symbiont to already be present in low abundance in the coral tissues prior to the bleaching event. During bleaching, the dominant symbiont is expelled, allowing the more stress-tolerant, background symbionts to proliferate during recovery. Symbiont shuffling may play an important role in scleractinian corals, but may be limited in its potential, as it requires a background presence of stress-tolerant symbiont types. As most corals have been found to harbor only one *Symbiodinium* type based on standard assays (Diekmann *et al.* 2002; Goulet 2006), the potential for shuffling has been thought to be small.

I hypothesize here that this notion is incorrect because the standard techniques used in symbiont genotyping have a very low sensitivity for background symbiont

populations and the majority of symbiont backgrounds may have eluded detection. I develop a new real-time PCR assay to investigate how prevalent background clades are in four common scleractinian corals on the Great Barrier Reef (Chapter 2).

EXPERIMENTAL TECHNIQUES & ASSAYS

Raising of specific coral-zooxanthella associations (custom holobionts)

Broadcast spawning of scleractinian corals (such as *Acropora millepora*) allows the collection of huge numbers of (azooxanthellate) eggs and sperm with little environmental impact. These in turn can be used to raise large numbers of specific coral-algal associations, which in this thesis will be referred to as custom corals or custom holobionts. Custom holobionts can be used to partition the influence of the host and symbiont on the coral colony's physiological characteristics. Host (population) effects can be investigated by raising custom holobionts from different coral species (or populations of the same species) with identical symbiont types. Similarly, symbiont effects can be investigated by raising offspring from the same parent colonies, but associated with different symbiont types. Lastly, identical custom holobionts can be outplanted at different locations to establish the effects of the local environmental conditions. A reciprocal design including multiple hosts, multiple symbiont types and multiple field locations allows optimal statistical analyses of all three effects in a factorial ANOVA framework.

The first step in the raising of custom holobionts is the acquisition of coral spawn. As the timing of broadcast spawning is known for many species on the GBR (Babcock *et al.* 1985), fecund colonies of the species of choice can be collected a few days before spawning and kept in tanks. Once spawning has occured, the eggs are fertilized in the water column within the first four hours (at least two colonies need to spawn synchronously, as self-fertilization generally does not occur). The embryos are then gently washed and transferred to large rearing tanks containing filtered seawater (1µM pore) and allowed to develop into planula larvae. After four days, meta-morphosis of the larvae into sessile coral polyps is induced by adding adequate substratum, e.g. autoclaved terra-cotta tiles that have been preconditioned in the field. The coral juveniles are able to establish symbioses with zooxanthellae from the water column, and specific types of *Symbiodinium* (either from culture or

freshly isolated from a host) are added to produce the desired custom holobionts. Once the symbiosis is established (ca. three weeks after the symbiont introduction), the custom holobionts can be outplanted to the field location. The terracotta tiles, to which the custom holobionts are attached, are mounted on a steel rod via a hole in the middle of the tile, and the steel rod is placed in the field suspended between two metal pickets in an area where *Acropora* spp. are common (Fig. 9).

Fig. 9. Terracotta tiles with custom holobionts placed at a field location where *Acropora* spp. are common.

Identifying closely related *Symbiodinium* types

Single Strand Conformation Polymorphism (SSCP) analyses (Fig. 10) of the ITS1, in combination with sequencing, has been a standard approach to identify *Symbiodinium* types in coral samples (van Oppen *et al.* 2001b). Small fragments of coral, containing zooxanthellae, are collected and the total DNA is extracted. Using *Symbiodinium*-specific, fluorescent-labeled primers, the *Symbiodinium* ITS1 region can be amplified by Polymerase Chain Reaction (PCR). After amplification, the PCR product is heated to dissociate the complementary strands, after which the sample is snap-cooled to cause the DNA strands to fold back on themselves. The acrylamide-based gel used for SSCP separates the molecules based on the 3-D structure of the folded, single DNA strands, which depends on their sequence — not their length.

By comparing the obtained bands with previously sequenced reference bands, the *Symbiodinium* type can be resolved. Advantages of this approach include the screening of many individuals in a cost-effective manner prior to sequencing

(although recent advances in sequencing technology have significantly reduced this advantage) and the ability to distinguish multiple symbiont types within one sample.

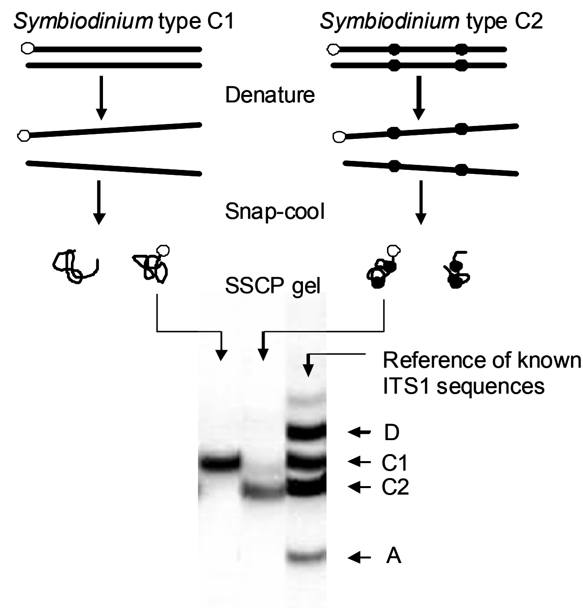

Fig. 10. Schematic representation of an SSCP analysis. ITS1 sequences of types C1 and C2 differ from each other by two base pairs (~ 350 bp long fragment) and produce different bands on the SSCP gel. • = mutation, o = fluorescent label (only on forward primer).

Detection and quantification of *Symbiodinium* background clades

Real-time PCR is a very sensitive assay for detection and quantification of low-abundance background clades in multi-clade associations. Each clade has its own specific primer pair, and a separate PCR reaction is set-up for each primer pair per sample. The reaction mixture includes a fluorescent reporter that only gives off a signal when interacting with double-stranded DNA. During the PCR reaction, the amount of DNA is measured through the fluorescence after each cycle (Fig. 11).
In the initial cycles, the fluorescent signal is too low to be detected. After a number of cycles, an exponential increase in the fluorescent signal is measured as the target DNA area doubles every cycle until the PCR reagents run out and a plateau is reached. The cycle in which the sample fluorescence crosses a fluorescent threshold

(set somewhere in the exponential phase) is called the threshold cycle (C_T) and this value is dependent on the amount of target DNA initially present in the sample. The relative abundance of different clades can be calculated by the difference in C_T of the different clade-specific reactions.

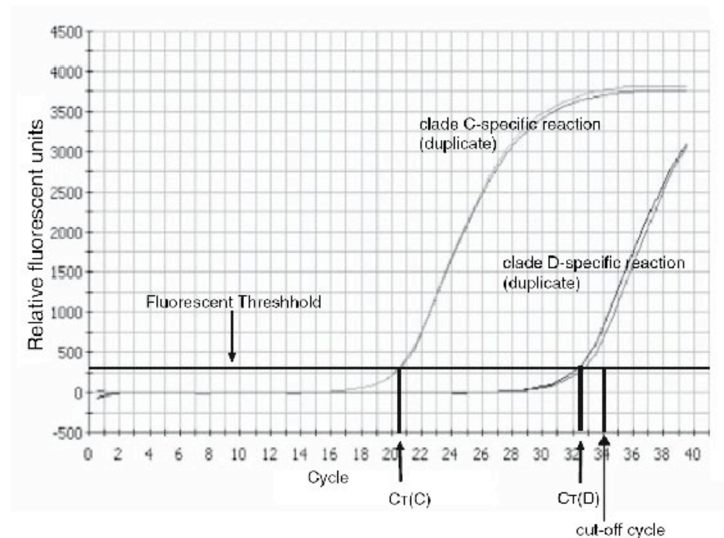

Fig. 11. Real-time PCR profile for the detection of *Symbiodinium* clades C and D in a coral sample. In this example, the average difference in C_T between the clade C and D-specific reactions (run in duplicate) is 12, indicating that there is $2^{12} = 4096$ times more clade C DNA than clade D DNA in the coral sample. The coral under investigation is, therefore, dominated by clade C with a very low (background) presence of clade D.

Measuring photosynthetic performance as a proxy for bleaching

Pulse Amplitude Modulated (PAM) fluorometry is a popular, non-invasive technique for measuring the "health" of *Symbiodinium* cells while in symbiosis with corals. The most commonly measured parameters are the maximum quantum yield (Fv/Fm) and the effective quantum yield (F/Fm'), which give indications of the efficiency of the photosynthetic machinery of the algal symbiont (Ralph *et al.* 2005). The technique involves the measurement of the fluorescence of chlorophyll a under different irradiance conditions (Fig. 12). Fv/Fm is measured after a period of darkness and with the photomachinery at rest. First, Fo is measured by exposing the

coral surface to a pulsating red light at a very low intensity (the measuring light), capable of inducing fluorescence but not inducing photosynthesis. Next, the coral is exposed to an ~1 sec burst of very high, white light, called a saturating pulse, and the maximum fluorescence (Fm) is measured. From these two measurements, the maximum quantum yield (Fv/Fm) can be calculated through the formula: Fv/Fm = (Fm-Fo)/Fm. Fv/Fm gives an indication of the maximum number of reaction centers in photosystem II (PSII) that are "open" (ready for light capture for photosynthesis) and heat-stress will lead to a reduction in this parameter.

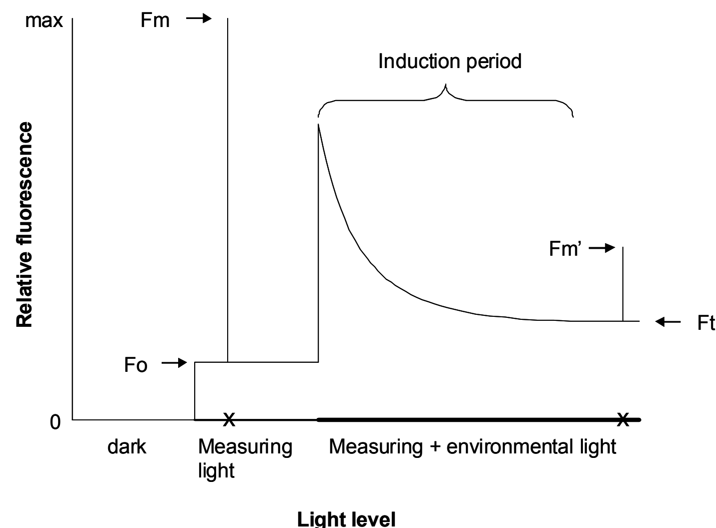

Fig. 12. Measuring maximum and effective quantum yields. X indicates the saturation pulse. Fo and Fm are measured in dark-adapted corals, Ft and Fm' are measured in light-adapted corals after the induction phase has passed.

Effective quantum yield (F/Fm') is measured similarly, but these measurements take place when the corals are under ambient environmental light conditions (either experimental or natural) and the photomachinery is active. Instead of Fo, Ft is measured, which is the fluorescence under the environmental light conditions (+ measuring light). Similarly, using the saturating pulse, Fm' is measured instead of Fm. Care must be taken to wait until the induction period has passed, during which the photomachinery has started up. The effective quantum yield is calculated according to the formula: F/Fm' = (Fm'-Ft)/Fm' and is typically

lower than the maximum quantum yield as a portion of the PSII reaction centers remain closed because of ongoing light capture. Again, heat-stress will cause a reduction in F/Fm'.

When combining Fv/Fm and F/Fm', the excitation pressure over photosystem II (Q) can be calculated using the formula: $Q = 1 - ([F/Fm']/[Fv/Fm])$, which gives an indication of the percentage of reaction centers that are closed under the environmental light conditions during the effective quantum yield measurements (Iglesias-Prieto *et al.* 2004). Q is considered a very important measure of heat-stress as the accumulation of excitation pressure over photosystem II is inherently associated with coral bleaching (Abrego *et al.* 2008).

OUTLINE OF THE THESIS

The popular view that most scleractinian corals harbor only one clade of *Symbiodinium* per colony is evaluated in C**hapter 2.** To do this required the development of a novel real-time PCR assay which boosted the detection sensitivity for multi-clade symbioses >100 fold. Application of this assay on a sample collection of four common scleractinian corals collected across the Great Barrier Reef revealed that previous studies have missed many multi-clade symbioses and indicated that the potential for symbiont shuffling is large.

Further technical refinements of the real-time PCR assay are described in **Chapters 3 and 4**, aimed to: (1) overcome the limitations associated with variability in ITS1 copy number, (2) include a measure of symbiont cell density per coral unit, and (3) increase the resolution of the assay to the type level.

Adult *Acropora millepora* colonies were experimentally bleached using a herbicide and exposed to high levels of an atypical *Symbiodinium* type over a 6-week recovery period (**Chapter 4)**. Screening of post-exposure DNA samples with the optimized real-time PCR assay revealed that no new symbioses had been established. However, results were inconclusive due to the presence of the "atypical" symbiont type at low levels at the start of the experiments in 30% of the colonies and the use of a low-infectious symbiont type (both issues were not known at the start of the experiment). Improvements to the experimental design are discussed.

In order to test specific host-symbiont responses (**Chapter 5**), custom holobionts were produced in the laboratory using two *A. millepora* populations and

six *Symbiodinium* types. The custom holobionts were reciprocally outplanted to two field sites differing in ambient temperature. It was established that the fitness (using growth, survival and heat-tolerance as proxies) of *A. millepora* on the GBR was primarily affected by the symbiont types present and secondarily by environmental factors working on growth and survival. In contrast, host population origin (and hence possible host genetic differences) had little to no effect and no environmental effect was found on the thermo-tolerance. As a result, trade-offs between thermo-tolerance and growth/survival related to *Symbiodinium* type were found to be dependent on the local environmental conditions.

We investigated the effect of a natural bleaching event on the symbiont community within an *A. millepora* population in **Chapter 6**. Before bleaching, 93.5% of the coral colonies harbored the thermo-sensitive *Symbiodinium* type C2, whereas up to six months after bleaching, 71% of the same coral colonies were dominated by the thermo-tolerant *Symbiodinium* type D. The high prevalence of background clades indicated that this change was due to symbiont shuffling. Indications for a drift back to the heat-sensitive C2 (post-bleaching reversal) were found after six months.

In **Chapter 7** the effects of *Symbiodinium* types C1 and D on growth of *A. millepora* is further investigated using custom holobionts outplanted at Magnetic Island. PAM fluorometry (rapid light curves) revealed that that C1 symbionts had a 87 % higher photosystem II (PSII) capacity than D symbionts *in hospite*. Incorporation of photosynthate by the host (as measured by ^{14}C-labelling) was 121 % higher for C1 holobionts than for D holobionts under identical environmental conditions. The advantage for C1 holobionts was lost in the presence of the herbicide diuron (DCMU), which blocked the electron transport and caused damage to PSII. Therefore, a strong link was suggested between photosynthetic capacity and nutritional benefit to the coral host, which explained why C1 holobionts grew twice as fast as D holobionts at the outplant location (as found in Chapter 5).

In **Chapter 8** the overall results, conclusions and prospects are summarized. The Dutch translation of this chapter is given in **Chapter 9**.

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Chapter 2

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

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Coral Reefs (2007) **26**: 449-457*

* This article received the "Best Paper Award for 2007" from *Coral Reefs*, journal of the International Society for Reef studies.

bionieuws
editie 15, 06-10-2007

Verhit koraal doet aan partnerruil

Iedere relatie kan kapot, zelfs een endosymbiotische. Koraal wisselt intracellulaire algen die voor fotosynthese zorgen in voor typen die beter tegen het warmere zeewater kunnen.

Door Lucas Wenniger
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Koraal vertoont risicospreidend gedrag. Rifkoralen beschermen zich tegen veranderende omstandigheden door met verschillende endosymbionten een symbiose aan te gaan. Dat denkt Jos Mieog, promovendus aan de Rijksuniversiteit Groningen. Hij dook op het Great Barrier Reef in Australië zelf zijn monsters op, en toonde aan dat koralen meestal meer dan één type symbiotische alg bevatten. Voorheen werd gedacht dat een koraal slechts bij uitzondering met verschillende algen samenleefde. Zijn recente artikel in Coral Reefs kreeg zoveel aandacht van Australische onderzoekers en natuurbeschermers dat de uitgever het nu vrij toegankelijk heeft gemaakt.

Endosymbiose is een delicate vorm van samenleven van twee organismen, waarbij de kleinere soort in de cellen van de gastheer leeft. De algen leveren in dit geval energie uit hun fotosynthese aan het koraal, en krijgen in ruil daarvoor de bescherming van het leven in de cellen van het koraal.

Om de koralen te screenen op verschillende stammen van endosymbiotische algen, gebruikte Mieog een gevoelige genetische techniek. Alle onderzochte algen die in de koralen leven zijn van het genus Symbiodinium, en zijn op basis van hun DNA te verdelen in verschillende claden, groepen van soorten. Met real time PCR kon hij laten zien dat koralen niet alleen een dominante clade van Symbiodinium bevatten, maar dat op de achtergrond in 78 procent van de monsters ook een andere clade aantoonbaar is.

Hittebestendig
Het huisvesten van verschillende claden biedt het koraal de kans om met de alg samen te leven die op dat moment het meest van pas komt, denkt Mieog. 'We weten dat jong koraal nog verschillende algen opneemt, waarvan dan één clade dominant wordt. Als het ouder wordt houdt het de voorkeur voor Symbiodinium van die clade. Als het koraal onder stress komt te staan, bijvoorbeeld door hoge zeewatertemperaturen, gooit het meer dan 90 procent van de algen eruit en wordt het bleek, het zogenaamde bleachen. Als deze bleaching niet te ernstig is kan een koraal herstellen, en soms is de nieuwe alg die in het koraal groeit van een andere clade. Wij denken dat de nieuwe dominante partner al op de achtergrond aanwezig was vóór de bleaching, en dat deze algen de symbiose overnemen omdat ze beter geadapteerd zijn aan een warmere zee.'

Mieog hoopt dat koraalriffen door deze symbiont shuffling beter bestand zullen zijn tegen klimaatverandering dan eerder werd gevreesd. 'We weten dat algen van een bepaalde clade minder gevoelig zijn voor hogere zeewatertemperaturen. Dat we die algen nu op de achtergrond van veel koralen aantreffen betekent dat ze in potentie kunnen shuffelen. Het overstappen op relatief hittebestendige algen kan een voordelig effect hebben voor het koraal op de weerbaarheid tegen warmtestress. Maar het blijft een complex probleem: koralen die vooral deze algen bevatten, groeien veel langzamer.'

ABSTRACT

Reef corals form associations with an array of genetically and physiologically distinct endosymbionts from the genus Symbiodinium. Some corals harbor different clades of symbionts simultaneously, and over time the relative abundances of these clades may change through a process called symbiont shuffling. It is hypothesized that this process provides a mechanism for corals to respond to environmental threats such as global warming. However, only a minority of coral species has been found to harbor more than one symbiont clade simultaneously and the current view is that the potential for symbiont shuffling is limited. Using a newly developed real-time PCR assay, this paper demonstrates that previous studies have underestimated the presence of background symbionts because of the low sensitivity of the techniques used. The assay used here targets the multi-copy rDNA ITS1 region and is able to detect *Symbiodinium* clades C and D with >100-fold higher sensitivity compared to conventional techniques. Technical considerations relating to intragenomic variation, estimating copy number and non-symbiotic contamination are discussed. Eighty-two colonies from four common scleractinian species (*Acropora millepora, Acropora tenuis, Stylophora pistillata* and *Turbinaria reniformis)* and 11 locations on the Great Barrier Reef were tested for background *Symbiodinium* clades. Although these colonies had been previously identified as harboring only a single clade based on SSCP analyses, background clades were detected in 78% of the samples, indicating that the potential for symbiont shuffling may be much larger than currently thought.

INTRODUCTION

Many marine invertebrates and protists (e.g., corals, anemones, jelly fish, giant clams, and Foraminifera) form obligate mutualistic symbioses with algae of the genus *Symbiodinium*. The genus is several tens of millions of years old and comprises eight phylogenetic clades (A-H) based on ribosomal and chloroplast DNA (Pochon *et al.* 2006). Each clade encompasses multiple strains or types (Baker 2003; Coffroth & Santos 2005). The high diversity of *Symbiodinium* is often linked to physiological performance. *In vitro* studies using genetically distinct *Symbiodinium* cultures have found differences in photo-acclimatory responses to light (Iglesias-Prieto & Trench 1997) and in growth (Kinzie *et al.* 2001; Robison and Warner 2006). Freshly isolated symbionts from different host species have shown distinct responses to heat-stress (Bhagooli & Hidaka 2003). *In hospite*, the genetic identity of the symbionts has been linked to a 2-3 fold difference in growth rates (Little *et al.* 2004) and 1-1.5°C difference in heat-tolerance (Rowan 2004; Berkelmans & van Oppen 2006) within a coral species. These results show that changes in a coral's zooxanthella community may result in changes in the physiology of the symbiosis.

There are two proposed ways by which changes in the symbiont population can occur: symbiont switching and symbiont shuffling (Baker 2003). Symbiont switching is the uptake of a new symbiont type from the environment, whereas in symbiont shuffling, the new symbiont does not come *de novo* from the environment, but is already present at low abundance in the coral tissues. Uptake of exogenous zooxanthellae by adult individuals has been shown experimentally in anemones (Kinzie *et al.* 2001) and in a soft coral (Lewis & Coffroth 2004), but has so far not been observed in scleractinian corals. Instead, a coral's temporal window for uptake of zooxanthellae may be narrow and restricted to the juvenile stage (Little *et al.* 2004). Symbiont shuffling may play an important role in scleractinian corals, as recent studies have shown that resident algal populations consisting of more than one type of alga may change in relative abundance: 1) on a seasonal basis (Chen *et al.* 2005); 2) while recovering from a natural bleaching event (Thornhill *et al.* 2006b); and 3) after bleaching following transplantation (Berkelmans & van Oppen 2006).

In a recent review, Goulet (2006) surveyed 442 species of both hard and soft corals from 43 published studies in the literature and concluded that the

simultaneous occurrence of multiple symbiont clades is low (23%) and, therefore, the promise of symbiont shuffling as a mechanism to cope with climate change, has been overestimated. While their conclusion is consistent with current data, absence of evidence is not necessarily evidence of absence. This study investigated whether the low value is an artifact caused by the low sensitivity of the most commonly used survey techniques for symbiont detection: Denaturing Gradient Gel Electrophoresis (LaJeunesse 2002), Single Strand Conformational Polymorphism (Fabricius *et al.* 2004), Restriction Fragment Length Polymorphism (Diekmann *et al.* 2002), and DNA fingerprinting (Goulet & Coffroth 2003a). None of these methods can detect clades which are present at levels below 5-10% of the total symbiont population, and direct sequencing of PCR products will also only detect the dominant symbiont.

Here, a highly sensitive, real-time PCR assay is presented that quantifies the nuclear ribosomal DNA (nrDNA) Internal Transcribed Spacer 1 (ITS1) of clade C symbionts vs. clade D symbionts within a single sample. The goal is to provide a new tool for a reassessment of the presence of low abundance, background or cryptic clades. The new assay was tested by resurveying a set of samples that were previously identified as having a single symbiont clade based on SSCP analyses and by verifying the nrDNA results with a similar assay based on chloroplast DNA. The implications of the results are discussed in terms of the current view about the importance of symbiont shuffling the prevalence and significance of cryptic symbionts.

MATERIALS AND METHODS

Collection and preparation of the coral samples

A collection of 82 scleractinian coral samples spanning 11 locations and four species (Fig. 1 and Table 1) was assayed for background clades. Small pieces were taken from 2-5 colonies/species and fixed in absolute ethanol. DNA was extracted using the DNeasy tissue kit (Qiagen) following the manufacturer's protocol for animal tissues and using 150 µL elution buffer. The DNA samples were the same as those used in van Oppen *et al.* (2005) (*Acropora millepora, Acropora tenuis* and *Stylophora pistillata*), and in Ulstrup *et al.* (2006) (*Turbinaria reniformis*).

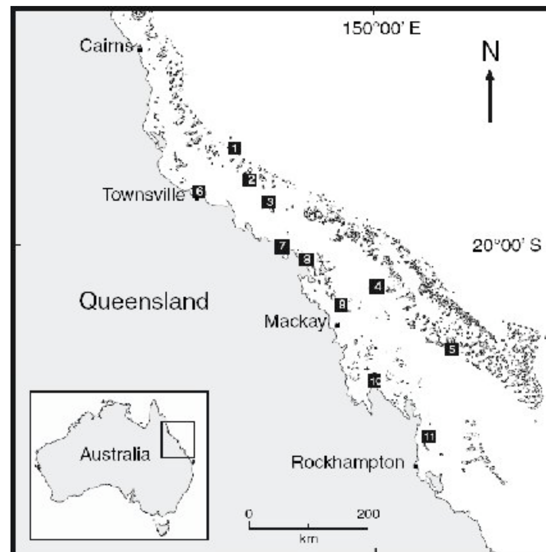

Fig. 1. Sampling locations. See Table 1 for location names, coordinates and sampling numbers.

The real-time PCR assay

Clade C and clade D-specific ITS1 primer pairs were obtained from Ulstrup & van Oppen (2003) (Table 2). A 25 µL real-time PCR reaction contained: 1x IQ SYBR Green supermix (BioRAD), 180 nM clade *Symbiodinium*-universal forward primer, 180 nM clade C or D-specific reverse primer and 2.5 µL coral DNA template (MilliQ H_2O in case of no-template controls). Amplifications were run on the ICycler IQ Real-Time PCR Detection System (BioRAD). After an initial heating step to activate the Taq-polymerase following the manufacturer's instructions, the profile consisted of: 40 2-step cycles of 15 s at 95°C and 1 min at 60°C. At the end of each run, a melt curve was generated by starting at 60°C and increasing the temperature by 0.5°C each 5 s for 70 cycles. Data collection took place during the 1 min at 60°C in each cycle, and during each temperature step of the melt curve.

The Cycle-Threshold (C_T) is the PCR cycle at which the fluorescence of a sample exceeds the chosen threshold limit. Setting the fluorescent threshold to a fixed value allows the comparisons of C_T values between runs. Duplicate clade C and D reactions were run for each sample along with negative (no-template) and positive controls in each run. The positive controls showed distinct peaks in the melt

curve for clades C and D. Any runs with peaks not within 1°C of these temperatures were discarded as having primer dimer signals (which occurred rarely). In some runs, the no-template controls showed a small signal during the last few cycles of the run, which is common in real-time PCR and caused by the formation of non-specific fluorescence. To avoid the inclusion of false positives, the end of the detection range was set three cycles prior to the C_T of the no-template controls that showed a non-specific fluorescence signal. Samples with C_T's above the cut-off were treated as false positives and scored as no background clade present. Runs were analyzed using the IQ software V3.1 (BioRAD).

Table 1. Species, locations and symbiont types/clades based on SSCP and real-time PCR.

Species	*Location name*	*Lat-Long*	*Fig. 1*	*Dominant type based on SSCP[1]*	*Dominant clade, back-ground clade based on real-time PCR*
Acropora millepora	Chicken Reef	18.5 S, 147.5 E	1	C2 (2)[1]	C(2)[1], D (1)[2]
	Darley Reef	19.1 S, 148.1 E	3	C2 (5)	C(5), D (5)
	Stone Island	20.0 S, 148.2 E	7	D (4)	D(4), C2 (4)
				C2 (1)	C(1), D (1)
	Credlin Reef	20.3 S, 150.0 E	4	C2 (5)	C(5), D (5)
	Miall Island	23.1 S, 150.5 E	11	C1, C2 (2)	C(2), D (2)
				D (2)	D(2), C (2)
Acropora tenuis	Chicken Reef	18.5 S, 147.5 E	1	C2 (5)	C(5), D (3)
	Darley Reef	19.1 S, 148.1 E	3	C2 (5)	C(5), D (4)
	Credlin Reef	20.3 S, 150.0 E	4	C1 , C2, C3 (5)	C(5), D (4)
	Miall Island	23.1 S, 150.5 E	11	C1 (3)	C(3), D (2)
Stylophora pistillata	Chicken Reef	18.5 S, 147.5 E	1	C1, Cn (5)	C(5), D (3)
	Darley Reef	19.1 S, 148.1 E	3	C1, Cn (4)	C(4), D (4)
	Credlin Reef	20.3 S, 150.0 E	4	Cn (5)	C(5), D (5)
	Brampton Island	20.5 S, 149.2 E	9	C1 (3)	C(3), D (3)
	Mumford Island	22.0 S, 149.5 E	10	C1, Cn (5)	C(5), D (5)
Turbinaria reniformis	Big Broadhurst Reef	18.5 S, 147.4 E	2	C1 (4)	C(4), D (1)
	Magnetic Island	19.1 S, 147.5 E	6	C1 (5)	C(5), D (3)
	Grassy Island	20.1 S, 148.4 E	8	C1 (4)	C(4), D (2)
	Heralds Reef Prong	21.3 S, 151.2 E	5	C1 (3)	C(3), D (2)
	Miall Island	23.1 S, 150.5 E	11	C1 (5)	C(5), D (3)

(n)[1]= number of samples analyzed, (n)[2] = number of samples with a background clade.

[a] van Oppen *et al.* (2005) and Ulstrup and van Oppen (2003)

Table 2. Nuclear and chloroplast primers used in real-time PCR.

Primer	***Sequence***
nITS1 universal forward[a]	5'-AAGGAGAAGTCGTAACAAGGTTTCC-3'
nITS1 C-specific reverse[a]	5'-AAGCATCCCTCACAGCCAAA-3'
nITS1 D-specific reverse[a]	5'-CACCGTAGTGGTTCACGTGTAATAG-3'
cp23S C forward	5'- GGGATAAAACTTGGGTAACATTC -3'
cp23S C reverse	5'- CCAATTAAACAGTGGTCTTAGGAG -3'
cp23S D forward	5'- AACCCCCGATTGGCCTAG -3'
cp23S D reverse	5'- CTTGATTGGGCCATTAAGCA -3'

[a] Ulstrup and van Oppen (2003)

D:C cell number ratios were calculated using the formula: D:C = $(2^{CT(C) - CT(D)})/K^{DC}$, where $C_T(C)$ is the threshold cycle for the clade C specific reaction, $C_T(D)$ the threshold cycle for the clade D specific reaction, and K^{DC} the ratio of average copy number per cell between clades D and C (see next subheading). The average D:C cell number ratio and SE were calculated for each species per location, including the samples in which no backgrounds were detected as zero. A typical run for one coral sample including calculations is shown in Fig. 2.

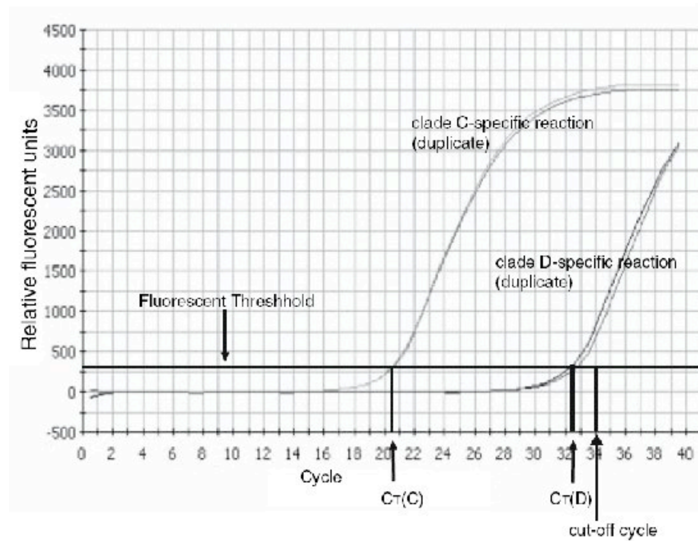

Fig. 2. A typical real-time PCR profile for the detection of a clade D background in a clade C dominated coral sample. The D-specific amplications begin later indicating the low, background abundance of this clade. The D:C cell nr. ratio is calculated as: D:C = $(2^{CT(C) - CT(D)})/K^{DC} = 2^{(20.5-32.5)}/3 = 8.1 \times 10^{-5}$. The sensitivity for this sample is $2^{(20.5-34)}/3 = 2.9 \times 10^{-5} = 0.003\%$.

Estimating average copy number

Ribosomal DNA loci can exhibit large variations in copy numbers between individuals (Zhang *et al.* 1990; Rogers & Bendich 1987; Govindaraju & Cullis 1992) thus affecting the translation from ITS1 copy number ratios to cell number ratios. To assess the extent of this potential bias, the cell-to-cell copy number variability within a C and D dominated coral colony was investigated, and the average copy number differences between colonies was determined from bulk cell analyses.

The cell-to-cell variability of ITS1 copy numbers within a clade was measured in a clade C-only and clade D-only population using single-cell real-time PCR. Clade C and clade D zooxanthellae were isolated from *A. tenuis* and *A. millepora*, respectively, obtained from Magnetic Island. Coral tissue was airbrushed off the skeleton into 1 μm filtered seawater (FSW). The slurry was spun down (400 g for 5 min) and the zooxanthella pellet washed three times with FSW. Cell suspensions were quantified using a haemocytometer and diluted in FSW to 1 cell per 2 μL. Ten μL were pipetted onto a microscope slide and viewed under a dissecting microscope (70 x magnification). Single cells were then collected in 0.5 μL of FSW using a pipette with a heat-elongated plastic pipette tip (to narrow the diameter of the tip), and were added directly to the real-time PCR reaction mix, which were thoroughly mixed by pipetting.

The 20 μL PCR reactions consisted of: 180nM universal Forward Primer (uFP), 180nM C or D-specific Reverse Primer (RP), 1 x SYBR Green PCR master mix (ABI), and 0.5 μL FSW containing 1 zooxanthella. Runs were performed on a Rotor-Gene RG-3000A (Corbett Research) and analyzed using the Rotor-Gene v6.0 software. The reaction profile was the same as described in the previous section. A standard curve was prepared from purified PCR products of ITS1 with known DNA concentrations. DNA used for the standard curves was diluted in MilliQ with 20% FSW to compensate for the FSW added to the single cell reactions.

To obtain an indication of average copy numbers per clade, zooxanthellae were isolated and quantified from four C-dominated (2 x *A. millepora*, 1 x *A. tenuis* and 1x *Pocillopora damicornis*) and 2 D-dominated (2 x *A. millepora*) colonies as described above. An aliquot containing 100,000 cells was spun down (400 g for 5 min) and the DNA extracted following an adapted version of a method used for the

black tiger shrimp, *Penaeus monodon* (Wilson *et al.* 2002). DNA isolations were performed in duplicate and DNA pellets were dissolved in 200 μL 0.01M Tris buffer pH 9. Two μL template was used in the real-time PCR reaction mixes, which were prepared and run as described above. Values were calculated against the standard curve (diluted with 0.01M Tris buffer pH 9) and divided by 1,000 (only 1/100 of the sample was used in the PCR) to obtain the mean ITS1 copy number per cell per colony.

Intragenomic variation

Ribosomal DNA copies within a genome evolve in concert (Arnheim *et al.* 1980), usually resulting in rapid sequence homogenization within individuals and populations through gene conversion and unequal crossing over (Dover 1982). However, the homogenization is assumed not to be complete in *Symbiodinium* (Apprill and Gates 2007) and the occurrence of intragenomic variants may affect the real-time PCR assay if they interfere with the correct binding of the clade C- and clade D-specific primers. To ensure that detected background clades were not due to intragenomic variants of ITS1 or real-time PCR artifacts, and to cross-test the accuracy of our ITS1 real-time PCR assay, twelve coral samples were re-analyzed using a second real-time PCR assay.

The 23S region of the chloroplast ribosomal DNA was quantified using two separate primer pairs (Table 2). Invitrogen's Platinum SYBR green 2 x PCR mastermix was used as more consistent results were obtained in this assay with this mastermix than with the ABI real-time PCR mastermix. The 20 μL reactions contained: 400nM C or D-specific forward primer, 400nM C or D-specific reverse primer, 1 x Platinum SYBR Green PCR master mix, 2 μL template. Cycle conditions were: 2 min at 50 ºC, 2 min at 95 ºC, followed by 15 s at 95 ºC and 30 s at 60 ºC for 40 cycles. Calculations were identical to the ITS1 assay except that no KDC was used.

RESULTS

Specificity, efficiency and sensitivity of the ITS1 real-time PCR assay

An initial PCR amplification of cloned clade C and D ITS1 PCR products (i.e., using plasmids as template) verified that the clade C and D reactions were specific to their respective clades. The fluorescent threshold was set at 300 relative fluorescent units in all Icycler runs. Clade C and D reactions with identical plasmid concentrations differed in their C_T values by less then 0.5, which allowed direct comparison of $C_T(C)$ and $C_T(D)$ of one sample.

In order to calculate the relative abundances of the two clades using the differences in C_T values, near-equal PCR efficiencies were required. PCR efficiency is 100% when the product doubles every cycle. Using a dilution series of a clade C and D sample, a C_T range of 18-32 was obtained for each clade. The ΔC_T was plotted against the Log of the relative template concentrations and the slope of the linear regression line was -0.092, which showed that PCR efficiencies were well-matched and around 93%.

Sensitivity is the ability to detect very low levels of DNA. The range of background clade detection was between the C_T of the dominant symbiont up to the cut-off cycle (set at 34, Fig. 2), thus the sensitivity varied per sample. On average, background clade copy numbers could be detected down to 0.004% of the total zooxanthella population, representing a ca. 1,000-fold greater sensitivity as compared to conventional assays. However, translating these values into relative abundances of the two clades requires caution because abundance is affected by copy number variability between individual zooxanthella cells (see next subheading). Thus, a more conservative overall estimate of the sensitivity is suggested, at minimally a 100-fold increase over conventional methods.

Copy number variability and intragenomic variants

The limit of detection for single-cell real-time PCR runs was set at 500 copies, as the negative controls showed relatively high non-specific fluorescence. The fluorescent threshold was set at 0.1 normalized fluorescence in all Rotorgene runs. ITS1 copy number per cell ranged from <500 to 22,000 (N=20) and from 2,300 to 12,000 (N=13) for clade C and D, respectively.

Based on bulk analysis of 100,000 isolated cells per colony, cell-average ITS1 copy number for clade C was 984 ± 109 (over 4 colonies) and 3181 ± 69 (over 2 colonies, mean ± SE) for clade D. Therefore, the K^{DC} is estimated at 3 and reflects the difference in average copy number between clades C and D.

The comparison between D:C ratios calculated from ITS1 and the cp23S (Table 3) showed good agreement within a factor of ten. Therefore, D:C cell nr. ratios should be interpreted as "order of magnitude" estimates. In two cases the assays disagreed, where the ITS1 assay detected a background clade but the 23S assay did not.

Table 3. Accuracy of ITS1 D:C ratio compared with an independent chloroplast marker.

Species	*Location*	*Cp 23S background (D:C cell nr. ratio)*	*ITS1 background (D:C cell nr. ratio)*	*Relative difference (ITS1/23S)*
Acropora millepora	Darley Reef	2.0×10^{-4}	6.8×10^{-5}	0.3
	Darley Reef	5.5×10^{-4}	1.9×10^{-4}	0.3
	Miall Island	4.5×10^{2}	8.9×10^{1}	0.2
	Stone Island	2.5×10^{2}	6.1×10^{1}	0.2
Acropora tenuis	Chicken Reef	0	0	-
	Darley Reef	2.0×10^{-5}	1.5×10^{-5}	0.7
	Credlin Reef	0	8.0×10^{-4}	**x**
Stylophora pistillata	Chicken Reef	0	0	-
	Darley Reef	3.0×10^{-4}	3.1×10^{-4}	1.0
Turbinaria reniformis	Grassy Island	0	0	-
	Miall Islands	1.6×10^{-1}	2.9×10^{-2}	0.2
	Magnetic Island	0	4.8×10^{-4}	**x**

Re-assessing the incidence of background clades in scleractinian corals

Using the ITS1 assay, background clades were detected in 78% of 82 colonies which, based on previous SSCP analysis, were originally thought to harbor only a single symbiont clade (Table 1). *A. millepora* and *S. pistillata* had the highest frequency of backgrounds (20 out of 21 and 20 out of 22 colonies, respectively). *A. tenuis* (13 out of 18) and *T. reniformis* (11 out of 21) were less successful in acquiring/maintaining backgrounds. For both *A. millepora* and *S. pistillata*, the only colonies without detectable background clades were found at Chicken Reef, whereas *A. tenuis* and *T. reniformis* had colonies without backgrounds at all locations.

Symbiodinium clade C was the dominant endosymbiont at all locations for three out of the four species (*A. tenuis, S. pistillata, T. reniformis*), with average D:C ratios ranging from 4.3 x 10^{-6} ± 1.5 x 10^{-6} (mean ± SE) for *S. pistillata* at Credlin Reef to 5.8 x 10^{-3} ± 5.7 x 10^{-3} for *T. reniformis* at Miall Island (Fig. 3b-d). Clade D dominance was found in 6 *A. millepora* colonies sampled at the inshore locations of Stone and Miall Islands (Fig. 3a). These colonies had a relatively high background level of clade C compared to the clade D backgrounds in the other samples (D:C ratio = 7.4 x 10^{1} ± 1.2 x 10^{1}). Again, it is cautioned that all D:C cell nr. ratios should be interpreted as "order of magnitude" estimates.

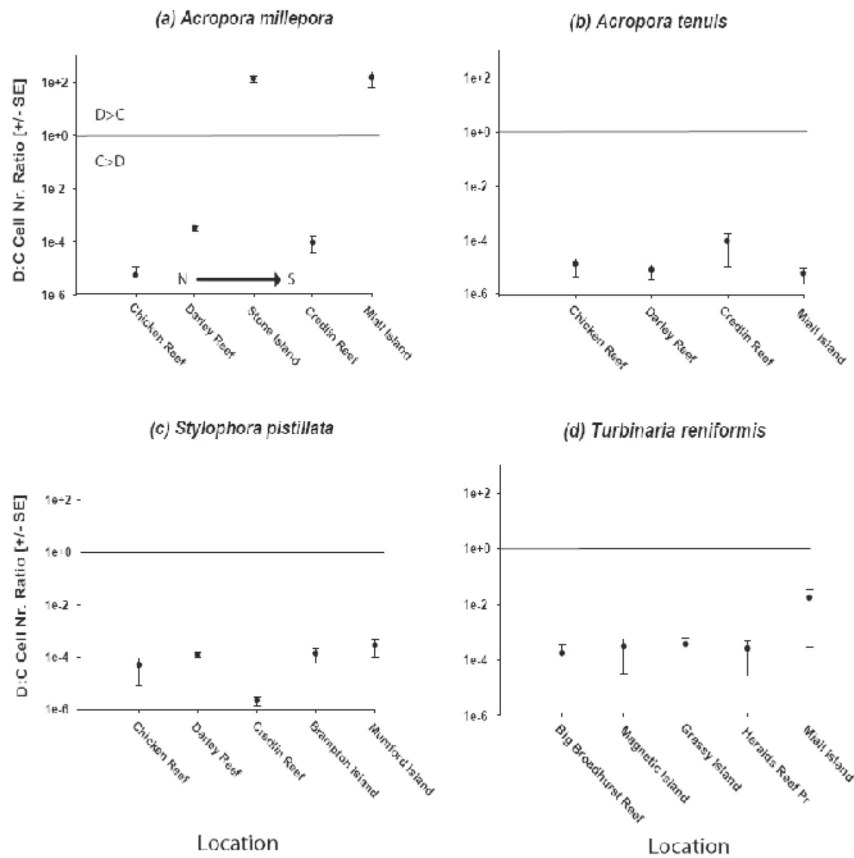

Fig. 3. The mean relative abundance of *Symbiodinium* clade D vs. clade C in the four coral species at each of the sampling locations. Values should be regarded as "order of magnitude" estimates only.

Symbiodinium clade D backgrounds were detected in 71% of the colonies. The average clade D background density was 1 D cell per 150-15,000 C cells. Scleractinian corals have been estimated to harbor ~1,500,000 zooxanthella cells.cm^{-2} of colony surface (Drew 1972), which translates into an average clade D background level of 100-10,000 cells.cm^{-2}.

No latitudinal gradient of D:C cell nr. ratio was apparent. Cross-comparison of off-shore and mid-shore reefs showed a tendency of C>D on offshore reefs and D>C on near-shore reefs in *A. millepora*, but not in the other species.

DISCUSSION

Sensitivity, accuracy and caveats of the ITS1 real-time PCR assay

Real-time PCR can detect background or cryptic clades over eight orders of magnitude and is at least 100 times more sensitive than previously used techniques. However, the high copy number variability found between symbiont cells within both clades with the "single-cell" analyses means that the conversion of ITS1 copy numbers into an estimate of the number of symbiont cells has limited accuracy. On the other hand, the average ITS1 copy number estimates of clades C and D, obtained from "bulked cell" analyses, were remarkably stable for each clade. These results indicate that cell-to-cell variation has only minor impact when enough cells are analyzed, but low density background clade measurements will be stronger affected. It should also be considered that the measured cell-to-cell variation might be an over-estimation of the real variation, caused by the difficult nature of single-cell amplifications. The ~3-fold discrepancy in average copy numbers between clades C and D led to the use of the correction factor K^{DC} in an effort to get background abundance estimates as accurate as possible. The relative difference between the ITS1 and 23S assays varied within 10-fold, which is still reasonable over the range measured, and indicates that D:C cell nr. ratios are meaningful when interpreted as "order of magnitude" estimates.

The high agreement between the ITS1 and 23S assays indicated that the measured background clade signals were not due to intragenomic variation or PCR artifacts. For the two samples where the assays did not agree, it is assumed that the

23S D-specific reactions failed as opposed to the absence of a D background, as it was noted that the 23S assay was generally less robust compared to the ITS1 assay.

As a result of the high sensitivity of the real-time assay, it is possible that non-symbiotic zooxanthellae were detected, present as ingested cells or surface contaminants. Abundances of genuine free-living *Symbiodinium* are unknown, although progress is being made in detecting non-symbiotic, environmental populations (Coffroth *et al.* 2006). However, given the average background abundance detected in this study of 100-10,000 cells per cm^2 of coral surface, it is unlikely, in our opinion, that these zooxanthellae were non-symbiotic.

Significance of background clades

Ninety-three percent of the colonies tested were dominated by clade C and 76% of these had a D background. A number of studies have shown that clade D symbionts are amongst the most thermo-tolerant types know to date, whereas clade C types are often relatively thermo-sensitive (Rowan 2004; Tchernov *et al.* 2004; Berkelmans & van Oppen 2006; Ulstrup *et al.* 2006). Clade D is also found on reefs that chronically experience unusually high temperatures (Fabricius *et al.* 2004) or that have recently been impacted by bleaching events (Baker *et al.* 2004), suggesting that temperature stress can favor clade D. It is proposed here that the clade D backgrounds detected in this study can potentially act as a safety-parachute, allowing corals to become more thermo-tolerant through symbiont shuffling as seawater temperatures rise due to global warming.

For species that have already been found to be dominated by clade D symbionts at some locations, such as *A. millepora* and *T. reniformis*, shuffling between these two clades seems a likely mechanism. Could shuffling act as a mechanism to acclimatize to increasing sea surface temperatures in more than a few species? At present this question remains open. It is likely that not all coral species will have a shuffling capacity, as certain coral species have a high symbiont stability (Goulet & Coffroth 2003b, Thornhill *et al.* 2006a, b) which may not allow them to shuffle even when recovering from bleaching (Thornhill *et al.* 2006a). However, the ratio between shuffling and non-shuffling species is currently unclear and needs further study.

The results of this study indicate that the potential for symbiont shuffling is higher than previously thought. However, symbiont shuffling is likely to represent a

trade-off and comes at a cost. First, coral colonies may have to bleach first before the background symbionts can proliferate (Baker 2001; Berkelmans & van Oppen 2006; Buddemeier & Fautin 1993), possibly causing high mortality in the process. Second, newly shuffled corals that have successfully recovered from bleaching are still likely to be impaired in growth and reproduction (Baird & Marshall 2002). Third, if the stressor disappears for a prolonged period of time, the corals may shuffle back to the original symbiont (Thornhill *et al.*, 2006b), leaving them again vulnerable to subsequent bleaching events. Lastly, the extra heat-resistance that corals may gain by shuffling (1-1.5 °C) may be insufficient to help these populations cope with the predicted increases in average tropical sea temperatures over the next 100 years (Berkelmans & van Oppen 2006). Nevertheless, symbiont shuffling is likely to play a role in the way some corals cope with global warming conditions, leading to new competitive hierarchies and, ultimately, help shape the coral community assemblages of the future.

CONCLUSIONS AND FUTURE DIRECTIONS

Real-time PCR can detect background clades over eight orders of magnitude, thus offering an assay that is at least 100 times more sensitive than previously used techniques. Furthermore, previous studies have failed to detect the majority of background clades because of the low sensitivity of the techniques used. This has led to an underestimation of the potential for symbiont shuffling.

The assay presented here should be used as a starting point to optimize the real-time PCR technique for coral-*Symbiodinium* research. First, at present it only distinguishes between clades C and D and not between types within clades, i.e., C_1, C_2, etc. Because *Symbiodinium* physiology is highly diverse and thermally tolerant types are known to exist within a single clade (Tchernov *et al.* 2004), future efforts will need to be directed towards the development of type-specific real-time PCR assays. Second, while rDNA-ITS continues to be the main marker in use, it has many unfavorable properties associated with its multi-copy nature. The development of novel single-copy markers for *Symbiodinium* should be a research priority and may become available from EST libraries that are currently under development.

The application of this technique in broad scale surveys for background *Symbiodinium* clades/types in many coral species, will allow the re-evaluation of the

prevalence of background symbionts. The next challenge will be to decipher how symbiont shuffling is regulated (by the coral host, through competition between symbiont types inside the host tissues, and/or by environmental factors), and to understand how many corals harboring multiple symbiont types are able to undergo temporal changes in their symbiont communities.

ACKNOWLEDGEMENTS

We thank K. Ulstrup for supplying the coral DNA collection for the species *T. reniformis*, and B. Küst (University of Groningen, the Netherlands) for help with the real-time PCR measurements. We also thank the four anonymous reviewers whose comments greatly improved the manuscript. This work was funded by NWO-WOTRO (The Netherlands), Project Nr. W84-576, and further supported by the Australian Institute of Marine Science.

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Chapter 3

Quantification of algal endosymbionts (*Symbiodinium*) in coral tissue using real-time PCR

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Molecular Ecology Resources (2009) **9**: 74-82

ABSTRACT

Understanding the flexibility of the endosymbioses between scleractinian corals and single-cell algae of the genus *Symbiodinium* will provide valuable insights into the future of coral reefs. Here, a real-time PCR assay is presented to accurately determine the cell densities of *Symbiodinium* clades C and D in the scleractinian coral *Acropora millepora*, which can be extended to other coral-symbiont associations in the future. The assay targets single to low-copy genes of the actin family of both the coral host and algal symbiont. Symbiont densities are expressed as the ratio of *Symbiodinium* cells to each host cell (S/H ratio, error within 30%), but can also be normalized to coral surface area. Greater accuracy in estimating ratios of associations involving multiple clades is achieved compared with previous real-time PCR assays based on high-copy ribosomal DNA loci (error within an order of magnitude). Healthy adult *A. millepora* containing ~1.4 x 10^6 zooxanthellae per cm^2 (as determined by haemocyto-meter counts) had S/H ratios of ca. 0.15, i.e., ~15 symbiont cells per 100 host cells. In severely bleached colonies this ratio decreased to less then 0.005. Because of its capacity to accurately determine both densities and ratios of multiple symbionts within one sample, the assay will open the door for novel research into the mechanisms of symbiont shuffling and switching.

INTRODUCTION

Reef-building scleractinian (stony) corals form obligate endosymbioses with single-cell algae of the dinoflagellate genus *Symbiodinium* (zooxanthellae). The algal genus is highly diverse, both genetically and physiologically, and is divided into eight phylogenetic clades based on ribosomal and chloroplast DNA (Pochon *et al.* 2006). Subclades, strains and types can be distinguished below the cladal level (LaJeunesse 2001, 2002; van Oppen *et al.* 2001b, 2005). Here we define a type as a taxonomic entity below the level of the eight known clades.

Members of six of the eight *Symbiodinium* clades (A-D, F, G) are known to associate with scleractinian corals (Baker 2003). It is not uncommon for the same coral colony to harbor multiple clades (reviewed by Baker 2003; Chapter 2) or types of symbionts simultaneously (Ulstrup & van Oppen 2003). Some species undergo seasonal change in their endosymbiont community (Chen *et al.* 2005) or exhibit dramatic changes after bleaching (Berkelmans & van Oppen 2006, Chapter 6). It is hypothesized that this symbiont flexibility enables the corals to respond to thermal stress events due to a relative increase in abundance of heat-resistant symbionts at the cost of heat-sensitive ones (symbiont shuffling), resulting in a rapid increase in thermo-tolerance (Buddemeier & Fautin 1993; Baker 2001; Baker *et al.* 2004; Berkelmans & van Oppen 2006; Chapter 2).

Investigations into the coral-algal flexibility have traditionally involved gel-based genotyping methods combined with manual (i.e., microscopic) algal cell counts. Gel-based genotyping can have high resolution with respect to identification, but has low sensitivity for the detection of trace-abundances of background symbionts, and only provides a rough relative abundance estimate of the different symbionts involved in the symbiosis (Fabricius *et al.* 2004). Manual zooxanthella counts give reasonably accurate estimates of symbiont densities (around 20%, Andersen 2005), but do not provide any way to distinguish between different symbiont clades; it is also highly labor-intensive and becomes increasingly unreliable with low cell numbers. Automated counting of *Symbiodinium* in coral tissue slurries (e.g. using a Coulter counter, flow cytometer or FlowCam^{TM}) suffers from reduced accuracy because of the inherent difficulty in accurately distinguishing zooxanthellae from host cell debris, irrespective of the technology (R. Berkelmans, unpublished data).

Recently, real-time PCR quantification assays have been introduced that measure the relative abundance of different symbiont clades based on the number of copies of specific DNA loci in a sample (Ulstrup & van Oppen 2003; Loram *et al.* 2007; Chapter 2). These studies targeted the nuclear ribosomal DNA (nrDNA) region, a high-copy locus for which a large amount of sequence data is available, facilitating the development of clade-specific primer pairs. Sensitivity for low-density clades was greatly improved, however accuracy was limited because of variable nrDNA copy numbers among cells, subclades and clades (Loram *et al.* 2007; Chapter 2). To improve accuracy, ideally single-copy DNA loci with high variation are targeted. However sequence information is scarce for *Symbiodinium* and no known single-copy genes are available to date.

In this study, we targeted the actin gene for two reasons: (1) actin sequence information including highly variable intron regions was already available for *Symbiodinium,* and (2) in most eukaryotes, actin is encoded by a gene family of small to moderate size (3-10 genes; Baird & Meagher 1987), and it has been reported as single-copy for two ciliates (Perez-Romero *et al*. 1999; Kuribara *et al*. 2006). By quantifying both *Symbiodinium* and coral host cell numbers, the assay not only determines the cell number ratio between different symbiont clades, but also provides a per clade density estimate expressed as a *Symbiodinium* cell/Host cell ratio (S/H ratio). Hence, the assay reduces the effort of quantifying symbiont densities in corals, and increases the accuracy of quantifying multiple symbiotic partners within one sample.

MATERIALS AND METHODS

Primer development

All available actin sequences for *Symbiodinium* were downloaded from GenBank (Table 1) and aligned in BioEdit (Tom Hall, Ibis Biosciences). Three scleractinian coral actin sequences were available from GenBank, and a search of the GenBank EST-library produced seven partial *A. millepora* actin sequences that contained the region of interest (Table 1).

Table 1. GenBank sequences used for initial actin alignment, n.a. = not assigned, N/A = not applicable, ? = undetermined.

GenBank accession number	***Symbiodinium clade/ coral species***	***DNA region***	***DNA used for sequencing***	***assignment after alignment***
AB086827	n.a.	actin gene	cDNA	Clade C
AB086828	n.a.	actin gene	cDNA	?
AB094432	n.a.	actin gene	cDNA	?
AB231899	Clade A	actin gene	cDNA	Clade A
AB231900	Clade F	actin gene	cDNA	Clade F
AB231901	Clade A	actin gene	cDNA	Clade A
DQ174766	Clade C	actin gene	cDNA	Clade C
DQ174767	n.a.	actin gene	genomic	Clade C?
DQ174768	n.a.	actin gene	genomic	Clade C?
DQ174769	n.a.	actin gene	genomic	Clade C
DQ174712	n.a.	actin gene	genomic	Clade A
DQ174713	n.a.	actin gene	genomic	Clade C
DQ174714	n.a.	actin gene	genomic	Clade C
DQ174715	n.a.	actin gene	cDNA	Clade C?
DQ174716	n.a.	actin gene	genomic	Clade F
DQ174717	n.a.	actin gene	genomic	Clade F
DQ174718	n.a.	actin gene	genomic	Clade F
DQ174719	n.a.	actin gene	genomic	?
DQ345314	n.a.	actin gene	genomic	Clade C?
DY579126	*Acropora millepora*	actin gene	cDNA	N/A
DY578549	*Acropora millepora*	actin gene	cDNA	N/A
DY579238	*Acropora millepora*	actin gene	cDNA	N/A
DY579393	*Acropora millepora*	actin gene	cDNA	N/A
DY582315	*Acropora millepora*	actin gene	cDNA	N/A
DY584374	*Acropora millepora*	actin gene	cDNA	N/A
DY585356	*Acropora millepora*	actin gene	cDNA	N/A
AF344401	*Acropora millepora*	PaxC intron1	genomic	N/A
AF344402	*Acropora millepora*	PaxC intron1	genomic	N/A
AF344403	*Acropora millepora*	PaxC intron1	genomic	N/A
AY083872	*Acropora millepora*	PaxC intron1	genomic	N/A
AY083873	*Acropora millepora*	PaxC intron1	genomic	N/A
AB086826	*Galaxea fascicularis*	actin gene	cDNA	N/A
AB094431	*Favites chinensis*	actin gene	cDNA	N/A
AY360081	*Stylophora pistillata*	actin gene	cDNA	N/A

Initial degenerate primers (Table 2, a-c) were designed with Primer3 software to amplify the region containing the three possible introns for all *Symbiodinium* clades (Fig. 1), in order to obtain the sequences for the particular C and D symbionts analyzed here. Sequence analyses of the ribosomal ITS1 regions classified these as belonging to C1 (GenBank accession No. AF380555) and D (EU024793), both common on the Great Barrier Reef (van Oppen *et al.* 2001b, 2005). Because of the high level of sequence divergence between the *Symbiodinium* clades, degenerate primers were made for highly conserved regions, which as a result amplified both *Symbiodinium* and coral actin genes. DNA isolated from juvenile *Acropora*

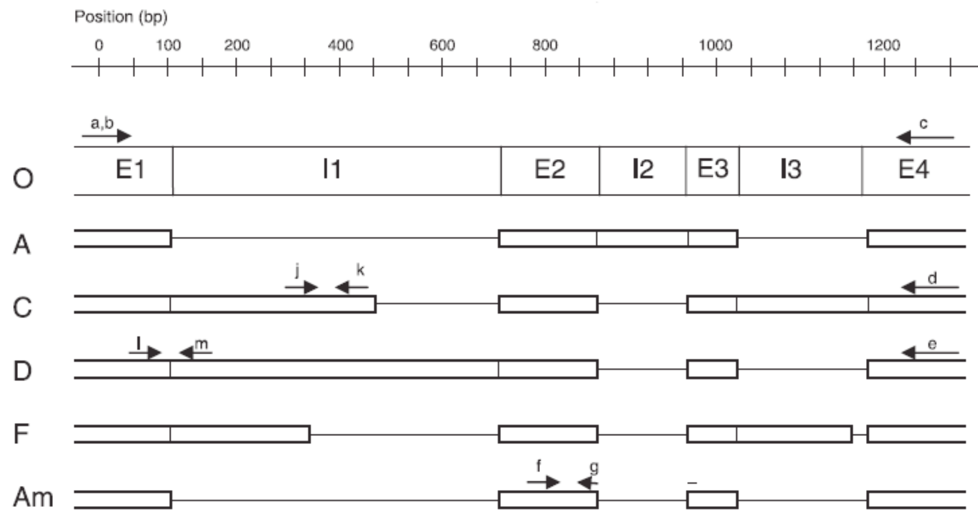

Fig. 1. Overview of the partial actin genes of *Symbiodinium* (clades A, C, D and F) and *A. millepora* (Am). Top line gives position in bp from start of the alignment, first bar (O) gives overview of exons (E) and introns (I). ▭ = present, — = absent. Arrows show the annealing sites of the actin primers given in Table 2.

millepora in symbiosis with *Symbiodinium* C1 and D, and one DNA sample isolated from *A. millepora* sperm (which does not contain algal symbionts), were used for initial PCR amplifications. Reaction volumes ranged from 15 to 50 µL and consisted of 500 nM forward primer, 500 nM reverse primer, HotMaster Taq buffer with 25 mM Mg^{2+}, 0.5-2 U Hotmaster Taq DNA Polymerase (Eppendorf), 1 µL template. Reaction conditions were according to the manufacturer's suggestions, with a 55°C

annealing step. The products were cloned (pGEM-T Vector system 1, Promega), sequenced (ABI 310 and 377 Gene Analyzers, Applied Biosystems), and aligned in BioEdit.
Because of low amplification success with these initial primers, new reverse primers were designed for clades C and D (Table 2, d and e). PCR products from these primers were also sequenced and added to the alignment, after which real-time PCR primers were designed (Fig. 1 and Table 2) using ABI Primer Express 3 software.

Table 2. Primers used in the development of the *Symbiodinium* quantification assay. For positions of the primers on the actin genes see Fig. 1.

Primer name	***Sequence (5'→3')***
Primers for actin gene sequencing	
a) universal actin forward primer 1	GGCTACTCCTTCACCACCAC
b) universal actin forward primer 2	CGGCTACTCCTTYACCACMA
c) universal actin reverse primer	TCRCCCTTGGAGATCCACAT
d) clade C actin reverse primer	ATGGTGGTTCCACCTGAAAG
e) clade D actin reverse primer	AAAGCACCACGTTGGCATAG
Primers for coral real-time PCR	
f) coral actin forward primer	TTCCCGATGGGCAAGTTATC
g) coral actin reverse primer	AAACAACGCCTCGGGACAT
h) coral PaxC forward primer	AAAATCAATACTGTTCCGCTAAGACAC
i) coral PaxC reverse primer	TCGACGAGGTGCTGTCCTCT
Primers for *Symbiodinium* real-time PCR	
j) C actin forward primer	CAGGATGACACATGCTGATGAA
k) C actin reverse primer	AATTGATGGATTTGTTGGAACTTGT
l) D actin forward primer	GTGAAATTGCGCGTGACATC
m) D actin reverse primer	AGTGCTCCCACTGTCCAACC

Real-time PCR assay

Real-time PCR reactions were run in duplicate on the ABI 7500 Fast System or ABI 7300 System (standard run mode), and analyzed with the Sequence Detection Software version 1.3. A 15 µL reaction consisted of: 500 nM forward primer, 500 nM reverse primer, ABI Power SYBR Green Mastermix, 1 µL DNA template. The fluorescent threshold was set at 0.1 for all runs, with baseline selection on automatic. S/H ratios were calculated with the formula: S/H ratio = ($2^{CT(H)-CT(S)}$/actin copies per cell) * E * 2, where $C_T(H)$ and $C_T(S)$ are the C_T values of the coral host and

symbiont-specific reactions, respectively, and E the extraction efficiency correction factor. The values were divided by actin copy number per cell, which was found to differ between *Symbiodinium* clades C and D (see results), multiplied by E to correct for the difference in extraction efficiencies of coral and *Symbiodinium* DNA (see results), and multiplied by two because *Symbiodinium* cells are thought to be haploid *in hospite* (Santos & Cofftroth 2003), whereas *A. millepora* cells are diploid. If no *Symbiodinium* were detected (one or both reactions negative), the S/H ratio was entered as 0. Absolute cell numbers were calculated according to copy number standards (see below).

Specificity tests were performed using a DNA extract from *A. millepora* sperm (no *Symbiodinium*) and cloned fragments (including all three intron sites) of *Symbiodinium* actin of C1 and D (~10^8 copies per reaction).

Efficiency tests were performed by using serially diluted DNA samples (*A. millepora* + *Symbiodinium*) and PCR products (*Symbiodinium*) as template. Melt curves were produced to check that only the desired locus was amplified. To further ensure that the C_T values of the different reactions could be compared directly, copy number standards were produced from cleaned PCR products. The DNA concentrations of the standards were determined with a picogreen assay (Boelen *et al.* 2002), calculated into copy numbers and diluted as necessary. A hundred thousand and 1000 copies were run per primer pair in triplicate.

Sample preparation and extraction efficiency

To obtain reliable S/H ratios, it is important that both the host and the symbiont DNA is extracted with high efficiency. First, a sodium dodecyl sulphate (SDS) method adapted from Wilson *et al*. (2002) with an overnight incubation at room temperature was used to extract healthy and bleached *A. millepora* juveniles in symbiosis with C1 and D. These samples were obtained from an aquarium-based heat-stress experiments, in which both groups were exposed to 32.5°C for 3 weeks (10-14 h light-dark cycle of approximately 140 micromoles of photons.$m^{-2}.s^{-1}$), until they were white-bleached. The algal actin copy numbers were normalized to surface area (determined by scaled digital photographs) and to host actin copy number (S/H ratio). When normalized to surface area, consistent results were obtained among the sample replicates, but the S/H ratios were highly variable within experimental

groups (see results). A CTAB-based DNA extraction protocol (adapted from Hoarau *et al.* 2007) was therefore trialed, which yielded consistent S/H ratios between replicates.

The CTAB extractions were performed as follows: a small fragment was cut (using scissors) from the coral and placed in a 2 mL vial to which 800 μL extraction buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA (pH 8), 100 mM Tris-HCl (pH 8), 20 $\mu g.mL^{-1}$ proteinase K) was added. Next, 3-5 glass beads (diameter = 3 mm) were added and the sample was macerated in the BioSpec MiniBeadbeater-96 for 5 min, after which the sample was incubated at 60 °C overnight while rotating. Next, 800 μL chloroform/isoamyl alcohol (24:1 v/v) was added, the sample was mixed thoroughly and centrifuged for 15 min at maximum speed in a tabletop centrifuge. The aqueous phase was transferred to a new tube, and 800 μL ice-cold isopropanol was added. The sample was gently mixed and incubated for 20 min at -20°C. Next, the sample was centrifuged again (max. speed) for 20 min after which the supernatant was discarded and replaced by 150 μL 70% ethanol. After a 5 min centrifugation the supernatant was pipetted off, the pellet was airdried for 5 min, after which the precipitated DNA was resuspended in 200-600 μL 0.01M TE pH 8. Samples were 10-fold diluted before use in the real-time PCR assay. To check that reproducible S/H ratios were obtained with this extraction protocol, 4-6 samples were taken in close proximity of each other from each of three healthy colonies harboring clade D symbionts, and for all samples the S/H ratio was determined.

To obtain an indication of the extraction efficiencies, the pellets remaining from the SDS extraction or the evaporated chloroform-phase of the CTAB extraction (which are normally discarded during the DNA extraction) were re-extracted. By comparing the number of DNA target copies in the first and second extraction samples with the real-time PCR assay, an estimate could be made of the extraction efficiencies for both the endosymbiont and host. Assuming a similar ratio between a second and third extraction, the expected total number of copies in a sample was calculated as the sum of the copies from three subsequent extractions. The number of copies obtained from the first extraction was divided by the total number of copies to obtain the extraction efficiency.

Actin copy numbers

To assess whether the targeted *A. millepora* actin locus is single-copy, its C_T value was compared with that of the PaxC intron 1, which is most likely a single copy locus (van Oppen *et al.* 2001a). *A. millepora* PaxC intron sequences were obtained from GenBank (Table 1) and real-time PCR primers were designed as described above. The coral actin primers and the PaxC intron primers were both tested on a sample dilution series spanning three logs.

For *Symbiodinium*, no such comparison could be made because of a lack of known single-copy loci, and hence, DNA was extracted from known *Symbiodinium* cell numbers to determine the actin copy number directly through absolute quantification. DNA was extracted (CTAB) from C1 and D *Symbiodinium* ethanol-preserved samples containing 750,000 and 75,000 cells, and the DNA was resuspended in 100 μL 0.01M TE pH 8. Next, the samples were 10-fold diluted to ensure a 100% efficient PCR reaction, and 1 μL was used for real-time PCR analyses (equivalent of 750 and 75 cells, respectively). Standard curves from 10^6 to 10^1 copies were produced from the clade C and D standards as described above. Number of actin copies per cell were calculated assuming an 83% efficient DNA extraction (see Results).

S/H ratios vs. manual counts

Six nubbins, about 2 cm long after the top 1 cm was removed, were taken from a single clade D dominated *A. millepora* colony. A small piece was cut off and used for CTAB DNA extraction to determine the S/H ratio. The remainder of the nubbin piece was used for a manual zooxanthella count. The tissue was removed and homogenized by airbrushing, and the concentration of zooxanthellae in the slurry was counted directly using a haemocytometer (average of eight chamber counts). The surface area of the nubbin was determined through the aluminum foil method (Marsh 1970).

S/H ratios of bleached and recovering samples

An opportunity presented by a natural bleaching event in the summer of 2005/06 at Miall Island (23.2 S, 150.5 E) was seized to test if recovery of bleached corals could be monitored by S/H ratios. Ten white, hand-sized coral fragments were transported to the Australian Institute of Marine Science in early April 2006, where they were allowed to recover for 5 weeks in an outdoor flow-through aquarium set-up under natural light conditions. Samples were taken at the beginning and at the end and the symbionts belonging to clades C and D were quantified. DNA was extracted from all samples using the CTAB protocol.

RESULTS

Sequencing of actin genes and development of real-time PCR primers

The degenerate primers produced products from ~ 500 up to 900 bp in the different samples. Amplifications from DNA isolated from *A. millepora* sperm (no symbionts) only produced 500 bp products, indicating that longer products were of *Symbiodinium* origin. A clone of a 500 bp fragment yielded a sequence that contained no introns and aligned well with other coral actin and EST sequences available from GenBank, although a number of sequence differences were present.

The alignment of the *Symbiodinium* actin gene sequences showed that intron positions and sizes differ among the clades (Fig. 1). PCR products of *Symbiodinium* C1 and D (amplified using the reverse primers d and e) could be sequenced directly (using primer d or e), but produced sequences that contained ambiguities, which could be due to allelic variation in the *Symbiodinium* population, and/or the occurrence of an actin gene family (see actin copy number). One clone per clade was sequenced and used to design the clade-specific real-time PCR primer pairs. The *A. millepora* actin sequence was aligned with the obtained *Symbiodinium* sequences, and areas of mismatch were used to produce *A. millepora* specific real-time PCR primers. All sequences used to design real-time PCR primers have been submitted to GenBank (accession no. EU312078-80).

The coral and *Symbiodinium* real-time PCR primer pairs were found to be species and (for *Symbiodinium*) clade-specific. Sample and PCR dilution series were

all near-100% efficient, and C_T values of copy number standards were within 0.5 from each other across the different primer pairs.

Actin copy numbers

The near identical results of the *A. millepora* actin and PaxC reactions strongly suggested that the targeted actin gene was single-copy (Fig. 2). The observed differences between the obtained *A. millepora* actin sequence and the *A. millepora* actin sequences available from GenBank may indicate that a specific member of an actin gene family was targeted.

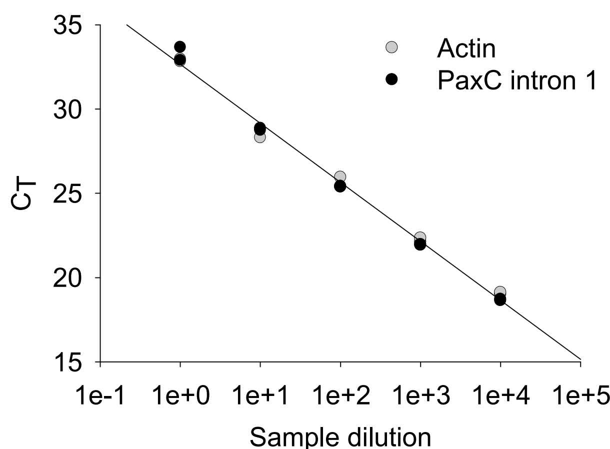

Fig. 2. A coral DNA sample dilution series comparing the results of coral actin and coral PaxC specific reactions (duplicates). Slope of the curves is -3.29, indicative of 100% efficient reactions. The virtually identical results (R^2 = 0.996 of combined dataset) indicated that both primer pairs targeted a single-copy locus.

For *Symbiodinium*, targeted actin gene copy numbers per cell were estimated at 7.0 ± 2.9 (SD) and 0.98 ± 0.66 for C and D, respectively. To ensure that these differences were not due to the primer pairs used, two more C-specific and 4 more D-specific real-time PCR primer pairs were designed for different parts of the clade C and D introns (primer sequences not shown). All gave similar results (average difference between clades C and D for all primer pairs: 8.4 copies ± 2.2. The

difference is within the error margin for real-time PCR analyses). The S/H ratios were divided by the actin copies per cell (C = 7, D = 1) to correct for this difference.

DNA extraction efficiency and assay accuracy

SDS and CTAB DNA extraction efficiencies for both the host and endosymbiont (Fig. 3) showed that the SDS method is more variable, though adequate for the detection of a bleaching response in juvenile corals when data is normalized against surface area (Fig. 4a). SDS extractions, however, were not suitable for S/H ratio determination (Fig. 4b), due to the fact that this ratio is affected by both the algal symbiont and coral host DNA extraction efficiencies, leading to unreliable results (high values and variation among replicates). Data are corrected by C and D having 7 and 1 copies, respectively. To correct S/H ratios for the difference in extraction efficiencies of coral and *Symbiodinium* DNA, the S/H ratios were multiplied by the ratio between the host and symbiont SDS extraction efficiencies (Fig. 3, $E_{SDS} = 93/60 = 1.55$).

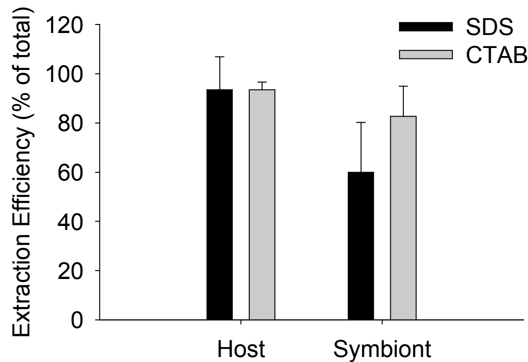

Fig. 3. Extraction efficiencies for coral host and algal endosymbiont DNA extracted with either a SDS- or CTAB-based method. N = 5 for all groups (± SD).

In contrast, samples extracted with the CTAB method showed good agreement in S/H ratios in 4-6 replicates from three different colonies, due to the better extraction efficiencies (Fig. 3 and 5). S/H ratios of CTAB-extracted samples were multiplied by 1.13 ($E_{CTAB} = 94/83 = 1.13$) to correct for the remaining difference between the host and symbiont extraction efficiencies. Standard deviations between replicates indicated that results are within an acceptable error margin of 30%.

Comparison of real-time PCR data with manual counts

An S/H ratio of 0.15 ± 0.047 (SD) corresponded to 1.4 x 10^6 ± 0.2 x 10^6 zooxanthella cells per cm^2, as determined by manual counts of the same branches (N=6). Hence, a healthy adult *A. millepora* harbors about 15 symbiont cells per 100 animal cells. This would naturally be variable as symbiont densities in healthy corals vary with species, water quality, season, location, depth and microhabitat (Drew 1972).

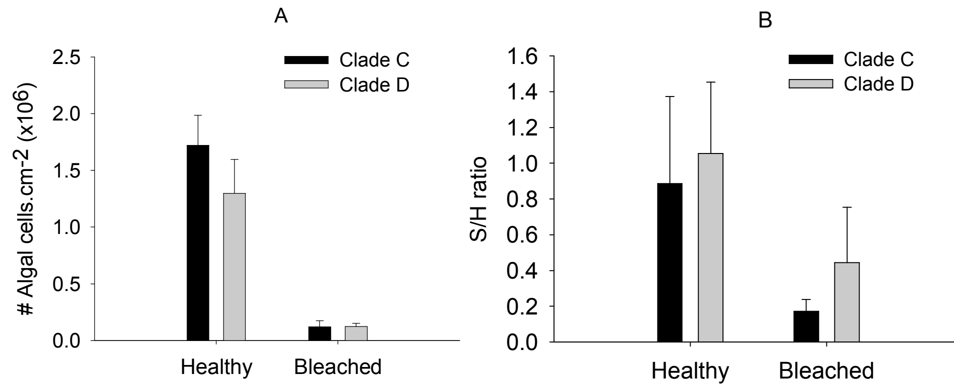

Fig. 4. Healthy and bleached *A. millepora* juveniles in symbiosis with either C1 or D, samples extracted with the SDS-based method. A: symbiont cell number normalized against colony surface area, B: symbiont cell number normalized against host cell number (S/H ratio). N = 5 for all groups (± SE).

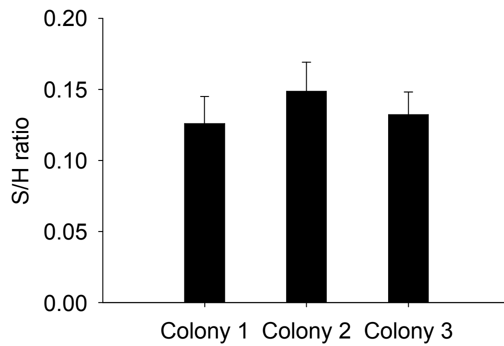

Fig. 5. Reproducibility of the S/H ratio when using CTAB-based DNA extraction and healthy coral samples in symbiosis with clade D symbionts (± SE).

Healthy, bleached and recovering corals

A comparison of the S/H ratios from healthy, bleached and recovering adult *Acropora millepora* showed a clear pattern ranging from approximately 0.15 in healthy corals (Fig. 5) to less than 0.005 in totally bleached corals, and up to 0.08 in recovering corals (Fig. 6). Note that for the recovering corals, the S/H ratio for all symbionts is the sum of the ratios for clades C and D, which are measured separately. It is known that these corals can harbor both C and D (van Oppen *et al*. 2005), and the dynamics of the recovery process is illustrated by some colonies recovering with mostly C, others with mostly D, and others with both in similar abundances.

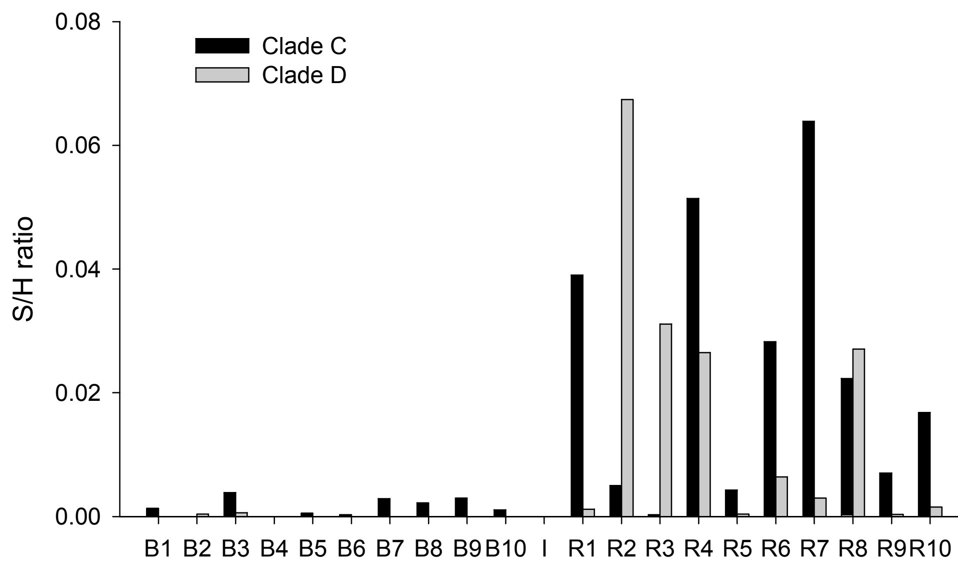

Fig. 6. Recovery of multiple *Symbiodinium* clades over a time-period of 5 weeks within the same coral colonies from bleached (B) to recovering (R).

DISCUSSION

Quantification of dinoflagellate abundances by real-time PCR is well developed for toxic dinoflagellates in environmental samples, such as *Pfiesteria piscicida* (Popels *et al.* 2003; Bowers *et al.* 2006; Coyne *et al.* 2006; Dyhrman *et al.* 2006; Lin *et al.* 2006). For *Symbiodinium in hospite*, only few studies have utilized real-time PCR, and the application has been limited to the determination of the relative abundances of two different clades within a single host (Ulstrup and van Oppen 2003; Loram *et al*. 2007; Chapter 2). These studies revealed that *Symbiodinium* types present in low abundances (below 5-10% of total) have missed detection in the past, and that multiple symbiont clades within one coral may be common. The real-time PCR assay presented here goes beyond these studies to include a fast method to establish *Symbiodinium* densities in coral tissues as well as a more accurate estimate of relative abundance of simultaneously occurring *Symbiodinium* clades.

The actin gene has proven very useful in these analyses, because it can be amplified in distantly-related species with the same degenerate primer pairs which target the conserved exon regions. The intron regions on the other hand are highly variable, making them ideal for clade-specific analyses. Actin sequences obtained from different types within clade C suggest that subclade specificity is possible (data not shown), and this warrants further investigation.

The finding that clade C has seven actin gene copies per genome and clade D only 1 is unlikely to be an artifact of the primers used, as all primer pairs tried gave similar results. This indicates that either the actin gene alone, larger parts of the genome or even the whole genome has experienced duplications in this clade. Two important benefits can be obtained from gene duplication: increased gene expression and increased complexity (Ohta 1991). At present it is not known if these copies are all identical (increased expression), or if a multigene family (increased complexity) and/or pseudogenes were co-amplified. Further research involving the cloning of the PCR product of (single) *Symbiodinium* cells needs to be undertaken to elucidate the functional meaning of this difference. For this assay, the multi-copy nature of clade C meant that a correction factor for this clade had to be established and included in the calculations of the S/H ratios. As a result, the uncertainty in copy numbers of other clades will complicate the efforts to expand the assay. If truly single-copy

markers (with high variability) become available for *Symbiodinium*, the assay described here can be further optimized by targeting these. Presently however, quantification of *Symbiodinium* by actin gene copy number is more accurate than by the high copy number nrDNA region which is organized in tandem repeats, facilitating unequal recombination events (e.g. Rogers & Bendich 1987). As a result, nrDNA copy numbers per cell vary between 1 to > 1000 between clades (Loram *et al.* 2007; Chapter 2), and are also highly variable between cells of the same clade and even the same type (Chapter 2). Previous relative abundance data were therefore considered to be "order of magnitude" estimates. Although it is unclear at this time how the multiple copies of the clade C actin gene are organized (in one or more clusters, on different parts of the same chromosome or on different chromosomes), actin copy numbers are expected to vary significantly less between closely related cells than the nrDNA copy numbers, and the accuracy of relative abundance estimates is expected to be within a factor two.

The real-time PCR approach provides two ways to express symbiont densities: per colony surface area or per host cell (S/H ratio). Per surface area reduces the number of real-time PCR runs by half, because there is no need to quantify the coral host cells. Instead, coral surface area needs to be determined, which can be difficult and time-consuming in corals with complex morphologies. Also, care must be taken when expressing absolute *Symbiodinium* numbers, as DNA losses during extraction, pipetting and degradation have to be taken into account. S/H ratios are preferred as both symbiont and host cell numbers can be determined simultaneously, and errors due to downstream DNA losses will cancel out as these are expected to be the same for both the host and the symbionts. For reliable S/H ratios it is important that host and symbiont DNA extraction efficiencies are similar, and the CTAB-based extraction protocol described here is advised. The S/H ratios had an error margin of ca. 30%, which is slightly less accurate than can be obtained with haemocytometer counts (typically 20% accuracy under optimal conditions; Anderson 2005). However, this is compensated by (1) the much higher time efficiency of the real-time PCR analyses, (2) the fact that only small amounts of tissue are required for real-time PCR, (3) the added ability to distinguish between algal clades, and (4) the improved accuracy of counts when cell densities are low, especially when using small (juvenile) and/or bleached coral samples.

In conclusion, the assay described here provides a major step forward in quickly and accurately quantifying zooxanthellae densities in coral tissues. Furthermore, the added ability to simultaneously quantify different clades of symbionts within one coral sample is of crucial importance in the quest for understanding the flexibility of coral-algal associations, and how this may affect the ability of corals to cope with global climate change.

ACKNOWLEDGEMENTS

We like to thank J.A. Kaandorp for his help with the shipping of the DNA samples. This work was funded by NWO-WOTRO (The Netherlands), Project Nr. W84-576, and further supported by the Australian Institute of Marine Science.

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Chapter 4

Investigating symbiont switching in *Acropora millepora*

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ABSTRACT

Coral reefs are under serious threat of climate change, but the impact can be delayed if the coral-algal symbioses can acclimatize or adapt fast enough to rising seawater temperatures. One way corals may respond is to change their resident symbiont community from a thermally sensitive to a more thermo-tolerant one, and, as a result, become more thermo-tolerant themselves. This study investigated the ability of bleached adult corals of the scleractinian species *Acropora millepora* to form a new symbiosis with environmentally acquired zooxanthellae (symbiont switching). Nine colonies of *A. millepora*, collected at an inshore location of the Great Barrier Reef, were divided into separate coral nubbins and spread over eight tanks. Four tanks were treated with the herbicide diuron to induce a bleaching response, the other four served as no-diuron controls. After 10 days of diuron exposure, a 27-day recovery period started, during which time two diuron and two control tanks were exposed to high levels of an atypical *Symbiodinium* type. Samples for DNA analyses were taken at the start, during and at the end of the experiment, and were analyzed using a type-specific real-time PCR assay. The results show that the coral nubbins underwent a moderate to severe bleaching response, followed by a significant recovery. No new associations were found during the 27 days of recovery, possibly suggesting an inability for switching. However, this result was inconclusive due to two important flaws in the experimental design (which were unknown at the time this experiment was designed): (1) 30% of the coral colonies already had a background presence of the atypical symbiont type, and (2) the atypical symbiont type turned out to have zero infectivity in our experimental set-up. Improvements to the experimental design are discussed for further investigations into this mechanism of symbiont change.

INTRODUCTION

Corals that produce hard calcium-skeletons (Order Scleractinia) are the eco-system engineers of coral reefs (Jones et al. 1994). These scleractinian corals form obligatory partnerships with single-cell algae of the genus *Symbiodinium*, also called zooxanthellae. The zooxanthellae are photosynthetically active, provide the host with up to 90% of its nutritional needs (Muscatine 1990), and promote skeletal growth (Gattuso et al. 1999). Loss of algal symbionts turns the coral tissue bright white, the process referred to as coral bleaching, and leads to starvation. If bleaching is severe and/or prolonged, the coral will die.

In recent years, an increase in the disruption of the coral-algal symbiosis has been witnessed worldwide (Hoegh-Guldberg 2004), a phenomenon that is strongly correlated with abnormally warm seawater temperatures associated with climate change (Hoegh-Guldberg 1999; Berkelmans 2002). It is now clear that climate change is seriously threatening the well-being of the coral reef systems as we know them today (Hoegh-Guldberg et al. 2007). Therefore, a focus of recent studies has been to identify ways of mitigating the effects of climate change on coral reefs through improved management. Information on reef connectivity (sources and sinks) and resilience (disturbance and recovery rates) can assist in the selection of marine protected areas (Hughes et al. 2003). An important variable in the estimation of reef resilience is the potential of corals to acclimatize or adapt to the rising seawater temperatures (Coles & Brown 2003), but very little is known about this aspect of coral reefs.

The genus *Symbiodinium* is diverse and comprises eight phylogenetic clades based on nuclear ribosomal and chloroplast DNA (Pochon et al. 2006). Each clade can be further divided into multiple subclades or types (e.g. LaJeunesse 2001; van Oppen et al. 2001). Genetic differences at the type-level (here defined as the taxonomic unit below the cladal level) have been shown to frequently correlate with physiological differences (Iglesias-Prieto & Trench 1997; Kinzie *et al.* 2001; Bhagooli & Hidaka 2003; Robinson & Warner 2006) and within- as well as between-clade differences can be large, the former sometimes larger than the latter (Tchernov et al. 2004). The zooxanthella type harbored by a coral host can, therefore, have a major effect on the physiology of the holobiont (coral + symbiont),

e.g. on growth (Little et al. 2004) and thermo-tolerance (Rowan 2004; Berkelmans & van Oppen 2006).

The adaptive bleaching hypothesis (Buddemeier & Fautin 1993; Fautin & Buddemeier 2004) states that bleaching provides an opportunity for coral to change their algal communities from a heat-sensitive population to a more heat-resistant one. Although this is a risky strategy, as bleaching episodes are generally followed by high mortality rates, there is now good evidence to show that the *in hospite* zooxanthellae community may change following bleaching in at least a few coral species (Baker 2001; Baker *et al.* 2004; Berkelmans & van Oppen 2006; Chapter 6). However, an important, unknown variable is the source of the "new" symbiont population (Baker 2003). There are two possibilities: (1) symbiont shuffling, in which the "new" symbiont is already present in the coral tissue at low background levels and becomes dominant following the bleaching event and impairment/loss of the previously dominant type, and (2) symbiont switching, in which the "new" symbiont is taken up *de novo* from the environment following the bleaching event and loss of the previously dominant type.

In Chapter 2, we found that a large number of corals on the Great Barrier Reef contain background levels of clade D symbionts — which are linked to high thermo-tolerance in most coral species (but also see Chen *et al.* 2003; Abrego *et al.* 2008) — even though their normal, dominant clade was C. This suggests that the potential for symbiont shuffling is large, whereas symbiont switching has been observed in anemones (Kinzie et al. 2001), in an octocoral (Lewis & Coffroth 2004) and in the hard coral *Porites divaricata* (Cofftroth *et al.* 2008). It remains unclear how widespread switching may be among hard corals.

Acropora millepora is a common reef-building coral with a horizontal transmission of symbionts. On the Great Barrier Reef (GBR), it is known to associate with *Symbiodinium* types A (*sensu* Chapter 5), C1, C2, C2*, C3, and D (*sensu* van Oppen *et al.* 2001; Little *et al.* 2004; van Oppen *et al.* 2005; Berkelmans & van Oppen 2006; Chapter 6) and has been shown to have the ability to shuffle zooxanthellae (Berkelmans & van Oppen 2006; Chapter 6). The flexibility of *A. millepora*'s symbiosis makes it a good candidate for testing the switching hypothesis.

The goal of the present study was to test whether experimentally bleached, adult *A. millepora* could take up experimentally offered atypical zooxanthellae,

thereby investigating whether symbiont switching is possible for this species, and, potentially, for hard corals of this genus more generally. We used an inshore population of *A. millepora* from the GBR, which harbored predominantly *Symbiodinium* type D, with background levels of type C1 (*sensu* van Oppen *et al.* 2001). Bleaching was artificially induced using DCMU (diuron) and the bleached corals were repeatedly exposed to high concentrations of symbionts of type C2* (*sensu* Berkelmans & van Oppen 2006). Changes in the symbiont community over the course of the experiment were monitored using clade and type-specific primers and real-time PCR. The results are discussed in terms of experimental procedures and caveats, and improvements are suggested for further investigations.

MATERIALS & METHODS

Experimental bleaching and recovery

Nine *A. millepora* colonies were collected at Cape Cleveland (GBR, 19.14 E, 147.03 S) in July 2005. Colonies were transported to AIMS and maintained overnight in an outdoor tank. Corals were divided into separate coral nubbins which were wedged in plastic clothes pegs. Pegs were attached to holders suspended on plastic racks (Fig. 1). The experiment consisted of four treatments (performed in duplicate): (1) diuron no C2*, (2) diuron plus C2*, (3) no diuron no C2* (control), and (4) no diuron plus C2* (control). Five nubbins of each colony were placed in each of the eight tanks.

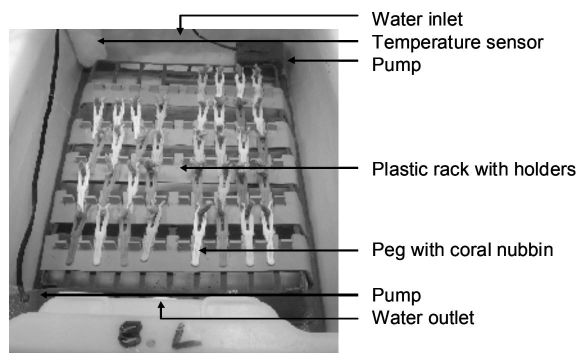

Fig. 1. Experimental tank set-up showing mounted coral nubbins.

Tanks were equipped with continuous flow-through of fresh seawater at 25°C. Light was provided by one metal halide lamp/tank (10,000 K color temperature, BLV Germany) with a spectrum suitable for photosynthesis. Light intensity was adjusted through a 50% shade cloth that maintained an underwater irradiation of 120-150 μmol photons.m^{-2}.s^{-1}. For details on the experimental procedures see Table S1 of the supplement (at the end of this Chapter).

Bleaching was artificially induced using DCMU (diuron), a herbicide that inhibits photosynthesis by blocking electron transport and causing damage to photosystem II (Jones et al. 2003) without affecting the coral host (Negri et al. 2005; Cantin et al. 2007). This approach reduces coral mortality rates as compared with the stress caused by increasing water temperatures (e.g. Berkelmans & van Oppen 2006). Corals were exposed to 100 μg.L^{-1} diuron for 10 days. To minimize the amount of diuron needed, the seawater was recirculated between the diuron-exposed tanks and two large sump tanks (one sump tank per two experimental tanks, ~1000 L). Photoinhibition was monitored by fluorescent measurements using a mini-PAM (Walz, Germany). Dark-adapted yield measurements (Fv/Fm) were taken under low light conditions (<2 μmol photons.m^{-2}.s^{-1}) approximately 1 h before the metal halide lamps were switched on. To increase the bleaching response, stress levels were increased by incremental removal of the shade cloths (Table S1).

Visually bleached corals were exposed to new symbiont cells from day 11 onwards. Type C2* symbionts were obtained from an adult *A. millepora* colony collected from Davies Reef (18.20 E, 147.08 W). Symbiont-containing coral tissue was freshly isolated by airblasting the tissue into a slurry followed by centrifugation (300 g for 5 min). C2*-algae were added to the appropriate tanks in densities of ~60 million cells per tank (ca. 40 L) per addition. In order to enhance the chances of algal uptake, the main tank circulation was stopped for 3-7 hrs (see Table S1) during which time local circulation was provided by mini-powerheads. After that, the seawater from the tanks receiving zooxanthellae was recycled using the sump tanks (~ 1000 L) as before with the diuron expose. Every 5-8 days, the seawater and algae were renewed. From day 14 onwards (four days after diuron treatment), freshly hatched *Artemia* (under sterile conditions) were added to the diuron-treated tanks on a regular basis to ensure the corals remained nourished (Table S1). Tissue samples for real-time PCR were taken: pre-bleaching (before diuron treatment), post-bleaching (directly after the diuron treatment, day 11), and at 19 and 28 days into the

recovery phase (day 29 and 38 of the experiment, respectively). Two nubbins per colony per treatment were taken. Nubbins were manually washed with a large volume of filtered seawater before storage in ethanol.

Real-time PCR primer development

Type-specific primers were developed using the intron region of the *Symbiodinium* actin (Chapter 3). The clade C partial actin gene was amplified for sequencing from a Cape Cleveland (C1) and a Davies Reef (C2*) *A. millepora*

Table 1. Primer sequences

Primer name	***Sequence (5'→3')***
Primers used for sequencing	
a) Universal actin forward primer 2	See Chapter 3
b) Clade C actin reverse primer 2	GGTTCCACCTGAAAGCACCAC
Primers used for real-time PCR	
c-h) clade/species-specific actin primers	See Chapter 3
i) C-specific actin FP	ACAAGGGGATGTGAGGTGTATGT
j) C2*-specific actin FP	ACCCCCTGCTAGTACCAAGTCA

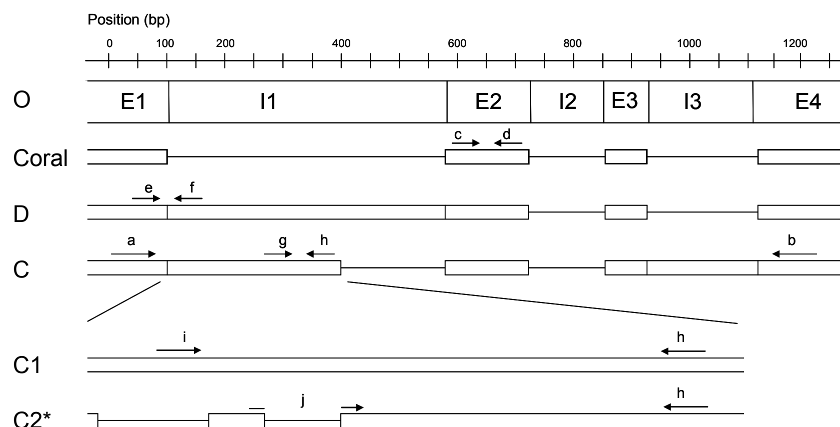

Fig. 2. Map of annealing positions of real-time PCR primers for *Symbiodinium* actin. For primer sequences see Table 1. O = overview of partial actin gene showing the exons (E) and introns (I). □ = present, - = absent.

sample using the universal actin forward primer 1 (Chapter 3 - Table 1) and a newly designed clade C actin reverse primer 2 that annealed in exon region 3 (Table 1, Fig. 2).

The PCR products were cloned, re-amplified and run on an agarose gel. Clone-inserts of anticipated length were sequenced and aligned; areas of dissimilarity between the two sequences were used to develop type-specific primers (Fig. 2).

Real-time PCR analyses

The top (~0.5 cm) of each ethanol-preserved coral nubbin was cut off and transferred into CTAB-extraction buffer as described in Chapter 3. Pellets were resuspended overnight in 500 μL 0.01M Tris buffer pH 9. Samples were diluted 25 x before use in the real-time PCR assay.

Reactions were run on the ABI 7300 using the standard run mode, including a melt curve. A 15 μL reaction consisted of: 500 nM (0.75 μL) Forward Primer, 500 nM Reverse Primer (0.75 μL), 7.5 μL ABI SYBR green mastermix (2 x concentrated), and 1 μL DNA sample. All samples were run in duplicate and melt curves were generated to check that the desired locus was amplified. The cut-off cycle was set at 40, and a sample was positive for a certain symbiont type only if both reactions showed a signal.

Primers were tested for type-specificity using the PCR clones and coral DNA samples known to contain types C1, C2, C2*, and D zooxanthellae as determined by SSCP (van Oppen *et al.* 2001; Berkelmans & van Oppen 2006), before the efficiencies of their reactions were assessed with the use of standard dilution series of both cloned PCR products and genomic DNA.

The D-specific primer pair is known to amplify a single-copy locus, whereas the C-specific primer pair amplifies a locus with seven copies (Chapter 3). To examine the copy number of the target gene of the clade C primer pairs developed here, clade C-specific reactions were run on DNA samples known to be dominated by C1 or C2* zooxanthellae. Next, the same DNA samples were measured with the primer pairs developed in this study, and the copy numbers were calculated from the differences in C_T values.

The sample collection was analyzed with D-specific and coral-specific primer pairs developed in Chapter 3, and the two primer pairs developed here for C1 and

C2*-specificity. Goals of these analyses were: (1) to determine the effectiveness of the diuron-treatment in reducing the zooxanthella densities (as determined by S/H ratios of C1 and D), (2) to monitor subsequent recovery, and (3) to test for the presence/absence of the externally administered C2* in the coral tissues. For C and D, S/H ratios were calculated as described in Chapter 3.

Statistical analyses

To test the effectiveness of the diuron-treatment and the subsequent recovery, S/H ratios of clade D (the original dominant type) were analyzed. C was not included in this part of the analysis as it occurred only at low background levels in some of the nubbins. Clade D S/H ratios were cube-root transformed to satisfy assumptions of distribution normality and variance homogeneity, and analyzed in a repeated measures ANOVA model with "Diuron Treatment" (yes/no) and "C2* Treatment" (yes/no) as the predictor variables. Because "no diuron" treatments were absent at T=29, two tests were performed. First, all treatments were compared (full factorial design) leaving out T=29. Next, the two groups that received the diuron treatment (C2* treatment yes and no) were compared at all time points. Significant Treatment x Time interactions were further investigated using Fisher's post-hoc tests to determine where the differences were located.

RESULTS

Real-time PCR primers

The amplified partial C1 actin sequence was similar to the one found in Chapter 3, although some differences were present in intron 1. For C2*, a partial actin sequence was obtained which showed two deletions at the beginning of intron 1 compared to C1 (Fig. 2). Both sequences have been submitted to GenBank (accession no. FJ456924 and FJ456925 for C1 and C2*, respectively). The dissimilarity between these two sequences allowed the design of sequence-specific forward primers, which were used in combination with a clade C-specific reverse primer (Fig. 2). The two primer pairs amplified with near-100% efficiency. The primer pair designed from the C1 sequence amplified C1, C2 and C2* samples but

not D samples, indicating that this primer pair was clade C but not type C1 specific. It is known that the actin gene is multi-copy in clade C (Chapter 3), and the sequence obtained from C1 *Symbiodinium* may represent a variant shared by all members of clade C. The primer pair developed from the C2* sequence amplified C2* samples and C2 samples, but not C1 samples or D samples, indicating that this primer pair was specific between types C1 and C2/C2*, but not between the more closely related C2 and C2* types. In this Chapter, they are named the C1 and C2* primer pairs, referring to the sequence used for their development. The C1 primer pair amplified a single-copy locus, whereas the C2* primer pair locus had two copies.

Pre-bleaching samples had C_T values for clade D of around 28, indicating that the sensitivity for C2 as background in D-dominated corals was around 0.05% of the relative abundance (using a C_T cut-off of 40, for calculation see Chapter 2). However, it has to be kept in mind that this sensitivity will drop proportionally with the loss of the algal symbionts during a bleaching response.

Bleaching and recovery

Pre-screening using SSCP (van Oppen *et al.* 2001) revealed that the Cape Cleveland *A. millepora* population was dominated by D with C1 present in some of the colonies at background levels. The diuron-treatment of the coral nubbins resulted in a significant bleaching response (Figs. 3 and 4). The large drop in Fv/Fm over the course of the first week indicated a strong photo-inhibition of the symbionts, and a visual census showed that >85% of the coral nubbins were pale to bleached at day 11. Bleached nubbins kept their tentacles extended during the diuron treatment and the recovery phase, indicating they were actively feeding and therefore capable of zooxanthella uptake. Fv/Fm values returned to normal approximately 11 days after the diuron-exposure had stopped (day 22 of the experiment) and maintained normal values until the end of the experiment. The “no diuron” treatments maintained Fv/Fm values between 0.80 and 0.64 throughout the experiment (data not shown). At the end of the experiment, the diuron-exposed coral nubbins had regained most of their color.

Pre-stress, the coral nubbins had S/H ratios for *Symbiodinium* D of around 0.1, which correlates to a normal symbiont abundance for a healthy coral (Chapter 3). At

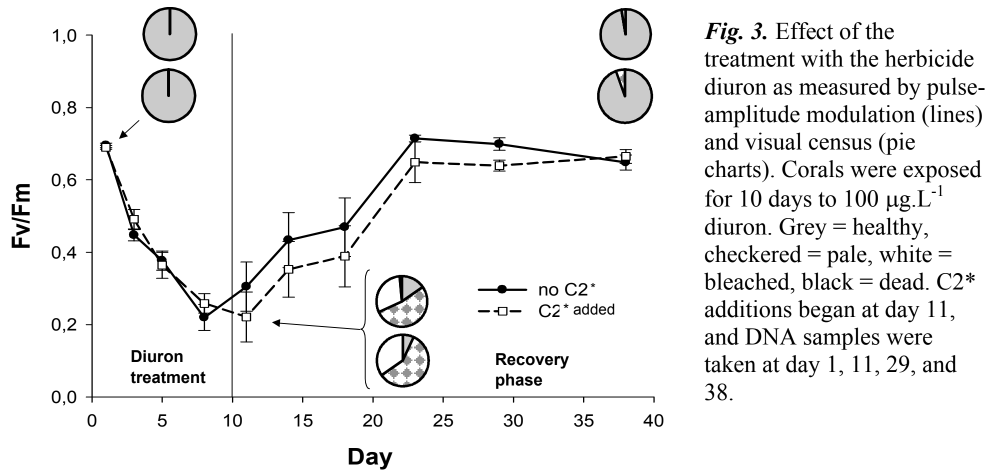

Fig. 3. Effect of the treatment with the herbicide diuron as measured by pulse-amplitude modulation (lines) and visual census (pie charts). Corals were exposed for 10 days to 100 μg.L^{-1} diuron. Grey = healthy, checkered = pale, white = bleached, black = dead. C2* additions began at day 11, and DNA samples were taken at day 1, 11, 29, and 38.

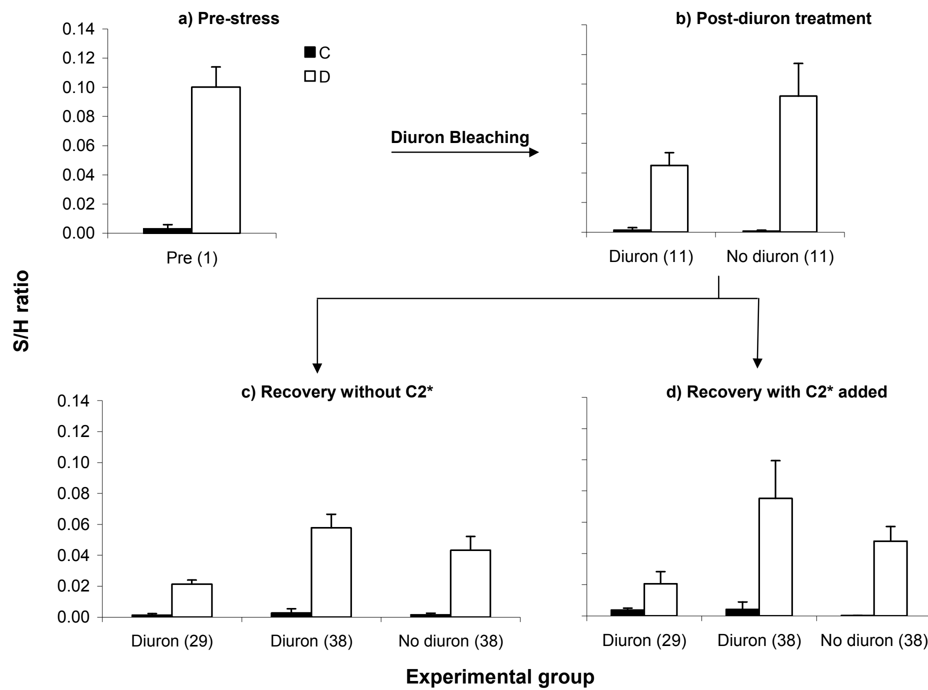

Fig. 4. S/H ratios (mean ± SE) of coral nubbins pre-stress (a), directly after the diuron treatment (b), and in the recovery phase (day 29 and 38) without (c) or with (d) the addition of C2* zooxanthellae. (n) = day of the experiment.

day 11 (after ten days of diuron stress), D zooxanthella abundances in the diuron-exposed treatments had dropped ($p<0.0001$) by about 50% (Fig. 4). This reduction was not seen in the "no diuron" group at day 11. At day 29 (19 days into the recovery phase), D abundances were lower than directly after the diuron exposure ($p<0.005$, no control values are available for this time point), at approximately 20% of the pre-diuron levels. D zooxanthellae abundances were higher at the end of the experiment (day 38) compared to day 29 ($p<0.001$). The unbleached nubbins ("no diuron" treatments) showed a ~ 50% reduction in their D abundances at the end of the experiment compared to the pre-treatment levels ($p<0.0001$), and harbored abundances of symbiont cells comparable to the diuron-treated groups.

Presence of C2*-like symbionts

Pre-stress analyses revealed that C2*-like (C2* primers can not distinguish between C2* and closely related symbiont types such as C2) symbiont types were unexpectedly present in three of the nine coral colonies (F, H and I) at background levels. Directly after the diuron treatment (day 11) and after 19 days of recovery (day 29), no C2*-like symbionts were detectable. However, at day 38 (28 days into the recovery phase), C2*-like symbionts were detectable again at low abundances in F, H and I of the no-diuron groups. No C2*-signal was detected in any of the other samples (Table 2).

Table 2. Presence/absence of C2*-like zooxanthellae in coral nubbins of the different treatments.

Colony	*Pre*	*Diuron with C2**		*Diuron no C2**		*No diuron no C2**	*No diuron with C2**
	Day 1	**Day 29**	**Day 38**	**Day 29**	**Day 38**	**Day 38**	**Day 38**
A	No	No	No	No	No	No	No
B	No	No	No	No	No	No	No
C	No	No	No	No	No	No	No
D	No	No	No	No	No	No	No
E	No	No	No	No	No	No	No
F	**Yes**	No	No	No	No	**Yes**	**Yes**
G	No	No	No	No	No	No	No
H	**Yes**	No	No	No	No	No	**Yes**
I	**Yes**	No	No	No	No	No	**Yes**

DISCUSSION

Coral population and symbiont type

This study employed, for the first time, a type-specific real-time PCR assay, to detect low abundances of C2*-like symbionts in corals. The natural occurrence of C2*-like symbionts at low density in the Cape Cleveland *A. millepora* population, as detected with this tool after the experiment was performed, showed that, in hindsight, this population was less than ideal for the experiment. It compromised the intent of the experiment which was to detect the presence of an atypical symbiont type in a population known to be devoid of this type before experimental manipulation. Types such as C2 and C2* were not expected in this population, as these symbiont types are mostly found at locations with clear water (van Oppen *et al.* 2005), and have a relatively low thermal tolerances (Berkelmans & van Oppen 2006). They have not been detected in *A. millepora* colonies from Magnetic Island, which is in close proximity to Cape Cleveland, and both locations frequently encounter high turbidity and summer seawater temperatures. At Magnetic Island, *A. millepora* associates with *Symbiodinium* D (Berkelmans & van Oppen 2006), a symbiont type considered to be adapted to these conditions (van Oppen *et al.* 2005; Chapter 6). Background levels of *Symbiodinium* type C1 were expected, at this symbiont type is common at Magnetic Island in a closely related species (*A. tenuis*) and can successfully associate with *A. millepora* at Magnetic Island after experimental manipulation (Little *et al.* 2004). However, the presence of low levels of C2*-like symbionts in the *A. millepora* colonies at Cape Cleveland indicates that C2*-like symbionts may also be commonly harbored at background levels by *A. millepora*, and possibly other *Acropora* spp., on inshore locations of the GBR. C2 has been previously found in one *Montipora* colony (van Oppen 2004) and one *Acropora tenuis* colony (Ulstrup & van Oppen 2003) at Magnetic Island, further supporting the presence of C2*-like symbionts at these locations.

A second factor affecting our experiment was the choice of symbiont type C2* as the atypical symbiont. Although unknown at the time, these symbionts have a very low infection success in our experimental set-up. Recently, a similar experimental set-up has proven very successful in infecting azooxanthellate *A. millepora* juveniles with symbiont types from three different clades (A, C1 and D),

but for unknown reasons, infections of juveniles with C2 and C2* symbionts failed (Chapter 5). Why uptake of C2*-like symbionts appears so different from types C1, D and A is unclear, but could be related to biological factors (such as developmental stage) or physical factors in the experimental set-up (such as light levels, water temperature, etc). Whatever the cause, their failure the infect azooxanthellate juveniles competent of taking up other types of zooxanthellae further compromised our study.

Bleaching response

Thermal bleaching can result in the loss of up to 90% of the zooxanthellae from a coral's tissues (Porter et al. 1989; Fitt et al. 1993). In our experiment, the ten-day diuron-exposure resulted in a 75% loss of symbiont cells, as shown by the S/H ratios at day 29 (after 19 days of recovery). Diuron-induced symbiont loss continued after the diuron-exposure was ceased because at day 11 only a 50% reduction in clade D zooxanthellae was measured. The continued loss of symbionts even after the stressor has been removed is not unusual (Hill & Ralph 2007). It is possible that >75% of the symbionts were lost at one stage between day 11 and day 29 and that some recovery had already occurred by day 29. This interpretation is supported by the complete recovery of the symbiont photomachinery (as measured by Fv/Fm) around day 22. Taken together, these results indicate that our experimental bleaching was successful, with a medium to large loss of symbiont cells and death of only one coral nubbin. The reduction in clade D abundance in the "no diuron" tanks at the end of the experiment may be explained by the use of the top 0.5 cm of the coral nubbin for S/H ratios. These surfaces receive the highest levels of irradiation, and irradiation levels in our experiment were most likely higher than in the natural environmental setting for these corals, which may have led to photo-bleaching. However, both the "no diuron" and the diuron-treated tanks had significantly higher clade D densities at the end of the experiment than the diuron-treated tanks at day 29, indicating that significant recovery had occurred in the diuron-exposed tanks.

A pale to bleached appearance was not necessarily correlated with the lowest symbiont abundance. This was most likely due to the fact that visual inspection was more a measure of photosynthetic pigment loss than of zooxanthellae loss. Symbiont cells can lose color as a result of a reduction in photosynthetic pigments (Jones

1997) and this process may have played an important part in the bleaching response of our coral nubbins. Pigment loss and recovery can explain why the nubbins in the diuron-treated tanks looked bleached at day 10 and pigmented at the end of the experiment, while their symbiont densities were similar. Alternatively, the differences in the visual census and S/H ratios may be a result of differences between the nubbin tops (0.5 cm), which were used for the S/H ratios, and the lower 3 cm, which were used for the visual census.

Conclusions and future directions

Our experimental set-up using diuron exposure was successful in inducing a moderate to severe bleaching response, followed by a significant recovery with almost no mortality. Establishing S/H ratios using real-time PCR provided an easy and powerful tool to follow the symbiont dynamics. However, the background presence of C2*-like symbionts in some of the experimental colonies and the general lack of infection with the C2* symbiont under experimental conditions confounded our results, making it impossible to definitely conclude whether symbiont switching is possible or not in *A. millepora*. Suggestions for improvements for future studies include: (1) ensuring the absence of backgrounds of the atypical symbiont types with real-time PCR analyses prior to the start of the experiment, (2) using a more infectious symbiont type, e.g. type A (Chapter 5), and (3) using mixtures of different symbiont types in order to optimize symbiont-host compatibility (Cofftroth *et al.* 2008). Further studies are urgently needed as knowledge about mechanisms such as symbiont switching is the key to assessing the acclimatization potential of coral reefs and their ability to cope with global warming.

ACKNOWLEDGEMENTS

We like to thank Eric Matson for help with the field work, and the AIMS mariculture staff for supplying newly hatched *Artemia* nauplii. This study was funded by a grant from NWO-WOTRO (project nr. W84-576) and further supported by the Australian Institute of Marine Science.

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SUPPLEMENT

Table S1. Experimental schedule. Light exposure was either with or without the application of shade cloth. Unshaded light levels were between 250 and 450 µmol photons.m^{-2}.s^{-1}, and shaded light levels were between 120-150 µmol photons.m^{-2}.s^{-1}.

Day	*Light (L:D)*	*Diuron*	*Algae added*	*PAM*	*Visual census*	*Artemia added*	*Sampled*
1	10:14	100µg/L		Yes	Yes		Yes
2	Diuron: 3h unshaded						
3	Diuron: 8h unshaded			Yes			
4	Diuron: 10h unshaded						
6		2/3 water change (+ Diuron)					
7	12:12 Diuron: 12h unshaded						
8				Yes			
10	All shaded	Stop Diuron exposure					
11			Yes, 3h no flow	Yes	Yes		Yes
12			Yes				
14				Yes		Yes	
15							
16			Yes, water change, 7h no flow				
17						Yes	
18			Yes	Yes			
19						Yes	
22			Water change			Yes	
23			Yes	Yes			
29							Yes
30				Yes			
31			Water change				
32			yes				
33						Yes	
38				yes	Yes		Yes

Chapter 5

The roles and interactions of symbiont, host and environment in defining coral fitness

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Submitted to *Public Library of Science ONE*

ABSTRACT

Reef building corals live in symbiosis with dinoflagellate algae (*Symbiodinium*) whose identity can influence the fitness of the coral holobiont. Manipulation of coral-algal associations during the initial phases of development can provide insights into the influence of *Symbiodinium* type on the physiology of the coral holobiont by partitioning the contributions of the algal symbiont and the coral host. Here, this approach was followed using two coral populations from thermally distinct locations and six *Symbiodinium* types spanning three phylogenetic clades (A, C and D). Azooxanthellate juveniles were reared from adult *Acropora millepora* colonies collected from the central and southern inshore Great Barrier Reef, Australia. The coral juveniles were exposed to one of six different *Symbiodinium* types, and following symbiont uptake, outplanted to the two field locations. The custom holobionts were monitored for growth and survival for 31-35 weeks, after which their thermal tolerances were experimentally assessed. The main findings were that: (1) not all symbiont types were taken up in an experimental environment and the coral-algal associations that did establish, were not always stable in the field, (2) *Symbiodinium* type was the most important predictor of holobiont fitness, as measured by growth, survival, and thermo-tolerance, (3) growth and survival, but not heat-tolerance, were affected by the local environmental conditions at the grow-out site, and (4) host population had little to no effect on holobiont fitness. Furthermore, *Symbiodinium* types C1 and D were found to be relatively thermo-tolerant, with type D conferring the highest tolerance in *A. millepora*, whereas type A conferred the lowest fitness in *A. millepora* juveniles as assessed by growth, survival and thermal tolerance. These results highlight the complex interactions that occur between the coral host, the algal symbiont, and the environment to shape the fitness of the coral holobiont. An improved understanding of the factors affecting coral holobiont fitness will assist in predicting the responses of corals to global climate change.

INTRODUCTION

The obligate symbiosis between reef-building corals and unicellular algae of the genus *Symbiodinium*, commonly referred to as zooxanthellae, is a key feature of tropical reefs. The algal endosymbionts are photosynthetically active, and provide up to 95% of the energy requirement of the coral host (Muscatine 1990). In return, the coral host offers protection from predation and an environment with increased inorganic nutrients (Muscatine & Porter 1977). The success of coral reefs, which thrive in nutrient-poor tropical waters, has been heavily dependent on this successful partnership. However, the coral-zooxanthella symbiosis is very sensitive to increases in temperature. Changes of as little as 1°C above average summer maximum can lead to a breakdown of the symbiosis. This breakdown results in expulsion and/or degradation of the algal partner causing the phenomenon known as coral bleaching (reviewed in Coles & Brown 2003). When bleaching is severe, and the symbiosis is unable to re-establish itself, the coral dies.

The genus *Symbiodinium* is highly diverse and consists of eight phylogenetic clades, each of which contains multiple subclades or types (Coffroth & Santos 2005; Pochon *et al.* 2006; Stat *et al.* 2006). Scleractinian corals form symbioses with six of these clades (A-D, F, G) (Baker 2003; Goulet 2006). Most importantly, the genetic diversity at the type level correlates with functional diversity in parameters such as growth, thermal tolerance and photosynthetic response to changes in irradiance (Iglesias-Prieto & Trench 1997; Kinzie *et al.* 2001; Bhagooli & Hidaka 2003; Little *et al.* 2004; Rowan 2004; Tchernov *et al.* 2004; Berkelmans & van Oppen 2006; Robinson & Warner 2006). Sub-lethal bleaching events may result in changes in the symbiotic relationships towards dominance of more thermo-tolerant *Symbiodinium* types (Jones *et al.* 2008). The change to a more heat tolerant type may, however, be a trade-off against other physiological characteristics, such as growth (Little *et al.* 2004).

Understanding the influence of the algal partner on the fitness of the holobiont is essential in terms of predicting the potential for acclimatization to global warming through changes in the algal symbiont community (Buddemeier & Fautin 1993; Baker 2001; Fautin & Buddemeier 2004). However, progress has been slow because of the difficulty in separating the responses attributable to the algal symbiont, the coral host, and the environment on the fitness of the holobiont. Comparative studies

have been performed on *Symbiodinium* cultures or fresh isolates (e.g. (Iglesias-Prieto & Trench 1997; Kinzie *et al.* 2001; Bhagooli & Hidaka 2003; Robinson & Warner 2006), but it is not possible to couple these findings to the *in hospite* situation. Other studies have compared the holobiont and the environment but were unable to discriminate between effects of the host and the symbiont (Baird & Marshall 2002; Bhagooli & Hidaka 2003; Bhagooli & Hidaka 2004). However, recent studies have utilized three ways to control for host and environmental factors as follows: (1) by taking advantage of rare populations in which conspecific host colonies harbor different symbionts in sympatry (Rowan 2004; Jones *et al.* 2008; Stat *et al.* 2008), (2) by comparing host colonies before and after a bleaching event that involved a symbiont community change (Berkelmans & van Oppen 2006), or (3) by experimentally infecting coral juveniles with different *Symbiodinium* types (raising custom holobionts) followed by outplanting and monitoring *Symbiodinium* communities in the field (Little *et al.* 2004; Abrego *et al.* 2008).

The last technique takes advantage of the fact that most corals produce azooxanthellate larvae, and each generation has to acquire algal symbionts anew from the environment early in their ontogeny (Richmond 1997). These juvenile corals appear to have a relatively dynamic association with *Symbiodinium*. Multiple types are initially taken up (Coffroth *et al.* 2001; Little *et al.* 2004) but later typically one type becomes dominant in the adults (Goulet & Coffroth 2003; Goulet 2006). The others are often not lost completely, but are reduced to low-abundance or background types that persist throughout the adult life (Chapter 2). Changes in the adult coral's *Symbiodinium* population may therefore be realized through an increase in the relative abundance of these background types. Alternatively, adult corals may take up new symbionts from the water column to establish a new symbiosis, which has been shown under experimental conditions for anemones (Kinzie *et al.* 2001), an octocoral (Lewis & Coffroth 2004), and a scleractinian coral (Coffroth *et al.* 2008). Lastly, symbiont change may be implemented from one generation to the next (Baird *et al.* 2007).

Here, we describe a comparative study involving custom holobionts raised from two populations of the common scleractinian coral *Acropora millepora* from thermally contrasting, inshore environments on the Great Barrier Reef (Australia), and six different *Symbiodinium* types from three phylogenetic clades (A, C and D). The aims of this study were: (1) to investigate whether different *Symbiodinium* types

are equally capable of establishing a symbiosis with *A. millepora* and whether experimentally induced symbioses are stable, and (2) to determine how fitness proxies (growth, survival and thermo-tolerance) varied with *Symbiodinium* type, coral host population, and local environmental conditions during development.

MATERIALS AND METHODS

Research locations

Two inshore reef locations were selected ca. 750 km apart: Magnetic Island (19.1 S, 147.5 E) in the central Great Barrier Reef (GBR), and Miall Island (23.1 S, 150.5 E) within the Keppel Islands group in the southern GBR. Note that throughout the text, we will refer to Miall Island as the Keppel Islands. The sites differ significantly in several aspects (Table 1). Importantly, spawning times of *Acropora millepora* colonies differ by one month between the two locations, which made it possible to perform the experiments on both populations within a single year.

Table 1. Comparison of Magnetic Island and the Keppel Island field locations and their *A. millepora* populations.

Factor	*Magnetic Island*	*Keppel Islands*
Mean Summer Seawater Temperature	29.2 ± 0.45[1]	27.0 ± 0.50[1]
Bleaching threshold	31.2°C – 5 days exposure 30.4°C – 20 days exposure[2]	29.5°C - 5 days exposure 28.8 – 20 days exposure[2]
Symbiont clade/type	D[1,3]	C2K (95%) + D (5%)[1,3]
Spawning time	October[4]	November[4]

[1] Berkelmans & van Oppen (2006)
[2] Berkelmans (2002)
[3] Jones *et al.* (2008), before 2006 bleaching event
[4] This study

Coral host populations and *Symbiodinium* types

Acropora millepora was chosen because its relationship to *Symbiodinium* types at the two research locations was already established (van Oppen *et al.* 2005; Berkelmans & van Oppen 2006), and aposymbiotic juveniles of this species had been successfully infected with different *Symbiodinium* types experimentally (Little *et al.* 2004).

Symbiodinium types were harvested from three coral species and three locations on the GBR (details are provided in the supplement at the end of this Chapter, Table S1) and identified based on the nuclear ribosomal DNA internal transcribed spacer 1 (ITS1) region using a combination of Single Strand Conformation Polymorphism (SSCP) and DNA sequencing (van Oppen *et al.* 2001; Ulstrup & van Oppen 2003). Initially, five *Symbiodinium* types were selected for the inoculations of juvenile corals (designated as C1, C2*, C2, C• and D) as these are abundant on the GBR (van Oppen *et al.* 2001; van Oppen *et al.* 2005; Berkelmans & van Oppen 2006) and are therefore ecologically relevant. C1, C2, C2* and D are normally found in adult *A. millepora*; C1 and D are predominantly found at inshore, more turbid locations and C2/C2* at cooler, clearer locations. To date, C• has never been found in *A. millepora* on the GBR or elsewhere, and is mostly found in maternally transmitting corals such as *Montipora* and *Porites* ssp. SSCP analyses revealed that the two *A. millepora* colonies from Davies reef, collected for their C2* type, harbored ~50% *Symbiodinium* type C2* and ~50% *Symbiodinium* type A (Fig. 1). Clade A is very rare on the GBR (van Oppen *et al.* 2001; LaJeunesse *et al.* 2003), and is mostly found in the southern GBR and higher latitude reefs (Gomez-Cabrera *et al.* 2008). The latter combination brought the total number of *Symbiodinium* types used in the inoculations to six; four types were offered in isolation while C2* and A were administered as a 50-50 mixture.

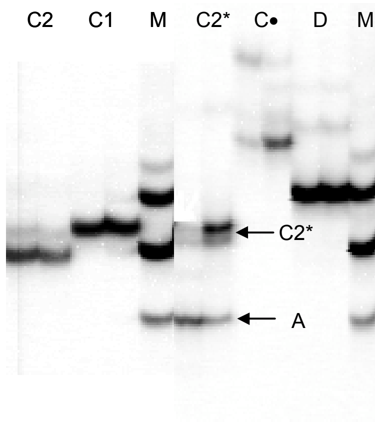

Fig. 1. SSCP profiles of the six *Symbiodinium* types used for tank inoculations (C2* is a mix of C2* and A). M = Marker of reference ITS1 sequences.

All ITS1 sequences obtained were identical to sequences available in GenBank (A AB207206, C1 AF380551, C2-AY643495, C2*-AY643497, C•-AY237300 and D EU024793). ITS1 genotypes A, C1, C2, C•, and D correspond to ITS2 genotypes A1, C1, C3, C15, and D1, respectively (LaJeunesse 2001; LaJeunesse *et al.* 2003; Jones *et al.* 2008). Three times during the following eight months of grow-out on the reef, a subset of the juvenile corals was genotyped from each group to verify that the symbiont type matched the one offered experimentally.

Raising custom holobionts

Twelve colonies of *A. millepora* were transported from Magnetic Island to the Australian Institute of Marine Science (AIMS) in mid October 2005, and kept in outdoor tanks. On the night of 20 Oct 2005, four corals showed pre-spawning signs ("setting" *sensu* Babcock et al. 1986) in the evening, and these were isolated in buckets. The released gametes were collected using 250 mL beakers and mixed together to ensure fertilization, before being transferred to 500 L larval rearing tanks. This procedure was repeated a month later with *A. millepora* from the Keppel Islands. All twelve colonies spawned on the night of 16 November 2006, and gametes were collected from eight colonies.

Embryos were reared in filtered seawater (1 μm) in five, round 500 L tanks. Densities were ca. 100,000 embryos/tank and water temperature was controlled at 27°C. After three days, (when the larvae started to swim around), autoclaved terracotta tiles were placed on plastic racks to provide settlement surfaces. Tiles where either pre-conditioned at Magnetic Island for six weeks, or sprinkled with crushed coralline algae to induce settlement.

Symbiodinium inocula were prepared from the corals listed in Table 2 by airbrushing followed by centrifugation of the coral-algal slurry (350 g for 5 min). These were added to the tanks of azoothanthellate, juvenile larvae on the third and seventh day after spawning. Ca. 50 million freshly isolated algal cells were added per tank during each inoculation. Because no *Symbiodinium* cells could be detected in the newly settled juveniles of tanks inoculated with C2*, C2 and C● after 12 days, an extra inoculation was performed for these groups.

Table 2. The *Symbiodinium* type, species and origin (including position) of the coral colonies used as the source for the *Symbiodinium* inoculations.

Clade/Type[+]	***Host species***	***Origin***	***Lat-Long***
C1	*Acropora tenuis*	Magnetic Island	19.1 S, 147.5 E
C2	*Acropora millepora*	Keppel Islands	23.1 S, 150.5 E
C2*/A	*Acropora millepora*	Davies Reef	18.5 S, 147.1 E
C•	*Porites australiensis*	Magnetic Island	19.1 S, 147.5 E
D	*Acropora millepora*	Magnetic Island	19.1 S, 147.5 E

[+] *sensu* van Oppen (2004), Berkelmans & van Oppen (2006)

At seventeen days after spawning, twenty squash preparations of coral polyps were made per tank and analyzed under a fluorescent microscope (Zeiss) to check that *Symbiodinium* cells were present in the coral tissues. Sixteen squash preparations per group were stored in 100% ethanol for later genetic analysis.

Outplanting

Each tile was labeled, photographed and mounted on a stainless steel rod that passed through a central hole in the tile. Up to nine tiles could be mounted per rod, plus two "edge"-tiles without coral juveniles. Tiles were separated by 2 cm sections of garden hose. The tile-laden rods were transported to Magnetic Island and the Keppel Islands in 100 L plastic containers filled with seawater; transport to the Keppel Islands included a continuous flow-through system. Rods were fixed horizontally between two star-pickets and suspended ~40 cm above the substratum in a zone where *Acropora spp.* were present.

Custom holobionts raised from corals originating from Magnetic Island were outplanted to Magnetic Island but not to the Keppels Islands due to logistical limitations. In contrast, custom holobionts raised from corals originating from the Keppel Islands were outplanted to both Magnetic Island and the Keppel Islands. The nomenclature of experimental groups consists of a three-letter code designating the location of the outplant, the location of the parental population, and the *Symbiodinium* type. For example, MKC1 means that the group was outplanted to Magnetic Island, and consisted of juveniles originating from the Keppel Islands population and *Symbiodinium* type C1.

Field monitoring of growth and survival

The field locations were visited three times during the grow-out phase, which ran for 31 (Keppel Islands hosts) or 35 (Magnetic Island hosts) weeks. Tile-laden rods were collected around noon and transported to the shore (close to the field site) where they were kept in plastic 100 L containers containing ~30 L of seawater (two rods/container). A small pump was added to provide water circulation. Tiles were removed from the rods one by one, placed in a plastic bowl containing a fixed water level and photographed from a fixed distance. Tiles were returned to the field the following morning.

Growth was estimated from changes in two-dimensional surface area (averaged per tile side) measured from the scaled digital photos using the software package Image-J (NIH Image), taking care to use only single, non-fused colonies. The total number of tile sides measured ranged from 8 to 15; the number of colonies per time point ranged from 34 to 342. Survival was determined by changes in colony number per tile surface (surface area per tile = 121 cm^2) over the experimental period. Up to 42 single polyps (numbers ranged from 5 to 42 polyps) were randomly selected from 10 to 14 tile sides on pre-field pictures (total numbers ranged from 165 to 247 colonies/group), and their survival or mortality was noted over the different time-points.

Laboratory heat-stress experiments

Tiles were shipped from the field to AIMS wrapped in wet bubble wrap and packed in cooler boxes (no seawater added). Once at the institute, all growth other than the coral colonies was removed using forceps and scalpels. Tiles were placed on plastic racks in 50 L tanks, and cleaned on an average of every four days during the experiment. The tanks were supplied with fresh filtered (1 μm) seawater via a flow-through system at a rate of 1 $L.min^{-1}$. Two small pumps per tank provided water movement. Water temperature was computer-controlled to within 0.05°C (Turner et al. 2002). Light was provided by one metal halide lamp per tank (10,000 K color temperature, BLV Germany) with a spectrum suitable for photosynthesis, at an underwater light intensity of 120-150 μmol $photons.m^{-2}.s^{-1}$. The design of the heat-stress experiments followed Berkelmans and van Oppen (2006). Tiles were

divided over four temperature treatments, each with three replicate tanks (total of 12 tanks). Each experimental group was represented by one tile per tank, and the number of coral juveniles on each tile ranged from 10 to 70 (average of 25). In order to assess the thermal tolerance of the maximum number of custom holobiont groups, two successive experiments were performed.

Experiment 1: (May-June 2006) This experiment involved the custom holobiont groups MMC1, MKC1, MMD and MKD. Juvenile corals were acclimated at 27°C (the ambient temperature at Magnetic Island in autumn) for 10 days with photoperiod increasing from 5L:19D to 8L:16D. The temperature was raised to the target temperatures (27° (control), 30.5°, 31.5° and 32.5°) over a period of three days and maintained for 18 days. During the experiment, the photo-period was further increased in two steps (Day 8 and 16) to 10L:14D.

Experiment 2: (July 2006) Four additional custom holobiont groups, KKA, KKC1, KKD and MKC1, were heat-stressed in July 2006. Only three temperature treatments were performed because an insufficient number of colonies were available for four. Juvenile corals were acclimated at 22°C (the approximate ambient temperature at the Keppel Islands in early spring) for 9 days with an increasing photo-period starting at 3L:21D to 10L:14D. At the end of the acclimation period, the temperature was raised to the target temperatures over a period of seven days (27° (control), 31° and 32.5°) and maintained for 15 days. Due to technical constraints on the maximum temperature difference between treatments, the control group temperature was increased to 27°C.

Photosynthetic performance

Photosynthetic performance was assessed as an indicator of thermal stress using a MAXI-imaging PAM (MAXI-iPAM; Walz, Germany), allowing a whole tile side to be measured at once. Tiles were kept clean to avoid interference from turf algae. For dark-adapted measurements of maximum quantum yield (Fv/Fm), tiles were kept for at least 14 hours in darkness before being transported from the tanks to the MAXI-iPAM under fluorescent light conditions of < 2 μmol photons.m^{-2}.s^{-1}. Light-adapted measurements of effective quantum yield (F/Fm') were taken 2-4 hours after the photoperiod had started. Tiles were placed (one at a time) under the MAXI-iPAM and exposed to 1 min actinic light of 146 μmol photons.m^{-2}.s^{-1} before

measuring F/Fm'. The excitation pressure over photosystem II (Q) was calculated according to the formula described in Iglesias-Prieto et al. (2004).

Q is a highly informative measure for photosynthetic performance that takes into account both the photochemical and non-photochemical processes (Iglesias-Prieto et al. 2004). It provides an indication of the ratio between open and closed reaction centers of photosystem II under the experimental irradiance level. A value of close to zero indicates that most of the reaction centers are open, suggesting light-limitation, and a value close to one indicates that almost all reaction centers are closed, suggesting photo-inhibition. Although still poorly understood, thermal bleaching of corals is inherently associated with an accumulation of excitation pressure within PSII (Jones *et al.* 1998; Smith *et al.* 2005). Therefore, a rise in Q with accumulating heat-stress is interpreted as an indicator of bleaching in corals (Abrego et al. 2008).

Real-time PCR and visual assessment

Six juvenile colonies (2 per tank) were sampled from each treatment for each *Symbiodinium* type before heating started (experiment 2 only) and one day after the last PAM measurements, to determine relative *Symbiodinium* cell densities. For this, the real-time PCR assay based on actin genes and described in Chapter 3 was followed, using SDS-based DNA extraction and normalization to coral surface area New real-time PCR primers for *Symbiodinium* type A were developed following the method described in Chapter 3 (see also the supplement). Densities were expressed in relative rather than absolute numbers, avoiding the estimation of DNA extraction efficiencies and actin gene copy numbers. This method assumes that extraction efficiencies were equal for all samples. *Symbiodinium* densities of juveniles in control treatments or pre-stress treatments (only available in the second heat-stress experiment) were set to 100%.

All colonies were visually scored at the end of the experiment as healthy, pale, or bleached, using tile color as visual reference. Mortality unrelated to bleaching, which may have been caused by accidental abrasion of coral juveniles during cleaning of the tiles, was judged by the presence of patchy tissue necrosis. These individuals were immediately removed to avoid the spreading of any disease and were not included in analyses.

Statistical analyses

For growth, mean colony surface areas were compared between custom holobiont groups. Colony surface areas were averaged per tile side to facilitate analyses and to be conservative. In the first test, all groups except KKA were compared at T=31 weeks. KKA was left out of this analysis because no other A group was present to test for host population or environmental effects. The data for MMC1 and MMD were interpolated per tile side to T=31 weeks by curve-fitting the data using all time points. The T=31 data were log-transformed to correct for heteroscedacity of variances. A general linear model ANOVA was used, specifying the following terms: Symbiont type, Host population, Outplant location, Symbiont type*Outplant location, Symbiont type*Host population.

To further analyze the effect of the three *Symbiodinium* types on growth at the Keppel Islands, a repeated measure model ANOVA was run on all data points (T=6, 13 and 31 weeks). Data were averaged per tile side and log-transformed as before. Symbiont type was specified as the predictor, with Time as the Within-Subjects factor. When a significant Symbiont type effect was found, a Fisher *post hoc* test was performed to determine which symbiont types were different.

Survival was analyzed for each outplant location with Kaplan-Meyer log-rank tests. As no satisfactory method of interpolation could be established for the survival data, pairwise comparisons of host populations were used for the groups outplanted to Magnetic Island (MMC1 x MMD and MKC1 x MKD) to test for an effect of Symbiont type. For the Keppel Islands, all groups were included in a first test for Symbiont type. Upon finding a significant effect, pairwise comparisons were performed to establish where the differences were located.

Analyses of the laboratory heat-stress experiments utilized PAM-fluorometry data and symbiont density data. Separate analyses were performed for each experiment since the stress-levels differed. Q fluorescence data were arcsine transformed and analyzed using repeated measures model ANOVAs. To adjust for differences in colony number per tile side, average Q values per tile side were calculated. For Experiment 1, a full factorial approach was used with Time as the Within-Subjects factor. The following terms were specified: Temperature, Symbiont type, Host population. For experiment 2 the data were analyzed in two steps: first, KKC1 and MKC1 were analyzed, with Time as the Within-Subjects factor and

Outplant location as the predictor. If no significant differences were found, all groups were included in a second analysis with Time as the Within-Subjects factor and Temperature and Symbiont type specified as predictors.

Symbiodinium density data were square-root transformed and analyzed using factorial ANOVAs. The same approach was used as described for the fluorescence data set.

RESULTS

Establishment and stability of symbioses with the different algal partners

Symbiodinium types C1, D and C2*/A successfully established symbioses with *A. millepora* from both populations as indicated by significant numbers of *Symbiodinium* cells in coral juvenile squash preparations (30 to 500 cells per 0.53 mm^2). In contrast, no *Symbiodinium* cells were observed in tissue squashes of juveniles from the C2 or C• tanks. SSCP analyses of the ethanol preserved tissue preparations established that the juveniles exposed to the mixture of C2* and A established a symbiosis with *Symbiodinium* A only (results not shown).

Genetic analyses of the outplanted juveniles at three time points throughout the 8 month study revealed that symbioses with C1 and D *Symbiodinium* were stable over the course of this study at both locations (see supplement, Table S2). In contrast, after 9-13 weeks only *Symbiodinium* D was found in MMA, MKA and the uninfected groups (those exposed to C2 or C•), apart from a single colony in the latter group at the Keppel Islands containing both C1 and D. KKA continued to harbor mostly *Symbiodinium* A for 31 weeks, but 30% of the colonies were found to harbor mixtures of A and C1 and/or D at the end of this period.

Growth and survival of outplanted juvenile corals

The effect of symbiont type on coral growth differed between the two outplant locations (Fig. 2a, b), as indicated by a significant interaction between the factors outplant location and symbiont type ($p<0.05$, Table 3a). At Magnetic Island, the C1 holobionts (MMC1 and MKC1) grew nearly twice as fast as the D holobionts (MMD and MKD) (Fig. 2a, $p<0.05$), whereas at the Keppel Islands no difference

was found between KKC1 and KKD (Fig. 2b). KKA however grew significantly slower than either KKC1 or KKD (Fig. 2b and Table 3b, p<0.001).

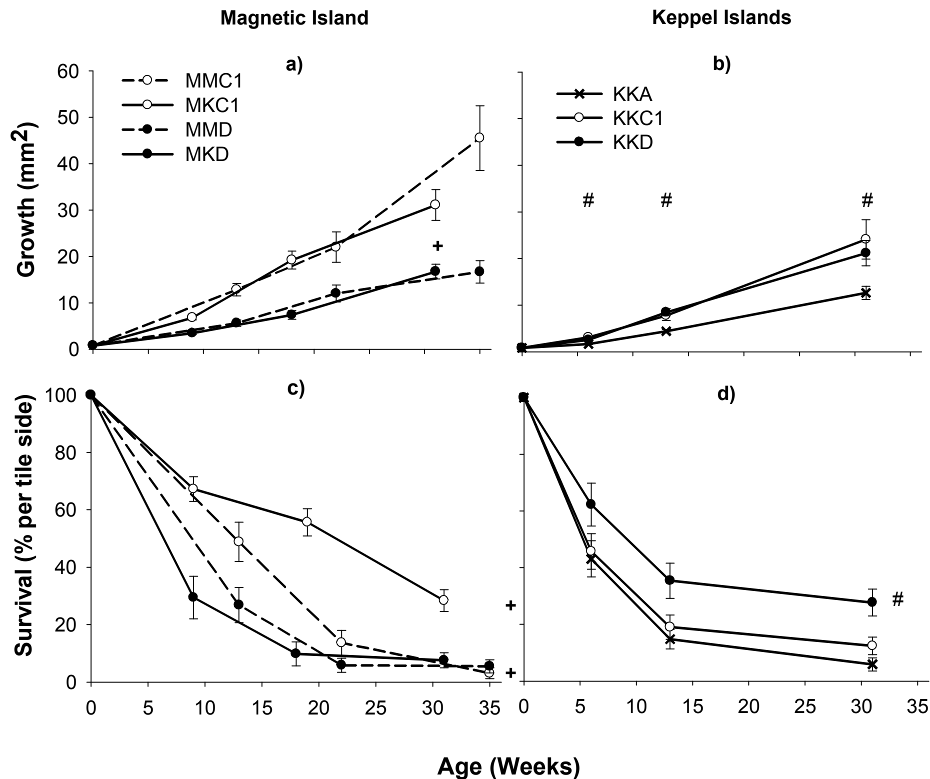

Fig. 2. Growth and survival of the custom holobionts at Magnetic Island (a + c) and the Keppel Islands (b + d). + indicates significant difference between C1 and D groups (p<0.05), and # indicates significant difference between KKA and KKC1/KKD (growth, p<0.05) or between KKD and KKA/KKC1 (survival, p<0.001).

Survival was also significantly affected by symbiont type. C1 holobionts survived better than D holobionts at Magnetic Island (Fig. 2c, MMC1 * MMD: p<0.05, MKC1 * MKD: p<0.001). This was especially evident in the first 12 weeks. As juveniles matured, host-correlated differences may also have been present between MMC1 and MKC1, with the latter indicating better survival than MMC1 (not statistically tested because of age difference). At the Keppel Islands, the pattern was the inverse of that at Magnetic Island, with survival being significantly higher

for KKD than for KKA and KKC1 (Fig. 2d, p<0.001). Hence, survival was also affected by the outplant location (Fig. 2c, d).

Laboratory heat-stress experiments

Experiment 1: There was a significant difference in the excitation pressure on PSII (Q) between C1 holobionts (MMC1 and MKC1) and D holobionts (MMD and MKD, Fig. 3 and Table 3c). In contrast, no significant effect of host population origin over time was found (Table 3c). At the intermediate temperatures (30.5 and

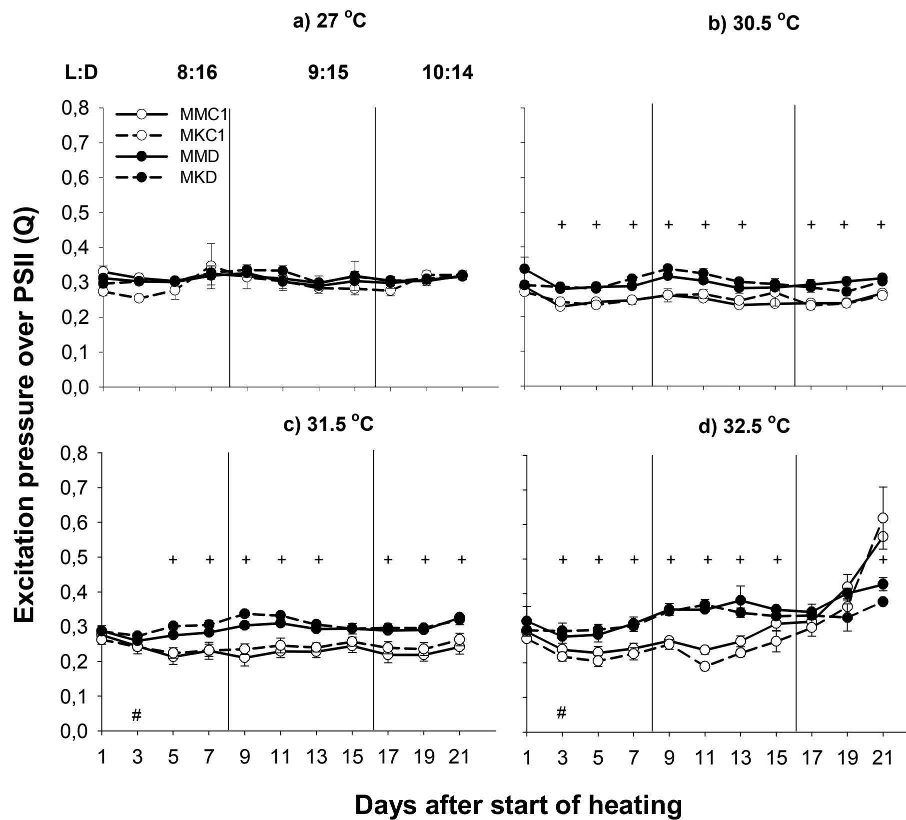

Fig. 3. PAM-results of heat-stress experiment 1 on the custom holobionts grown out at Magnetic Island. L:D = light-dark regime, # = target temperature is reached, + = significant difference between C1 and D groups (p<0.05).

31.5°C, Fig. 3b, c), the Q of C1 holobionts was lower than that of D holobionts ($p<0.05$). Exposure to 32.5°C (Fig. 3d) initially resulted in lower Q in C1 holobionts compared to D holobionts, but after ~11 days of exposure, Q rapidly increased in the C1 holobionts. This response was not seen in the D holobionts. At the end of the experiment (T-21 days), Q was significantly higher in the C1 holobionts than in D holobionts ($p<0.05$). These results indicated that the C1 holobionts were more heat-stressed than the D holobionts. The difference in thermo-tolerance was further supported by a stronger reduction in Fv/Fm for C1 holobionts than for D holobionts at 32.5°C (supplement, Fig. S2).

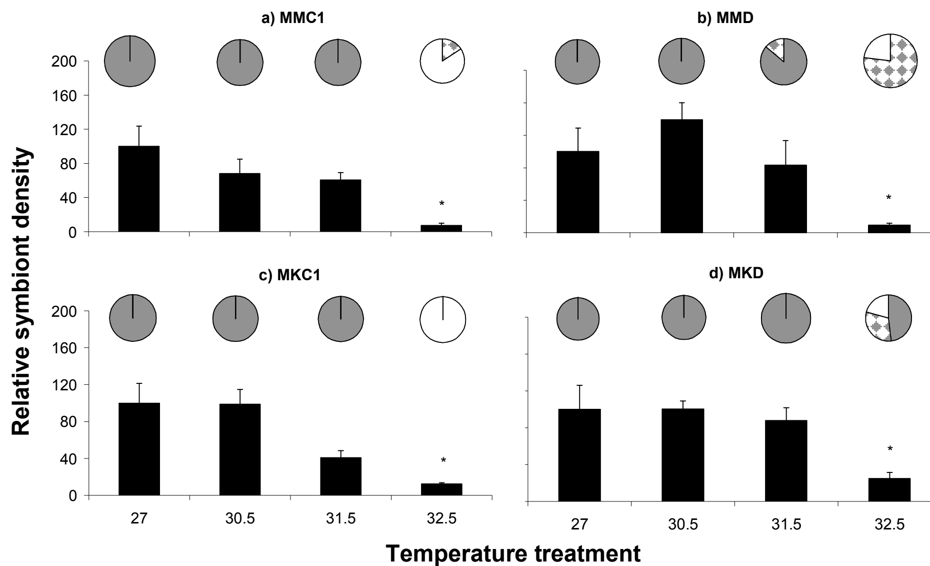

Fig. 4. Relative algal symbiont densities (bars) and coral condition (pies) of the custom holobionts at the end of heat-stress experiment 1. Grey = healthy, checkered = pale, white = bleached. * = significantly different from lower temperatures within a group ($p<0.05$).

Symbiodinium cell density measurements showed significant temperature-related reductions by the end of the experiment in all groups (Fig. 4 bar graphs, Table 3d, $p<0.0001$), indicating that both C1 and D holobionts experienced significant bleaching at the highest temperature. No Temperature*Symbiont effect was found for *Symbiodinium* density (Table 3d), but the visual appearance of the C1

holobionts suggested a stronger bleaching response at 32.5°C than the D holobionts (Fig. 4 pie graphs).

Experiment 2: There was also a strong symbiont effect on Q under heat-stress in experiment 2 (Fig. 5 and Table 3f). No significant effect of outplant location was found; the KKC1 and MKC1 treatments responded in similar manner at all temperatures and time points (Table 3e). No significant differences in Q were found at 27°C or 31°C (Fig. 5a, b). At the highest temperature (32.5°C, Fig. 5c), Q rapidly increased for KKA after ~1 week and approached values of 1 by the end of the experiment. This coincided with a sharp drop in maximum quantum yield (supplement, Fig. S3), indicating severe heat-stress by KKA early in the experiment.

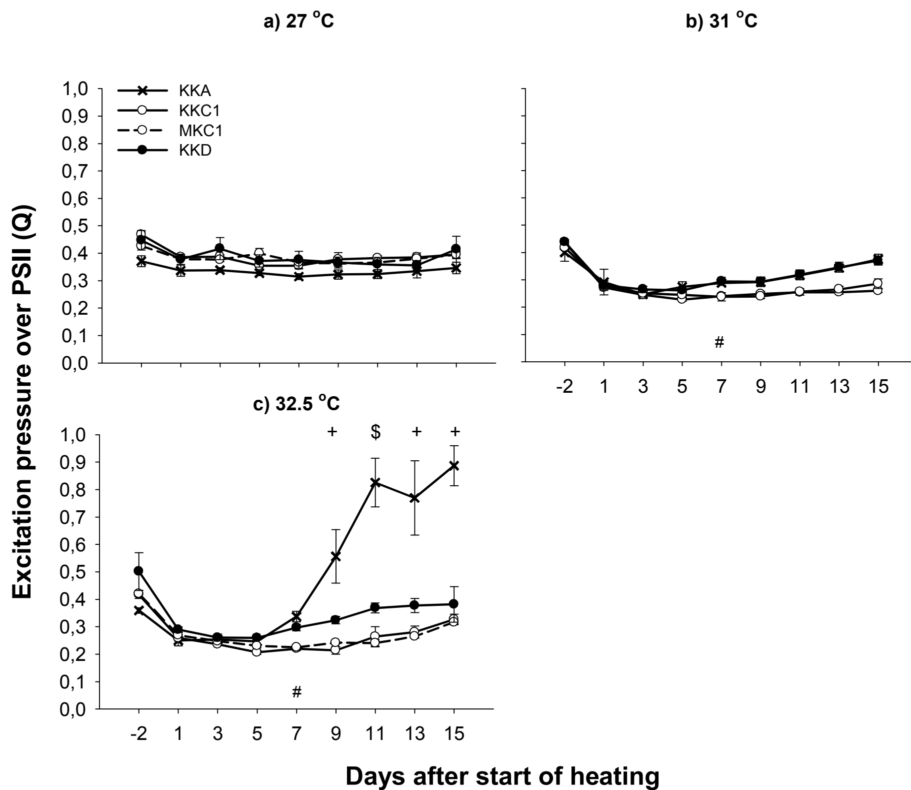

Fig. 5. PAM-results of heat-stress experiment 2 on three custom holobiont groups from Keppel Islands (KKA, KKC1, KKD) and one from Magnetic Island (MKC1). # = target temperature is reached, + = significant difference between A and C1/D ($p<0.001$), $ = significant difference between all three symbiont types ($p<0.05$).

Variance around mean Q for KKA after 9 days was relatively high, due the presences of nine colonies within the KKA group that were less heat-stressed. These colonies were sampled at the end of the experiment, and upon genotyping, were found to harbor a residual community of type D *Symbiodinium* (results not shown). By comparison, Q values of KKC1/MKC1 and KKD were much less affected by the cumulative heat-stress ($p<0.001$, Table 3f). Fv/Fm showed relatively small and similar reductions for KKC1/MKC1 and KKD (supplement, Fig. S3).

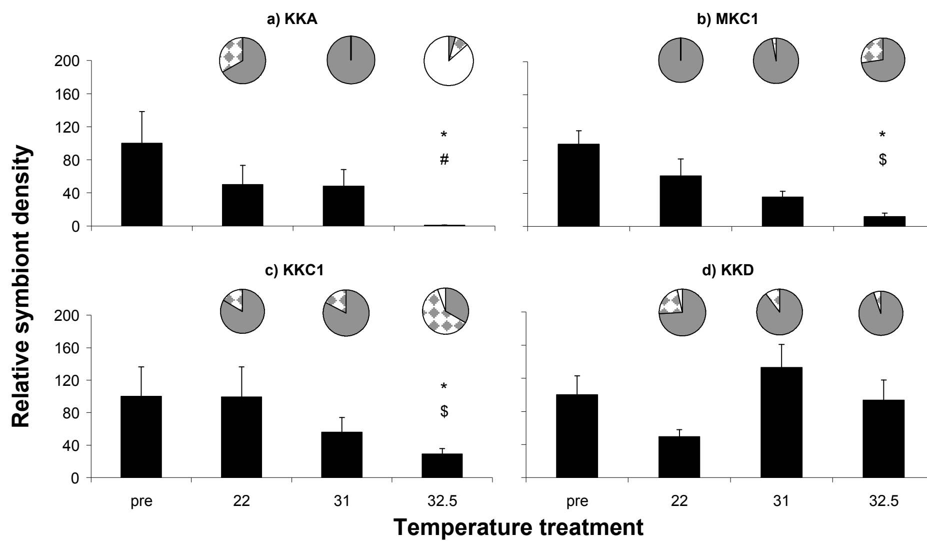

Fig. 6. Relative algal symbiont densities (bars) and coral condition (pies) of the custom holobionts at the end of heat-stress experiment 2. Grey = healthy, checkered = pale, white = bleached. * = significantly different from lower temperatures within the same group ($p<0.05$), # = significantly different from C1 and D symbioses at the same temperature ($p<0.05$), $ = significantly different from A and D symbioses at same temperature ($p<0.05$).

Density measurements of *Symbiodinium* again showed no significant outplant location differences (KKC1 vs. MKC1) across the different temperatures (Fig. 6b,c, Table 3g). However, there was a significant Temperature*Symbiont type interaction with A holobionts more affected than with C1 holobionts, which in turn were more affected than D holobionts at the highest temperature (Table 3h). At 32.5°C, almost no *Symbiodinium* type A cells could be detected in A holobionts at the end of the experiment (Fig. 6a) while *Symbiodinium* C1 cell densities were also significantly reduced in the C1 holobionts (MKC1 and KKC1; Fig. 6b, c). Symbiont cell densities

Table 3. ANOVA results. GLM = general linear model, RM = repeated measures, F = factorial.

Group	*Type 3 SS*	*Df.*	*F*	*p*
(a) GLM-ANOVA T=31 Surface area all groups - KKA				
Symbiont	0.393	1	6.195	0.015*
Host pop	0.002	1	0.036	0.850
Environment	0.041	1	0.651	0.422
Symbiont*Envir	0.350	1	5.521	0.021*
Symbiont*Host pop	0.050	1	0.783	0.379
(b) RM-ANOVA Surface area groups from Keppel Islands only				
Time	14.84	2	307.20	0.000*
Symbiont	1.47	2	11.00	0.000*
Time*Symbiont	0.11	4	1.10	0.37
(c) RM-ANOVA 1st Heat-stress experiment: Q				
Time	0.272	10	31.280	0.000*
Time*Temp	0.496	30	18.980	0.000*
Time*Symbiont	0.099	10	11.410	0.000*
Time*Host pop	0.016	10	1.790	0.061
Time*Temp*Symbiont	0.234	30	8.970	0.000*
Time*Temp* Host pop	0.024	30	0.940	0.567
(d) F-ANOVA 1st Heat-stress experiment: Rel. symbiont densities				
Temp	615.100	3	41.890	0.000*
Symbiont	18.500	1	3.780	0.056
Host pop	6.900	1	1.410	0.239
Temp*Symbiont	8.200	3	0.560	0.646
Temp*Host pop	10.400	3	.07410	0.549
Temp*Host pop*Symbiont	23.800	3	1.620	0.191
(e) RM-ANOVA 2nd Heat-stress experiment KKC1 & MKC1: Q				
Time	0.050	7	21.200	0.000*
Time*Temp	0.020	14	4.300	0.000*
Time*Outplant loc.	0.004	7	1.800	0.091
Time*Temp*Outplant loc.	0.005	14	1.100	0.342
(f) RM-ANOVA 2nd Heat-stress experiment all groups: Q				
Time	1.131	7	32.420	0.000*
Time*Temp	1.172	14	16.800	0.000*
Time*Symbiont	0.964	14	13.810	0.000*
Time*Temp*Symbiont	1.510	28	10.820	0.000*
(g) F-ANOVA 2nd Heat-stress experiment KKC1 & MKC1: Rel. symbiont densities				
Temp	284.300	3	12.820	0.000*
Outplant loc.	3.300	1	0.450	0.508
Temp*Outplant loc.	32.000	3	1.440	0.245
(h) F-ANOVA 2nd Heat-stress experiment all groups: Rel. symbiont densities				
Temp	222.800	3	8.770	0.000*
Symbiont	209.500	2	12.360	0.000*
Temp*Symbiont	271.300	6	5.340	0.000*

were only marginally lower in the KKD treatment over the three temperature regimes (Fig. 6d). This was in agreement with the visual appearances of the holobionts: KKA was almost completely bleached, KKC1 and MKC1 were becoming pale and KKD appeared mostly healthy.

DISCUSSION

Factors affecting coral holobiont fitness

The notion that symbiont type can strongly affect holobiont physiology is now well-established (Little *et al.* 2004; Rowan 2004; Tchernov *et al.* 2004; Berkelmans & van Oppen 2006; Sampayo *et al.* 2008). However, the effect of symbiont type on thermo-tolerance can be coral species-specific (Abrego *et al.* 2008), and upper thermal tolerance limits may differ between conspecific corals, harboring the same symbiont type, due to adaptation/acclimatization to local environmental conditions (Ulstrup *et al.* 2006). Acclimatization is often reversible, but may be fixed early in the ontogeny, which is referred to as developmental plasticity or irreversible non-genetic adaptation (Kinne 1962). Developmental plasticity in thermo-tolerance has been found organisms such as *Drosophila* sp. (Gibert & Huey 2001) and zebra fish (Schaefer & Ryan 2006), but its importance for corals is unknown.

This study shows that for the scleractinian coral species *A. millepora*, *Symbiodinium* identity is the strongest predictor for coral holobiont fitness. In contrast, almost no host population effects were evident in the three parameters measured (growth, survival, heat-tolerance), even though the populations originated from contrasting environments (Table 1). Growth and survival, but not heat-tolerance, were secondarily shaped by environmental conditions experienced during early development from a single-polyp to a multi-polyp stage at the outplant locations, indicating that developmental plasticity was not an important factor in the thermo-tolerance of *A. millepora* after metamorphosis.

The environmental factors determined whether there were realized trade-offs in associating with different symbiont types. The trade-off found at Magnetic Island between thermo-tolerance and growth/survival when associating with C1 vs. D (see also Little *et al*. 2004), did not occur in the Keppel Islands. This variability in the realization of trade-offs has important implications for the potential of symbiont

shuffling (Baker 2003; Fautin & Buddemeier 2004) as a mechanism to induce a change in coral holobiont physiology, as it is directly linked to the occurrence of post-stress reversals (Thornhill *et al.* 2006b). Furthermore, trade-offs are identified as an important factor affecting the potential for evolving bleaching resistance in corals (Day *et al.* 2008), and understanding differences in symbiont-linked trade-offs between reefs may therefore be important in the assessment of reef resilience.

A *Symbiodinium* thermal-tolerance ranking for *A. millepora*

The A holobionts were identified as the least thermally tolerant of the three *Symbiodinium* types tested, based on the inability to maintain the association at Magnetic Island, and the sharp increase in Q for KKA (not seen in KKC1/MKC1 or KKD), their bleached appearance, and the greatest reductions in Fv/Fm and relative symbiont density. Although subtle, the combined results from experiments 1 and 2 indicated that C1 holobionts are less thermo-tolerant than the D holobionts in *A. millepora*. In Experiment 1, the sharp increase in Q of C1 holobionts towards the end of the experiment indicated stronger photoinhibition for this group compared to the D holobionts. This was supported by a stronger reduction in Fv/Fm and a more bleached appearance for C1 holobionts than for the D's. However, the relative zooxanthella densities were similarly reduced for all holobiont groups. At the lower cumulative heat-stress level of experiment 2, the relative symbiont densities (and to some degree, the visual assessment) indicated that only the C1 holobionts were bleaching. For this experiment, however, no differences in thermo-tolerance between the C1 and D holobionts were obvious from the Q or Fv/Fm measurements, possibly because of lower accumulated heat stress. The apparent incongruence between the different measures of thermal tolerance may be explained by the fact that samples for relative symbiont density determinations were taken one day after the last PAM-measurements, leading to a stronger heat-stress effect on symbiont densities than on the fluorescent measures. Alternatively, loss of symbiont cells due to heat-stress may have preceded large responses in the fluorescent parameters. Whatever the cause, the difficulty in separating the thermo-tolerance of C1 holobionts and D holobionts indicated that the differences were small.

The consistently lower Q of C1 holobionts at relatively low levels of accumulated heat-stress compared to A and D holobionts may indicate that C1 is

adapted to higher light levels than either A or D (Iglesias-Prieto *et al.* 2004). Why this pattern is only visible at higher temperatures remains unclear, as Q includes both photochemical and non-photochemical quenching and is therefore affected by multiple, indistinguishable processes (e.g. electron transport rates, D1 repair, xanthophyll cycling, etc.).

We were unable to raise holobionts with the generalist symbiont type C2 (Lajeunesse 2003 *sensu* C3), one of the main symbiont types on the GBR (LaJeunesse *et al.* 2003; LaJeunesse *et al.* 2004a; van Oppen *et al.* 2005). However, it is known that both C1 and D are more common than C2 at relatively warm, inshore locations (van Oppen *et al.* 2001; van Oppen *et al.* 2005), C2 confers a 1-1.5°C lower thermo-tolerance in adult *A. millepora* than D (Berkelmans & van Oppen 2006), and both C1 and D increased in relative abundance at the expense of C2 after a natural bleaching event (Jones et al. 2008). Taken together, these results strongly suggest that C2 has a significantly lower thermal tolerance in *A. millepora* than both C1 and D, although its relative tolerance in comparison to A remains uncertain. Therefore, we can rank the symbiont types according to thermo-tolerance, when in symbiosis with *A. millepora*, as D > C1 >> C2/A. Interestingly, Abrego *et al.* (2008) found that *Acropora tenuis* had a higher thermo-tolerance with C1 rather than with D, indicating that this ranking is affected by the coral host species. Further studies to establish the generality (i.e. valid for many coral species) of this or other *Symbiodinium* thermal tolerance rankings would facilitate assessments of acclimatization capacity of coral reefs.

Type A

Type A (as identified by the ITS1 sequence) belongs to subclade A1 (LaJeunesse 2001) which has been found worldwide (e.g. the Caribbean, Red Sea, French Polynesia, Bermuda, Japan, the Great Barrier Reef) in a variety of hosts including scleractinian corals, zoanthids, jellyfish and giant clams (LaJeunesse 2001; Savage *et al.* 2002; Reimer *et al.* 2006; Magalon *et al.* 2007; this study). Recently, it has been suggested that members of the clade A lineage may be more adapted to a free-living life-style and have opportunistic interactions with cnidarian hosts such as corals that more resemble parasitism (Stat *et al.* 2008). This conclusion was based on: (1) the relative rarity of coral-clade A associations (e.g. LaJeunesse *et al.* 2004a),

(2) the presence of clade A in corals with a reduced health (Toller *et al.* 2001; LaJeunesse 2005; Stat *et al.* 2008), (3) a low carbon translocation to hosts when in symbiosis compared to clade C (Stat *et al.* 2008), (4) a low diversity within clade A, suggesting an opportunistic lifestyle (Stat *et al.* 2008), and (5) the outcompeting by clade A symbionts of other clades in culture (LaJeunesse 2002). Our results support the notion that clade A coral associations are correlated with low coral health, and are of a relatively unstable, opportunistic nature. In contrast to our results, Robison & Warner (Robinson & Warner 2006) found that A1 (obtained from the upside-down jellyfish *Cassiopea xamachana*) was relatively thermo-tolerant based on experiments on long-running *Symbiodinium* cultures. However, no clade C or D *Symbiodinium* were included in these experiments, and *Symbiodinium* cells are known to respond differently in culture compared to *in hospite* (Bhagooli & Hidaka 2003).

Specificity in uptake of experimentally delivered symbionts

The majority of coral-*Symbiodinium* partnerships, including those with *A. millepora*, exhibit horizontal symbiont transmission (Richmond 1997). An advantage of this mode of transmission is that coral juveniles have the potential to form partnerships that are best adapted to local environmental conditions (Buddemeier & Fautin 1993; Baird *et al.* 2007). Several studies have shown that the initial infection by algal symbionts of cnidarian juvenile hosts is relatively non-specific, often exceeding the symbiotic diversity found in adult hosts, and symbiont specificity subsequently develops early in the ontogeny of the host (reviewed by Thornhill *et al.* 2006a). At the cladal level, certain *Symbiodinium* types from clades A, C and D were similarly successful in infecting juveniles of *A. millepora* (this study) and *A. longicyathus* (Gomez-Cabrera et al. 2008). In contrast, *Symbiodinium* C2, C2* and C• did not manage to infect the *A. millepora* juveniles in our experiments. Hence, symbiont infectivity can not be explained by phylogenetic relatedness, suggesting that research into the regulation of molecular signaling involved in the initial establishment of the symbioses (Wood-Charlson *et al.* 2006) needs to be performed at the sub-clade level.

The inability of C• (C15 *sensu* Lajeunesse *et al.* 2003, 2004b) to establish a symbiosis was not surprising, as this type has mostly been found in the maternally

transmitting coral genera *Montipora* and *Porites* (LaJeunesse *et al.* 2003; Fabricius *et al.* 2004; LaJeunesse *et al.* 2004b; van Oppen 2004) and direct symbiont transfer from generation to generation favors the evolution of specialist symbiont lines (Douglas 1998). However, the failure of the C2/C2* types to infect the coral juveniles was unexpected, since these are among the most common ones found in adult populations of *A. millepora* on the GBR (van Oppen *et al.* 2005). We have no explanation for these results, and can only hypothesize that: (1) the physical conditions of our experimental setup were unfavorable for C2 and C2*, and/or (2) *Symbiodinium* C2 and C2* are taken up at a later developmental stage in nature. Interestingly, the 'uninfected' juveniles that were outplanted to the Keppel Islands mostly took up *Symbiodinium* D in the first few months and no *Symbiodinium* C2 was found in any of the genotyped samples, supporting the hypothesis that development stage might play a role in establishment of the C2/C2* symbioses.

Stability of the different custom holobiont

When outplanted (ca. three weeks after metamorphosis), the juveniles were still able to acquire *Symbiodinium*, as the uninfected groups (as determined by squash preps) established symbioses in the field (mostly with type D). Furthermore, the instability of the A holobionts indicated that the initial infection did not necessarily prevent subsequent infections in the field. The differences in holobiont stability may therefore be explained by the differences in competitiveness or specificity between the symbiont types rather than by a limited window of opportunity for infection (Little *et al.* 2004). As indicated by our results, C1 and D may be competitively close in *A. millepora*. Once a C1 or D dominated symbiosis has been established, the other type may not be able to proliferate (if the symbiosis is not disrupted first) due to the competitive exclusion principle, which states that two complete competitors cannot co-exist (Hardin 1960). However, if a large differences in fitness between symbiont types exist, such as between A and D, an established symbiont type may be outcompeted by a new infection. At Magnetic Island, A holobionts were associated with very low fitness (possibly due to relatively high water temperatures at this location), which resulted in MMA and MKA harboring only type D after 13 weeks, which they acquired in the field. At the Keppel Islands, A could be maintained by the juveniles, but C1 and D were

competitively stronger (higher growth) and, as a result, types C1 and D were also present in KKA holobionts at significant relative abundances, which would be expected to further increase over time. It has to be kept in mind that the inoculations with type A were at only half the density of the C1 and D inoculations (50-50 mix with C2*), which might have increased the chance of other types becoming dominant, although this can not explain the difference between Magnetic Island and the Keppel Islands.

The establishment of mostly D symbioses by the experimentally uninfected juveniles at both the Keppel Islands and Magnetic Island cannot be explained by the results of this study. At both locations, C1 symbionts are present in *Acropora* spp. (Little *et al.* 2004; Jones *et al.* 2008), and our results suggest that these symbionts are close or even superior competitors to D in absence of extreme heat-stress. Furthermore, at the Keppel Islands adult *A. millepora* colonies mostly harbor C2, suggesting that a change in the dominant *Symbiodinium* type must take place during juvenile development, possibly induced by changing local environmental conditions due to growth (cryptic juveniles vs. exposed adults) or by other factors relating to host development. Interestingly, azooxanthellate juveniles that were experimentally exposed to a 50-50 mix of C1 and D all started with mixed infections, and over time were equally likely to become dominated by type C1 or D at Magnetic Island, whereas the large majority became dominated by C1 at the Keppel Islands (Box 2). These patterns are not consistent with predictions based on growth data. Obviously, the initial establishment and subsequent succession of *Symbiodinium*-coral associations are complex interactions that are far from resolved.

CONCLUSIONS

This study reveals that the fitness of juvenile colonies of *A. millepora* at two sites on the GBR is primarily affected by the *Symbiodinium* types they harbor, and secondarily by local environmental factors. In contrast, host population origin, and hence possible host genetic differences, had little to no effect on growth, survival or thermal tolerance. Moreover, no evidence for developmental plasticity of thermo-tolerance was detected. C1 and D holobionts of *A. millepora* were both relatively thermally tolerant, with D conveying the highest thermal tolerance in association with *A. millepora*. C1 holobionts had higher growth and survival rates at one

location, but not the other. Therefore, trade-offs between thermo-tolerance and growth/survival rate, mediated by the *Symbiodinium* type, are clearly important, and locally differing environmental conditions can weaken or strengthen these trade-offs. Type A was identified as a poor symbiotic partner with more opportunistic characteristics. Lastly, differences in symbiont infectivity can not be explained by their phylogenetic relatedness, and holobiont stability in early ontogeny may be mediated by mechanisms such as competitive exclusion rather than host specificity or a very narrow window of opportunity for symbiont infections.

BOX 2: MIXED CUSTOM HOLOBIONTS

Newly settled *A. millepora* juveniles from the Magnetic Island and Keppel Islands populations were exposed to mixes of *Symbiodinium* types A, C1, C2, C2*, C•, and D in the ratio 0.5:1:0.5:1:1:1. After the symbioses were established, they were outplanted to both field locations. Up to 14 samples per group were taken pre-field (approx. 2 weeks after settlement), after 2-3 months (February) and after 7-8 months (July). DNA was extracted as before, and their *symbiodinium* community was examined with the use of SSCP gels, estimating relative abundances based on band densities (Fabricius *et al.* 2004).

Before outplanting, all custom holobionts had mixed symbiont communities of mainly C1 and D, with type A at low background levels (not visible for the hosts from Magnetic Island) (Fig. B1). This was expected as C2, C2* and C• failed to infect the *A. millepora* juveniles in the single-symbiont treatments, and type A was supplied at a lower relative abundance than C1 and D. Outplanting to the field locations resulted in several interesting trends. First, colonies tended to drift towards dominance of one symbiont type at both locations (Fig. B1, lower pie charts). Second, type C1 and D were similarly successful at Magnetic Island, whereas at the Keppel Islands C1 became clearly dominant over time, and type A was lost within the first few months at both locations (Fig. 1B, both upper and lower pie charts). These results show that symbiont succession is dependent on the environment, suggesting that symbionts compete for space within coral tissues. As discussed in chapter 5, type A is a poor competitor and was quickly lost from the mixed holobionts. C1 and D may be close competitors at Magnetic island, explaining why both symbionts were maintained in the mixed custom holobionts at similar levels. At the Keppel Islands, not a single colony was found dominated by type D whereas many were dominated by type C1, which could be the result of the environment favoring C1.

BOX 2: CONTINUED-1

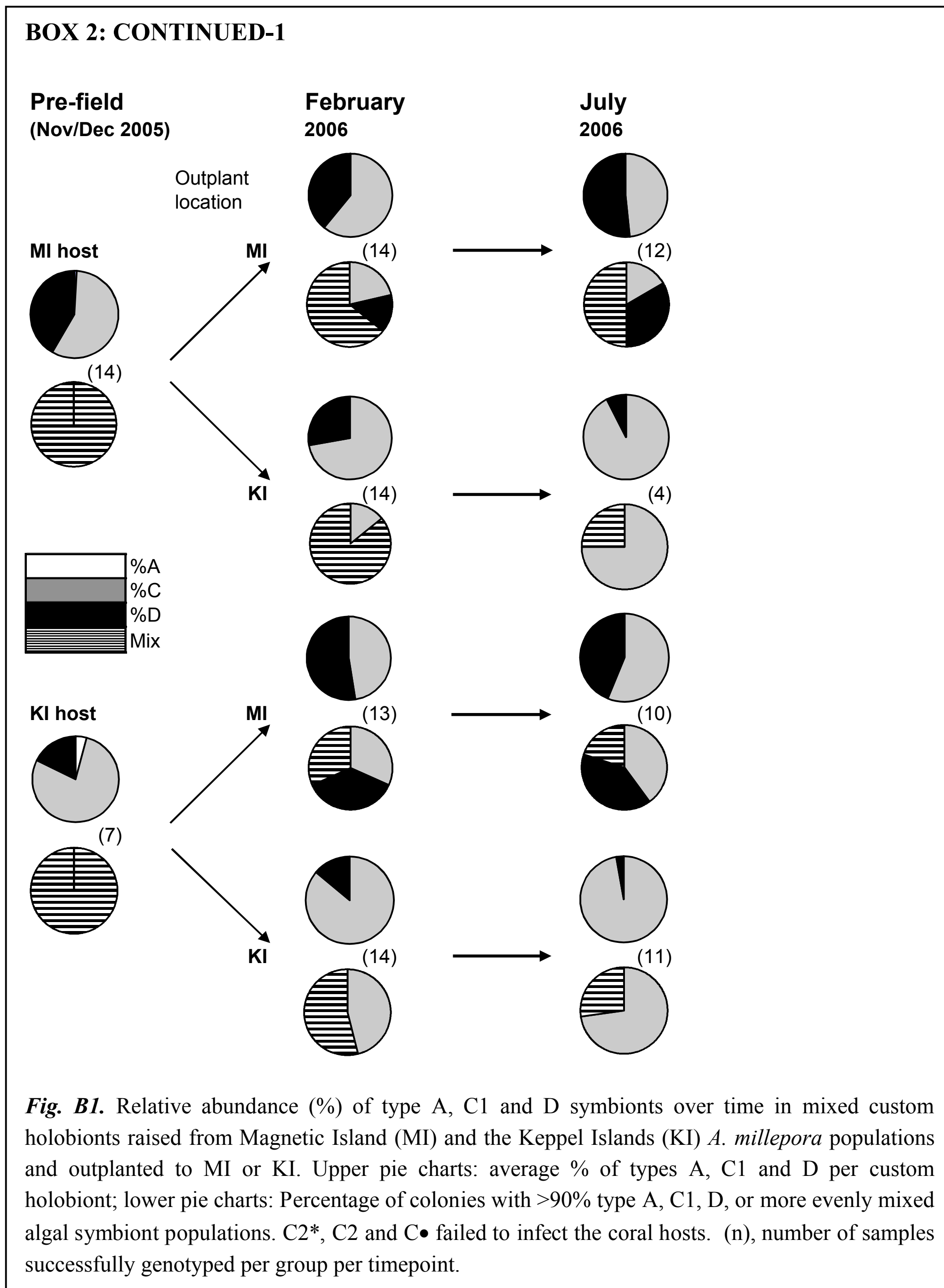

Fig. B1. Relative abundance (%) of type A, C1 and D symbionts over time in mixed custom holobionts raised from Magnetic Island (MI) and the Keppel Islands (KI) *A. millepora* populations and outplanted to MI or KI. Upper pie charts: average % of types A, C1 and D per custom holobiont; lower pie charts: Percentage of colonies with >90% type A, C1, D, or more evenly mixed algal symbiont populations. C2*, C2 and C• failed to infect the coral hosts. (n), number of samples successfully genotyped per group per timepoint.

BOX 2: CONTINUED-2

The fact that both C1 and D could become the dominant symbiont type over time at Magnetic Island may indicate that either the micro-environment and/or stochastic processes played an important role in algal succession. These processes may have had little effect at the Keppel Island because of the large competitive advantage for C1. The competition dynamics between C1 and D however are not easily explained from the separate physiological parameters measured in this chapter. At Magnetic Island, the higher growth and survival rates of C1 would be expected to lead to a competitive advantage for C1 in the absence of bleaching pressure, which was the case during the outplant period. However, C1 and D were equally successful at this location. Furthermore, the adult population of *A. millepora* harbors exclusively D at Magnetic Island (Berkelmans & van Oppen 2006), and the uninfected juveniles that were planted at this location all formed symbioses with type D (this chapter). At the Keppel Islands, C1 and D holobionts had about equal growth rates, temperatures were below to the bleaching threshold, but D holobionts had higher survival rates. This indicates that D may be relatively more fit here, but the mixed infections showed that it was outcompeted by C1 at this location. These results show that the competitive interactions of *Symbiodinium* inside their hosts are largely unresolved, and this urgently needs further investigation.

ACKNOWLEDGEMENTS

We would like to thank A. P. Negri for help with spawning and coral larvae cultures and J. A. Kaandorp for his help with shipping DNA samples. We are also grateful to the crews of the RVs Lady Basten and Cape Ferguson, and the people who provided field assistance, A. Jones, D. Abrego and A. McDonald in particular. Thanks also to the AIMS workshops for technical support, and GBRMPA for the permits needed to conduct this study. This research was financed by the Dutch National Science Foundation, NWO-WOTRO (Project Nr. W84-576), and further supported by the Australian Institute of Marine Science.

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Wood-Charlson EM, Hollingsworth LL, Krupp DA, Weis VM (2006) Lectin/glycan interactions play a role in recognition in a coral/dinoflagellate symbiosis. *Cellular Microbiol* **8**:1985-1993

SUPPLEMENT

Real-time PCR

Table S1. Primers used for *Symbiodinium* type A actin gene sequencing (a+b) and for *Symbiodinium* A, C and D real-time PCR analyses (c-h). Annealing sites of the primers are given in Fig. S1.

Primer name	***Sequence (5'→3')***
Primers for actin gene sequencing	
a) Universal actin forward primer 1[a]	GGCTACTCCTTCACCACCAC
b) Clade A actin reverse primer	GCAGTCAGCTCCTTGGTCAT
Primers for *Symbiodinium* real-time PCR	
c) A actin forward primer	GCCGGATGGCAACATCAT
d) A actin reverse primer	CCGGTTGTGGCAGGACAT
e) C actin forward primer[a]	CAGGATGACACATGCTGATGAA
f) C actin reverse primer[a]	AATTGATGGATTTGTTGGAACTTGT
g) D actin forward primer[a]	GTGAAATTGCGCGTGACATC
h) D actin reverse primer[a]	AGTGCTCCCACTGTCCAACC

[a] Chapter 3

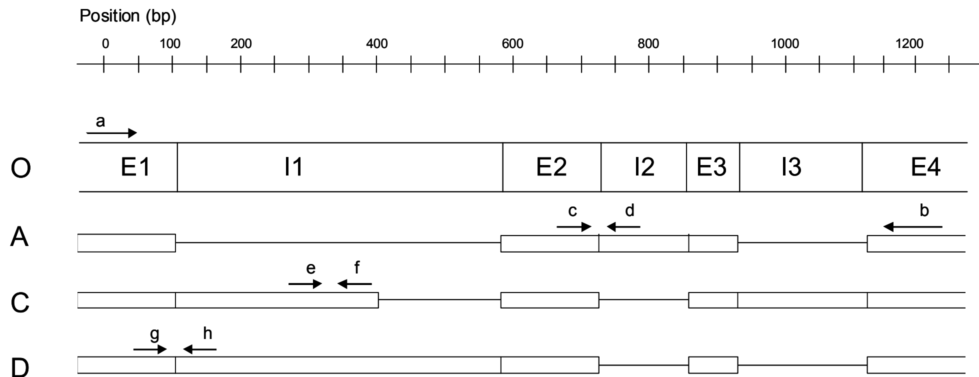

Fig. S1. Overview of the partial actin genes of *Symbiodinium* clades A, C and D. Top line gives position in bp from start of the alignment, first bas (O) gives overview of exons (E) and introns (I). = present, - = absent. Arrows show the annealing sites of the actin primers given in Table S2.

The actin gene of *Symbiodinium* type A was sequenced and real-time PCR primers were designed and tested as described for the *Symbiodinium* C and D actin real-time PCR primers in Chapter 3. The type A partial actin gene sequence is

submitted to Genbank (accession no. FJ456926). Primers for sequencing and real-time PCR are given in Table S1. The real-time PCR primers were found to be clade A specific, and amplified with near 100% efficiency. The position of the A specific real-time PCR primers in relation to the C and D specific primers is given in Fig. S1.

Periodic genotyping of outplanted custom holobionts

Table S2. Temporal confirmation (by SSCP and sequencing) of *Symbiodinium* genotype in outplanted custom holobionts. See Materials and Methods for group nomenclature. Juvenile corals that were inoculated with C2 and C• but failed to take them up are marked "un". *Symbiodinium* types presumably taken up in the field are marked in bold.

Age (Weeks)	*Group*	*Nr. genotyped*	*Symbiodinium type*
At Magnetic Island			
9	MKC1	7	All C1
9	MKD	12	All D
9	MKA	7	All D
9	MKun	13	All **D**
13	MMC1	7	All C1
13	MMD	12	All D
13	MMA	9	All **D**
13	MMun	19	All **D**
31	MKC1	5	All C1
31	MKD	8	All D
35	MMC1	8	All C1
35	MMD	8	All D
At Keppel Islands			
6	KKA	7	All A
6	KKC1	7	All C1
6	KKD	10	All D
6	KKun	6	All **D**
10	KKA	4	3 A, 1 **D**
10	KKC1	5	All C1
13	KKA	8	5 A, 1 A+**D,** 1 A+**C1+D,** 1 **C1+D**
13	KKun	12	**11D, 1 C1+D**
31	KKA	14	10 A, 3 A+**D,** 1 **C1+D**
31	KKC1	11	All C1
31	KKD	11	All D

Maximum quantum yields of the custom holobionts during heat-stress

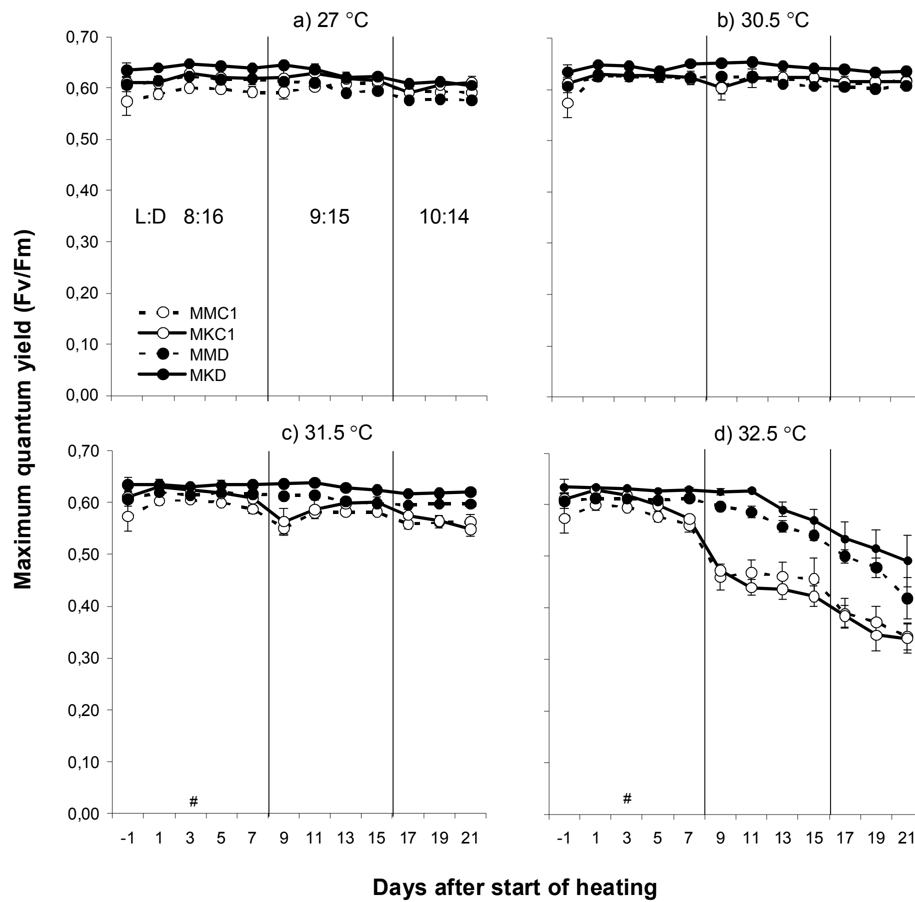

Fig. S2. Maximum quantum yields of the custom holobionts outplanted to Magnetic island during heat-stress experiment 1. L:D = light-dark regime, # = target temperature is reached.

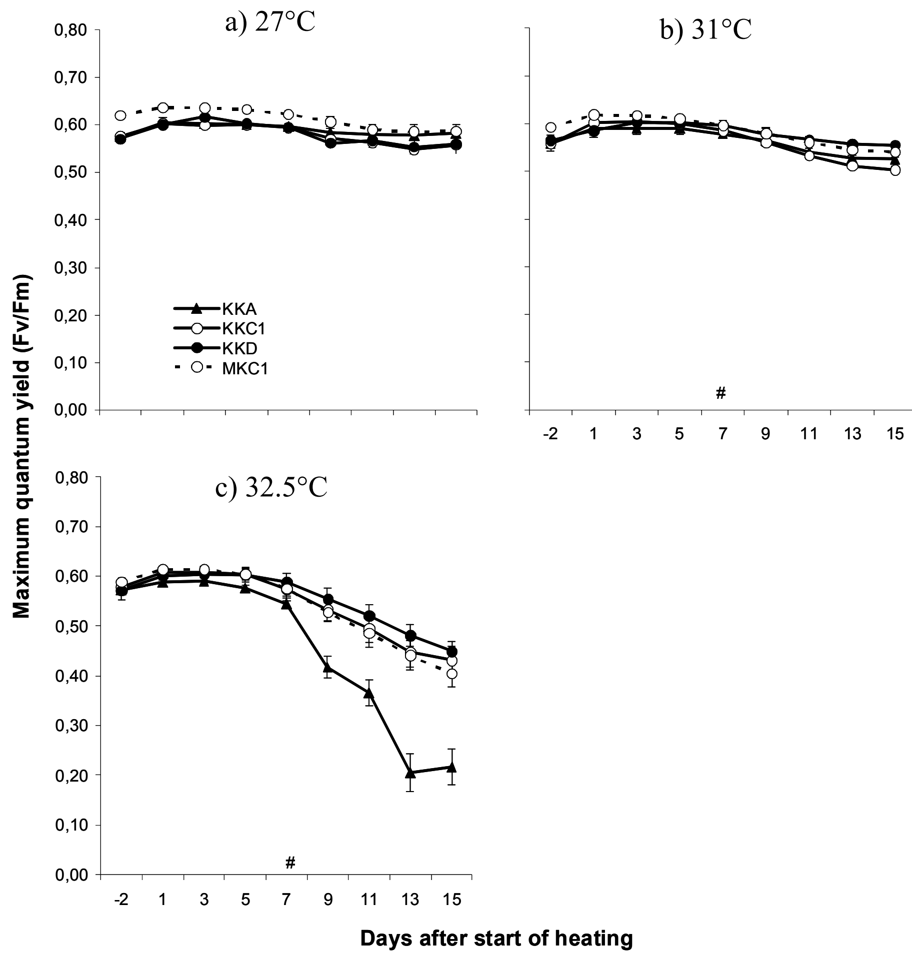

Fig. S3. Maximum quantum yields of three custom holobiont groups outplanted to the Keppel Islands (KKA, KKC1 and KKD) and one to Magnetic Island (MKC1) during heat-stress experiment 2. # = target temperature is reached.

Chapter 6

A community change in the algal endosymbionts of a scleractinian coral following a natural bleaching event: field evidence of acclimatization

Alison M. Jones, Ray Berkelmans, Madeleine J. H. van Oppen, **Jos C. Mieog** & William Sinclair.

Proceedings of the Royal Society B (2008) **275**: 1359-1365

NRC Handelsblad Saturday 12 April & Sunday 13 April 2008, *Wetenschap & Onderwijs*

WISSELING VAN ALGSYMBIONT MAAKT KORAAL HITTEBESTENDIG

Gooi die oude alg eruit

Koralen verbleken door temperatuurstijging. Sommige koralen blijken te herstellen door een spontane algenruil.

Paul Janknegt

DE KORAALSOORT *Acropora millepora* op het Australische Great Barrier Reef kan zich aanpassen aan de stijgende temperatuur van de zeeën, zo hebben Australische en Nederlandse onderzoekers ontdekt. Een meerderheid van die koralen kan de hittegevoelige alg waarmee ze samenleven en die hen van voedsel voorziet, inruilen voor een hittetoleranter type (*Proceedings of the Royal Society B*, 19 maart).

Harde koralen, waartoe *Acropora millepora* behoort, leven in zeer voedselarme wateren en zijn daarom sterk afhankelijk van inwonende symbiotische algen. De eencellige algen leven binnenin de koraalcellen waar ze door middel van fotosynthese suikers produceren, waar het koraal van leeft.

"Opwarming van zeeën is dodelijk voor koralen", vertelt de Groninger promovendus Jos Mieog, een van de auteurs van het artikel. "Tienduizend jaar lang was de gemiddelde watertemperatuur stabiel en opeens stijgt het tot wel drie graden per eeuw. Normaal duurt het generaties om je daaraan aan te passen, zodat vele koralen zullen uitsterven. Maar *Acropora millepora* heeft laten zien dat het door middel van partnerruil toch in staat zich binnen drie maanden te wapenen tegen volgende hittegolven."

VERBLEKING Het wisselen van symbiont is echter een zeer riskante operatie; ongeveer dertig procent van de koralen overleefde de wissel niet. Tijdens de ruil raakt het koraal bijna al zijn voedselproducerende algen kwijt, waardoor het bleek uitslaat en verhongert.

Toch lijkt het verblekingsproces belangrijk te zijn voor een succesvolle en levensreddende partnerruil. "We denken dat *Acropora millepora* op het moment dat het zich vestigt en een symbiose vormt, meerdere typen algen van het geslacht *Symbiodinium* bij zich draagt. Die lijken met elkaar te concurreren om dominantie", legt Mieog uit. Indertijd heeft een hittegevoelig type de dominantiestrijd gewonnen en ervoor gezorgd dat de rest zich niet kan profileren. Daarom moet het koraal eerst de dominante symbiont eruit zetten waarna de hittetolerante symbiont de overhand kan krijgen."

De verbleking van het rif op de onderzoekslocatie was een puur toevallige gebeurtenis, vertelt de koraalonderzoeker. "We hadden net 460 koralen in de hele baai gemerkt en de aanwezige symbionten geïnventariseerd, maar dat was eigenlijk voor een heel andere studie. Toen veranderde binnen een aantal weken het roodbruin gekleurde rif in een geestbleke doodse onderwaterwoestijn. Drie maanden later was de meeste kleur weer terug, wat ons de uitgelezen kans gaf om na te gaan wat er veranderd was ten opzichte van de oorspronkelijke situatie.

"Verbleking komt vaker voor maar omdat je de symbiontsamenstelling moet weten net voordat dit plaatsvindt, is het hiervoor nog nooit iemand gelukt om partnerruil op zo'n grote schaal in de natuur vast te stellen."

BIJPRODUCTEN Wat het verschil in hittegevoeligheid tussen symbiotische algen bepaalt moet nog onderzocht worden, maar Mieog denkt dat de productie van gifstoffen een grote rol speelt. "Fotosynthese gaat gepaard met de productie van bijproducten die schadelijk zijn voor het koraal. Normaal worden die afgevangen, maar bij hoge temperaturen gaat dit mis en lekken ze het koraalweefsel in. De hittegevoelige symbiont lekt onder stress waarschijnlijk meer van deze gifstoffen dan de tolerante soort, waardoor het koraal eerder verbleekt."

Mieog waarschuwt voor al te veel euforie na deze ontdekking. "We weten niet of andere soorten ook in staat zijn om van partner te ruilen en of hittetolerante symbionten wel overal voorkomen. Ook is het nog onduidelijk hoe permanent de verandering is en of de tolerantie wel genoeg is, gezien de voorspelde temperatuurstijging."

Maar volgens de onderzoeker toont het onderzoek wel aan dat bepaalde koralen snel kunnen reageren op het broeikaseffect. "En dat levert in ieder geval tijdwinst op."

Het harde koraal 'Acropora millepora' verbleekt als hittegevoelige algen in de koraalcellen sterven. Door partnerruil met een hittetolerante alg kan het koraal zich tegen hittegolven wapenen. Boven: gebleekt en gezond A. millepora. Linksboven: duikers onderzoeken gezond koraal. Links: gebleekt koraal.

FOTO'S ABBI MCDONALD/JOS MIEOG

ABSTRACT

The symbiosis between reef-building corals and their algal endosymbionts (zooxanthellae of the genus *Symbiodinium*) is highly sensitive to temperature stress, which makes coral reefs vulnerable to climate change. Thermal tolerance in corals is known to be substantially linked to the type of zooxanthellae they harbor and, when multiple types are present, the relative abundance of types can be experimentally manipulated to increase the thermal limits of individual corals. Although the potential exists for this to translate into substantial thermal acclimatization of coral communities, to date there is no evidence to show that this takes place under natural conditions. In this study, we show field evidence of a dramatic change in the symbiont community of *Acropora millepora*, a common and widespread Indo-Pacific hard coral species, after a natural bleaching event in early 2006 in the Keppel Islands (Great Barrier Reef). Before bleaching, 93.5% (n = 460) of the randomly sampled and tagged colonies predominantly harbored the thermally sensitive *Symbiodinium* type C2, while the remainder harbored a tolerant *Symbiodinium* type belonging to clade D or mixtures of C2 and D. After bleaching, 71% of the surviving tagged colonies that were initially C2 predominant changed to D or C1 predominance. Colonies that were originally C2 predominant suffered high mortality (37%) compared with D-predominant colonies (8%). We estimate that just over 18% of the original *A. millepora* population survived unchanged leaving 29% of the population C2 and 71% D or C1 predominant six months after the bleaching event. This change in the symbiont community structure, while it persists, is likely to have substantially increased the thermal tolerance of this coral population. Understanding the processes that underpin the temporal changes in symbiont communities is the key to assessing the acclimatization potential of reef corals.

INTRODUCTION

Coral reefs owe their success to the symbiosis between reef-building corals and intracellular, phototrophic dinoflagellates of the genus *Symbiodinium* (zooxanthellae) that supply up to 95% of the coral host's energy requirements (Muscatine 1990). Under stressful environmental conditions, such as abnormally high water temperatures in combination with high light, this symbiosis can break down and the algae are lost in a process known as 'bleaching'. Such conditions have occurred on reefs globally (Hoegh-Guldberg 1999; Wilkinson 2004) and are predicted to become more frequent as a result of global warming (Donner *et al.* 2005; Hoegh-Guldberg *et al.* 2007). Therefore, coral bleaching is considered one of the biggest threats to coral reefs (Marshall & Schuttenberg 2006).

Nuclear ribosomal and chloroplast DNA markers show that the genus *Symbiodinium* is highly diverse. The genus is currently divided into eight distinct clades (categorized as A–H), each containing multiple subclades, strains or types (Coffroth & Santos 2005; Pochon *et al.* 2006; Stat *et al.* 2006). This level of genetic diversity appears to be matched by appreciable levels of physiological diversity within and between clades. For instance, symbiont types differ in their photosynthetic response to light (Iglesias-Prieto *et al.* 2004) and temperature stress (Robinson & Warner 2006). Reef-building corals can form associations with members of six of the eight *Symbiodinium* clades (A–D, F and G; reviewed by Baker 2003) and some of these associations seem to be more flexible than others (van Oppen *et al.* 2004). *Symbiodinium* C is the most common symbiont type in *Acropora* corals on the Great Barrier Reef (van Oppen *et al.* 2001; LaJeunesse *et al.* 2004; Smith 2004) and certain types within this clade have been shown to be particularly sensitive to heat stress (Berkelmans & van Oppen 2006). *Symbiodinium* clade D is common in Acropora corals on shallow and inshore reefs and has been shown to be relatively tolerant to high temperatures (Glynn *et al.* 2001; Baker 2004; Fabricius *et al.* 2004; van Oppen *et al.* 2005b; Ulstrup *et al.* 2006).

Some corals are known to harbor multiple types within a single colony (Rowan & Knowlton 1995; Rowan *et al.* 1997; Ulstrup & van Oppen 2003), which may allow for changes in the relative abundances of each symbiont type under influence of the environment (symbiont 'shuffling'). One way in which this change can occur is by the predominant, thermally sensitive symbiont population being replaced by a

population of thermally tolerant symbionts that arise from the presence of less abundant 'background' symbionts (Baker 2003). As a result, the entire coral colony becomes more thermally tolerant. This acclimatization mechanism has been shown to occur in at least one population of *A. millepora* on the Great Barrier Reef after transplantation to a different thermal environment (Berkelmans & van Oppen 2006). However, only a few species of coral have been shown to shuffle their symbiont communities (Goulet & Coffroth 2003; Thornhill *et al.* 2003; Goulet 2007) and the longest symbiont monitoring study to date indicates stability rather than wholesale changes in symbiont communities (Thornhill *et al.* 2006). Symbiont 'switching', i.e. the acquisition of new symbionts from the surrounding environment (Baker 2003), may be another way by which corals can achieve a functional change in their predominant symbiont population, but so far this has not been demonstrated in scleractinian corals. Since bleaching is predicted to become more frequent as a consequence of climate change (Dunbar *et al.* 1994; Hoegh-Guldberg 1999; Hoegh-Guldberg *et al.* 2007), shuffling to more heat-resistant symbiont types may be an important acclimatization mechanism, but it must operate at the scale of populations and communities if reefs are to acclimatize and become more resistant to subsequent events (Buddemeier & Fautin 1993; Buddemeier *et al.* 2004). To date, this has not been shown to occur in a natural setting.

In this study, we characterize the *Symbiodinium* community in an inshore population of *Acropora millepora* and compare the *Symbiodinium* community in the same tagged colonies before and after a natural bleaching that took place in 2006. This is the first field study that follows changes in *Symbiodinium* genotypes in specific colonies over 3 years that includes a natural bleaching event. We show a dramatic shift in the symbiont community within this host population as a result of the disturbance, which is likely to have increased its thermal tolerance. We argue that if this shift is sustained and is community wide, the reefs in this area are likely to have substantially increased their capacity to withstand the next bleaching event.

MATERIAL AND METHODS

Study site

The study site is a reef flat adjacent to Miall Island (23°09′ S, 150°54′ E), which is one of 15 islands in the Keppel Island group, in the southern inshore Great Barrier Reef. Miall Island, like many of the islands in this group, has an extensive reef flats on its leeward shore with an average coral cover of approximately 50%, dominated by colonies of the corymbose, Indo-Pacific stony coral *A. millepora* (van Woesik & Done 1997). The region suffered moderate to severe mass bleaching (more than 60% corals bleached) in February 2002 (Berkelmans *et al.* 2004) and severe bleaching in January/ February 2006 (89% corals bleached; R. Berkelmans & A. M. Jones 2006, unpublished data).

Coral sampling

To determine the *Symbiodinium* community composition before the bleaching, 460 colonies were tagged on the reef flat at Miall Island between September 2004 and March 2005. A small (2–3 cm) branch was sampled from the central area of each colony and placed in a labeled bag for subsequent storage in 100% ethanol. Symbiont changes were monitored in a subset of 79 tagged colonies that survived the bleaching three and six months (May and August, respectively) after the bleaching event in January/February 2006. The subset of 79 colonies was chosen haphazardly from surviving colonies and comprised 58 with predominantly C2-type (no background types detected), 15 with predominantly D-type (no background types detected) and six with both C2 and D types present. To minimize confounding of temporal trends in symbiont community by intra-colony variation in symbiont types, we sampled from the same area within each colony on each sampling occasion and ensured that only the tips of branches were used for DNA extraction. Mortality in the *A. millepora* population was assessed six months after the bleaching event in August 2006 by visually estimating the percentage of live and dead coral tissue on 159 haphazardly chosen tagged colonies using pre-bleaching photos of each colony as a reference.

Genotyping and sequencing

DNA was extracted from coral tissue based on the method of Wilson *et al.* (Wilson *et al.* 2002). A combination of single-strand conformation polymorphism (SSCP) analysis, cloning and DNA sequencing was used for symbiont identification. The internal transcribed spacer 1 (ITS1) region was amplified as described by van Oppen *et al.* (2001). SSCP analysis was used to identify the predominant symbiont type in each colony and estimate the relative abundance of *Symbiodinium* types within each sample when more than one type was identified. Relative abundances of less than 5–10% are not detected using SSCP (Fabricius *et al.* 2004). SSCP bands that were faint compared with another more intense band in the same sample were identified as background and predominant types, respectively. The presence of two equally intense bands in the same sample was interpreted as the colony hosting equal amounts of each type. Fabricius *et al.* (2004) found that this was a reliable method for estimating the relative abundance of different *Symbiodinium* types. SSCP profiles were assigned to symbiont type by comparing to reference samples of known identity and by cloning and sequencing in the case of novel SSCP profiles.

Statistical analysis of symbiont community changes

Counts of colonies of *A. millepora* before the bleaching were analyzed using a Pearson's chi-squared contingency table to compare the frequencies of colonies with different combinations of predominant symbiont types C2, C1 and clade D with the null hypothesis that there were no differences in the observed and expected cell frequencies. In addition, two separate multinomial loglinear regressions were used to test for significant changes in the (1) predominant and (2) low-level background symbiont types in the subset of 79 colonies three and six months after bleaching with the null hypotheses that there were no differences in the log ratios of the observed and expected cell frequencies. Predominant symbiont types (C2, D and C1) and background types (C2, D, C1, D, multiple types and no background types) before bleaching and three and six months after bleaching were fixed factors in the analyses, and cases were weighted by the number of colonies of each type. The parameter estimates derived from the multinomial loglinear regressions were used to

show the nature of any significant changes. All statistical analyses were performed with SPSS v. 15.0.

RESULTS

Phylogenetic analysis of *Symbiodinium* ITS1

Four different bands were observed on the SSCP gels using the ITS1 region (Fig. 1). The ITS1 regions of 8 representative samples were sequenced and aligned with sequence from GenBank (Table 1). Types C1, C2, and D, as previously described by van Oppen *et al.* (2001), were identified. Type D*, appearing on SSCP gels in a small number of samples taken before bleaching, differed by a single base pair from type D. Sequencing of clones revealed additional sequence variation, but it is assumed that these represent intragenomic variants (Thornhill *et al.* 2007). In total, twenty sequences have been submitted to GenBank (accession no. EU189435 – EU189455).

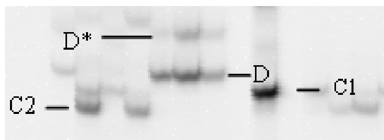

Fig. 1. The four *Symbiodinium* types of clades C and D, as visualized by SSCP, found before and after bleaching in the *Acropora millepora* colonies at Miall Island.

Symbiont diversity at Miall Island before and after bleaching

Before the bleaching in 2006, *A. millepora* at Miall reef associated predominantly with *Symbiodinium* type C2 (93.5%, sensu (van Oppen *et al.* 2001) and to a much lesser extent with *Symbiodinium* clade D (3.5%) or mixtures of C2 and D (3.0%; $\chi_1^2 = 398$, $p < 0.001$, $n = 460$, Fig. 2a). Cloning and sequencing of five clade D and six clade C ITS1 PCR products (370 bp in length) showed that these differed by 1–6 bp within clades, which are assumed to represent intragenomic variation. By late February 2006 when bleaching was at its most intense, the relative difference in bleaching susceptibility between corals predominated by C2 and D was clearly evident, with the former bleaching white and the latter normally pigmented

(Fig. 3). Tagged corals harboring a mix of *Symbiodinium* C2 and D were mostly pale in appearance.

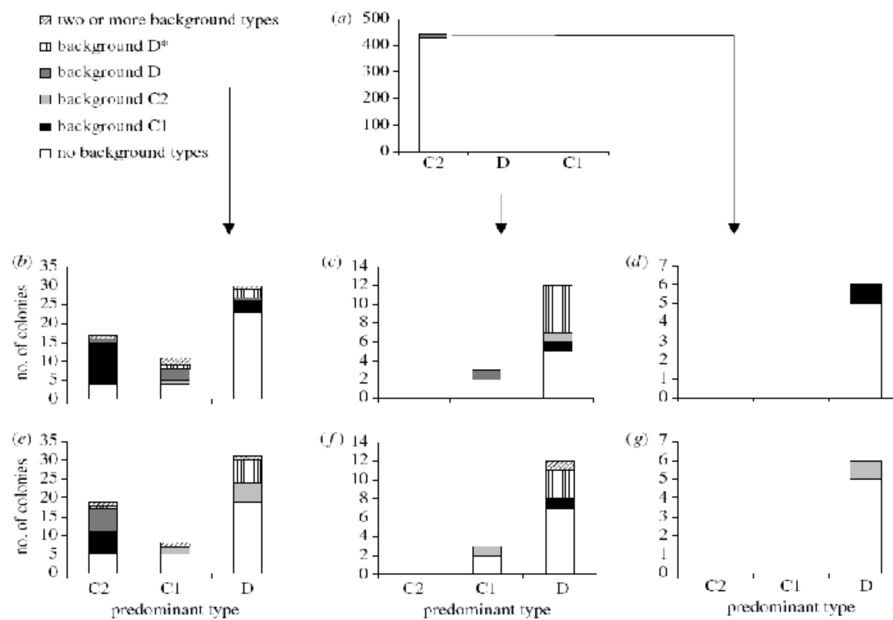

Fig. 2. *A. millepora* colonies sampled (a) prior to bleaching between September 2004 and March 2005 (n = 460), (b-d) three months after bleaching in May 2006 (n = 79), (e-g) six months after bleaching in August 2006 (n = 79) at Miall Island reef (Keppel Islands, southern Great Barrier Reef). (b,e) Original C2 type (n=58), (c,f) original D type (n=15), (d,g) original C2/D type (n=6).

Fig. 3. A stark landscape of 100% bleached *A. millepora* colonies predominated by *Symbiodinium* clade C2 and unbleached colonies with clade D symbionts at Miall Island reef in the Keppel region of the southern Great Barrier Reef during the Januari/Februari 2006 summer bleaching confirm the differential bleaching susceptibility of corals with these symbiont types.

In May 2006, three months after the bleaching, a major shift to thermally tolerant type D and C1 symbiont communities occurred in the surviving colonies (Fig. 2b-d). Of 58 C2-predominant colonies monitored post-bleaching, 30 became predominant in type D symbionts (most without detectable levels of C2 symbionts. Type C1 was not detected in any of the SSCP gels of samples just before the bleaching. By May 2006, however, C1 became the predominant type in 11 out of the 58 colonies and was clearly evident as a background type in 11 out of 17 colonies that remained C2 predominant. C2-predominant colonies without other detectable background symbiont types (lower detection limit 5–10%) made up only four out of the 58 colonies three months after bleaching. Of the 15 original D-predominant colonies monitored post-bleaching, 12 retained their D predominance while three changed to C1 predominance. All six colonies that initially hosted C2 with background clade D became D predominant by May 2006. A variant of D which we called D* was not apparent in any colonies prior to bleaching but was detectable at low levels in 10% of colonies after bleaching (Fig. 2b, c). The appearance of previously undetected C1 and D* led to an increased diversity of symbiont types three months after bleaching.

By August 2006, six months after bleaching, the proportion of predominant symbiont types in each of the three initial groups of colonies (C2 or D predominant and C2 with D) remained stable, but there were substantial changes in the mix of background types (Fig. 2e-g). In the group that changed from C2 to D predominance, none had detectable background levels of C2 in May but C2 reappeared in five colonies in August. In addition, the other two groups also showed a slight increase in the background occurrence of C2 in August, possibly suggesting the start of a drift back to pre-bleaching C2 predominance. By contrast, more colonies had C1 in May compared with August while the abundance of D* increased from May to August (Fig. 2b, e). The loglinear regressions showed that a significant change occurred in the predominant symbiont types of the colonies at Miall Island. The C2-predominant colonies were more likely to have changed to clade D predominance than to have remained unchanged or changed to C1 predominance in both May and August ($Z = -15.0$, $p < 0.001$, d.f. = 10). Type C2 colonies were more likely to occur with clade D than any other type or combination of types (C1, C2, D or D) in May ($Z = 29.7$, $p < 0.001$, d.f. = 70) and August ($Z = 34.4$, $p < 0.001$, d.f. = 70).

While the symbiont community change in surviving colonies was dramatic (71% changed predominance from C2 to D or C1), selective mortality also played a substantial role in shifting the symbiont community in the coral population. Of 159 colonies monitored for survival, 147 were initially C2 predominant and of these, 54 colonies suffered 100% mortality and a further 34 suffered more than 50% partial mortality (Fig. 4). Only one of 15 colonies that were initially D predominant died. The difference in mortality between clades was statistically significant ($p = 0.043$, $\chi_1^2 = 4.1$), confirming their differential thermal tolerance.

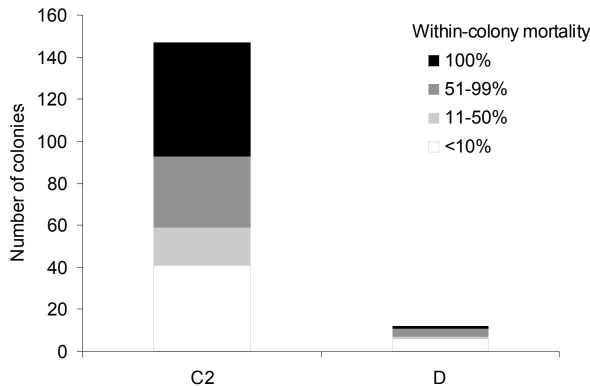

Fig. 4. A summary of the partial mortality in tagged *A. millepora* colonies at Miall Island (n=159). Thirty seven percent of colonies with type C2 *Symbiodinium* suffered 100% mortality compared to only 8% of clade D (χ_1^2 = 4.1, p = 0.043, d.f. = 1). D, colonies with clade D; C2, colonies with type C2 and background clade D; C2/D, colonies with both type C2 and clade D.

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In terms of relative contribution to symbiont community change, selective mortality accounted for 37% of the change while altered symbiont-type predominance accounted for 42% of the change (n = 159). Just over 20% of the original C2-predominant population survived and maintained C2 as their predominant symbiont (cf. 93.5% prior to bleaching).

DISCUSSION

We have shown field evidence of a dramatic shift in the symbiont community in a reef-building coral as a result of bleaching. The balance of the symbiosis shifted from a predominant association between *A. millepora* and *Symbiodinium* type C2 to a predominance of type D and to a lesser extent to predominance of type C1. This

shift resulted partly from a change of symbionts within coral colonies that survived the bleaching event (42%) and partly from selective mortality of the more bleaching-sensitive C2-predominant colonies (37%). While these numbers are event, population and location specific, they do confirm that several interrelated processes play a role in shaping reef symbiont communities after bleaching episodes (Baker 2003). We propose that symbiont shuffling is a more likely explanation for the observed shift in symbiont communities than switching (i.e. de novo uptake) because (i) all 14 colonies that harbored low levels of D-type symbionts prior to the bleaching event survived and changed from C2 to D predominance, (ii) SSCP analysis is known to lack the sensitivity to detect symbiont types at a relative abundance of less than 5–10% and (iii) cloning and sequencing a subset of samples before bleaching revealed D and C1 below the detection limits of SSCP, the presence of which predicted their appearance after bleaching if shuffling was the mechanism of change. This is supported by the observation of novel symbiont types three and six months after the bleaching. Although de novo uptake cannot be ruled out, mathematical modeling of the recovery of symbiont populations after bleaching suggests that such rapid changes are more easily explained by upward and downward regulations of existing symbiont populations (Jones & Yellowlees 1997).

As a direct result of the shift in symbiont community, the Miall Island *A. millepora* population is likely to have become more thermo-tolerant. We base this conclusion on the experimental evidence of Berkelmans & van Oppen (2006) who found that differences in thermal tolerance in *A. millepora* from the same area is driven by symbiont type rather than the host coral. Furthermore, a shift from bleaching-sensitive type C2 to clade D increased the thermal tolerance of this species by 1–1.5 °C. These findings are supported by our observation of differential bleaching susceptibility between C2-and D-predominant colonies during the 2006 bleaching event. We suggest that *A. millepora* colonies that host predominantly C1-type symbionts are also more thermally tolerant than their counterparts with C2. Unbleached colonies of the staghorn coral *Acropora formosa* sampled in February 2006 at Miall Island harbored predominantly C1 symbionts whereas white-bleached colonies of this species hosted C2. These observations, together with the high occurrence of C1 in acropid corals (van Oppen *et al.* 2001) at one of the most thermo-tolerant reefs on the Great Barrier Reef (Berkelmans 2002), suggest that C1 may confer thermal tolerance to some species, just like D-type symbionts. Given the

direct experimental evidence of increased thermal tolerance of *A. millepora* with D-type symbionts and the circumstantial evidence of similar thermal tolerance in this species with C1-type symbionts, the symbiont community change documented in this study is therefore likely to have resulted in increased thermal resistance for the majority of the *A. millepora* population. If the symbiont community drifts back to C2 predominance, the increased thermal tolerance will be lost. A drift back to pre-bleaching symbiont types was suggested for *Montastraea annularis* in the Florida Keys (Thornhill *et al.* 2006), and there are signs of a similar drift back to pre-bleaching C2 predominance in this study six months after bleaching.

Our results strongly support the re-interpreted adaptive bleaching hypothesis of Buddemeier *et al.* (2004), which postulates that a continuum of changing environmental states stimulates the loss of bleaching-sensitive symbionts in favor of symbionts that make the new holobiont more thermally tolerant. However, such a change may come at a physiological cost such as loss of photosynthetic efficiency (Rowan 2004) leading to lower energy reserves (Hoogenboom *et al.* 2006; Loram *et al.* 2007) and slower growth (Little *et al.* 2004). Our field observations provide the first extensive colony-specific documentation and quantification of temporal symbiont community change in the field in response to temperature stress, suggesting a population-wide acclimatization to increased water temperatures. If this shift is sustained and extends to other species, the reefs in this area are likely to have substantially increased their capacity to withstand the next bleaching event. However, at this stage, it is unknown whether the increased thermal tolerance, even if it persists, will necessarily translate into increased reef resilience, particularly if growth and carbonate accretion are depressed to levels whereby bioerosion outweighs net accretion.

This study highlights the importance of improving our understanding of multi-clade symbiotic partnerships (Baker & Romanski 2007). Our results show an increase in the diversity of symbionts after bleaching together with a considerable change in the make-up of the symbiont community within individual colonies over time scales as short as three months. This increase in the diversity and variation of symbionts has not been previously shown following a bleaching event. Most studies that have followed the *Symbiodinium* community during bleaching (Glynn *et al.* 2001; Guzman & Cortes 2001; Baker 2003; van Woesik *et al.* 2004) have not used molecular techniques sensitive enough to detect the low-density symbiont genotypes

and genetic variations of rDNA types (Apprill & Gates 2007). A recent study has shown that the majority of scleractinian corals are likely to harbor symbiont types at levels that are undetectable using electrophoretic genetic techniques (Chapter 2), suggesting that symbiont flexibility may also be more common than previously thought. Subtle seasonal and spatial shifts in symbiont populations that occur as a result of even minor changes in environmental variables such as temperature and light may underwrite the more permanent, climate-driven shifts following dramatic bleaching events (Thornhill *et al.* 2006). Smith (Smith 2005) found that four months before a major bleaching event in early 2002, 20 out of 20 *A. millepora* colonies at Miall Island were predominant in type C2, while van Oppen *et al.* (2005a) found that five months after the 2002 bleaching event, six out of 19 were predominant in type D. Although the sample sizes in these studies are small, these results suggest that the *A. millepora* symbiont community underwent a similar shift towards clade D predominance as a result of the 2002 bleaching event and then drifted back to C2 predominance 4 years later just prior to the 2006 bleaching event. This poses the question of why some coral populations retain thermally tolerant symbionts while others revert back to former sensitive types. Baird *et al.* (2007) hypothesize that symbiont community shuffling to clade D may persist only as a result of enduring changes in environmental conditions, e.g. repeated warm summers. This may be evident at Magnetic Island, where temperatures exceed 30.5°C during most summers (Berkelmans 2002) and *A. millepora* have harbored exclusively clade D symbionts over many years (van Oppen *et al.* 2001; Berkelmans & van Oppen 2006). Conditions similar to those currently occurring at warm reefs such as Magnetic Island have been projected to occur on in the southern Great Barrier Reef by 2020–2030 (Done *et al.* 2003). Understanding the role of these backgrounds symbionts and the process and conditions under which they are up-and down-regulated is the key to assessing the acclimatization potential of coral reefs and their ability to withstand future thermal stress events in an era of climate change.

ACKNOWLEDGEMENTS

We gratefully acknowledge the crews of the Lady Basten and Cape Ferguson, Scott Gardner, Deanna Gardner and Peter Williams from Keppel Reef Scuba Adventures for their assistance with fieldwork. J.C.M. was supported by a grant

from NWO-WOTRO, the Netherlands (Project no. W84-576) and R.B. by funds from the CRC Reef. We also thank the Great Barrier Reef Marine Park Authority for their permits allowing sampling at Miall Island for this study.

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Chapter 7

Juvenile corals can acquire more carbon from high-performance algal symbionts

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Coral Reefs (2009) **28**: 405-414

ABSTRACT

Algal endosymbionts of the genus *Symbiodinium* play a key role in the nutrition of reef-building corals and strongly affect the thermal tolerance and growth rate of the animal host. This study reports that the capacity of photosystem II ($rETR_{MAX}$) was 87% greater in *Acropora millepora* juveniles associated with *Symbiodinium* C1 than for those associated with *Symbiodinium* D. Furthermore, the ^{14}C photosynthate incorporation (energy) into the tissues of the same corals was twice as high for C1 corals as for D corals in a laboratory experiment. Both differences were lost in the presence of diuron (DCMU — a herbicide that limits electron transport), further supporting the link between photosynthetic capacity and host photosynthate incorporation. These findings advance our current understanding of symbiotic relationships between corals and their symbionts, providing evidence that enhanced growth rates of juvenile C1 corals may result from greater translocation of photosynthates from *Symbiodinium* C1. This may translate into a competitive advantage for juveniles harbouring *Symbiodinium* C1 under certain field conditions, since rapid early growth typically limits mortality.

INTRODUCTION

Zooxanthellae (symbiotic dinoflagellates of the genus *Symbiodinium*) are critical to the survival of reef-building corals, providing a major source of energy from photosynthesis for cell maintenance, growth and reproduction of their coral hosts (Muscatine *et al.* 1984; Crossland *et al.* 1991). DNA sequencing studies using 28S, ITS1, ITS2, 23S and mtCOX1 loci have uncovered a high diversity of at least eight clades within the genus *Symbiodinium* (van Oppen *et al.* 2001; LaJeunesse 2002; Santos *et al.* 2002; Baker 2003; Pochon *et al.* 2004; Rowan 2004; Takabayashi *et al.* 2004; Coffroth & Santos 2005; Apprill & Gates 2007). A review by Goulet (2006) revealed that most hard and soft corals may reportedly contain only one *Symbiodinium* type. However, sensitive molecular detection methods have recently revealed that a considerable number of anthozoans can harbor several algal types simultaneously (Ulstrup & van Oppen 2003; Apprill & Gates 2007; Loram *et al.* 2007; Chapter 2).

Physiological traits of the coral host are at least partly shaped by the dominant symbiont type present within its tissues. For instance, the genetic type of symbiont within juveniles of *Acropora millepora* and *A. tenuis* has been linked to a 2-3 fold increase in growth rate within the first 6 months of development on the reef (Little *et al.* 2004). Furthermore, adult *A. millepora* corals on the Great Barrier Reef (GBR) have been shown to acquire a 1-1.5 ºC increase in thermal tolerance by shuffling the dominant symbiont type present within coral tissues (Berkelmans & van Oppen 2006). Likewise, in Guam, colonies of *Pocillopora* spp. associating with different symbiont clades exhibited differences in thermal stress tolerance (Rowan 2004). The capacity for photoacclimation and tolerance to high irradiance stress has also been linked to the genetic type of *Symbiodinium* spp., both in culture and within multiple coral host species (Warner *et al.* 2006). A recent study shows that symbiont type can affect the incorporation of algal-derived photosynthetic carbon (^{14}C) into host tissues of an anemone (Loram *et al.* 2007), further supporting the notion that symbiont type can affect growth and resilience to stress. The ability of corals to associate with a diverse range of symbiont types (van Oppen *et al.* 2001; Baker 2003; Rowan 2004) may provide ecological advantages to the host colony, enabling it to colonize a variety of reef habitats and survive a changing global climate.

Acropora millepora is typical of most broadcast spawning corals, acquiring symbionts from the environment just prior to or following larval metamorphosis. It associates with *Symbiodinium* D on reefs surrounding Magnetic Island, a nearshore island in the central section of the GBR. However, it commonly harbors *Symbiodinium* C1, C2 and C3 on inner-, mid- and outer-shelf reefs (van Oppen *et al.* 2005). On the GBR, *Symbiodinium* D has a predominantly inshore distribution and hence experiences high temperatures, turbidity and pollution events relatively frequently. Its rarity on mid- or outer-shelf reefs is possibly due to higher light intensities common at these sites (van Oppen *et al.* 2005).

We compared the effects of *Symbiodinium* types C1 and D in symbiosis with *A. millepora* on: (1) the translocation of carbon based energy from the photosymbiont to the coral host, and (2) the photosynthetic performance of the coral holobiont. Comparisons were performed under normal and stress conditions. Stress was induced by exposure to the herbicide diuron, an environmentally relevant contaminant that inhibits photosynthesis by blocking electron transport and causing damage to photosystem II (PSII) (van der Meulen *et al.* 1972; Jones *et al.* 2003). Unlike high temperature and irradiation stresses, diuron exposure influences the symbiont's performance without directly affecting the coral host (Schreiber *et al.* 1997; Negri *et al.* 2005; Cantin *et al.* 2007), therefore enabling the effects of photoinhibition to be distinguished between symbiont types.

Our experiments were performed under controlled light and temperature conditions using 9-month old *A. millepora* juveniles that had a common parentage and had been experimentally infected with either *Symbiodinium* C1 or D immediately following metamorphosis (i.e. custom corals, Chapter 5). Translocation of carbon based energy was followed through ^{14}C incorporation by the coral host. Photosynthetic performance was determined by the relative electron transport rates of photosystem II ($rETR_{MAX}$). The pigments of the light-harvesting complex and the xanthophyll carotenoids were analyzed to gain further insight into the differences of the *Symbiodinium* C1 and D photomachineries and their responses to the diuron-stress.

MATERIALS & METHODS

Raising C1 and D corals

Following spawning, gametes were collected from eight hermaphroditic colonies of *A. millepora* and mixed for fertilization. Larvae were raised in 500 L tanks with filtered seawater (1 µm). Four days after spawning, when larvae were first observed to exhibit settlement behavior, preconditioned (for 16 weeks on the reef at Magnetic Island), autoclaved (to kill any *Symbiodinium* spp. present on the tiles) terracotta tiles were placed as settlement surfaces on the bottom of the tanks. *Symbiodinium* types C1 (GenBank Accession No. AF380555) and D (GenBank Accession No. EU024793), based on ITS1, were selected for experimental infection of juveniles because both associate with *A. millepora* on inshore reefs of the GBR (van Oppen *et al.* 2005).

Symbiodinium C1 and D were obtained from adult colonies of *A. tenuis* and *A. millepora* at Magnetic Island, respectively, by airbrushing the coral tissue and isolating the *Symbiodinium* cells from the coral-algal slurry by centrifugation (350 g for 5 min). These isolated zooxanthellae were offered to larvae and newly settled juveniles three and five days after spawning at ca. 10^8 cells.tank^{-1}. Infection of the coral juveniles was confirmed by microscopic observation of squash preps indicating the presence of symbionts within the coral tissue. *Symbiodinium* genotypes were confirmed following infection prior to field deployment by Single Strand Conformation Polymorphism (SSCP) and the potential presence of unexpected background types was tested using quantitative PCR at the end of the experiment (see below).

The custom corals were allowed to develop for a further two weeks in the laboratory, after which the tiles were attached vertically to racks on a fringing reef (Nelly Bay, Magnetic Island) in a zone where *A. millepora* is common. Racks were randomly arranged to minimize any effects of partial shading during this grow-out period. The corals were collected nine months later and acclimated horizontally under identical natural illumination (75% shading, max 350 µmol.photons.m^{-2}.s^{-1}) in an outdoor flow-through aquarium for two days prior to experimental testing. In this set-up, C1 and D corals originated from crosses involving the same parent corals,

thereby minimizing potential host genetic differences that may have influenced the physiology of the custom corals (Coles & Brown 2003; Chapter 5).

Experimental treatments

The C1 and D corals were randomly distributed over nine glass tanks (4 L filtered sea water, 0.25 μm), with an average of 3 colonies per symbiont type per tank. Treatments were: 0 $\mu g.L^{-1}$ (control), 1 $\mu g.L^{-1}$, or 10 $\mu g.L^{-1}$ diuron (DCMU: 3,(3,4-dichlorophenyl)-1,1-dimethylurea) for ten hours (tank triplicates). Tanks were placed under metal halide lamps exposing the corals to a constant illumination of 180-200 μmol $photons.m^{-2}.s^{-1}$. These light levels are similar to those the corals would experience in the natural reef habitat during development.

Pulse amplitude modulation (PAM) fluorometry

All fluorescence measurements were taken with a Diving-PAM (Walz, Germany). The 2 mm fiber-optic probe was held at a consistent distance of 2 mm directly above each juvenile coral using the manufacturer-supplied leaf clip. After 2 h of light exposure, the custom corals were placed for 2 min in the dark to allow substantial re-oxidation of the primary electron acceptor (Q_A) (Schreiber 2004) while minimizing the total time required for the measurement of the rapid light curves (RLCs). RLC measurements were taken in the dark on three juveniles for each symbiont type and diuron treatment (n=3), taking a total of 90 min. The RLC's were measured using a pre-installed software routine, where the actinic measuring light was incremented over eight steps (0, 44, 72, 116, 147, 222, 283, 428 and 653 μmol $photons.m^{-2}.s^{-1}$), each with a duration of 10 s. Each tank was immediately returned to the normal light regime following each RLC.

RLCs can be used to assess the current capacity of PSII as a function of irradiance (Schreiber *et al.* 1997). RLCs are constructed by plotting the effective quantum yield (as measured with a PAM fluorometer) against PAR. The relative electron transport rate (rETR) obtained from RLCs provides a reliable approximation of relative electron flow through PSII when absorbance between samples is identical (Genty *et al.* 1989). In our experiments this was assumed since all juveniles: (1) had similar colony heights (2-4 mm), (2) received the same

irradiance, (3) contained the same quantity of symbionts (see below) and pigment concentrations in control treatments (see Results section), and (4) were tested using the same host coral species with identical corallite shapes thus reducing the influence of light scattering based on coral skeleton morphologies. The maximum relative electron transport rate of PSII ($rETR_{MAX}$) reflects the present state of photosynthesis and is strongly dependent on the immediate light pre-history of the sample (Schreiber 2004). These measurements differ from traditional photosynthesis-irradiance (P-E) curves derived from gas exchange measurements which effectively describe how the entire photosynthesis apparatus acclimates to different light intensities and are less dependent on light pre-history. RLCs calculated within this study were used comparatively among the different symbiont and diuron treatments to reflect differences in the current photosynthetic performance under the experimental light conditions.

Maximum quantum yield (Fv/Fm) values, an indicator for photosynthetic efficiency (Genty *et al.* 1989), were obtained from dark adapted symbionts (n=3 juvenile corals) following each RLC and an additional 10 min dark adaptation.

Radio-labeled ^{14}C incorporation

Following the fluorescence measurements, the volume of filtered seawater was reduced to 1 L and 1 mL of NaH14CO3 (specific activity 74 $MBq.mL^{-1}$, Amersham Biosciences, USA) was added to each tank. The water level was subsequently raised to 2 L to ensure equal distribution of radiolabel throughout the tank, and ^{14}C incubation was carried out for 6 hrs. Next, each tile was removed from the experimental light exposure and rinsed twice with fresh filtered sea water for 5 min to remove unincorporated ^{14}C from the surface of coral tissues. Digital images of the juveniles were taken from a standardized height on a tripod and the perimeter of each juvenile coral was then traced using image analysis software (Optimas, Media Cybernetics, Silver Spring, MD, USA). Surface area of each juvenile coral was calculated using Optimas (Negri *et al.* 2005). The coral juveniles were removed from the terracotta tiles with a scalpel, snap frozen in liquid nitrogen and stored at –80°C for later analyses. Tissue was removed from each juvenile coral (n = 9) by airbrushing and the host tissue was separated from the symbionts by centrifugation (490 g for 5 min). Host tissue samples (100 µL) were acidified with 0.1M HCl (100

μL) prior to scintillation counting, to remove unincorporated ^{14}C. Host tissue samples were counted on a 1450 Microbeta Plus scintillation counter (Perkin Elmer) for 2 min to determine disintegrations per minute (dpm). *Symbiodinium* cells were resuspended in 10% formalin and cell densities were determined with a haemocytometer. The radio labeled photosynthate incorporation was expressed as radioactivity per unit area of juvenile coral per dinoflagellate cell ($dpm.zoox^{-1}.cm^{-2}$) to standardize for the variations in the size of individual juveniles and the *Symbiodinium* densities they hosted.

***Symbiodinium* cp23S-rDNA real-time PCR**

To determine the relative abundance of symbiont types within *A. millepora* juveniles (immediately prior to ^{14}C studies), a sub-sample of *Symbiodinium* cells from each tissue slurry (above) was taken and fixed in absolute ethanol. Symbiont DNA was extracted following a previously published DNA isolation method (Wilson *et al.* 2002). Relative symbiont abundance within the *A. millepora* juveniles was determined using the cp23S-rDNA real-time PCR assay described in Chapter 2. Relative abundances (as a percentage of total copies per reaction) were calculated from the absolute number of copies of each *Symbiodinium* type per sample using standard lines.

Pigment analysis by HPLC

Chlorophylls and carotenoids were extracted sequentially by sonication (Cole Parmer Ultrasonic Processor, Extech Equipment, Victoria, Australia) of *Symbiodinium* cells (suspended in 100% acetone) that were separated from the host tissue (n=8 juvenile corals). High performance liquid chromatography (HPLC) was used to analyze the extracts on a Waters 600 HPLC, combined with a Waters PDA 996 photodiode array detector, on a 3 μm, 50 x 4.6 mm Phenomonex C 18 Gemini 110Å column (Phenomonex, NSW, Australia). A two-solvent gradient with a flow rate of 1 $mL.min.^{-1}$ for 18 min was used to separate the pigments. Percentages of the solvents A and B were, respectively: 0 min: 75, 25%; 0 to 5 min linear gradient to: 0, 100%; 5 to 10 min hold at: 0, 100%; 10 to 11 min linear gradient to: 75, 25%; 11 to 18 min hold at 75, 25%. Solvent A was 70:30 v/v methanol:28 mM tetrabutyl

ammonium acetate (TBAA, 1.0M aq. Sigma-Aldrich, Australia) and solvent B was 50:50 v/v methanol:acetone. Chlorophyll a, c2, peridinin and diadinoxanthin standards were obtained from the International Agency for ^{14}C Determination (DHI, Denmark). The peaks reported were identified by comparison of retention times and absorption spectra with standards and published data (Wright & Jeffrey 1997).

Data analysis

Two-way ANOVAs ($\alpha = 0.05$) were used to test the effect of *Symbiodinium* type and diuron concentration on $rETR_{MAX}$, Fv/Fm, ^{14}C incorporation and total pigment concentrations. Fisher's LSD post-hoc tests were used to identity statistical differences between and within treatments. Data were tested for assumptions of normality and homogeneity of variances; no transformations were required. All figures and curve-fittings to determine the characteristic parameters of the rapid light curves (Ralph *et al.* 2002) were created using Sigmaplot 2001 for Windows (v. 7.1, SPSS Inc.). Statistica v. 6.0 (StatSoft, Inc. Oklahoma, USA) was used for all statistical analyses. Rotor-Gene Analysis Software v. 6.0 (Corbett Research, NSW Australia) was used for all real-time PCR analysis.

RESULTS

Mean control cell densities were: C1 = 5.5 ± 0.4 (SE) x 10^5 cells.cm^{-2}, and D = 4.6 ± 0.8 x 10^5 cells.cm^{-2} (not significantly different). Real-time PCR assays confirmed that the raising of C1 and D corals was successful: C1 corals were estimated to contain a 98 – 100% relative abundance of C1, while D corals contained a 94 – 100% relative abundance of D (n = 27 for each group).

C1 corals produced, on average, 87% greater relative electron transport rates through PSII ($rETR_{MAX}$) than D corals in the absence of diuron (Fig. 1, 2A; Table 1). RLCs also revealed that the minimum saturating irradiance (E_K) was not significantly different between C1 and D corals (Fig. 1; Table 1), suggesting that the photosynthetic characteristics of the two genetically distinct symbionts were similarly acclimatized to low-light conditions. This is not surprising since the corals were raised for 9 months in the frequently turbid inshore waters of Magnetic Island.

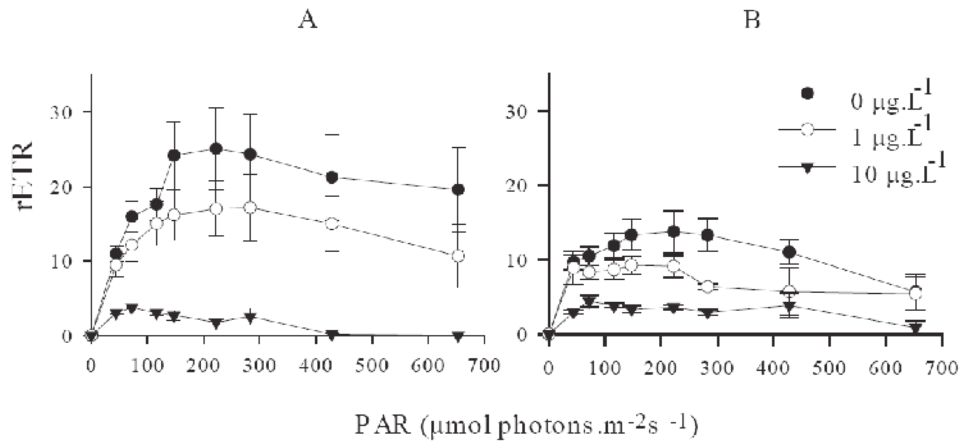

Fig. 1. Rapid light curves from C1 and D corals. Relative electron transport rate (rETR) as a function of photosynthetically active radiation (PAR, µmol photons.$m^{-2}.s^{-1}$) derived from colonies hosting *Symbiodinium* type C1 (A) and *Symbiodinium* type D (B) exposed to 3 diuron treatments (0, 1 and 10 µg.L^{-1}). n = 3 juveniles, mean ± SE.

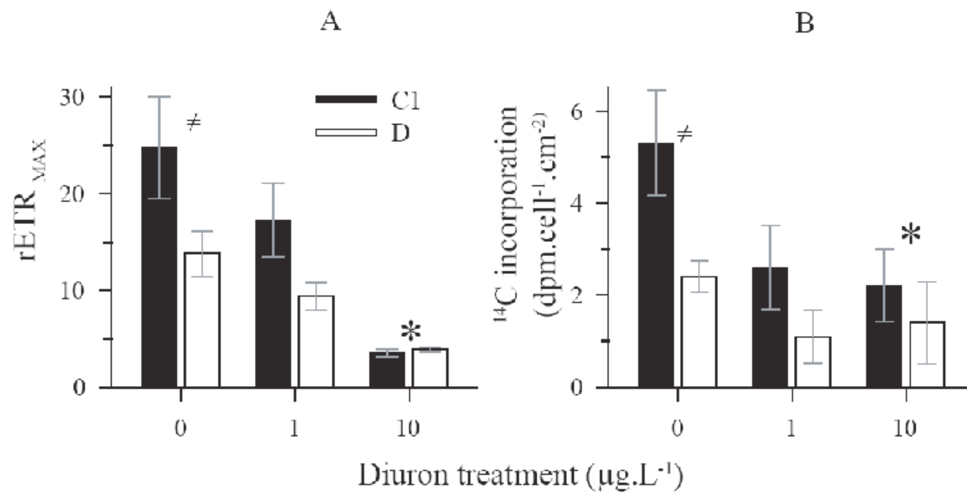

Fig. 2. A: photosynthetic capacity of PSII in C1 and D corals as measured by relative maximum electron transport rates (rETRmax) derived from rapid light curves (n = 3 juveniles, mean ± SE); B: photosynthate incorporation into C1 and D corals as measured by uptake of ^{14}C into host tissue (n = 9 juveniles, mean ± SE). * indicate significant differences ($p<0.05$) between the diuron treatments and the control (0 µg.L^{-1}), and ≠ indicate significant differences between symbiont types within a treatment.

Exposure of the corals to diuron significantly reduced the $rETR_{MAX}$ in both groups (Fig. 1 and 2A). At 10 $\mu g.L^{-1}$ diuron, the $rETR_{MAX}$ was reduced by 86% in C1 corals and by 71% in D corals. The maximum quantum yields (Fv/Fm) in dark adapted corals were identical for each symbiont type in the absence of diuron (Fig. 3; Table 1), indicating similar efficiencies of excitation energy capture by PSII for each symbiont (Genty *et al.* 1989). Diuron exposure caused similar reductions in Fv/Fm in both symbionts (Fig. 3; Table 1), indicating equivalent damage to the D1 protein of PSII in both symbiont types (Schreiber 2004).

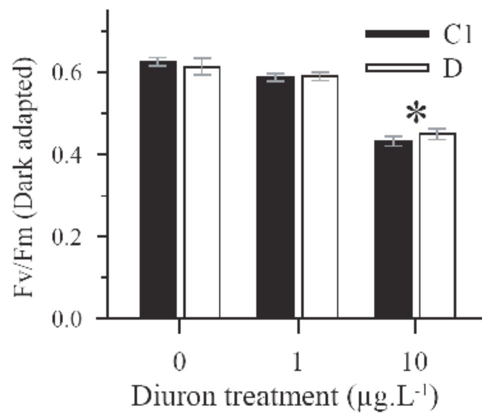

Fig. 3. Maximum quantum yields (Fv/Fm) for C1 and D corals exposed to 3 diuron treatments (n=3 juveniles, mean ± SE). * indicate significant differences ($p<0.05$) between the diuron treatments and the control (0 $\mu g.L^{-1}$).

Incorporation of radio labeled photosynthate (^{14}C, energy) into the host tissue was 121% greater within C1 corals than within D corals in the control treatments (Fig. 2B; Table 1). Diuron exposure reduced the incorporation of photosynthates into the tissues of both C1 and D corals (Fig. 2B). C1 corals exhibited a 58% drop in photosynthate accumulation when exposed to 10 $\mu g.L^{-1}$ diuron whereas D corals showed a 42% drop (Fig. 2B).

The pigments, chlorophyll a, c2 and peridinin, which constitute the major light harvesting complex (LHC) within dinoflagellates, were detected with a molar ratio of 1:0.3:0.5 within the controls. Diadinoxanthin was the major xanthophyll carotenoid, along with low concentrations of diatoxanthin. No differences in light harvesting and xanthophyll pigments were evident between symbiont types in the absence of diuron (Figs. 4). The 10 $\mu g.L^{-1}$ diuron treatment for 10 hrs resulted in an 108% increase in the total light harvesting and an 114% increase total xanthophyll pigments in C1 corals, whereas pigment concentrations did not change in D corals

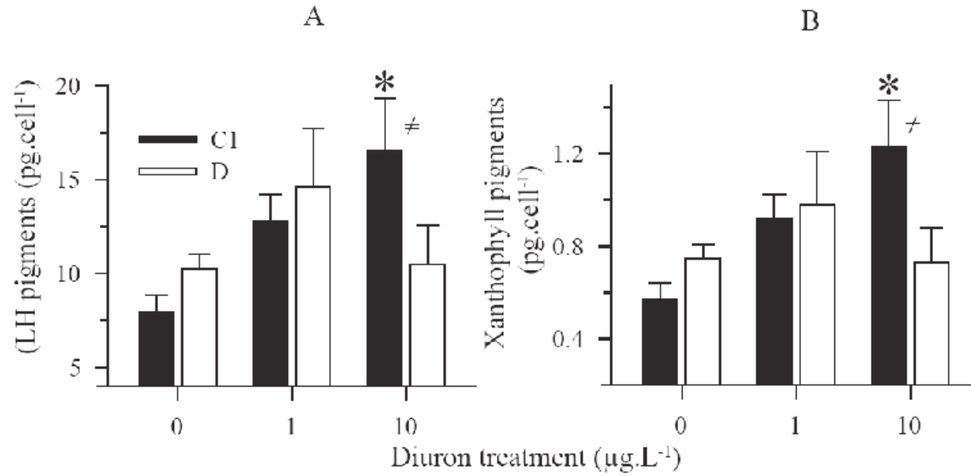

Fig. 4. Pigments of C1 and D corals exposed to 3 different diuron treatments (0, 1 and 10 $\mu g.L^{-1}$). A: concentrations of total light harvesting pigments (LH), comprising chlorophyll a and c2 and peridinin ($pg.cell^{-1}$, mean ± SE); B: concentrations of xanthophyll pigments, diadinoxanthin and diatoxanthin ($pg.cell^{-1}$, mean ± SE). * indicate significant differences ($p<0.05$) between the diuron treatments and the control (0 $\mu g.L^{-1}$) and ≠ indicate differences ($p<0.05$) between symbiont type.

Table 1. Two-way ANOVA results.

Group	*Type 3 SS*	*Df.*	*f*	*p*
rETR(MAX)				
Symbiont	171.02	1	6.85	0.02*
Diuron	737.57	2	14.76	0.00*
Symbiont*Diuron	103.18	2	2.07	0.23
E_K				
Symbiont	873.22	1	2.38	0.15
Diuron	3439.53	2	4.68	0.03*
Symbiont*Diuron	1252.90	2	1.71	0.22
Fv/Fm				
Symbiont	0.00017	1	0.05	0.82
Diuron	0.66	2	103.76	0.00*
Symbiont*Diuron	0.0039	2	0.61	0.52
^{14}C incorporation				
Symbiont	33.84	1	6.69	0.02*
Diuron	41.54	2	4.11	0.01*
Symbiont*Diuron	8.53	2	0.84	0.41
LH pigments				
Symbiont	1.82	1	0.25	0.62
Diuron	46.45	2	3.14	0.05
Symbiont*Diuron	37.56	2	2.54	0.09
Xanthophyll pigments				
Symbiont	0.023	1	0.52	0.47
Diuron	0.25	2	2.76	0.07
Symbiont*Diuron	0.26	2	2.91	0.07

(Fig. 4; Table 1). No changes in pigment ratios were observed for either symbiont type at any of the diuron concentrations. The time frame used to rinse each juvenile coral at lower light intensity (approx 15-30 µmol photons.m^{-2}.s^{-1} for 10-15 min) prior to freezing may have influenced the xanthophyll pigment ratios (conversion of diatoxanthin back to diadinoxanthin in the absence of light stress (Brown *et al.* 1999), however, this is unlikely to have influenced the total xanthophyll pool.

DISCUSSION

C1 corals exhibited a 121% greater capacity for translocation of photosynthates to the coral tissue, and had an 87% greater relative electron transport rate through photosystem II compared to D corals under identical environmental conditions. This indicates that the genetic identity of *Symbiodinium* spp. can influence the nutritional benefits available to a coral holobiont provided through photosynthesis. *Acropora tenuis* and *A. millepora* juveniles in a previous study exhibited 2-3 times faster growth rates when associated with C1 compared to those associated with D at the same field site where the custom corals were reared in the present study (Little *et al.* 2004, Chapter 5). This symbiont effect on coral growth may result from the difference in photosynthate translocation between C1 and D corals as demonstrated here. The differences in carbon based energy transfer between symbiont types may, therefore, provide a competitive advantage to corals when associating with *Symbiodinium* C1 compared to D. This is particularly true during their early life histories, as greater energy investment into rapid tissue and skeletal growth can prevent overgrowth of juveniles by competitors and mortality from grazers (Hughes & Jackson 1985; Chapter 5).

The simultaneous reduction in photosynthetic capacity and ^{14}C incorporation into coral tissues of C1 and D corals exposed to 10 µg.L^{-1} diuron supports a strong link between these two processes. Under conditions of severe electron transport inhibition (10 µg.L^{-1} diuron) and at the PAR levels of this experiment, C1 corals received the same ^{14}C allocation from their symbionts as D corals. Therefore, C1 corals may lose their potential for more rapid growth and any competitive advantage over D corals under stressful conditions that limit electron transport.

The actual relationship between host photosynthate incorporation and photosynthetic capacity is complex, and resolving it will be (experimentally)

challenging. First, the self-shading of symbionts may lead to an over-estimation of rETR compared with oxygen evolution (Hoogenboom *et al.* 2006). For our experiment it was assumed that self-shading did not affect the relative comparisons, as the heights, corallite morphology, symbiont numbers and pigment concentrations were similar in C1 and D corals between control treatments, leading to a consistent effect between symbiont types. Second, electron transport through PSII, as measured by PAM fluorometer, and whole-organism photosynthesis (O_2 or CO_2 flux), as measured by respirometry, are not likely to be equivalent over a wide range of PAR (Ulstrup *et al.* 2006). Third, Hoogenboom *et al.* (2006) demonstrated that the saturation of O_2 evolution in corals can occur at lower PAR than rETR saturation, which is indicative of a non-assimilatory electron flow through PSII. Fourth, the efficiency of photosynthate transfer to the host and specific molecular allocation of fixed carbon can differ between different symbiont types. For instance, in the sea anemone *Condylactis gigantea*, the percentage photosynthetically fixed ^{14}C translocated and specifically incorporated into the lipid fraction of the host was significantly higher in A anemones compared to B anemones under normal conditions (25°C). ^{14}C uptake in the present study was only measured in the host tissue, not in the symbiont, and specific molecular allocation was not investigated. Future studies should take these variables into account.

The identical maximum quantum yields (Fv/Fm) in dark adapted samples for each symbiont type in the absence of (diuron-induced) stress indicate similar efficiencies of excitation energy capture by PSII for each symbiont type (Genty *et al.* 1989), consistent with previous reports (Rowan 2004; Berkelmans & van Oppen 2006; Warner *et al.* 2006). Reductions in Fv/Fm at 10 $\mu g.L^{-1}$ diuron were similar for both symbiont types tested in this study, indicating equivalent levels of damage to the D1 protein of PSII (Genty *et al.* 1989). Marine and freshwater algal species have been shown in culture to display inter-species differences in sensitivity to low diuron concentrations within toxicity tests (Nash *et al.* 2005). It is plausible that longer exposure to diuron and exposures at higher irradiances might reveal similar differences in diuron sensitivity between symbiont types, analogous to the PSII damage observed during longer exposure experiments to thermal stress. For example, far greater reductions in Fv/Fm were observed in C2 symbionts in adult *A. millepora* exposed to elevated seawater temperatures than for the more thermally tolerant D symbionts in the same species (Berkelmans & van Oppen 2006). A

similar difference between clades C and D was found in *Pocillopora* spp. reared at 32°C (Rowan 2004). It has to be kept in mind, however, that relationships between photoacclimation and growth are not always consistent (Warner *et al.* 2006).

Photosynthetic capacity can be enhanced by an increase in light harvesting pigments, which capture the photons at the beginning of the electron transport chain (Walters 2005). Xanthophyll carotenoid pigments are used for non-photochemical quenching (NPQ), preventing oxidative damage from radical oxygen species and reducing damage caused by high light and herbicide exposure (Muller *et al.* 2001). In the control treatments, no differences in pigments between C1 and D corals were found, indicating that the higher $rETR_{MAX}$ of C1 corals was not the result of pigment adjustments. However, the 10 $\mu g.L^{-1}$ diuron treatment resulted in doublings in both the light harvesting and xanthophyll pigments in *Symbiodinium* C1, whereas pigment concentrations did not change in type D symbionts. Under these conditions, a large proportion of the PSII reaction centers remains inactive due to photoinhibition and damage of the D1 protein (Jones *et al.* 2003). It is possible that rapid pigment biosynthesis was stimulated in *Symbiodinium* C1 in an attempt to compensate for the reduced electron transport. This type of rapid pigment biosynthesis has been reported for high light acclimated green alga *Dunaliella salina* following a 12 h transition to low illumination (Masuda *et al.* 2002). However, this apparent upregulation of pigments was unable to compensate for reduced rETR, since electron transport was reduced under these conditions to the same level as for *Symbiodinium* D.

In conclusion, this study identified the potential energetic consequences to the coral host of association with genetically distinct types of the algal endosymbiont, *Symbiodinium*, that differ intrinsically in their photophysiology. It demonstrated that photosynthetic performance, as measured by photosynthate incorporation (carbon-based energy) and PSII relative electron transport, was significantly greater within *Symbiodinium* C1 compared to *Symbiodinium* D, which might explain the influence that symbiont type has previously been shown to have on juvenile coral growth (Little *et al.* 2004, Chapter 5). Therefore, coral juveniles associated with high-performance algal symbionts are predicted to have a competitive advantage under certain field conditions, as rapid early development typically limits mortality. These findings advance the understanding of the dynamic relationship between the coral host and its symbiotic partner.

ACKNOWLEDGEMENTS

We thank Jason Doyle and Lesa Peplow for technical advice, Andrew Baird for comments on the experimental design and Peter Ralph for critical reading of the manuscript. This work was supported by a grant from AIMS@JCU, and J.C.M. was supported by a grant from NWO-WOTRO (Project no. W84-576).

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Chapter 8

Summary and conclusions

SUMMARY

Scleractinian corals are ecosystem engineers and the growth and maintenance of coral reefs relies heavily on the success of these organisms. A key aspect of their success is the symbiotic relationship with single-cell algae of the genus *Symbiodinium* — also known as zooxanthellae. These algal (endo)symbionts provide the coral host with most of its nutrition and enhance the calcification process (skeletal growth), allowing it to thrive in oligotrophic waters. However, climate change is threatening this association, as seawater temperatures of only 1°C above the long-term mean summer maximum can lead to a disruption of the symbiosis. When this happens, the zooxanthellae are expelled from the host tissues. If zooxanthella loss is severe, corals turn bright white as their calcium skeleton becomes visible through the now-transparent host tissue. This process is, therefore, referred to as coral bleaching. Severe and/or prolonged bleaching can result in colony mortality.

Zooxanthellae are, however, not all the same. A huge diversity of phylotypes has been identified within the genus *Symbiodinium* (presently eight “clades” are distinguished, with multiple “types” within each clade) and many of these symbiont types are strongly correlated with differences in holobiont (coral host plus algal symbionts) physiological performance — heat tolerance in particular. Thus, it has been hypothesized that association with different symbiont types provides physiological flexibility to corals and, therefore, a mechanism to respond to rising seawater temperatures. Bleaching — as stated in the Adaptive Bleaching Hypothesis (ABH) — provides an opportunity for corals to change their resident symbiont community from a heat-susceptible one into a more heat-tolerant one, and, as a result, become more thermo-tolerant themselves.

The central aim of this thesis was to assess the potential of the mechanisms described by the ABH to: (1) induce changes in the symbiotic communities of coral, and (2) mitigate the effects of global warming on coral reefs.

The first question that was asked was:

What is the source of the new, heat-tolerant symbiont type that becomes dominant while the coral is recovering from bleaching?

Two possible sources were identified: (1) the new symbiont is already present al low abundance in the coral host before bleaching, and increased in relative abundance (symbiont shuffling), or (2) the new symbiont is taken up from the environment (symbiont switching).

In **Chapter 2** the potential for symbiont *shuffling* was assessed. At the beginning of the study (April 2004), it was generally believed that most corals only harbored one clade of symbionts, implying a low potential for symbiont shuffling because the mechanism requires a diverse *in situ* symbiotic community. However, we questioned this notion as a possible artifact of the limited detection abilities of the techniques commonly used in symbiont diversity studies. Electrophoretic techniques have a high resolution for discriminating different symbiont types, but a low sensitivity for detecting symbiont types that occur at (very) low, background levels — symbiont types occurring in relative abundances below 5-10% of the total symbiont community would therefore have missed detection.

We developed a novel, real-time PCR assay that boosted the sensitivity for background clade detection over 100-fold. This assay was then used to re-screen a sample collection consisting of four common species of hard corals collected across the Great Barrier Reef (GBR) of Australia. These samples had been previously analyzed using standard electrophoretic techniques and only one symbiont clade per sample had been detected in all cases. Using our new real-time PCR assay, we were able to show that 78% of the corals sampled actually did harbor a background clade, indicating that the potential for symbiont shuffling was much larger than previously thought. Notably, most corals predominantly harbored *Symbiodinium* clade C, with the clade D occurring at background levels, and clade D zooxanthellae are generally considered to be more thermo-tolerant.

The real-time PCR assay was further developed in **Chapters 3 and 4**. The initial assay used the ITS1 region, which exists in the genome in many tandem-

repeated copies. The number of copies can vary significantly between closely relate cells (e.g. within a type), which resulted in a loss of accuracy because of uncertainties in the translation of measured ITS1 copy numbers to symbiont cell numbers. In the optimized assay, introns of the actin genes were targeted (which were established to be single-to-low copy number loci in the symbiont genome), which increased the accuracy of the assay an estimated 10-fold. Another improvement was the introduction of a symbiont density measure — the symbiont cell/ host cell ratio (S/H ratio) — by determining the cell numbers of both the coral host and the algal symbionts within a DNA sample. The high variability of the intron region also allowed type-specific analyses within clade C.

In **Chapter 4**, the real-time PCR assay was used to investigate the potential for symbiont *switching*. Nine colonies of the common reef-building coral *Acropora millepora* — which harbored predominantly *Symbiodinium* clade D with a type C1 background — were experimentally bleached using the herbicide DCMU (diuron). The corals were then allowed to recover in the presence of high levels of environmental zooxanthellae belonging to *Symbiodinium* type C2*. Real-time PCR analyses showed that the corals underwent a moderate to severe bleaching response, followed by significant recovery. However, unforeseen problems related to the used coral population and the environmentally administered atypical symbiont type led to inconclusive results; improvements to the experimental design are discussed to assist future studies evaluating this mechanism of symbiont change.

The next set of questions was:

> **To what extent does the symbiont type shape coral fitness, and what are the relative contributions of the coral host and the local environmental conditions?**
>
> **Are there trade-offs between heat-tolerance and other favorable characteristics (such as high growth rate) and what defines them?**

Previous studies convincingly showed how symbiont type shaped certain physiological parameters. However, it was still largely unknown how strong the

influence of the symbiont type was on the holobiont's fitness compared to coral host population and/or the local environmental conditions, and how these factors affected each other. In order to study all three factors simultaneously, custom holobionts were prepared, i.e., coral-*Symbiodinium* associations from which the genetic make-up of both the coral host and the algal symbiont were experimentally controlled **(Chapter 5)**. *A. millepora* colonies were collected from two contrasting locations and allowed to spawn in the laboratory. Azooxanthellate juveniles were then exposed to six types of *Symbiodinium* spanning clades A, C and D. Once symbioses with the newly settled coral juveniles were established, the custom holobionts were (partially reciprocally) outplanted back to the two contrasting locations of the two original *A. millepora* populations. Over the next 7-8 months, their growth and survival were monitored, after which the custom holobionts were returned to the lab for the assessment of their thermo-tolerance. The results showed that symbiont type was the strongest predictor for holobiont fitness. In contrast, almost no host population effects were evident on growth, survival, or heat-tolerance. D holobionts were the most thermo-tolerant, whereas A holobionts were the most thermally sensitive.

The ABH predicts that trade-offs are likely to exist between thermo-tolerance and other favorable characteristics, explaining why thermo-tolerant symbionts are not dominant in the absence of heat-stress. We found a trade-off between growth (and, to a lesser extend, survival) and heat-tolerance. However, this trade-off was dependent on the environment, as growth (and survival), but not heat-tolerance, was secondarily shaped by the local environmental conditions.

The next question asked was:

What is the field evidence for the ABH?

In a collaborative project led by fellow PhD candidate Alison Jones (Central Queensland University, Australian Institute of Marine Science), seventy-nine corals of an *A. millepora* population situated on a southern inshore reef of the GBR were randomly tagged and followed through a natural bleaching event (**Chapter 6)**. Prior to bleaching, the population harbored predominantly thermo-sensitive *Symbiodinium* C2 (93.5%), while the remaining corals harbored a more tolerant *Symbiodinium* type

belonging to clade D or mixtures of C2 and D. After bleaching, 71% of the surviving colonies that harbored C2 changed to D or C1 predominance. Corals that were associated with C2 before bleaching had higher mortality rates (37%) than colonies that already were associated with D (8%). In total, only about 18% of the original *A. millepora* population survived unchanged, showing the magnitude of the effect of the bleaching event on the S*ymbiodinium* community structure for this population. The change towards more thermally tolerant symbiont types, as predicted by the ABH, is likely to have substantially increased the thermal tolerance of this coral population. This study showed, for the very first time, that symbiont change after bleaching does happen at large scales under field conditions.

The last question investigated in this thesis was inspired by the results described in Chapter 5:

> **What is the cause of the large difference in growth between *A. millepora* juveniles harboring C1 or D at Magnetic Island?**

In **Chapter 7**, a second collaborative project was conducted with fellow PhD candidate Neal Cantin (James Cook University, Australian Institute of Marine Science). This study investigated the link between the photosynthetic and photosynthate incorporation in 9-month old C1 and D corals (*A. millepora*). The results showed that C1 corals had an 87% greater PSII photosynthetic capacity than D corals, and that this was correlated with a 121% higher translocation of photosynthate to the coral host. Exposure to diuron (DCMU, a herbicide that inhibits electron transport) resulted in a loss of the difference between C1 and D holobionts for both photosynthetic capacity and photosynthate translocation, further supporting the link between these two processes. We concluded that the greater carbon delivery from *Symbiodinium* C1 allowed faster growth, which most likely provided the C1-holobionts with a competitive advantage since rapid early development typically limits mortality (see Chapter 5).

CONCLUSIONS

The severe damage to coral reefs caused by climate change over the last decades has raised major concerns about the future of these ecosystems (Hoegh-Guldberg 1999; Hoegh-Guldberg *et al.* 2007). The research described in this thesis has found significant support for the ABH (Buddemeier & Fautin 1993; Buddemeier *et al.* 2004) as a mechanism — available to at least certain corals — for responding to the threat of rising seawater temperatures. Our evidence includes: (1) the high incidence of (thermally tolerant) clade D backgrounds, that may allow corals to shuffle their symbionts after bleaching, in four of the most common scleractinian species on the GBR, (2) the realization that symbiont type (at least in *A. millepora*) is the most important predictor of holobiont fitness, and (3) a *Symbiodinium* community change in an *A. millepora* population following a natural bleaching event, resulting in an increased thermo-tolerance for that population. These results showed that changing algal symbionts is an important mechanism for reef acclimatization in an era of climate change.

Many aspects of the ABH are still unknown, limiting our ability to predict the acclimatization potential, and further studies are urgently needed. First, the analyses of symbiont backgrounds have to be expanded to include other coral species and locations. We must also move to the subcladal level with these surveys, as physiological differences between *Symbiodinium* types within a clade can be as important as differences between types from different clades (Tchernov *et al.* 2004). Second, the generality of symbiont shuffling has to be determined, i.e., how many coral species can actually do it and do the same symbiont changes have similar effects in different coral species (e.g. Abrego *et al.* 2008)? Third, symbiont switching needs to be further examined; if it can happen on a similar (or larger) scale as shuffling, reef resilience would be significantly affected. Fourth, the influence of more symbiont types on the holobiont physiology needs to be examined, which can be accomplished by raising custom holobionts. Finally, the possibility of symbiont change over generations, rather than during the coral colony's life, should be investigated (Baird *et al.* 2007).

The capacity of the ABH to mitigate the effects of global warming must not be overestimated for several important reasons. First, coral colonies may have to bleach first before symbiont change can take place (Buddemeier & Fautin 1993; Baker 2001; Berkelmans & van Oppen 2006), possibly causing high mortality in the process. Second, newly shuffled (or switched) corals that have successfully recovered from bleaching are still likely to be impaired in growth and reproduction (Baird & Marshall 2002). Third, if the stressor disappears for a prolonged period of time, the corals may change back to the original symbiont (Thornhill *et al.* 2006), leaving them again vulnerable to the next round of bleaching events. Last, the maximum extra heat-resistance that corals may gain by changing their symbionts may only be 1-1.5°C (Berkelmans & van Oppen 2006), which will be insufficient within the coming century if the most recent predictions of the Intergovernmental Panel for Climate Change are accurate (1.5-4°C increase in the tropics by the end of this century; IPCC 2007).

On a more optimistic note, symbiont change is likely to play a positive role in the way some corals cope with global warming conditions, leading to new competitive hierarchies and, ultimately, helping to shape the coral community assemblages of the future. Most importantly, an increase in reef thermo-tolerance of 1-1.5°C buys time — approximately 50 years (Donner *et al.* 2005) — in which measures for the reduction of greenhouse gas emissions can be implemented so that, hopefully, a catastrophic effect of climate change on coral reefs is avoided.

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Chapter 9

Nederlandstalige samenvatting en conclusies

SAMENVATTING

Koralen van de orde Scleractinia zijn belangrijke rifbouwers en de aanwas en onderhoud van koraalriffen is in grote mate afhankelijk van het succes van deze organismen. Een sleutelaspect in hun succes is de symbiotische relatie met eencellige algen van het genus *Symbiodinium* — ook wel zoöxanthellen genoemd. Deze endosymbionten voorzien de koraalgastheer van het meerendeel van zijn voedsel en versterken het calcificatieproces (de groei van het skelet), wat hem in staat stelt succesvol te gedijen in voedselarme wateren. De symbiose wordt echter ernstig bedreigd door de klimaatsopwarming, want zeewatertemperaturen van slechts 1°C hoger dan de gemiddelde maximum zomertemperatuur (over de lange termijn) kan de associatie al verstoren. De zoöxanthellen worden dan uit het weefsel van de koraalgastheer geworpen. Wanneer een koraal het overgrote deel van zijn zoöxanthellen verliest wordt hij spierwit omdat het kalkskelet zichtbaar wordt door het - nu transparante - gastheerweefsel. Daarom wordt dit proces koraalverbleking genoemd. Sterke en/of langdurige verbleking kan leiden tot sterfte van de koraalkolonie.

Zoöxanthellen zijn echter niet allemaal hetzelfde. Een enorme diversiteit van phylotypen is geïdentificeerd binnen het genus *Symbiodinium*: acht “clades” worden momenteel onderscheiden, met meerdere “typen” binnen elke clade. Vaak is er een sterke correlatie tussen zoöxanthella type en fysiologische eigenschappen van de koraal holobiont (koraalgastheer plus endosymbionten) — in het bijzonder hittebestendigheid. Dit heeft aanleiding gegeven tot de hypothese dat associatie met verschillende zoöxanthella typen een koraal fysiologische flexibiliteit kan geven, wat een mechanisme kan zijn om te reageren op een stijging in zeewatertemperaturen. Verbleking, zoals beschreven word in de Adaptieve Verbleking Hypothese (AVH), geeft koralen een kans om hun endosymbiont-gemeenschap om te ruilen van een hittegevoelige naar een meer hittebestendige gemeenschap om daarmee zelf meer hittebestendig te worden.

Het centrale doel van dit proefschrift was om het potentieel te bepalen van de mechanismen beschreven in de AVB om: (1) veranderingen in de endosymbiontische gemeenschappen van koralen te induceren, en (2) de effecten van klimaatsopwarming op koraalriffen te matigen.

De eerste onderzoeksvraag was:

Wat is de oorsprong van de nieuwe, hittebestendige endosymbiont die dominant wordt als een koraal herstelt van verbleking?

Twee mogelijkheden werden geïdentificeerd: (1) de nieuwe endosymbiont zit al in zeer lage concentraties in de weefsels van de koraalgastheer voor de verbleking en neemt toe in relatieve dichtheid tijdens herstel (endosymbiont-omwisseling), of (2) de nieuwe endosymbiont wordt opgenomen uit de omringende waterkolom (endosymbiont-omschakeling).

In **hoofdstuk 2** werd het potentieel voor endosymbiont-*omwisseling* onderzocht. Aan het begin van deze studie (april 2004) werd over het algemeen aangenomen, gebaseerd op de toen beschikbare literatuur, dat het merendeel van de koralen zoöxanthellen van slechts één clade bevatten. Dit gaf aan dat het potentieel voor endosymbiont-omwisseling laag was, want dit mechanisme heeft een diverse endosymbiont-gemeenschap binnen een koraalkolonie nodig. We vroegen ons echter af of deze opvatting wellicht veroorzaakt werd door een technisch artefact met betrekking tot de detectiegevoeligheid van de gebruikte electroforese-technieken. Deze technieken hebben een hoge resolutie voor het discrimineren van verschillende clades van zoöxanthellen (en typen binnen de clades), maar een erg lage gevoeligheid voor het detecteren van extra endosymbiont clades/typen die aanwezig zijn in lage achtergronddichtheden — endosymbiont clades/typen die aanwezig zijn in relatieve dichtheden van 5-10% van de totale endosymbiont-gemeenschap werden dus niet gedetecteerd.

Een nieuwe, kwantitatieve PCR test werd ontwikkeld die de gevoeligheid voor achtergrondclades met meer dan 100 keer vergrootte. Deze test werd gebruikt om een collectie van monsters van vier algemene hardkoraal soorten, verzameld over de breedte van het Groot Barrière Rif (GBR) in Australië, te heranalyseren. De

collectie was eerder al geanalyseerd met de standaard electroforese-technieken, en in alle gevallen werd slechts één endosymbiont clade per monster gedetecteerd. De nieuwe test liet echter zien dat 78% van de monsters een endosymbiont-achtergrond bevatte van een andere clade, wat aangaf dat het potentieel voor endosymbiont-omwisseling veel groter was dan voorheen werd aangenomen. Opmerkelijk was ook dat de meeste koralen voornamelijk clade C zöoxanthellen bevatten met op de achtergrond clade D zoöxanthellen, die over het algemeen wordt gezien als meer hittebestendig.

De kwantitatieve PCR test van hoofdstuk 2 werd verder ontwikkeld in de **hoofdstukken 3 en 4**. De initiële test gebruikte een DNA regio genaamd ITS1, welke in het genoom voorkomt in een groot aantal kopieën. Het aantal kopieën kan sterk variëren tussen nauw verwante cellen (b.v. binnen een endosymbiont type), wat een gereduceerde precisie van de test veroorzaakte door de onzekerheid in de vertaling van gemeten ITS1 kopieën naar endosymbiont-cel aantallen. In de nieuwe test werden intronen van het actine gen gemeten (deze bleken in één of slechts enkele kopieën in het genoom van zoöxanthellen voor te komen), wat de precisie van de test met ongeveer 10 keer verhoogde. Een andere aanpassing was de introductie van een endosymbiont-dichtheidsbepaling — de endosymbiont-cel/gastheercel ratio (E/G ratio) — door in de DNA monsters het aantal cellen van zowel de endosymbiont als gastheer te bepalen. De hoge variabiliteit van de intronensequenties binnen een clade maakte het ook mogelijk om type-specifieke (i.p.v. clade-specifieke) analyses uit te voeren.

De kwantitatieve PCR test werd gebruikt in **hoofdstuk 4** om het potentieel van endosymbiont-*omschakeling* te onderzoeken. Kolonies van het algemeen voorkomende koraal *Acropora millepora*, die voornamelijk clade D zoöxanthellen bevatten met een type C1 achtergrond, werden experimenteel gebleekt met behulp van het herbicide DCMU (Diuron). Tijdens de herstelfase werden hoge concentraties van endosymbiont-type C2* toegevoegd aan het zeewater. Kwantitatieve PCR analyses lieten zien dat de koraalkolonies een matig tot sterke verbleking ondergingen, gevolgd door een significant herstel in de endosymbiont dichtheden. Er waren echter onvoorziene complicaties, veroorzaakt door de selectie van koraalpopulatie en het toegevoegde atypische endosymbiont, wat ertoe leidde dat de

resultaten moeilijk interpreteerbaar waren. Verbeteringen aan de experimentele opzet worden besproken om toekomstige studies te helpen met het bestuderen van endosymbiont-omschakeling.

De volgende onderzoeksvragen waren:

> **In hoeverre bepaald het endosymbiont type de fitness van een koraal en wat zijn de relatieve contributies van de koraalgastheer en de locale omgevingsfactoren?**
>
> **Bestaan er afwegingen tussen hittebestendigheid en andere gunstige endosymbiont-gerelateerde karakteristieken (bv. snelle groei) en waarvan zijn die afhankelijk?**

In eerdere studies was overtuigend aangetoond dat endosymbiont type een sterk effect kan hebben op fysiologische parameters van de holobiont. Het was echter grotendeels onbekend hoe sterk de invloed van koraalpopulatie en/of de locale omgevingscondities zijn relatief aan het endosymbiont effect, en hoe deze factoren elkaar beïnvloedden. Om deze drie factoren tegelijkertijd te kunnen bestuderen werden experimentele holobionten geproduceerd, d.w.z. koraal-endosymbiont associaties waarvan de genetische opmaak van zowel de koraalgastheer als de endosymbionten experimenteel werden gecontroleerd. Deze experimenten worden besproken in **hoofdstuk 5**. Kolonies van *A. millepora* werden verzameld van twee verschillende locaties net voor de jaarlijkse reproductie, waarbij massale hoeveelheden ei- en zaadcellen worden afgegeven aan de waterkolom. De ei- en zaadcellen werden verzameld, en de eicellen werden gecontroleerd bevrucht per koraalpopulatie. Nadat de eicellen waren opgegroeid tot (asymbiotische) koraalpoliepen, werden ze blootgesteld aan zes verschillende endosymbiont-typen van drie verschillende clades (A, C en D). De zo gevormde experimentele holobionten werden uitgezet op de twee lokaties waar de moederkolonies waren verzameld. De volgende 7 à 8 maanden werden de groei en sterfte bijgehouden, waarna de hittebestendigheid werd bepaald in het lab. De resultaten lieten zien dat het endosymbiont-type de sterkste voorspeller was van de fitness van de experimentele holobionten. De koraalpopulatie, aan de andere kant, had zo goed als

geen invloed op de groei, sterfte en hittebestendigheid. D-holobionten waren het meest hittebestendig, terwijl A-holobionten het meest hittegevoelig waren.

De AVH voorspelt het bestaan van afwegingen tussen hittebestendigheid en andere gunstige endosymbiont-gerelateerde karakteristieken, waarmee verklaard kan worden waarom hittebestendige endosymbionten niet dominant aanwezig zijn bij afwezigheid van hittestress. Wij vonden bewijs voor een afweging tussen groei (en, in mindere mate, sterfte) en hittebestendigheid. Deze was echter afhankelijk van de omgeving, omdat groei (en sterfte), maar niet hittebestendigheid, secondair werd beïnvloed door de locale omgevingsfactoren.

De volgende vraag in dit promotieonderzoek was:

Welk bewijs is er te vinden voor de AVH op het rif?

Een gezamenlijk onderzoek geleid door collega-promovendus Alison Jones (Central Queensland University, Australian Institute of Marine Science) volgde de endosymbiont-gemeenschap van een *A. millepora* populatie op een rif aan de zuidkant van het GBR in Australië. Negenenzeventig kolonies werden gemerkt en monsters werden genomen voor, tijdens en na een natuurlijke verblekingsperiode (**hoofdstuk 6**). De koraalpopulatie was voor de verbleking voornamelijk geassocieerd met het hittegevoelige endosymbiont-type C2 (93.5% van de kolonies, de overigen hadden D of een mix van C2 en D zoöxanthellen). Na de verblekingsperiode was 71% van de kolonies, die eerst geassocieerd waren met C2, verandert in een associatie met D of C1. Koralen die initieel C2 huisvestten hadden een hoger sterfte (37%) dan kolonies die D hadden (8%). In totaal was slechts 18% van de overlevende kolonies onveranderd t.o.v. voor de verbleking. Het is waarschijnlijk dat de verandering naar meer hittebestendige endosymbionten, zoals voorspeld door de AVH, een substantiële verhoging van de hittebestendigheid van de koraalpopulatie tot gevolg had. Dit was de eerste studie die liet zien dat verandering van endosymbiont-gemeenschappen kon gebeuren op de grote schaal onder natuurlijke condities.

De laatste vraag die werd onderzocht in deze dissertatie werd geïnspireerd door de resultaten beschreven in hoofdstuk 5:

Wat veroorzaakt het grote verschil in groeisnelheid tussen *A. millepora* kolonies die geassocieerd zijn met C1 of D zoöxanthellen?

In **hoofdstuk 7** wordt een tweede gezamenlijk onderzoek beschreven, geleidt door collega-promovendus Neal Cantin (James Cook University, Australian Institute of Marine Science). Deze studie keek naar de connectie tussen the fotosynthetische activiteit en incorporatie van fotosyntheseproduct in 9-maanden oude C1- en D-holobionten van *A. millepora*. De resultaten lieten zien dat C1-koralen een 87% hogere fotosynthetische capaciteit hadden dan D-koralen en dat dit verschil gecorreleerd was met een 121% hogere translocatie van fotosyntheseproduct naar de koraalgastheer. De verschillen in fotosynthetische capaciteit en fotosyntheseproduct-translocatie verdwenen door toevoeging van DCMU, een herbicide dat de elektrontransportketen blokkeert, wat de link tussen de twee processen verder onderbouwde. We konden concluderen dat de hogere groeisnelheid van C1-koralen zeer waarschijnlijk veroorzaakt werd door de hogere fotosynthetische capaciteit en fotosyntheseproduct-translocatie van C1-zoöxanthellen, en dat C1-koralen een competitief voordeel over D-koralen hebben omdat een snelle vroege ontwikkeling typisch gerelateerd is aan een lager sterftecijfer (zie hoofdstuk 5).

CONCLUSIES

De ernstige schade die de afgelopen jaren aan koraalriffen is toegebracht door de klimaatsopwarming heeft ernstige zorgen opgeroepen over de toekomst van deze ecosystemen (Hoegh-Guldberg 1999; Hoegh-Guldberg *et al*. 2007). In dit proefschrift zijn sterke aanwijzingen gevonden dat de mechanismen van de AVH (Buddemeier & Fautin 1993; Buddemeier *et al*. 2004) beschikbaar zijn voor tenminste sommige koraalsoorten en koraalriffen in staat kunnen stellen te reageren op de opwarming van de oceanen. Onze aanwijzingen zijn: (1) de aanwezigheid van (hittebestendige) clade D achtergronden, die gebruikt kunnen worden voor endosymbiont-omwisseling, in vier van de meest algemene scleractinische koraalsoorten op de GBR, (2) de realisatie dat endosymbiont-type (voor *A. millepora*) de belangrijkste voorspeller is van holobiont fitness (gemeten via groei, sterfte en hittetolerantie), en (3) een verandering in de endosymbiont-gemeenschap

van een koraalpopulatie gemeten na een natuurlijke verblekingsperiode, welke resulteerde in een verhoging van de hittebestendigheid van die populatie. Deze resultaten laten zien dat het aanpassen van endosymbiont-gemeenschappen een belangrijk mechanisme is voor de acclimatisatie van koralen in een tijd van klimaatsverandering.

Veel aspecten van de AVH zijn echter nog onbekend, wat ons potentieel voor het voorspellen van koraalacclimatisatie limiteert, en verdere studies zijn dan ook hard nodig. Ten eerste moeten veel meer koraalsoorten en locaties getest worden op de aanwezigheid van endosymbiont-achtergronden. Deze tests moet verder ontwikkeld worden zodat de zoöxanthellen op het subclade-niveau kunnen worden onderscheiden, daar typen van eenzelfde clade evenveel fysiologisch kunnen verschillen als typen van verschillende clades (Tchernov *et al*. 2004). Ten tweede, de algemeenheid van endosymbiont-omwisseling moet worden bepaald, d.w.z. hoeveel koraalsoorten kunnen het en hebben dezelfde veranderingen hetzelfde effect in verschillende koraalsoorten (e.g. Abrego *et al*. 2008)? Ten derde, het mechanisme van endosymbiont-omschakeling moet verder worden onderzocht; als dit mechanisme bestaat op een vergelijkbare (of grotere) schaal dan endosymbiont-omwisseling kan dit zeer significant zijn voor de veerkracht van koraalriffen. Ten vierde, de invloed van meer endosymbiont-typen op de fysiologie van koraalholobionten moet onderzocht worden, wat gedaan kan worden via het maken van experimentele holobionten. Tenslotte moet de mogelijkheid dat endosymbionts-veranderingen eerder over generaties dan tijdens de levensspan van een koraalkolonie tot stand komen verder onderzocht worden (Baird *et al*. 2007).

De capaciteit van de AVH om de gevolgen van de klimaatsveranderingen te verminderen moet echter niet overschat worden voor verschillende belangrijke redenen. Ten eerste, koraalkolonies moeten wellicht eerst verbleken voordat een verandering kan optreden (Buddemeier & Fautin 1993; Baker 2001; Berkelmans & van Oppen 2006), een proces dat gepaard gaat met een hoog sterfterisico. Ten tweede is het waarschijnlijk dat nieuw omgewisselde (of omgeschakelde) koralen, die net succesvol van verbleking zijn hersteld, voor een langere periode geschaad blijven in hun groei en voortplanting (Baird & Marshall 2002). Ten derde, wanneer de stressfactor over een lange periode verdwijnt, dan kunnen koralen weer terug

veranderen naar hun originele endosymbiont type (Thornhill *et al*. 2006), waardoor ze weer kwetsbaar worden in nieuwe verblekingsperiodes. En tenslotte, de maximale verhoging in hittebestendigheid die koralen kunnen winnen door endosymbiont-verandering is mogelijk slechts 1 - 1.5°C (Berkelmans & van Oppen 2006), wat in deze eeuw al onvoldoende is als de projecties van de Intergovernmental Panel for Climate Change uitkomen (1.5 - 4°C stijging in de tropen aan het eind van deze eeuw; IPCC 2007).

Op een meer optimistische toon, endosymbiont-veranderingen zullen zeer waarschijnlijk een positieve rol spelen in de manier waarop sommige koraalsoorten het hoofd bieden aan de klimaatsopwarming, wat zal leiden tot nieuwe competitieve hiërarchieën en, uiteindelijk, de samenstelling van de koraalgemeenschappen zal beïnvloeden. Het belangrijkste is echter dat een verhoging van 1-1.5°C in de hittebestendigheid van koraalriffen tijdwinst zal opleveren — ongeveer 50 jaar (Donner *et al*. 2005) — waardoor maatregelen voor het reduceren van de broeikasgas-emissies geïmplementeerd kunnen worden, zodat, hopelijk, een desastreus effect van de klimaatsverandering op koraalriffen wordt ontweken.

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Dankwoord/Acknowledgments

En dan opeens sta je voor de laatste uitdaging van je promotieonderzoek: het schrijven van het dankwoord. Als meest gelezen onderdeel van het proefschrift wil je er natuurlijk iets moois van maken en tegelijkertijd hoop je niemand te vergeten. Al snel blijkt echter dat het onmogelijk is om iedereen bij naam en toenaam te noemen, dus wie wil je specifiek bedanken, en waarvoor dan wel? Na veel spitwerk in mijn geheugen is hieronder mijn beste poging, en ik kan verder alleen hopen dat ik niemand tekort doe.

Om te beginnen bij het begin, wil ik graag eerst Madeleine van Oppen en Ray Berkelmans bedanken. ***Madeleine****, jij gaf me de kans om als vrijwilliger bij AIMS ervaring in koraalonderzoek op te doen, wat uiteindelijk tot mijn promotieonderzoek leidde. Ik heb de grote mate van vrijheid in mijn onderzoek, gecombineerd met je onvoorwaardelijke open-deur beleid, zeer gewaardeerd. We konden uitstekend van gedachten wisselen over het onderzoek, en ik kwam altijd gemotiveerd en wijzer uit onze gesprekken. Heel erg bedankt voor alle steun die je me gegeven hebt.* **Ray,** I would like to thank you for your great supervision, specifically all your input on how to perform the heat-stress experiments and your shared expertise with respect to the field work. I greatly valued your support (especially when things went wrong), and our discussions about the research and other aspects of (scientific) life.

Dan natuurlijk mijn twee promotoren, Jeanine Olsen en Wytze Stam. **Jeanine**, I greatly appreciate the opportunity you have given me to do this research in your lab. I want to specifically thank you for the large amounts of your time you have given me to discuss my work, to help me with the analyses, and to help writing the manuscripts. Although we sometimes had to get used to each other again after my returns from Australia, I have always really appreciated your input and supervision. ***Wytze****, erg bedankt voor je hulp met manuscripten, kritische vragen tijdens proefpraatjes en, bovenal, de financiën.*

A stimulating working environment is very important, and therefore a big thanks to my colleagues from MarBEE, Steven Ferber, Andreas Zipperle, Malia Chevolot, Galice Hoarau, Jim Coyer, Klemens Eriksson, Katrien Sieben, Henning Reiss, Denise Haydar, Jan Veldsink, Stella Boele-Bos, Gezien van Roon en Joukje de Vries, for assisting me when I needed it, and making fun both during and after working hours. I specifically would like to thank Klemens, for his assistance with my stats, and Galice, for sharing his thoughts whenever I asked for them (and also when I didn't ask). *Jan en Stella, erg bedankt voor jullie hulp met het labwerk. Ook wil ik graag de koffietafel, bestaande uit de collega's van Ocean Ecosystems aangevuld met een aantal MarBEE collega's, zeer bedanken voor de nodige afleiding elke dag. Gezien de inhoud van sommige discussies, waar ik elke dag weer naar uit keek, is het misschien maar het beste om hier verder geen namen te noemen...*

Thanks of course also to my Master's student, Silvia Martinez-Bleuler. I certainly kept you busy with the spawning work at AIMS (including carrying Nally bins full of seawater), but you took to the challenge with gusto. I really enjoyed working with you, and I'm sure you'll do well with your own PhD now.

A great many people need to be thanked at AIMS, and I regret that I can only name a few of you. From the PCII-lab, I would specifically like to thank Lesa Peplow and Beth Ballment: the PCII-lab was great to work in thanks to all your efforts. Andrew Negri, your help with the larval rearing was invaluable, and thanks for staying "cool" when I caught my own thumb while fishing during your field trip. My PhD colleagues in Australia also were a great help. David Abrego and Alison Jones, is was great that we could do so much field work together, thank you for letting me tag along so many times. Alison Jones, Neal Cantin, and Meir Sussman, I greatly appreciated our collaborations, and the results were well worth the effort! Thanks also to the many volunteers who helped out in the field in a variety of ways. I further received a huge amount of support from the technical staff at AIMS, including those at the workshops, the store, marine ops, the research vessels, mariculture and the security. They've all been incredibly co-operative and helpful, and therefore my heartfelt thanks.

From JCU, I would specifically like to thank Bette Willis and Phil Osmond for their help in making my field work possible when the AIMS rules were getting to restrictive for a PhD student.

Het verdelen van een leven over twee continenten bleek niet altijd even makkelijk. Ik ben al mijn vrienden in Nederland dan ook erg dankbaar voor hun begrip als ze weer een (groot) aantal maanden niets van mij hoorden. Broer, schoonzus en nicht, het is een enorme steun om zo'n fantastische familie-band te hebben, en gelukkig is er Skype. Mam en pap, bedankt dat jullie er altijd voor mij zijn, en ik waardeer erg dat jullie mij altijd gestimuleerd hebben om mijn eigen weg te zoeken. Het zal moeilijk blijven om zo ver weg van jullie te wonen (gelukkig houden we van vlieg-vakanties!).

Lastly, Abbi, your role in my life has been ever increasing since we first met. I can't express in words how important your encouragement, understanding, support, and, above all, your love, have been in these last four years, and I'm eagerly looking forward to building a life together in Australia.

PUBLICATION LIST

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BIOGRAPHY

Jos was born on December 29th 1977 in Amersfoort (The Netherlands). Ten days later he and his family moved to Hengelo, where he later attended primary, secondary and high school. He received his VWO diploma in 1996 from the *Openbare Scholengemeenschap Bataafse Kamp*. In the same year he started his degree in Biology at the University of Nijmegen. During the Master's phase he undertook three research projects, two of which took him abroad. In his first project with the Department of Animal Physiology in Nijmegen, he spent four months in Greece investigating the effects of stress on the physiology of two commercially important marine fish species. For the second project, also in Nijmegen with the Department of Biochemistry, he studied the enzymatic properties of the gastric H^+,K^+-ATPase in genetically modified human-embryonic kidney cells. Finally, he went off to Australia for six months to investigate the carbon concentrating mechanism of the giant clam in the Molecular Sciences Department of the James Cook University (JCU) in Townsville. Upon completion of his Master's degree in 2002, Jos took up a volunteer position for one year at the Australian Institute of Marine Science (AIMS), where he worked with Dr. Madeleine van Oppen (who he had met at JCU) and Dr. Ray Berkelmans on the phylogeny of *Symbiodinium* (the unicellular algae that live in symbiosis with many tropical coral species). Keen to continue with this line of research, Jos started discussing the possibility of a PhD in this field. Eventually, this resulted in a collaborative PhD project (funded by NWO-WOTRO) shared between the University of Groningen (where Madeleine had completed her PhD under Prof. dr. Jeanine Olsen and Prof. dr. Wytze Stam) and AIMS (where Madeleine is now a Principal Research Scientist). The project started in April of 2004 and the results are described in this thesis.

In his early days at AIMS, he met a beautiful Aussie girl named Abbi who stole his heart. On 17 January 2009, four years after their first kiss, they got married on the beach in Australia. Jos and Abbi have since taken up residence in Brisbane. Jos has been working on getting the last paper of his PhD into publication and securing a post-doctoral position in the lab of Prof. Hoegh-Guldberg at the University of Queensland.